Cutin

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Cutin:xyloglucan transacylase (CXT) activity covalently links cutin to a plant cell-wall polysaccharide

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Highlights

- CXT activity catalyses ester bond formation between cutin and xyloglucan.
- CXT activity is found to be widespread in plants.
- CXT activity may contribute to cuticle integrity and epidermal strength.

Abstract

The shoot epidermal cell wall in land-plants is associated with a polyester, cutin, which controls water loss and possibly organ expansion. Covalent bonds between cutin and its neighbouring cell-wall polysaccharides have long been proposed. However, the lack of biochemical evidence makes cutin–polysaccharide linkages largely conjectural. Here we optimised a portfolio of radiochemical assays to look for cutin–polysaccharide ester bonds in the epidermis of pea epicotyls, ice-plant leaves and tomato fruits, based on the hypothesis that a transacylase remodels cutin in a similar fashion to cutin synthase and cutin:cutin transacylase activities. Through in-situ enzyme assays and chemical degradations coupled with chromatographic analysis of the $^3$H-labelled products, we observed that among several wall-related oligosaccharides tested, only a xyloglucan oligosaccharide ($[^3]$HXXXGol) could acquire ester-bonds from endogenous cutin, suggesting a cutin:xyloglucan transacylase (CXT). CXT activity was heat-labile, time-dependent, and maximal at near-neutral pH values. In-situ CXT activity peaked in nearly fully expanded tomato fruits and ice-plant leaves. CXT activity positively correlated with organ growth rate, suggesting that it contributes to epidermal integrity during rapid expansion. This study uncovers hitherto
unappreciated re-structuring processes in the plant epidermis and provides a step towards the
identification of CXT and its engineering for biotechnological applications.

**Keywords**: Epidermis remodelling, cutin, xyloglucan, ester bond, transacylase, cell
expansion, polyester–polysaccharide conjugates

**Abbreviations**
CM, chloroform/methanol; CMNaOH, sodium hydroxide in CMW; CMW,
chloroform/methanol/water; CXT, cutin:xyloglucan transacylase; TLC, thin-layer
chromatography; PCW, primary cell wall; HFA, hydroxy-fatty acid; PXT,
polymer:xyloglucan transacylase; MFW, methanol/formic acid/water; XET, xyloglucan
endotransglucosylase.
**Introduction**

Cutin, a polyester-based matrix, is localised between the cell wall and the wax layer of the land-plant shoot epidermis, forming a protective cuticle together with wax. The composition of cutin in diverse plants has been studied intensively (e.g. Diarte *et al.*, 2019), as cutin biosynthesis (Schnurr *et al.*, 2004; Yeats *et al.*, 2012; Jakobson *et al.*, 2016) and its regulation (Martin *et al.*, 2017), the transmembrane transport of its precursors (Fabre *et al.*, 2016), and its physiological roles, such as protecting plants from microbial invasion (Isaacson *et al.*, 2009; Fawke *et al.*, 2019), UV damage (Rozema *et al.*, 2009) and desiccation (Bourgault *et al.*, 2020) as well as defining organ boundaries (Kurdyukov *et al.*, 2006). However, significant gaps remain in our knowledge of cutin—especially concerning its possible restructuring in the apoplast *in vivo*. Until recently, cutin polymerisation was the only documented apoplastic cutin reaction (Yeats *et al.*, 2012; Girard *et al.*, 2012). Investigating post-synthetic restructuring reactions was difficult because of technical obstacles, especially the lack of adequately sensitive methods to detect proposed rare covalent bonds between cutin and neighbouring polymers. Nevertheless, remodelling seems tenable in view of the fact that the neighbouring primary cell wall (PCW) is highly dynamic, so comparable enzymic reactions may also occur in cutin layer. This idea was strengthened by our recent discovery of a cutin:cutin endo-transacylase activity (Xin *et al.*, 2021).

In the present study, we sought biochemical evidence for the long-mooted hypothesis that cutin and nearby PCW polymers are covalently inter-linked. Evidence in favour of this hypothesis came from studies of partial hydrolysis with cutinase coupled with immunolabelling, showing that cutin can mask pectin, hemicelluloses (including xyloglucan) and cellulose (Philippe *et al.*, 2020). Earlier studies also suggested that cutin protrudes into PCW (Norris and Bukovac, 1968; López-Casado *et al.*, 2007). Additionally, pectinases and cellulases have been used to isolate cutin (Orgell, 1955; Lendzian *et al.*, 1986). The most direct evidence supporting our hypothesis is that partial cleavage of lime fruit cutin with cold HF released a conjugate consisting of a hydroxy-fatty acid (HFA) tetramer in which a secondary hydroxy group was ether-linked to the O-6 position of an unidentified hexose disaccharide (Tian *et al.*, 2008). Moreover, transcriptomic studies showed that SHINE1, 2 and 3 (proposed redundant transcription factors affecting *cus1* expression) also modulate the expression of various pectin-modifying genes (e.g. those encoding pectate lyases) (Shi *et al.*, 2011), suggesting a link between cutin biosynthesis and PCW remodelling. Also, alterations of PCW quantity and morphology are observed in SHINE3-silenced tomato fruit epidermis.
(Shi et al., 2013). Similar correlations between cutin-biosynthesis transcription factors and PCW modification were also reported for cutin deficient 2 (CD2), a cutin biosynthesis transcription factor distinct from the SHINEs (Issacson et al., 2009; Martin et al., 2016). Together, these observations support a relationship between cutin metabolism and wall polysaccharide metabolism and raise the possibility of cutin–polysaccharide interactions. The physiological significance of cutin–polysaccharide bonding could be to interlink epidermal components, thereby mechanically reinforcing the shoot epidermis — which plays a pivotal role in modulating organ expansion (Kutschera, 2008).

As reviewed by Fich et al. (2016), three models of cutin–polysaccharide covalent bonds are available (Fig. 1a, models 1–3).

[1] Cutin-to-polysaccharide esterification, i.e. a –COOH group of cutin is esterified to a free –OH group in the polysaccharide. We propose that this type of linkage could be formed by a transacylase breaking an HFA–HFA ester bond within cutin (donor substrate) and transferring the newly formed carboxy terminus onto a polysaccharide hydroxy group (acceptor substrate) (Fig. 1b). Such a reaction would be comparable to the transacylase (oxalyltransferase) activity that can transfer an oxalyl group from oxalyl-threonate onto a of a range of carbohydrates (Dewhirst & Fry, 2018).

[2] Polysaccharide-to-cutin esterification, i.e. a –COOH group of an acidic polysaccharide (e.g. pectin) is esterified to a free –OH group of the cutin. This could theoretically occur by a pectin methylesterase-like enzyme acting in transacylation mode — i.e. cleaving a GalA methylester (donor substrate), releasing methanol, and transferring the newly formed carboxy group onto a cutin hydroxy group (acceptor substrate).

[3] Polysaccharide–cutin ether bonding by condensation of an –OH group of cutin with an –OH group of a polysaccharide. Fich et al. (2016) proposed such a bond but did not suggest a mechanism for its formation. A cross-linking role for aromatic moieties e.g. p-coumaroyl residues was doubted because of the scarcity of aromatics in the structure of cutin (Fich et al., 2016).

In addition, we can propose a fourth model:

[4] polysaccharide→cutin glycosidic bonding, i.e. a linkage is formed between the reducing terminus of a polysaccharide a free hydroxy group of cutin. A bond of this type could
theoretically be formed by a hetero-transglycanase activity [comparable to the known
polysaccharide hetero-transglucanase activities (Simmons et al., 2015; Strtilová et al.,
2020)], cleaving a glycosidic bond within the polysaccharide (donor substrate) and then
creating a new glycosidic bond to the cutin (acceptor substrate). The product could contain,
for example, a xyloglucan-(1→16)-HFA linkage, where the HFA moiety is part of a cutin
molecule (Fig. 1a, model 4).

be alkali-stable.

Concerning model [1], based on the transacylation reaction catalysed by cutin synthases
(CUS1 and related isoforms; donor substrate, HFA–glycerol ester; acceptor substrate, nascent
cutin), we propose that similar transacylases could catalyse the formation of cutin-to-
polysaccharide ester bonds. A comparable transacylase activity was recently detected in the
plant epidermis [donor, cutin; acceptor, radioactive 16-hydroxyhexadecanoid acid (a free
HFA); Xin et al., 2021]. In contrast, we were unable to detect an enzyme activity capable of
using radioactive homogalacturonan methyl ester as donor and cuticular polymeric cutin as
acceptor as predicted by model [2] (Xin et al., 2021); therefore, we reject model [2]. Models

Any cutin–polysaccharide covalent bonds are likely to be present in low quantities (a)
because the two linked polymers are physically separated (cuticle versus wall), and (b)
because a single cutin–polysaccharide covalent bond per molecule is all that is required to
cross-link two such macromolecules. To enable the detection of enzymes capable of creating
rare linkages of the type envisaged, we devised and optimised a portfolio of highly sensitive
radiochemical assays based on in-situ and ex-situ feeding of 3H-labelled oligosaccharides that
model the major PCW polysaccharides (xyloglucan and pectin) while using endogenous
native cutin in the cuticle as the other substrate. Three thick-cuticled plant models were
chosen: pea epicotyl (Li et al., 2016), ice-plant leaf (Hylotelephium spectabile) and tomato
fruit (whose cutin is dominated by 10,16-dihydroxyhexadecanoic acid residues; Kosma et al.,
2010), thus spanning three organs and range of dicot phylogeny. We applied appropriate
chemical degradations coupled with chromatographic analyses to investigate the bond types
formed. Interestingly, in all three plant models, cutin (acyl donor) formed ester bonds with a
specific motif of xyloglucan (acyl acceptor), favouring model [1].
Materials and methods


Plant materials. *H. spectabile* (ice-plant), *Pisum sativum* (pea; cv. meteor) and *Solanum lycopersicum* (wild-type cv. M82 and cd1-knockout) were grown as in Xin et al. (2021). The cd1 seeds were kindly donated by Prof. J.K.C. Rose (Cornell University, USA). Briefly, *H. spectabile* and tomatoes were greenhouse grown with 16 h light (21°C) and 8 h dark (18°C); pea seedlings were grown in well-watered vermiculite in continuous darkness at 25°C and the epicotyls (here taken to mean the first three internodes above the cotyledons) were harvested at 7 d as default.

Assay of radioactivity. For measurement of total $^3$H incorporation into methanol-insoluble epidermal material, the MFW-washed and re-dried epidermis was added to 2 ml of scintillation fluid (0.5% w/v PPO and 0.05% w/v POPOP in toluene; chosen to avoid the organic bases used in many commercial scintillants, which may cleave esters), mixed overnight, and assayed for $^3$H in a Beckman scintillation counter. For solutions or moist solids, the specimen (with water added, if necessary, to bring the volume up to at least 0.5 ml) was mixed with 10 volumes of OptiPhase HiSafe (Fisher) scintillation fluid before scintillation counting. The scintillation-counting efficiency of $^3$H was ~7% for dry samples (epidermis or chromatography paper) and ~35% in solutions. Chromatogram lanes (paper and TLC) were either cut into small strips, which were assayed for $^3$H by scintillation-counting, or scanned in an AR2000 radioisotope thin-layer analyser (LabLogic, Sheffield).

In-situ CXT enzyme assay using endogenous cutin and enzymes. Epidermis specimens were obtained as in Xin et al. (2021). Briefly, epidermis containing endogenous enzymes and cutin was isolated from plants by manual peeling (*H. spectabile* leaves and tomato fruit) or by rolling under a glass rod to extrude internal (non-epidermal) tissues and epidermal protoplasm (pea epicotyl). Epidermis was stored frozen, then thawed at 0°C and either washed in cold reaction buffer [25 mM succinate (Na$^+$), pH 6.5] to remove intracellular metabolites (yielding native epidermis) or incubated in water at 100°C for 1 h (yielding...
denatured epidermis). Native epidermis was briefly blotted dry and cut into small pieces, then incubated with 0.1–12 kBq of radiolabelled substrates (e.g. \([^3]H]XXXGol\) in the 25 mM pH 6.5 buffer (routinely 6 ml per g fresh weight) at 20°C for up to 24 h. All enzymic reactions were stopped by addition of 10 volumes of freshly prepared methanol/formic acid/water (MFW) (5:1:5, v/v/v). To thoroughly remove unincorporated alcohol-soluble radiochemicals, we dried the epidermis onto Whatman No. 1 paper, which was run as a ‘chromatogram’ in MFW for 5–7 d.

**Ex-situ CXT enzyme assay with native plant enzymes.** As in Xin *et al* (2021), denatured epidermis (containing cutin but no active endogenous enzymes) was treated by sequential applications of acetone, boiling chloroform, and chloroform/methanol (CM) (2:1, v/v), removing intracellular substrates, pigments and wax, and dried. Additionally, in some experiments, polysaccharides were also removed by incubation of epidermis with 0.1% (w/v) purified Driselase in pH 4.5 buffer (pyridine/acetate/water, 1:1:98, v/v/v) at 37°C for 5 days, prior to the organic solvent treatments above. Apoplastic enzymes were then extracted as described by Xin *et al* (2021). Briefly, the isolated pea epidermis was ground in liquid nitrogen then homogenised in extraction buffer [35 mM succinate (Na\(^+\)), pH 6.5, containing 1% w/v polyvinylpolypyrrolidone] at 2 ml / g fresh weight with shaking at 4°C for 2 h. The suspension was then centrifuged at 4°C for 15 min at 3000 × g. The supernatant (300 µl), containing solubilised enzymes, was incubated with 10 mg of thoroughly dried, denatured epidermal particles together with 0.53 kBq \([^3]H]XXXGol\) (radioactive xyloglucan heptasaccharide) at 20°C for up to 1 d. Reaction termination and wash procedures were as for *in-situ* experiments.

**Quantifying radioactive CCT product.** To look for the transacylase products we were interested in, a chemical and enzymic degradation strategy was employed (Fig. 2). A portion of the MFW washings (which contained remaining free \[^3]H\]oligosaccharides and low-M\(_r\) products, if any) was assayed for \(^3\)H; in some cases, the remainder was run by thin-layer chromatography (TLC; Merck silica-gel plates) in 1-butanol/acetic acid/water, 2:1:1, v/v/v, with three ascents, followed by AR2000 radioisotope scanning or fluorography as in Fry (2000) (Fig. S1).

The MFW-washed epidermis samples were then washed sequentially in toluene, CM (2:1, v/v) and chloroform/methanol/water (CMW, 10:10:3, v/v/v), removing small, hydrophobic by-products (Fig. 2). To remove possible protein–[^3]H]oligosaccharide conjugates, 3 U/ml
proteinase K from porcine liver (Sigma) was incubated with the MFW etc.-washed epidermis samples at pH 8.8 [50 mM ammonium (acetate)] at 37°C for 3 days, cleaving peptide bonds.

To look for cutin-to-[\(^{3}\text{H}\)]oligosaccharide transacylase product, we hydrolysed ester bonds in chloroform / methanol / 4 M aqueous NaOH (CMNaOH, 10:10:3, v/v/v). The reaction was stopped by addition of acetic acid (5 mol per mol NaOH), and an aliquot of the products was dried, re-dissolved in MFW, and assayed for \(^{3}\text{H}\) (Fig. 2), quantifying the transacylase products. A second aliquot was re-dissolved in 3 ml 0.5% aqueous chlorobutanol (labelled ‘transacylase product stock’). Compounds in this stock were mixed with non-radioactive internal markers [6 mg each of glucose, a xyloglucan oligosaccharide mixture (DP7–9) and dextran] and size-fractionated on a Bio-Gel P-2 column (gel-permeation chromatography; 120 ml bed volume; flow-rate 0.5 ml/min in 0.5% aqueous chlorobutanol, collecting 50 fractions at 3 ml/fraction). A 2-µl aliquot of each fraction was tested by TLC as above followed by thymol staining (Jork et al., 1994), revealing which P-2 fractions contained the internal markers. Additional aliquots were assayed for \(^{3}\text{H}\), revealing the proposed transacylase products. Interesting fractions were pooled and dried in a SpeedVac, then half the pool was analysed by descending paper chromatography (on Whatman No. 3, in butan-1-ol/pyridine/water, 4:3:4, v/v/v, for 46 h). The other half was subjected to a second alkaline hydrolysis (in 1 M aqueous NaOH), acidified with excess acetic acid and then analysed by paper chromatography as above.

To test for radioactive XET reaction products, we hydrolysed the xyloglucan in the CMNaOH-resistant residues with either (a) 0.1% (w/v) xyloglucan endoglucanase (XEG) in pH 4.5 buffer (pyridine/acetate/water, 1:1:98, v/v/v) at 20°C overnight, or (b) 2 M TFA for 1 h at 120°C. In each case, the supernatant was assayed for \(^{3}\text{H}\).

**CXT activity calculation and statistical analysis.** CXT products are expressed as Bq of CMNaOH-releasable radioactivity per kBq of supplied \([^{3}\text{H}]\text{XXXGol}\) per (usually) 50 mg fresh weight of epidermis. Statistical analysis was by Student’s \(t\)-test.

**Results**

*Evidence for a polymer:xyloglucan transacylase (PXT) activity in situ with \([^{3}\text{H}]\text{xyloglucan heptasaccharide as acceptor substrate})*

To detect the enzymic formation of polymer–xyloglucan (e.g. cutin–xyloglucan) bonds in situ in the plant epidermis (Fig. 1), we incubated cutinised epidermal walls with a radiolabelled
xyloglucan-heptasaccharide ([glucitol-1-\(^3\)H]XXXGol) and looked for incorporation of radioactivity into polymeric products (i.e., those insoluble in acidified aqueous methanol, unlike free [\(^3\)H]XXXGol). The model acceptor substrate employed (an oligosaccharide-\(^3\)H]alditol) was of low molecular weight, unlike wall polysaccharides, but resembled wall polysaccharides in lacking a reducing group, like the majority of residues within a polysaccharide molecule. In addition, using a small acceptor substrate facilitates penetration into the wall/cuticle, and avoids the problem of avid hydrogen-bonding between an exogenous [\(^3\)H]polysaccharide and other wall polymers.

In pea and ice-plant epidermis, \(^3\)H incorporation was indeed observed (Fig. 3a), and the process was heat-labile, demonstrating an endogenous enzyme activity that can produce polymer–[\(^3\)H]XXXGol bonds.

The formation of methanol (MFW)-insoluble radioactive products in this experiment (Fig. 3a) could be attributed to any or all of: a polymer:xyloglucan transacylase (PXT) activity (Fig. 1b), an unknown enzyme capable of making ether bonds (Fig. 1a model [3]), or a transglycanase activity (XET). The key distinguishing difference is that hypothetical ether bonds (model [3]) and XET-generated glycosidic bonds will be alkali-stable whereas ester bonds (model [1]) will be broken by cold alkali (Fig. 2 and 3a).

Therefore, following removal of all methanol-soluble free [\(^3\)H]XXXGol and its smaller degradation products, we applied alkali (CMNaOH, i.e. NaOH in CMW, aiding cuticular penetration) (Fig. 2). Interestingly, CMNaOH was able to solubilise some \(^3\)H from the methanol-insoluble products formed by undenatured epidermis (~2% and 20% of the total radioactive polymeric products in pea and H. spectabile respectively; Fig. 3a). The yield of such ester-bonded material was dependent on the pH of the medium used during the in-situ incubation, peaking at pH 7.5; in contrast, at pH 3.5 in native epidermis, and in heat-denatured controls at pH 7.5, the ester yield was negligible (Fig. 3a). These results suggest that some of the exogenous [\(^3\)H]XXXGol molecules had had epidermal polymers enzymically linked to them via ester bonds, catalysed by a PXT activity.

A pH value of 6.5 was chosen for subsequent assays because it afforded satisfactory quantities of the PXT product and is within the physiological pH range of apoplast. Moreover, pea epicotyl epidermis was chosen as the main cutin and enzyme source because of the pea seedlings’ fast growth and high yield of PXT product (albeit low relative to XET products).
The yield of ester-bonded radioactive products was time-dependent, plateauing by ~2 h (Fig. 3b), probably because the $[^3]H$XXXGol was partially hydrolysed (Fig. S1a,b). This loss of $[^3]H$XXXGol appeared to be due to the action of endogenous $\alpha$-D-xylosidase (Guillen et al., 1995) plus $\beta$-D-glucosidase (Miyamoto et al., 1997; Franková & Fry, 2011), resulting in the sequential formation of $[^3]H$XXGol and $[^3]H$XGol (Fig. S1a,b) within ~6 h, agreeing with the time taken to reach a plateau of PXT product formation (Fig. 3b). An alternative — denaturation of PXT — played a minor role as shown by the continuing ability of ‘old’ (previously incubated) epidermis to produce ester-linked products in situ when supplied with fresh $[^3]H$XXXGol (Fig. S1c).

Acceptor substrate specificity

A survey of seven oligosaccharides showed that XXXGol (a xyloglucan-derived heptasaccharide with a Glc$_4$ backbone) was by far the most effective acceptor substrate for PXT activity (Fig. 3c). The second-best acceptor was the nonasaccharide XXFGol, which also has a Glc$_4$ backbone but has an additional fucosyl-galactose side-chain; and the third-best was XXGol, which has a Glc$_3$ backbone. Oligosaccharides with a Glc$_2$ backbone (XGol and cellobiitol) and monomeric glucitol were not effective. This size-discrimination on XXXGol’s building blocks helps to confirm our explanation of PXT products plateauing (Fig. 3b). The pectin-related octasaccharide Aras$_8$-ol was also not an acceptor substrate.

Ex-situ assay of transacylase activity

As an alternative to the above in-situ approach to detecting PXT activity, we extracted pea epidermal enzymes and incubated them ‘ex situ’ with denatured pea epidermis plus $[^3]H$XXXGol. Ester-linked polymer–$[^3]H$XXXGol products were again formed (Fig. 3d), indicating that the PXT activity survived extraction. Inclusion of a detergent, a reducing agent or a chelating agent enhanced the extraction and/or stability of the PXT activity (Fig. 3d). DTT, the reducing reagent, tripled the detected activity, possibly by limiting the oxidation of essential cysteine residues. EDTA’s effect suggests that the enzyme may not require divalent metal cofactors. And the non-ionic detergent, Triton X-100, might be expected to enhance the extraction of a cuticular enzyme.
Nature of the donor substrate

The above results indicated the formation of ester-bonded polymer-[\(^3\)H]XXXGol products by a polymer:xyloglucan transacylase (PXT) activity. Next, we investigated the nature of the ‘polymer’ that acts as donor substrate.

The products formed in situ at pH 6.5 by pea epicotyl epidermis were fractionated in more detail by a series of solvents and digestions (Fig. 2; Table 1). Acidified aqueous methanol (MFW) solubilised most of the radioactivity, much of which was still in the form of starting material (Fig. S1a). After thorough washing in MFW, subsequent treatments in neutral ‘lipid’ solvents (toluene, CM, CMW) released no additional radioactivity (Table 1). Products that can be released by such solvents, and especially by CMW [a recommended solvent for glycolipids (Behrens et al., 1971; Breil et al., 2017)], would include glycolipid structures such as wax-oligoesters (Kunst and Samuels, 2003) linked to XXXGol, or esters formed by cutin:xyloglucan transacylase action very close to the non-carboxy terminus (Fig. 1b) of cutin donor substrate (making small products such as HFA–XXXGol or HFA–HFA–XXXGol).

The absence of radioactivity in these extracts argues against the formation of such structures.

Cell-wall (glyco)proteins were also theoretically possible acyl donor substrates, since they possess ‘activated’ carboxy groups in the form of amide (including peptide) bonds. However, after the lipid solvents, Proteinase K released very little radioactivity from both native and denatured samples (Table 1), indicating that proteins were not the donor substrates of the observed transacylation reaction(s).

Another potential acyl donor substrate would be the methyl-esterified GalA residues of homogalacturonan, which could potentially create uronoyl ester linkages with other carbohydrates in the form of, for example, homogalacturonan-[\(^3\)H]XXXGol (Fig. 1a, model [2]), perhaps catalysed by a pectin methylesterase acting as a transacylase rather than as a hydrolase (Lenucci et al., 2005). However, as stated in introduction, our previous work on in situ epidermal incubations (Xin et al., 2021) indicated that exogenous, fully methyl-esterified [\(^3\)H]GalA\(_8\)-ol did not become covalently attached to any cell-wall components. [If there had been a pectin:xyloglucan transacylase activity, the radiolabelled Me\(_8\).GalA\(_8\)-ol would have been expected to form ester bonds to cell-wall xyloglucan.]

Since the acyl donor is not wax esters, (glyco)proteins or pectins, the most likely alternative in a cuticularised epidermal cell-wall preparation is the aliphatic polyester cutin, the only
insoluble polymer left that processes abundant activated carboxy groups. The PXT activity detected thus far is therefore considered to be a cutin:xyloglucan transacylase (CXT) activity.

Elucidation of CXT’s mode of action

To investigate how endogenous cutin might ester-bond to exogenous $[^3H]XXXGol$, we used gel-permeation chromatography (GPC) on Bio-Gel P-2 to size-profile the alkali-releasable radioactive product. The simplest predicted product would be free $[^3H]XXXGol$, released from cutin–$[^3H]XXXGol$ conjugates.

The most prominent alkali-released $^3H$-labelled product (peak I; Fig. 4a) had a higher apparent $M_r$ than XXXGol, eluting near the void volume ($V_0$) upon GPC. We verified that XXXG (the non-radioactive internal marker) and $[^3H]XXXGol$ do co-elute on Bio-Gel P-2 (Fig. S2), so the non-radioactive internal markers (Fig. 4a inset and horizontal blue lines) would have correctly indicated the DP of any radiolabelled oligosaccharides. Alkaline hydrolysis of the products formed in situ in heat-denatured epidermal material gave 25-fold less radioactivity in peak I (Fig. 4a), indicating that it was enzymically generated. Thus, the exogenous $[^3H]XXXGol$ underwent a structural change, leading to a higher apparent $M_r$, after ester-bonding with cutin. We discount the alternative possibility that lipid-like material present in the CMNaOH hydrolysate was adsorbing the $[^3H]$oligosaccharides and thus giving them an artefactually high apparent $M_r$ since the sample was loaded onto the column along with internal marker oligosaccharides, which did elute in the expected fractions (Fig. 4a inset).

A second enzymically generated product (peak II) eluted close to the totally included volume ($V_i$), in the GPC fractions containing internal markers of $DP$ 1–3 (Fig. 4a).

Peaks I and II were further analysed by paper chromatography (Fig. 4b). Peak I, which on GPC had eluted with apparent $DP>9$, now revealed three relatively mobile radiolabelled compounds (apparent $DP<9$), Ia, Ib and Ic, that approximately co-migrated on paper chromatography with $DP=7$, -5 and -3 markers respectively. Conversely, peak II, which on GPC had eluted with apparent $DP \leq 3$, ran on paper chromatography as a single broad peak of $^3H$ in the $DP=5–7$ zone. Such inconsistencies between the size estimates based on GPC and on paper chromatography may suggest that the alkali-released products were not simple oligosaccharides.
To test whether the original alkaline hydrolysis step (with chloroform/methanol/water as solvent) might have been incomplete, we repeated the alkaline hydrolysis on peaks I and II, this time in aqueous NaOH. The former peak I now still showed peaks Ia and Ib, but peak Ic had disappeared. Peak Ic might thus be a relatively CMNaOH-resistant acylated \([^3\text{H}]\text{XXGol}\) conjugate, solubilised from the cell wall/cuticle by CMNaOH and migrating faster than simple XXGol owing to its hydrophobic acyl moiety, but hydrolysed to \([^3\text{H}]\text{XXGol}\) by subsequent aqueous NaOH. Peak II remained unchanged after aqueous NaOH (Fig. 4c).

**Developmental changes in CXT activity: positive correlation with cell expansion rate**

It is interesting to explore the physiological significance of CXT activity, for example whether CXT activity has any correlation with cell expansion as we hypothesised. Tomato fruit is a good model because it undergoes dramatic expansion during part of its development. CXT activity increased during rapid expansion (5–30 days post-anthesis, while the fruit diameter was increasing at ~0.5 cm per five days), and then diminished once expansion had ceased (mature green to dark red) (Fig. 5a). *H. spectabile* leaf epidermis showed a similar trend: epidermis from large (~12 cm), expanding leaves had higher CXT activity than that from smaller leaves (3–5 cm) (Fig. 5b). However, pea epicotyl epidermis showed little difference between 4, 7 and 10 days after sowing (Fig. 5c), when the mean epicotyl lengths were about 7, 16 and 17 cm (thus when the epicotyls were rapidly, barely and not expanding) respectively.

Thus, in two specimens of the three tested, CXT activity positively correlated with organ expansion rate, supporting our hypothesis that CXT activity functions during, and contributes to, epidermal cell expansion.

**CUS1 is not the enzyme responsible for CXT activity**

The biochemical evidence in this paper supports model [1] — cutin and \([^3\text{H}]\text{XXXGol}\) being linked via ester bonds catalysed by CXT activity. Thus, as a candidate for the enzyme responsible, the only well-studied cutin transacylase, CUS1, was heterologously produced (with a C-terminal His6-tag) and purified as in Yeats *et al.* (2012) and Xin *et al.* (2021). However, the CUS1 did not catalyse any reaction between cutin and \([^3\text{H}]\text{XXXGol}\) (Fig. S3b). Also, the *cdl* mutant was compared with wild-type (cv. M82) for in-situ CXT activity and the
results point to the same conclusion (Fig. S3a). Hence, we conclude that CXT activity is not due to the CUS1 protein.

Discussion

Ester bonds link cutin to xyloglucan

Cutin, the hydrophobic polyester present in the growth-limiting shoot epidermis, is far less well understood than PCW polysaccharides, especially with regard to in-vivo re-modelling mechanisms. Cutin–polysaccharide covalent linkages have been investigated here, four models for which are considered (Fig. 1a). The data presented in this paper favour model [1].

Using highly sensitive radiochemical assays combined with appropriate chemical degradations in tandem with a series of chromatographic analyses, we obtained the first clear evidence for bonding between cutin and an oligosaccharide model ([3H]XXXGol) of the cell-wall polysaccharide, xyloglucan. Specifically, the methanol-insoluble endogenous epidermal polymer, cutin, became bonded exogenous [3H]XXXGol, as predicted by the existence of a CXT activity that can use XXXGol as its acceptor substrate (Fig. 1b). The newly formed bonds were cold-alkali-labile, indicating that they were esters. The alkali was applied in a hydrophobic (chloroform/methanol-rich) solvent, and at a low concentration — precautions preventing hemicellulose solubilisation. The bonding was observed in in-situ and ex-situ assays and in three contrasting epidermal samples, and is thus botanically widespread.

Oligosaccharides unrelated to xyloglucan were not substrates (Fig. 3c), so the CXT activity was xyloglucan-specific. Other xyloglucan-derived oligosaccharides than XXXGol were also tested, and their order of effectiveness as acceptor substrates was XXXGol (DP7) >> XXFGol (DP9) >> XGol (DP3). This order broadly concurs with that for XET activity (Fry et al., 1992), so it was important to prove that the reported ‘transacylase’ products were not XET products. The evidence was: (1) XXFGol is a good substrate for XET activity, usually as effective as XXXGol (Fry et al., 1992), but was only a weak substrate for CXT activity (Fig. 3c); (2) no [3H]polysaccharide was detected in the chloroform/methanol/water/alkali-released fraction (Fig. 4b,c), whereas XET activity uses exogenous [3H]XXXGol to generate a [3H]polysaccharide which is insoluble in CMW.

Multiple evidence indicated that the acyl donor substrate for the observed ‘CXT’ activity was indeed cutin. First, cutin is a major polymeric component of the epidermis that would provide abundant activated carboxy groups. Secondly, (glyco)proteins were discounted as alternative
potential apoplastic acyl donors: proteins possess various acyl residues [peptide-bonded amino acid residues of the backbone; the primary amides (R−CONH$_2$) of glutamine and asparagine side-chains; and potentially also isopeptide bonds formed by transglutaminases (Serafini-Fracassini and Del Duca, 2008)], which would be energetically capable of ester-bonding to a hydroxy group in $[^3]$HXXXGol via a novel transacylase activity e.g:

\[ R−CONH_2 + HO−XXXGol \rightarrow R−CO−O−XXXGol + NH_3. \]

Yet proteinase K solubilised negligible $^3$H from both native samples and denatured controls, indicating that proteins were not the anchorage points to which the $[^3]$HXXXGol became attached during the observed transacylation reaction. Thirdly, methylesterified pectic homogalacturonan domains could potentially form ester bonds with XXXGol:

\[ HG−CO−O−CH_3 + HO−XXXGol \rightarrow HG−CO−O−XXXGol + CH_3OH \]

where HG is homogalacturonan. However, this amounts to model [2] of Fig. 1a and is not discussed further because it was ruled out previously by research under comparable experimental conditions (Xin et al., 2021).

Not ether or glycosidic bonds

Fig. 1a also considers models [3] and [4]. Our experimental strategy (Fig. 2) would detect cutin–XXXGol ester bonds (model [1]), as already discussed. It would also detect any hypothetical XXXGol–cutin ether bonds (model [3]), which would result in $^3$H incorporation into alkali-resistant epidermal polymers. Such ether bonding could be plausible except that no known enzyme is capable of forming an ether bond with an oligosaccharide. Furthermore, since an ether bond would not be cleaved by any of the treatments applied in this study (Fig. 2) owing to its stability to alkali, acid and high temperature (Hua et al., 2016), we conclude that even such a bond was formed, it was not the main product (Table 1). Our conclusion is inconsistent with the NMR study that detected ether bonds between cutin and oligohexoses in lime fruit epidermis (Tian et al., 2008). The apparent discrepancy could be explained by the different organs used: we studied 7-day-old pea epicotyls which had recently been very rapidly expanding (Fig. 5c), whereas Tian et al. (2008) used lime fruit (presumably fully grown).

Our experimental strategy (Fig. 2) would not detect glycosidic bonding between the exogenous XXXGol and cutin (model [4]) because such bonding, formed by a
transglycosylation reaction, would require a cleavable glycosidic linkage within the XXXGol molecule as donor substrate, and the product would not be radiochemically detectable because the $[^3]H$glucitol moiety of the XXXGol would be lost as part of the small, methanol-soluble leaving group. Our experimental strategy would (and did; Fig. 3a) detect the expected polysaccharide–$[^3]H$XXXGol glycosidic bonding, catalysed by XET activity using the exogenous $[^3]H$XXXGol as acceptor substrate (Franková & Fry, 2013).

The ester bonding mechanism refocuses our understanding of epidermal dynamics and architecture

XXXG and XXFG are abundant motifs in natural xyloglucan. However, $[^3]H$XXXGol was the preferred exogenous acyl acceptor. This may suggest that the additional fucose and galactose residues on the third xylose imposed steric hindrance for the accessibility of the free hydroxy group to which the cutin acyl group could have been attached.

Based on imaging evidence (Jeffree, 2006; Philippe et al., 2020), pectin appears to be the closest polysaccharide to cutin. However, no covalent bonding of cutin to radioactive pectic galacturonan oligomers was detected (Xin et al., 2021). One of the side-chains of rhamnogalacturonan-I was represented by $[^3]H$Ara$_8$-ol in our study and had a very poor acyl acceptor ability (3% of the $[^3]H$XXXGol rate) (Fig. 3c). These observations suggest that cutin protrudes into the PCW deeper than the more superficial pectic fraction.

We note that the restructuring of cuticle/PCW components enabled by CXT activity would be reversible:

$$\text{●●●●} - CO - O - \text{●●●●} + \text{HO}-\text{●●●●●●●●} \leftrightarrow \text{●●●●} - CO - O - \text{●●●●●●●●} + \text{HO}-\text{●●●●}$$

where ● is an HFA residue of cutin and ◆ is a repeat unit (e.g. XXXG) of xyloglucan. Thus, CXT action, making or breaking a cutin–xyloglucan heteropolymer, could play roles in cuticle assembly and/or transient loosening in vivo.

CXT activity was detected in all three dicot species tested, representing three orders. It will be interesting to test whether in poalean monocots the cutin tends to ester-bond with their predominant hemicelluloses, such as mixed-linkage glucan (Scheller and Ulvskov, 2010), rather than xyloglucan which is a minor hemicellulose in grasses and cereals.

Characteristics of CXT activity informs future enzyme identification endeavours
The optimum pH value, substrate specificity, and effects of a reducing reagent, chelator or detergent (Fig. 3a, c, d) can be utilised in future screens for the protein(s) responsible for CXT activity.

The only well-studied extracellular cutin transacylase, CUS1 (Girard et al., 2021; Yeats et al., 2012), was shown not to be responsible for CXT activity. Furthermore, the protein responsible for CXT is distinct from the recently discovered cutin:cutin endo-transacylase (Xin et al., 2021) based on enzymological differences (e.g. pH optima) (Fig. 3a) and activity patterns during tomato fruit ontogeny and H. spectabile leaf development (Fig. 4a, b). We searched the Tomato Expression Atlas (http://tea.solgenomics.net/) for genes whose expression correlates with that of CUS1 (Solyc11g006250). There are 401 such genes with a correlation score of ≥0.7, of which 14 appear to have potentially relevant roles (transacylases, esterases), or are unknown wall proteins (Table S1). Of these 14, six have expression patterns correlating closely with CUS1, exhibiting high expression in the outer epidermis, and in no other tissues, between 10 d post-anthesis and the mature green stage (compare Fig. 5a). Three of them appear to serve roles unrelated to cutin remodelling: Solyc09g014350 encodes a transacylase responsible for the synthesis of the 2-monoacylglycerol precursors of cutin (Petit et al., 2016); Solyc11g008630 encodes a transacylase reported to act only on aromatic acyl residues (Escamilla-Treviño et al., 2014); and Solyc01g094010 may be an α/β-hydrolase involved in the de-methylesterification of auxin (as suggested for the arabidopsis homologue, at2G45600, on https://www.arabidopsis.org/index.jsp].) Solyc09g091800 encodes an unknown protein with a probable signal peptide (Fig. S4), but it is very short (~14 kDa) compared with known transacylases (e.g. GDSL proteins; see below; ~40 kDa), hydrophobic, and highly glycine-rich (Fig. S4); these features suggest that it may be a lipid-transfer protein rather than an enzyme.

The remaining two candidate genes (Solyc02g077330 and Solyc07g049430) encode GDSL proteins, and are therefore of particular interest. GDSL proteins, which include CUS1 itself, are a large family of ‘esterase/lipase/transacylases’ typically having a Gly-Asp-Ser-Leu (GDSL) active site near the N-terminus. The ‘L’ in GDSL is variable, and in the two examples highlighted here, the actual sequence is GDSS (Fig. S4). GDSL proteins characteristically cleave an ester substrate, forming an acyl–serine bond, which can then be attacked by H₂O, effecting hydrolysis, or by an alcohol, effecting transacylation. Some GDSLs catalyse both hydrolysis and transacylation (San Segundo et al., 2019). Of the 108 arabidopsis GDSLs, 99 have signal peptides predicted to target the protein to the endoplasmic
reticulum, Golgi apparatus or apoplast (Ling, 2008). Indeed, the GDSL proteins encoded by Solyc02g077330 and Solyc07g049430 are both predicted to be secretory proteins owing to the presence of a signal peptide (http://www.cbs.dtu.dk/services/SignalP/) (Fig. S4), suggesting that they are active in the apoplast. The fact that the expression of these two GDSLs is maximal in the outer epidermis of the growing tomato fruit (Table S1), coincidently with the accumulation of CXT activity, suggests that one or both of them could encode CXT. The finding that red light and jasmonate up-regulate a GDSL gene (GER1) in rice, coincidently with inhibiting coleoptile elongation (Riemann et al., 2007), suggests a role in wall extension, as we speculate for CXT activity. It will be interesting in future work to recombinantly express the two apoplast-targeted GDSLs of interest and test for their CXT activity by the radiochemical assay developed here. CXT’s physiological significance is suggested by its tendency to increase during fast organ expansion and to diminish after growth cessation (Fig. 4). These observations imply that CXT activity plays a role during organ expansion, which could be mechanical reinforcement through “stitching” cutin onto xyloglucan. Comparably, the newly reported mixed-linkage glucan:xyloglucan and cellulose:xyloglucan heteropolymers may also provide mechanical strength to plants (Herburger et al., 2020). The cutin–xyloglucan interlinking trait might be important for reducing fruit cracking (Khanal and Knoche, 2017) which causes dramatic economic loss. The discovery of CXT activity is also exciting in the context of biotechnology because it paves a road to develop a novel biocatalyst that specifically creates polyester–polysaccharide heteropolymers for human needs (Winnacker, 2017). Such a biocatalyst would permit a green, cheap and kinetically controllable manufacturing opportunity for food packing, drug delivery vehicles and implants.

Conclusion

A novel covalent linkage mechanism between cutin and xyloglucan in the cuticle/PCW was discovered via biochemical evidence. Ester-linked cutin–xyloglucan heteropolymers generated by CXT activity are suggested to reinforce the epidermis and their making and breaking appears to correlate positively with growth rate. The identity of the enzyme responsible for CXT activity remains unknown, but the characterisations of CXT activity provides a valuable reference for future studies. Overall, these findings open a door to
understand interactions between the hydrophobic cutin and hydrophilic PCW, and introduce a new role for xyloglucan in epidermal mechanics, as well as inspire potential future biocatalyst development.

Acknowledgements

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Author Contributions

SCF initiated the project. SCF plus AX designed the experiments and wrote the paper. AX conducted the experiments.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi **********

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### Table 1. Products formed in situ from $[^3\text{H}]\text{XXXGol}$ by pea epicotyl epidermis.

In three independent experiments, blot-dried pea epicotyl epidermis (100 mg; native or heat-denatured) was incubated with ~6 kBq $[^3\text{H}]\text{XXXGol}$ in 600 µl buffer (pH 6.5) at 20°C for 1.5 h. The radiolabelled products were then fractionated as in Fig. 2.

<table>
<thead>
<tr>
<th>Extractant [applied sequentially except where stated ‘either…or’]</th>
<th>Native epidermis</th>
<th>Denatured epidermis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble in acidified aqueous methanol* (MFW)</td>
<td>52.6 ± 4.7</td>
<td>76.1 ± 7.8</td>
</tr>
<tr>
<td>Soluble in toluene</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Soluble in chloroform/methanol (2:1)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Soluble in chloroform/methanol/water (10:10:3) (CMW)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Solubilised by aqueous Proteinase K</td>
<td>1.3 ± 0.9</td>
<td>1.2 ± 1.2</td>
</tr>
<tr>
<td>Solubilised by CMNaOH (ester-bonded products)</td>
<td><strong>2.5 ± 0.3</strong></td>
<td><strong>0.1 ± 0.0</strong></td>
</tr>
<tr>
<td>Either solubilised by XEG (XET products)</td>
<td>16.2 ($n=1$)</td>
<td>0.0 ($n=1$)</td>
</tr>
<tr>
<td>Final insoluble residue**</td>
<td>0.8 ($n=1$)</td>
<td>0.0 ($n=1$)</td>
</tr>
<tr>
<td>Or solubilised by hot TFA (XET products)</td>
<td>13.1 ± 2.9 ($n=2$)</td>
<td>0.0 ± 0.0 ($n=2$)</td>
</tr>
<tr>
<td>Final insoluble residue**</td>
<td>0.4 ± 0.2 ($n=2$)</td>
<td>0.0 ± 0.0 ($n=2$)</td>
</tr>
<tr>
<td>Total</td>
<td>66.1 ± 1.6</td>
<td>76.6 ± 0.1</td>
</tr>
</tbody>
</table>

* MFW includes only the solutes in the initial MFW extraction, performed in vials. The epidermis was subsequently washed more thoroughly by irrigation with MFW by a paper chromatography setup, where the chromatographically mobile radioactivity was not assayed. This explains why the total is <100%.

** ‘Final insoluble residue’ under XEG and TFA rows indicate the insoluble $^3\text{H}$ remaining after the treatment above.
Legends to figures

Figure 1. Three models of cutin–polysaccharide covalent linkages and a proposed mechanism of formation.

(a) Proposed structures. Black, blue, green and pink scaffolds are cutin, xyloglucan, pectin and methanol, respectively. Triangles represent carboxy groups; chevrons are alcohol groups (mostly not shown in the polysaccharides); thus a triangle plugged into a chevron represents an ester. In model [1], cutin forms an ester bond with an –OH group in a polysaccharide (e.g. xyloglucan). In model [2], a –COOH group of an acidic polysaccharide (partially methyl-esterified homogalacturonan is shown) forms an ester bond with an –OH group of a cutin hydroxy-fatty acid residue. In model [3], two alcohol groups are condensed to form an ether bond. In model [4], the polysaccharide is glycosidically linked to an –OH group of cutin.

(b) Proposed mechanism for the formation of the model [1] product. Cutin is cut in mid-chain by a transacylase (E), with which a portion of the cutin forms a transient covalent complex. When an appropriate polysaccharide (modelled by [3H]XXXGol) is present, the transacylase then transfers its cutin fragment onto it, forming a new ester bond (ping-pong mechanism).

Cutin is represented as a polyester chain randomly composed of 10,16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid. [3H]XXXGol (radiolabelled heptasaccharide) comprises three 1,4-linked β-D-glucose residues as the backbone with a 4-linked D-[3H]glucitol (yellow star; Gol) at the end. An α-D-xylopyranose residue is 1,6-linked to each glucose residue.

Figure 2. Work-flow to analyse the high-Mr enzymic product.

[3H]Oligosaccharide-incubated epidermis samples were washed in MFW, removing un-bonded 3H and low-Mr products, if any. Aliquots of the supernatant were quantified by scintillation counting and qualitatively analysed by chromatography. The MFW-insoluble residues (high-Mr material) were treated with neutral organic solvents, dissolving any small radiolabelled cutin or wax conjugates. Subsequently, proteinase K was applied, removing any peptide-associated 3H. To release any [3H]oligosaccharides still ester-bonded to polymers such as cutin, we applied alkali (CMNaOH). The supernatant was analysed quantitatively and qualitatively as for the MFW-soluble products (sometimes with a second round of alkaline hydrolysis). XET products were also brought into solution with XEG or TFA. The final
resistant residue (potentially unhydrolysed XET products and/or cutin–[^3]H]XXXGol ether conjugates) was also assayed.

**Figure 3.[^3]H Oligosaccharide incorporation by pea and *H. spectabile* epidermis.**

(a) **pH dependency in situ.** Upper graph (pea epicotyl epidermis): 50 mg of buffer-washed (native) or water-boiled (denatured), blot-dried epidermis (i.e., containing endogenous donor substrates with or without active enzymes) was incubated with 0.85 kBq[^3]H]XXXGol (acceptor substrate) at 20°C for 1 d in 300 µl buffer, pH 3.5–7.5. Lower graph (*H. spectabile* leaf epidermis): the sample was incubated as above but with 0.81 kBq[^3]H]XXXGol. In both cases, methanol-insoluble products were fractionated: blue, ^3^H subsequently solubilised by CMNaOH (i.e., putative ester-linked[^3]H]XXXGol); grey, ^3^H not solubilised by alkali (i.e., XET products). Bars indicate range (n=2).

(b) **Time-course of in-situ[^3]H]XXXGol incorporation by pea epicotyl epidermis.** Details as in a, but 0.77 kBq[^3]H]XXXGol was supplied, the pH was a constant 6.5, and the epicotyl epidermis was better freed of contaminating vascular strands (n=1).

(c) **In-situ** comparison of various[^3]H-oligosaccharides as acceptor substrates. Details as in b, but compounds tested as the acceptor substrate were: [^3]H]XXXGol (0.55 kBq, n=3),[^3]H]XGol (0.33 kBq, n=3),[^3]H]XFGol (0.63 kBq, n=3),[^3]H]cellobiitol (0.56 kBq, n=3),[^3]H]glucitol (0.69 kBq, n=3),[^3]H]XXFGol (0.32 kBq, n=2), or[^3]H]Ara-ol (0.86 kBq, n=2). Solid lines, native; dashed lines, denatured. Bars indicate SE (n=3) or range (n=2);

(d) **Ex-situ** assay of putative transacylase activity. Enzymes were extracted from pea epicotyl epidermis with or without the addition of 1% (v/v) Triton X-100, 10 mM DTT or 5 mM EDTA, then incubated with 10 mg (dry weight) of denatured, dewaxed epidermis (as acyl donor) plus 0.53 kBq[^3]H]XXXGol (acyl acceptor) in 300 µl pH 6.5 buffer for 1 d. CMNaOH-extractable radioactive products were then measured. Bars indicate range (n=2).

**Figure 4. Chromatographic analysis of in-situ transacylation products formed by pea epicotyl epidermis from[^3]H]XXXGol.**

Buffer-washed, blot-dried pea epicotyl epidermis (80 mg), with or without prior denaturation, was incubated *in situ* with 5.2 kBq[^3]H]XXXGol (acyl acceptor) at 20°C for 1.5 h in 480 µl
buffer, pH 6.5. Remaining methanol-insoluble epidermal polymers were alkali-hydrolysed with chloroform/methanol/NaOH for 24 h, cleaving (most) ester bonds.

(a) Products in the CMNaOH-hydrolysate were size-fractionated on Bio-Gel P-2 along with non-radioactive internal markers [dextran, xyloglucan oligosaccharides (DP 3, 7, 8 and 9) and glucose]. The markers were revealed by TLC in BAW, 2:1:1 (inset); the respective fractions are indicated by horizontal blue lines. The graph shows radioactive products [●, from native; ○, from denatured epidermis].

(b) Lower profiles: Aliquots of peaks I and II were then analysed by paper chromatography in BPW for 46 h (with scintillation counting). Upper profiles: external markers run on the same chromatograms (with AR2000 scanning).

(c) Further aliquots of peaks I and II were dried, re-hydrolysed in 1 M aqueous NaOH at 20°C for 1 d, and re-run by paper chromatography as in b.

Figure 5. Developmental effects on in-situ cutin-to-[3H]XXXGol transacylase activity.

(a) Tomato fruit (cv. M82) epidermis at various stages of fruit development. In-situ CXT activity was assayed in 50 mg blot-dried epidermis with 0.42 kBq [3H]XXXGol as acceptor substrate. Radioactive products that became ester-bonded to polymeric cutin in 3 h at pH 6.5 were assayed. The ripening stages (after expanding stage as labelled) are described after Shakya and Lal (2018): MG, mature green (growth stopped); Br, breaker (10% colour change); DR, deep red. Bars indicate SE (n=3).

(b) H. spectabile epidermis from small leaves (3–5 cm lamina length; still expanding) and large leaves (~12 cm; still expanding); 1.0 kBq [3H]XXXGol. Other details as in a. Bars indicate SE (n=3).

(c) Pea epidermis from epicotyls 4, 7 and 10 d after sowing (rapidly, barely, and not expanding, respectively; 0.72 kBq [3H]XXXGol. Other details as in a. Bars indicate SE (n=3).
List of Supplementary data

Fig. S1. Enzymic degradation of exogenous [$^3$H]XXXGol during in-situ assays with pea epidermis.

Fig. S2. Bio-Gel P-2 analysis of [$^3$H]XXXGol.

Fig. S3. Cutin synthase, CUS1, has negligible CXT activity in situ and ex situ.

Fig. S4. *In-silico* prediction of signal peptides in the products of *Solyc02g077330*, *Solyc07g049430* and *Solyc09g091800*.

Table S1. Potential transacylase genes expressed in correlation with *CUS1*. 
Acidified methanol (MFW) → Remaining substrate + low-M, \(^3\text{H}\)-labelled products

Neutral organic solvents, sequentially: toluene; chloroform/methanol (2:1) (CM); chloroform/methanol/water (10:10:3) (CMW) → Oligoester-\(^3\text{H}\) esters

Neutral organic solvents-insoluble \(^3\text{H}\)

Proteinase K → Protein-\(^3\text{H}\) esters

Proteinase K-insoluble \(^3\text{H}\)

Chloroform/methanol/4 M NaOH (CMNaOH) → The putative CXT products

CMNaOH-insoluble \(^3\text{H}\)

XEG or TFA → XET products

XEG- or TFA-insoluble \(^3\text{H}\) (remaining XET products and/or cutin-\(^3\text{H}\) ethers)
Xin & Fry, Fig. 5

**a** Tomato

![Bar chart showing CXT activity (Bq/kBq/50 mg FW/3 h) for different stages of tomato development. The stages are 6-7 DPA, 10-12 DPA, 15-18 DPA, 20-22 DPA, 30-32 DPA, MG, Bl, and DR.](image)

**b** *H. spectabile*

![Line graph showing CXT activity (Bq/kBq/50 mg FW/3 h) for small and large leaves native and denatured at different incubation times (0, 12, 24 h).](image)

**c** Pea

![Bar chart showing CXT activity (Bq/kBq/50 mg FW/3 h) for different days after sowing (4, 7, 10).](image)
Fig. S1. Enzymic degradation of exogenous [3H]XXGol during in-situ assays with pea epidermis.
(a) Enzymic degradation of [3H]XXGol in situ during 0–6 h incubation. MFW-soluble products (from Figs 3b and 3c) were analysed by TLC (BAW, 2:1:1, v/v/v, three ascents) and scanned for 3H in the AR-2000.
(b) As (a) but 0–24 h products and detection by fluorography. N, native epidermis; D, denatured.
(c) Only slight loss of acyltransferase activity during in-situ incubation. Buffer-washed and blot-dried pea epidermis was incubated with 6.0 nM [3H]XXGol (acyl acceptor) added either immediately and incubated for (i) 0 h or (ii) 1.5 h, or (iii) added after an 1.5 h pre-incubation (cold) without substrate and then incubated for a further 1.5 h. Bars indicate SE (n=3). **: P < 0.01. *: P < 0.05.
Fig. S2. Bio-Gel P-2 analysis of [\( ^{3}H \)XXXGol].

[\( ^{3}H \)XXXGol] was mixed with 'cold' markers (non-radioactive dextran, oligosaccharide (DP9, 8, 7, 3) and glucose), and the mixture was fractionated on Bio-Gel P-2 as in Fig. 4a.
Fig. S4. In-silico prediction of signal peptides in the products of the tomato genes Solyc02g077330, Solyc07g049430 and Solyc09g091800.

The prediction was made via http://www.cbs.dtu.dk/services/SignalP/. The first 70 amino acid residues of each predicted protein are shown. Sec/SPI, secretion signal peptide; CS, cleavage site; OTHER, the probability that the sequence does not have Sec/SPI. Glycine residues in the predicted mature proteins are circled in red. The GDSS sequence (variant on GDSL) is in the blue box.