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Download date: 05. Apr. 2019

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1   **Outlook on protein S-acylation in plants – what are the next steps?**

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8

9   Submitted:

10   Tables: 0

11   Figures: 3

12   Word count: 6135

13   running title: Outlook on S-acylation in plants

14

15   Highlight: S-acylation is an emerging dynamic post-translational regulatory modification of

16   proteins. This perspective highlights recent work in the area, illustrates emerging methods

17   and outlines future research direction in the field.

18 **Abstract**

19 S-acylation, also known as palmitoylation, is the reversible post-translational addition of fatty

20 acids to proteins. Historically thought primarily to be a means for anchoring otherwise soluble

21 proteins to membranes, evidence now suggests that reversible S-acylation may be an

22 important dynamic regulatory mechanism. Importantly S-acylation also affects the function

23 of many integral membrane proteins making S-acylation an important factor to consider in

24 understanding processes such as cell wall synthesis, membrane trafficking, signalling across

25 membranes and regulating ion, hormone and metabolite transport through membranes. This

26 review summarises the latest thoughts, ideas and findings in the field and charts the direction

27 of future work to enable progress to be made in understanding the role of this enigmatic

28 regulatory protein modification.

29

30 **Key words**

32   **The cellular context of S-acylation**

33   The eukaryotic cell is separated into a range of compartments and organelles by multiple

34   distinct membrane bilayers. In contrast to the historical view of membranes being largely

35   homogenous and static structures, data in the last few decades has revealed that membrane

36   composition and structure is very diverse, highly regulated on scales from a few nm to many

37   µm and can be extremely polarised within a cell (Abankwa *et al.*, 2007; Jarsch *et al.*, 2014;

38   Tian *et al.*, 2007). Membranes are increasingly found to act as signalling platforms for proteins

39   and may themselves form part of the signalling process in the form of lipid derived second

40   messengers. To achieve this degree of coordination in membrane organisation and function

41   proteins, and their interaction with membranes, must also be tightly controlled.

42

43   To date four main ways of promoting protein interaction with membranes have been

44   described; transmembrane domains, charged amino acid patches on a proteins surface, lipid

45   binding domains and the addition of fatty groups to proteins. The subject of this perspective

46   piece, S-acylation, falls into the latter category. S-acylation involves adding a variety of acyl

47   chains, primarily palmitic or stearic acid (Sorek *et al.*, 2007), to cysteine residues through a

48   thioester bond. Due to the addition of palmitic acid, S-acylation has historically been referred

49   to as palmitoylation, but as a result of the range of acyl groups that are now known to be

50   added it is more correctly termed S-acylation (Batistic *et al.*, 2008; Sorek *et al.*, 2007). Unlike

51   other lipid based posttranslational modifications of proteins such as N-myristoylation

52   (addition of 14 carbon myristate) or prenylation (addition of polyisoprene farnesyl or

53   geranylgeranyl groups) S-acylation is rapidly and readily reversible (Sorek *et al.*, 2007), giving

54   it the potential to act as a switch or regulatory modification in much the same way as has

55   been described for phosphorylation or ubiquitination. S-acylation is also much more common

56   that any of the other lipid based modifications of proteins with conservative estimates

57   suggesting that over 10% of the proteome, and therefore >30% of the membrane proteome,

58   may be S-acylated in eukaryotes (Hemsley *et al.*, 2013; Martin and Cravatt, 2009; Roth *et al.*,

59   2006). Mutants in the S-acylating enzymes themselves frequently have severe pleiotropic

60   phenotypes indicating a substantial requirement for S-acylation in plants (Hemsley *et al.*,

61   2005; Lai *et al.*, 2015; Li *et al.*, 2016; Qi *et al.*, 2013). Despite these outwardly important factors

62   suggesting that S-acylation is likely to be very important in cellular protein function, very little

63   is actually known about how S-acylation is regulated, exactly how many proteins are S-

64   acylated, how specificity of S-acylation is determined and what exactly its effects on proteins

65   are.

66

67   Two recent reviews on S-acylation in plants cover many of the individual proteins known or

68   hypothesised to be S-acylated (Hemsley, 2015; Hurst and Hemsley, 2015) and this review will

69   therefore only cover the more recent additions to this ever growing body of knowledge.

70   Instead, the main focus will be on where the gaps in our knowledge are, the direction of future

71   research in this area, what tools and resources are available to study S-acylation in plants and

72   what we can glean from other systems.

73

74   **Concepts in S-acylation**

75   S-acylation has often been described as a way of firmly attaching otherwise soluble or

76   peripherally membrane associated proteins to membranes (Batistic *et al.*, 2008; Traverso *et*

77   *al.*, 2013) as the degree of membrane association provided by an S-acyl group is, to all intents

78   and purposes, permanent on physiologically relevant timescales (Shahinian and Silvius, 1995).

79   More recently, and particularly with the advent of S-acylation proteomics, it is accepted that

80   integral membrane proteins account for at least 50% of the S-acylated proteome (Hemsley *et*

81   *al.*, 2013; Martin and Cravatt, 2009; Roth *et al.*, 2006). These discoveries highlight the fact

82   that S-acylation must be doing something within the cell beyond acting as a membrane anchor

83   because integral membrane proteins clearly aren't able to become more membrane

84   associated as a result of S-acylation.

85

86   Many proteins require S-acylation to traffic through the endomembrane system and reach

87   their destination membrane (Abrami *et al.*, 2008). It is hypothesised that the S-acyl group

88   helps to sort the protein into the endoplasmic reticulum (ER) or Golgi exit sites that have lipid

89   compositions similar to their destination membrane as the S-acyl group is preferentially

90   soluble in those membrane lipid environments (Patterson *et al.*, 2008). In some cases S-

91   acylation acts to protect proteins from the ER quality control mechanisms. This is proposed

92   to occur by S-acylation promoting tilting of transmembrane (TM) helices that are otherwise

93   longer than the ER membrane is thick. This prevents hydrophobic mismatch between the TM

94   domain and the ER membrane that would otherwise be recognised by the ER quality control

95   machinery (Abrami *et al.*, 2008). In other cases S-acylation acts to obscure a ubiquitination

96   site and prevent premature or inappropriate degradation (Valdez-Taubas and Pelham, 2005).

97   In the case of G-protein coupled receptors (GPCRs) S-acylation can directly alter sensitivity of

98   receptor signalling, primarily by altering the conformation of an intracellular loop responsible

99   for downstream signalling protein binding (Qanbar and Bouvier, 2003). These concepts have

100 recently been reviewed in great depth (Blaskovic *et al.*, 2013; Hemsley, 2015; Hurst and

101 Hemsley, 2015) but as more S-acylated proteins are identified it is becoming apparent that

102 we really know very little about what S-acylation does within the cell.

103

104 **Recent developments in plant S-acylation research**

105 A recent proteomics study using poplar suspension culture identified a range of proteins as

106 being S-acylated (Srivastava *et al.*, 2016), many of which are poplar orthologues of identified

107 S-acylated Arabidopsis proteins (Hemsley *et al.*, 2013). This provides independent, cross-

108 species support for the S-acylation of a number of groups of plant proteins. Two functional

109 categories of S-acylated proteins readily highlighted in both studies are cell surface receptors

110 and cell wall synthesis enzymes. Following up on this it has been shown that the 18 cellulose

111 synthase A family (CesA) subunits making up the cellulose synthase complex (CSC) are

112 multiply S-acylated, making it potentially the most heavily S-acylated complex ever described

113 in any organism. The effects and implications of this will be discussed later. Interestingly,

114 disrupting S-acylation of AtCESA7, one of the 3 CesA paralogs that combine to make up the

115 secondary cell wall CSC 18mer, traps the CSC in the Golgi and renders it non-functional. This

116 occurs despite the S-acylation status of AtCESA4 and AtCESA8, the other two secondary cell

117 wall CSC subunits, remaining broadly unaffected. This indicates that the whole complex must

118 be S-acylated for insertion into the plasma membrane (Kumar *et al.*, 2016).

119

120 Given the importance of cell surface receptors in almost all aspects of plant biology,

121 understanding the role of S-acylation in their function is likely to be an expanding area of S-

122 acylation research. A very recent study (Alassimone *et al.*, 2016) indicates that the receptor-

123 like cytoplasmic kinase SGN1 is S-acylated. SGN1 localises to the cortical side of endodermal

124 cells and is required for specifying the position of the casparian band in the root endodermis.

125 Plants lacking SGN1 are unable to form an intact casparian band and regulate apoplastic flow

126 in the root. Interestingly an SGN1 mutant lacking putative S-acylation sites is cytoplasmic and

127 unable to rescue the *sgn1⁻* phenotype indicating that plasma membrane localisation is

128 essential for SGN1 to perform its role. Critically SGN1 localisation appears to depend upon

129 cycles of S-acylation and de-S-acylation to maintain its polar localisation (Alassimone *et al.*,

130 2016) and this will be discussed later.

131

132 **Understanding the process of S-acylation**

133 We have known for over ten years now that the enzymes that add S-acyl groups to proteins,

134 Protein S-acyl Transferases or PATs, exist in plants (Hemsley *et al.*, 2005) and that the majority

135 of S-acylation does not occur spontaneously (Roth *et al.*, 2006). Like all PATs identified to date

136 plant PATs are polytopic integral membrane proteins and are characterised by the presence

137 of a DHHC motif domain which is presumed to contain the active site. In Arabidopsis 24 PATs

138 have been identified (Batistic, 2012; Hemsley *et al.*, 2005). Each is found only on a subset of

139 membrane compartments within the cell (Figure 1) indicating some form of spatial

140 organisation of the S-acylation machinery (Batistic, 2012). Interestingly, the majority of

141 animal (21/24) and yeast (5/7) PATs are found predominantly at the ER or Golgi (Ohno *et al.*,

142 2006). The Golgi has thus been proposed to act as an S-acylation centre in animals, primarily

143 concerned with exporting and sorting proteins from the Golgi to the plasma membrane (Rocks

144 *et al.*, 2010). The majority of plant PATs however are found at the plasma membrane (12/24)

145 with 8 PATs Golgi/ER localised, 2 on non-Golgi derived vesicle populations and 2 at the

146 tonoplast (Batistic, 2012). While plants do possess the capability for S-acylation at the

147 ER/Golgi it is appears that S-acylation at the plasma membrane plays a much greater role in

148 plants than in animals and the whole regulatory role of S-acylation in plants may be very

149 different to that of animals and fungi.

150

151 A number of Arabidopsis PAT mutants have been characterised phenotypically and, although

152 their losses have profound pleiotropic effects (Hemsley *et al.*, 2005; Lai *et al.*, 2015; Li *et al.*,

153 2016; Qi *et al.*, 2013), no plant PAT has yet been convincingly linked to a substrate protein.

154 This situation is not particularly unique to plants, although a few mammalian and yeast PAT-

155 substrate pairings have been identified. One potential issue that clouds the study of PATs is

156 that they exhibit low specificity when over expressed, particularly in heterologous systems

157 (Batistic, 2012) where membrane localisation and environment of PAT and substrate may not

158 be appropriate. As a result the absolute specificity of PATs is often questioned and it seems

159 likely that specificity is, at least in part, dictated by whether a PAT and potential substrate

160   reside in the same membrane and/or microdomain compartments. Some support to this idea

161   is provided by the mammalian PAT DHHC5 in neurons. In an unstimulated neuron DHHC5 is

162   sequestered by PSD-95 and Fyn kinase at the synaptic membrane away from its substrate δ-

163   catenin that resides in the dendritic spine. Upon neuronal stimulation DHHC5 is

164   phosphorylated by Fyn which promotes relocation to the dendritic spine. DHHC5 is then able

165   to S-acylate δ-catenin (Brigidi *et al.*, 2015). PATs also appear to have very few recognised

166   protein-protein interaction motifs that may help with substrate recruitment or recognition. It

167   is of course possible that specificity is provided by accessory proteins. This theory is supported

168   by the requirement of ERF4/SHR5 for yeast Ras S-acylation mediated by the PAT ERF2 (Lobo

169   *et al.*, 2002). In humans GCP16 is a protein cofactor for the RAS PAT DHHC9 (Swarthout *et al.*,

170   2005) and Selenoprotein K is required for CD36 and inositol-1,4,5-triphosphate receptor S-

171   acylation by the DHHC6 PAT (Fredericks *et al.*, 2014). While by no means demonstrated to be

172   a universal mechanism, accessory proteins are a factor worth bearing in mind when designing

173   experiments to identify plant PAT-substrate pairings, particularly if using heterologous

174   systems where the adaptor is likely not present (e.g. yeast systems) or over expression of

175   PATs where stoichiometry with adaptors is not maintained. Given the number of T-DNA

176   insertion alleles now available in Arabidopsis a worthwhile strategy to identify enzyme-

177   substrate pairings may be to directly assay protein S-acylation state in PAT mutant

178   backgrounds if an antibody is available. Alternatively the AGROBEST method (Wu *et al.*, 2014)

179   or similar approaches may be used to introduce an epitope-tagged form of the S-acylated

180   protein of interest to a panel of PAT mutants followed by assays of S-acylation state.

181

182   The catalytic mechanism of PATs is still a matter of some debate. Evidence currently supports

183   a model where the cysteine in the DHHC core motif forms an acyl-enzyme intermediate

184   before transferring the acyl group to a target cysteine and regenerating the initial PAT enzyme

185   – a so called ping-pong mechanism (Jennings and Linder, 2012). To form the initial acyl-

186   enzyme intermediate acyl-CoA must be cleaved by nucleophilic attack of the acyl-CoA

187   thioester. Based on the current model this nucleophile is provided by the deprotonated

188   thiolate form of the DHHC cysteine. The pKa of free cysteine thiol side chains is ~8.4 with

189   cytosolic pH maintained at ~pH 7.5. The majority of cysteine in the cell would therefore be

190   expected to be found in the thiol form and be much less potent as a nucleophile. However,

191   the immediate amino acid environment surrounding a cysteine can raise or lower its pKa

192 dramatically to lie anywhere in the range of 3.5-10 thereby stabilising either the thiol or
193 thiolate forms. This is best typified by cysteine proteases. In this case the active site cysteine
194 is deprotonated by a spatially near histidine residue (Drenth *et al.*, 1968), lowering its
195 effective pKa. In some cases an aspartic acid residue can act to deprotonate the histidine
196 making it more effective at deprotonating the cysteine thiol. Looking at the DHHC (Asp-His-
197 His-Cys) motif in PATs it is tempting to speculate that a similar mechanism may be at work
198 here (Mitchell *et al.*, 2010), either in forming the initial acyl-enzyme intermediate or in
199 deprotonating the substrate target cysteine *in trans* to allow it to efficiently attack the acyl-
200 cysteine thioester in the PAT. It may even be the case that both options exist; formation of
201 the acyl-enzyme intermediate occurs followed by substrate binding causing reorientation of
202 the His and/or Asp residues towards the substrate cysteine thereby promoting
203 deprotonation, nucleophilic attack of the acyl-enzyme thioester and transfer of the acyl
204 group. This would go some way towards providing a degree of specificity to PATs, not just at
205 the protein level but also at the level of individual cysteines. However, in the absence of any
206 structural data on a PAT or PAT-substrate (either protein or acyl-CoA) pair from any species
207 this remains speculation.
208
209 If knowledge about the PATs is limited in plants, what we know about de-S-acylating enzymes
210 is even worse. De-S-acylating enzymes, known as acyl-protein thioesterases or APTs, have
211 been described from animal (Duncan and Gilman, 1998; Lin and Conibear, 2015), yeast
212 (Duncan and Gilman, 2002) and toxoplasma (Child *et al.*, 2013) systems and are all members
213 of the serine hydrolase superfamily. Arabidopsis contains approximately 180 serine
214 hydrolases but none of them show particularly strong homology to known APTs from other
215 systems. Despite this plants must contain some enzymes capable of removing S-acyl groups
216 from proteins as Type-I ROP small GTPases are known to undergo activity state dependant
217 cycles from S-acylated to non-S-acylated forms in a rapid and tightly regulated manner (Sorek
218 *et al.*, 2007).
219
220 **S-acylation as a dynamic and regulatory modification**
221 Historically S-acylation has been viewed as a largely static modification, acting as a surrogate
222 transmembrane domain or accessory anchor to promote tighter association with membranes
223 in conjunction with poly-basic domains, prenylation or N-myristoylation. An emerging body

224   of work from the mammalian field suggests that many S-acylated proteins undergo regulated

225   S-acylation or de-S-acylation in response to various factors and that this is essential for their

226   function (Brigidi *et al.*, 2015; Christopherson *et al.*, 2003; Roy *et al.*, 2005). In plants the only

227   proteins where S-acylation state is confirmed to change are the Type-I ROPs as typified by

228   ROP6 (Figure 2A). GTP bound active ROP6 is S-acylated while GDP bound inactive ROP6 is not

229   (Sorek *et al.*, 2007). It is not known however whether de-S-acylation promotes GTP hydrolysis

230   or vice-versa nor what the exact role of S-acylation is in ROP function. Interestingly

231   constitutively active forms of ROP6 promote short bulbous root hair formation. Mutation of

232   the S-acylated cysteines to serine in constitutively active ROP6 largely supresses these

233   phenotypes indicating that S-acylation is required for constitutively active ROP6 to exert its

234   effect (Sorek *et al.*, 2010). Non-S-acylated ROP6 also displays very different physical

235   properties in terms of detergent solubility making it likely that S-acylated and non-S-acylated

236   forms of ROP inhabit different membrane environments (Sorek *et al.*, 2007). S-acylation may

237   therefore be responsible for maintaining foci of active ROP6 where it is needed and de-S-

238   acylation ensures that inactive ROP6 is rapidly removed from the site of action to prevent

239   negative or inhibitory effects. Similarly, but without the spatial changes in ROP6 distribution,

240   switching of ROP6 S-acylation state may alter the membrane environment of the complex by

241   recruiting different lipids, leading to a change in protein composition based on their individual

242   physical properties. Finally, S-acylation of ROP6 may alter ROP6 conformation thereby

243   promoting or hindering interaction with regulators and effectors of ROP6 function.

244

245   Some recent work indicates that receptor-like cytoplasmic kinases (RLCK) may also be

246   dynamically S-acylated. The SGN1 RLCK aids in casparian strip positioning and production.

247   SGN1, based on mutagenesis and inhibitor data, appears to be S-acylated at the N-terminus.

248   While wild type SGN1 is found solely on the epidermis facing side of the plasma membrane

249   of endodermal cells. SGN1 that is not S-acylated is exclusively cytosolic. After treatment with

250   the S-acylation inhibitor 2-bromopalmitate and the protein synthesis inhibitor cycloheximide

251   wild type SGN1 was observed in the cytoplasm indicating that SGN1 is removed from the

252   membrane. Treatment with Brefeldin A did not alter SGN1 distribution indicating that SGN1

253   does not undergo endocytosis as part of the observed redistribution process. These data

254   together suggest that SGN1 undergoes cycles of de-S-acylation and S-acylation as part of its

255   normal life cycle (Alassimone *et al.*, 2016). While no function was ascribed to these cycles it

256 is possible that it plays a part in signalling, either acting as part of a transduction relay by
257 moving into the cytoplasm or as a signalling strength modulator by removing itself from a
258 signalling complex. Another option is that SGN is de-S-acylated at the edges of its desired
259 distribution and re-S-acylation acts to trap it back where SGN1 activity is required thereby
260 creating a polarised distribution of signalling (Figure 2B).

261

262 The regulatory effect of S-acylation on protein function is probably the most exciting area for
263 future study. As suggested above we know very little about this in plants and it is impractical
264 to test for changes in known individual S-acylated proteins under every suspected condition.
265 As a result one of the big hurdles to overcome is proteomic analysis of dynamic S-acylation.
266 It would be particularly interesting to compare S-acylated proteome profiles of plants under
267 conditions or stimuli that promote rapid cellular responses (e.g. pathogen elicitation of
268 defences) to see which proteins increased or decreased in S-acylation state. This will not be a
269 trivial task, requiring quantification of S-acylated peptide/protein species and total
270 peptide/protein abundance for each protein of interest. This is required to confidently state
271 that a stimulus specific change in S-acylation state has been observed rather than just a
272 change in abundance of the protein. Practical considerations behind this are discussed in the
273 "methods and resources" section below.

274

275 **S-acylation and membrane microdomains**
276 As discussed above, membranes appear not to be homogeneous structures, rather they seem
277 to be heterogeneous mosaics composed of hundreds of different lipid, sterol and protein
278 types that almost certainly self-assemble based on physical properties to form proteolipid
279 complexes termed microdomains (Abankwa *et al.*, 2007; Suzuki *et al.*, 2012). Changes in the
280 biophysical properties of these complexes by changing protein-protein interactions, protein
281 conformation or protein post-translational modification state could therefore reasonably be
282 expected to change the overall character and composition of these microdomains (de
283 Almeida and Joly, 2014). S-acylation is essentially the addition of long chain fatty acids to
284 proteins; these same fatty acids form the core of the membrane bilayer. It is therefore not
285 surprising that S-acylation has been proposed to be one of the ways by which the cell can
286 change which membrane lipids a protein associates with, which microdomain it therefore
287 occupies and subsequently which other proteins are available for it to interact with. To further

288 complicate matters there is evidence that S-acylated proteins can be modified with acyl
289 chains of varying lengths and saturation (Kordyukova *et al.*, 2008; Sorek *et al.*, 2007). Whether
290 this is regulated at the protein or site level, or a function of acyl-CoA prevalence in a particular
291 cell type, has not been satisfactorily addressed. This could however be an additional
292 mechanism whereby S-acylation with certain length acyl chains could drive proteins into one
293 microdomain environment or another. Remorins are a large family of proteins with both S-
294 acylated and non-S-acylated members. They are also one of the best characterised families of
295 proteins known to form microdomains *in planta*. S-acylated and non-S-acylated remorins all
296 form microdomains indicating that S-acylation is not a prerequisite for microdomain
297 formation or occupancy. Mutant forms of normally S-acylated remorin that can no longer be
298 S-acylated still form microdomains (Konrad *et al.*, 2014) but it is not clear whether they are
299 the same microdomain observed for the WT version of the protein in terms of size,
300 composition, lifetime or mobility.

301

302 Recent work on the cellulose synthase complex (CSC), an 18-mer with 144 transmembrane
303 domains, indicates that it is heavily S-acylated with a proposed 70-110 S-acyl groups per 18-
304 mer (Kumar *et al.*, 2016). It is highly likely that S-acylation to this extent will have a profound
305 effect on the composition of the membrane environment surrounding the complex (Figure
306 3). The CSC is integral to the plasma membrane and extrudes cellulose microfibrils into the
307 extracellular environment to form the cell wall. This extrusion process propels the complex
308 through the plane of the plasma membrane and it has been hypothesised that CSCs form
309 highly specialised microdomains through their S-acylation, allowing them to recruit accessory
310 proteins and move unhindered through the plasma membrane (Kumar *et al.*, 2016).

311

312 **Direct effects of S-acyl groups on membranes**
313 Plant root hairs, pollen tubes and mammalian filopodia are all tip growing structures known
314 to be highly sensitive to perturbations in S-acylation (Gauthier-Campbell *et al.*, 2004; Hemsley
315 *et al.*, 2005). Filopodial formation was previously presumed to be a result of protein function.
316 However, a study using short protein regions containing only the S-acylation sites of GAP-43,
317 paralemmin, PSD-95 or PSD-93 fused to GFP demonstrated that filopodia could be induced
318 by GAP-43 or paralemmin but not PSD-95 or PSD-93 S-acylated regions (Gauthier-Campbell
319 *et al.*, 2004). The precise mechanism behind this effect is not known but it may be a result of

320   S-acyl group intercalation into the membrane resulting in altered membrane tension (Raucher

321   and Sheetz, 2000) or stabilisation of membrane microdomains that recruit factors for

322   filopodial growth. This role, independent of a described protein function, is an exciting

323   possibility for non-canonical effects of S-acylation on cellular processes and it is not

324   unreasonable to assume that this could also occur in plants.

325

326   **Interactions of S-acylation with other thiol modifications?**

327   Cysteine residues are among the most potent nucleophiles in proteins and are highly redox

328   sensitive. As a result many reversible post-translational modifications occur on cysteine

329   residues in addition to S-acylation. These include S-nitrosylation, S-glutathionylation,

330   sulfhydration, sulfenylation and disulphide bond formation. It is entirely possible that one or

331   more of these modifications occur on any given protein's S-acylation site to prevent S-

332   acylation from occurring and, of course, the reciprocal situation is also possible. Only one

333   documented example is known of this occurring and involves the neuronal protein PSD-95. S-

334   acylated PSD-95 clusters in synapses but upon neuronal stimulation becomes de-S-acylated

335   and is removed from clusters. NOS is also synthesised in response to neuronal stimulation

336   and blocks the recently de-S-acylated cysteines through nitrosylation thereby preventing re-

337   S-acylation. Once stimulation stops NOS synthesis reduces, PSD-95 de-nitrosylation occurs

338   and S-acylation is restored (Ho *et al.*, 2011). It will be interesting to see if and how all of these

339   cysteine PTMs interact with each other. There is however no reason to believe that every

340   cysteine that can be S-acylated is also a target for these other modifications or vice versa.

341

342   **Non-cannonical S-acylation**

343   As interest in S-acylation has progressed discoveries of proteins that are S-acylated but do not

344   fit the classical "S-acylation and membranes" interpretation have been described. BET3, a

345   transport protein particle component involved in vesicular trafficking, is known to be S-

346   acylated in all eukaryotes examined (Hemsley *et al.*, 2013; Turnbull *et al.*, 2005). Intriguingly

347   the S-acyl group, rather than acting as a membrane anchor, acts as a hydrophobic scaffold

348   and is essential for correct folding of BET3. Similarly the TEAD transcription factor also uses

349   an S-acyl group as a structural core rather than for membrane attachment (Noland *et al.*,

350   2016). In both cases S-acylation appears to be part of the maturation process and does not

351   require DHHC PATs to occur. These data indicate that the simple explanation of S-acylation

352   promoting some form of membrane association or interaction cannot always be taken for

353   granted. In some S-acylation proteomics experiments histones have also been identified.

354   While initially assumed to be false positives, S-acylation of histone H3 variants at Cys110 has

355   been confirmed (Wilson *et al.*, 2011) and this site is conserved in plants. The role of histone

356   S-acylation is less clear, particularly as histone H3 Cys110 is buried deep within the

357   nucleosome structure. It has been suggested that S-acylation may be acting as a glue between

358   histone monomers or may be acting to support nucleosome interaction with the perinuclear

359   envelope; a location associated with heterochromatin and silenced genes. No DHHC PATs

360   have been reported to localise within the nucleus; this indicates that histone S-acylation

361   either occurs before nuclear import, is self-catalysed or uses hitherto unknown S-acylating

362   enzymes.

363

364   **Methods and Resources**

365   As interest in S-acylation expanded the number of tools available to address its function and

366   action has also increased. Most of this more recent work has been developed in mammalian

367   systems but adaptation to plant work should be trivial. The main methods available are

368   outlined below along with any known pitfalls or likely changes required for plant work, as well

369   as thoughts on future procedures or adaptations that may prove of use.

370

371   *Mutagenesis* – Mutagenesis of cysteine residue to serine or alanine is the standard method

372   for determining sites of S-acylation and for investigating the effects of loss of S-acylation on a

373   proteins function. Serine is the substitution favoured by most of the community as cysteine

374   and serine differ only by one atom (sulfur in cysteine, oxygen in serine) in the R-group terminal

375   SH/OH. This maintains amino acid size but some researchers have concerns that serine is able

376   to act as a nucleophile in a similar manner to cysteine. While there is no evidence in the

377   literature to support this, some researchers prefer to substitute alanine for cysteine to ensure

378   that no nucleophilic activity exists at the site.

379

380   *Microscopy* – Coupled with mutagenesis of candidate S-acylated cysteine residues microscopy

381   has been a mainstay of S-acylation research for many years. However, it does have limitations.

382   While microscopy can be useful for examining the effects of S-acylation on otherwise soluble

383   proteins (Batistic *et al.*, 2008) or integral membrane proteins that show trafficking defects in

384 the absence of S-acylation (Kumar *et al.*, 2016), many integral membrane proteins show no

385 localisation change in the absence of S-acylation (Hemsley *et al.*, 2013; Konrad *et al.*, 2014).

386 Microscopy is therefore more commonly used nowadays to support the biochemical methods

387 outlined below rather than as primary evidence itself. More advance microscopy techniques

388 such as fluorescent recovery after photobleaching (FRAP) have been used to examine the

389 contribution of S-acylation to ROP6 membrane affinity and association dynamics (Sorek *et al.*,

390 2010).

391

392 *Acyl-biotin exchange and acyl-Resin Assisted Capture* – The mainstays of plant S-acylation

393 research, having been used to look at single proteins (Konrad *et al.*, 2014; Kumar *et al.*, 2016;

394 Qi *et al.*, 2013) and at proteomes (Hemsley *et al.*, 2013; Srivastava *et al.*, 2016), and combined

395 with mutagenesis to map sites of S-acylation (Hemsley *et al.*, 2013; Konrad *et al.*, 2014; Kumar

396 *et al.*, 2016). These assays are based on the use of neutral hydroxylamine (~pH7.2) to cleave

397 the acyl thioester revealing a free sulfhydryl. This can be labelled with sulfhydryl reactive

398 biotin and pulled down by streptavidin (Acyl-biotin Exchange; ABE) (Drisdel and Green, 2004;

399 Hemsley *et al.*, 2008) or directly immobilised to sulfhydryl reactive resin (acyl-Resin Assisted

400 Capture; acyl-RAC) (Forrester *et al.*, 2011; Kumar *et al.*, 2016). S-acylation state is then

401 determined by western blot. For quantitative analysis of altered S-acylation states relative

402 levels of S-acylation between mutant constructs or between treatments can be determined

403 (Kumar *et al.*, 2016). Various protocols for these assays are available for use in plants

404 (Hemsley *et al.*, 2008; Hemsley *et al.*, 2013; Kumar *et al.*, 2016) and bench protocols of the

405 most recently developed and improved variants, as used in the author's laboratory, are

406 available upon request. As these are indirect assays, and essentially report on the presence

407 of thioesters, care must be taken to exclude the detection of non-S-acylation related

408 thioesters such as those found in nitrilase, E2 ubiquitin ligases and many enzymes involved in

409 lipid synthesis (Hemsley *et al.*, 2013; Roth *et al.*, 2006). However, thioesters are not

410 particularly common in proteins and as many of the false positives in these assays are well

411 known and characterised the assays can be used so long as care is exercised, appropriate

412 controls used and independent lines of evidence (subcellular localisation, membrane

413 fractionation, metabolic labelling, inhibitor treatment, etc.) used to support claims. Proteomic

414 approaches using these methods, particularly those trying to compare S-acylation of the

415 proteome between conditions or stimuli or in mutant backgrounds, can suffer in accuracy due

416   to the large number of handling step required introducing sample to sample variation. Recent

417   work in Arabidopsis indicates that stable isotope labelling in culture (SILAC) is now viable

418   (Lewandowska *et al.*, 2013) and could be the solution to eliminating this source of inaccuracy

419   and variation. SILAC allows for samples to be combined before the processing steps of S-

420   acylated protein enrichment and would lead to more sensitive, accurate and quantitative

421   analyses of dynamic S-acylation in plants.

422

423   *Acyl PEG Exchange* – A recent addition to the field using similar chemistry to ABE and acyl-

424   RAC, Acyl PEG Exchange (APE) (Yokoi *et al.*, 2016) substitutes S-acyl groups for various weights

425   of PEG allowing for separation of S-acylated and non-S-acylated forms by SDS-PAGE based on

426   differences in molecular weight. These assays have the advantage that the total number of S-

427   acyl groups can be quantified and the relative abundance of each S-acylated form and non-S-

428   acylated form determined.

429

430   *Metabolic labelling* – These method have been highly successful in animals but only one

431   report in plants has been published (Boyle *et al.*, 2016). This method originally used tritiated

432   palmitic acid fed to cell cultures (Martin and Busconi, 2000) but in mammalian and yeast

433   systems the use of alkyne derivatives of fatty acids such as 15-hexadecynoic acid/Alk14 and

434   17-octadecynoic acid (17-ODYA)/Alk16 is now commonplace (Martin and Cravatt, 2009).

435   These alkyne derivatives enable labelling of S-acylated proteins with a range of reporters

436   (such as biotin or fluorophores) by copper catalysed click-chemistry (CuAAC). A recent

437   publication has now shown that this approach is feasible in Arabidopsis protoplast systems

438   (Boyle *et al.*, 2016) and provides an orthogonal and independent route to testing for protein

439   S-acylation.

440

441   *Proximity ligation assay* – This method allows subcellular localisation of S-acylated forms of

442   proteins to be determined by microscopy. Using alkyne fatty acids described above S-acylated

443   proteins are labelled in vivo. Cells are then fixed and S-acylated proteins labelled with biotin.

444   Antibodies against biotin and the protein of interest are then used to set up a proximity

445   ligation assay (PLA). This allows for very sensitive and highly accurate detection of the exact

446   subcellular localisation of the S-acylated forms of a protein compared to the total cellular

447   population of the protein of interest. This method has only recently been published (Gao and

448   Hannoush, 2014) but has the potential to be a game changing technique for addressing the

449   functional consequences of S-acylation.

450

451   *Inhibitors* – 2-bromopalmitate (2-bromohexadecanoic acid) is frequently used to inhibit S-

452   acylation (Batistic *et al.*, 2008; Hemsley *et al.*, 2005; Lavy *et al.*, 2002) but is reported to have

453   off-target effects (Davda *et al.*, 2013), especially over longer treatment times (>2-3 hours),

454   and can interfere with fatty acid synthesis and N-myristoylation (Webb *et al.*, 2000). Recent

455   work provided evidence that 2-bromopalmitate also inhibits de-S-acylation, further clouding

456   data interpretation (Pedro *et al.*, 2013). Tunicamycin (Patterson and Skene, 1995) inhibits N-

457   glycosylation and cerulenin (Lawrence *et al.*, 1999) inhibits fatty acid and sterol biosynthesis

458   but are also reported to inhibit S-acylation. Interpretation of data obtained using any of these

459   inhibitors should therefore be treated with caution unless validated by mutational or

460   biochemical analysis. This demonstrates the field's urgent need for specific inhibitors of S-

461   acylation. A number of inhibitors of mammalian (Adibekian *et al.*, 2010; Dekker *et al.*, 2010;

462   Martin and Cravatt, 2009) and toxoplasma (Child *et al.*, 2013) de-S-acylating enzymes of

463   varying potency and specificity have been described, but whether they are effective in plants

464   has not been determined.

465

466   *Direct detection of S-acylation* – Two methods to directly detect S-acylation, one that

467   identifies the nature of the S-acyl group and the other that identifies S-acylated peptides,

468   have been described. To detect the S-acyl group attached highly purified S-acylated protein is

469   hydrogenated using platinum (IV) oxide. This cleaves and trans-esterifies the S-acyl group

470   away to form the ethyl ester derivative of the fatty acid which can subsequently be separated

471   by gas chromatography and identified by mass spectrometry. Using this method the identities

472   of the S-acyl groups on ROP6 (Sorek *et al.*, 2007) and CBL1 (Batistic *et al.*, 2008) have been

473   shown to be a mixed population of palmitic and stearic acid, with stearic acid predominating.

474   While this method is very accurate for identifying the nature of the S-acyl group it does not

475   directly identify where a protein is S-acylated; mutagenesis must still be used to map the site.

476   A recent development promises to allow for direct detection of S-acylated peptides from

477   tryptic digests (Ji *et al.*, 2013). Although only performed so far on model peptides, the

478   prospect of this method to allow direct reading of the S-acylation state of sites within

479   individual proteins or even proteomes is very exciting and would allow S-acylation proteomics

480   to be pursued in the same manner as phosphorylation or ubiquitination. One potential hurdle

481   to the implementation of S-acylation proteomics is the increased instability of S-acyl

482   thioesters at pH >8. This may cause problems when performing overnight digestion using

483   trypsin where S-acyl groups could be lost. Alternative digestion strategies that preserve acyl-

484   thioesters may therefore need to be employed, such as lower pH, to achieve peptides with

485   intact S-acyl modifications. To be fully realised it is likely that new mass spectrometry

486   compatible separation strategies would also need to be developed. Due to the highly

487   hydrophobic nature of S-acyl groups dominating the character of any given peptide, achieving

488   effective separation of S-acylated peptides on reverse phase media is virtually impossible. For

489   dealing with the hundreds or thousands of S-acylated peptides likely generated by proteomics

490   experiments a separation strategy based on the character of the peptide backbone while

491   negating or minimising the effects of the S-acyl group would be desirable. A possible solution

492   would be to use hydrophilic interaction liquid chromatography (HILIC). This has the advantage

493   of being orthogonal to reverse phase systems and is able to separate hydrophobic molecules

494   on the basis of small differences in polarity. HILIC can also be used with the same

495   water/acetonitrile buffer systems as reverse phase chromatography meaning that

496   compatibility with standard mass spectrometry set ups is maintained.

497

498   *Prediction of S-acylation* – Computational prediction of S-acylation is very difficult as there is

499   nothing approaching a consensus sequence for S-acylation. However, a few attempts have

500   been made (Xie *et al.*, 2016), but these predictions should in no way be accepted without

501   experimental proof. A few general observations can help narrow down and prioritise

502   candidate cysteine residues, particularly if structures are known. **1.** S-acylation only occurs on

503   cysteines found on intracellular, cytosolic regions of proteins. **2.** Cysteines for S-acylation by

504   PATs need to be accessible (i.e. not buried in the protein interior or in transmembrane

505   domains) **3.** Cysteines need to be capable of being positioned close to the membrane surface

506   for PAT mediated S-acylation to occur. **4.** S-acylation appears to occur more often in regions

507   of predicted disorder or in/adjacent to α-helices. β-sheets appear to be rarely S-acylated. **5.**

508   S-acylation frequently occurs close to transmembrane domains, sites of N-myristoylation

509   (Batistic *et al.*, 2008; Traverso *et al.*, 2013) or prenylation (Sorek *et al.*, 2007).

510

511   *Databases* – The first true database of S-acylation, SwissPalm, was recently made available

512   (Blanc *et al.*, 2015). At the time of writing the database was being actively maintained and

513   updated but had not yet incorporated the recent work on Poplar. SwissPalm nonetheless

514   represents a very valuable resource bringing integration of S-acylation prediction, topology

515   data, species homologues and proteomics together. The Aramemnon database (Schwacke *et*

516   *al.*, 2003) focussed on plant membrane proteins also now includes published Arabidopsis S-

517   acylation proteomics data (Hemsley *et al.*, 2013) as well as predictions.

518

519   **Conclusions**

520   The data discussed here illustrate the huge steps forward made over the last 10 years in

521   understanding the role of S-acylation in plant cellular function. As the field is still relatively

522   young much of the fundamental knowledge is still waiting to be discovered, but there is a

523   feeling of having reached a watershed where it is now readily obvious that S-acylation has a

524   major role to play within the cell.

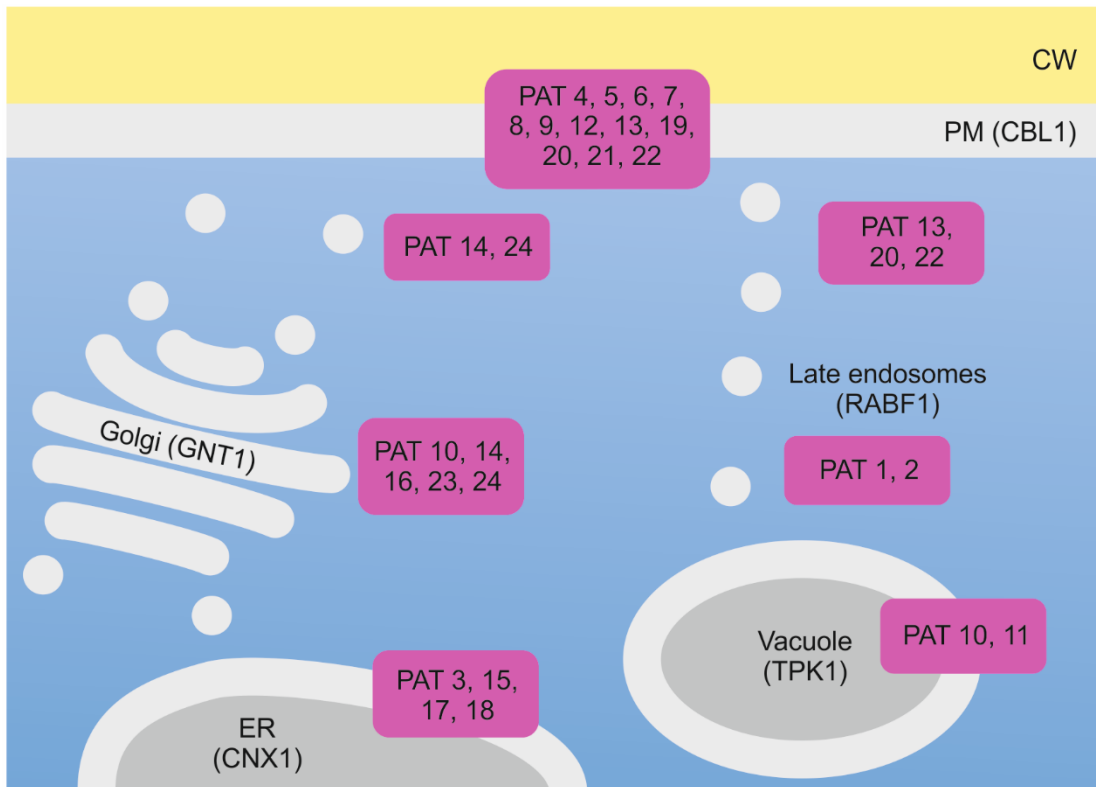525

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Figure legends

544

545   **Figure 1. S-acyl transferases are found in multiple membrane compartments within the cell.**
546   Known localisations of protein S-acyl transferases (PATs) are shown in cartoon form. PAT
547   numbering uses the system of Batistic 2012. Markers used to define membrane
548   compartments are indicated in parentheses (CNX1 – calnexin 1, GNT1 – N-acetylglucosaminyl
549   transferase 1, CBL1 – calcineurin B-like 1, RABF1 – Rab GTPase F1 (ARA6), TPK1 – two-pore
550   potassium channel 1).

551

Key: ⟨ S-acyl group   ⟨ prenyl group

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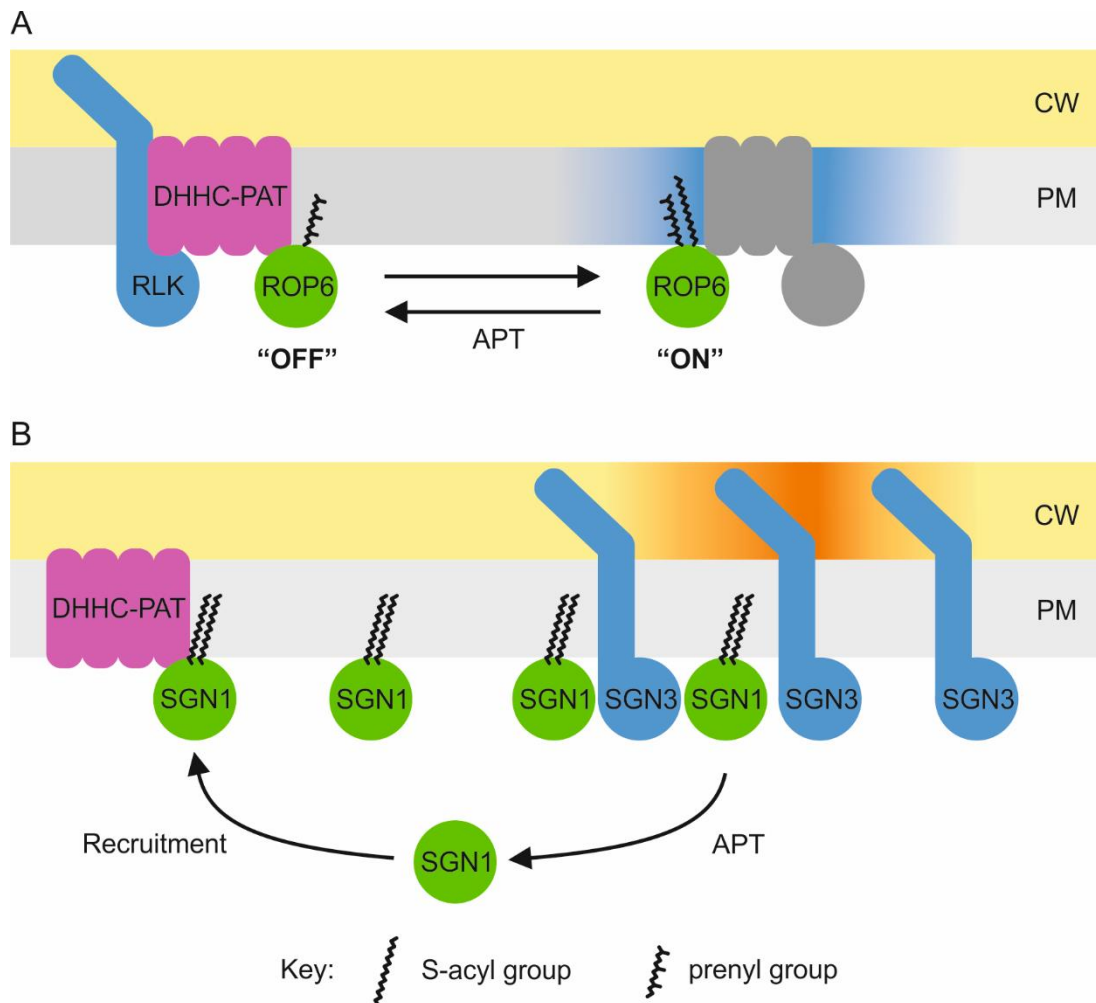
Figure 2. A. ROP6 undergoes activation state dependant S-acylation cycles potentially leading to changes in membrane microdomain composition or occupancy. ROP6 is prenylated but not S-acylated in its inactive GDP bound form ("OFF"). In many cases ROPs are activated by receptor-like kinases (RLK) and become S-acylated by an as yet unknown S-acyl transferase (DHHC-PAT). Active GTP bound and S-acylated ROP6 ("ON") may partition into a different membrane microdomain environment (blue shading) due to a change in its physical properties or may alter the membrane environment around the existing complex by recruiting different lipid species. Both situations would alter the proteins available for interaction (dark grey) with activated ROP6. Upon GTP hydrolysis ROP6 becomes de-S-acylated by an unknown acyl protein thioesterase (APT) and is thought to return to its resting state complex. This S-acylation cycle is therefore proposed to aid in regulating downstream signalling outputs and preventing inappropriate signalling in the absence of ROP6 activation. **B. SGN1 polar distribution is maintained by cycles of S-acylation.** The SGN1 receptor-like cytoplasmic kinase is attached to the plasma membrane by two S-acyl groups. SGN1 is found only on the

567   cortical side of endodermal cells and, in conjunction with the receptor-like kinase SGN3,

568   defines the zone of casparian band formation (orange shading). SGN1 polar distribution is

569   hypothesised to be maintained by recruitment to the cortical-facing plasma membrane by an

570   unknown S-acyl transferase (DHHC-PAT) and removal of SGN1 from the plasma membrane at

571   the limits of its desired distribution by the actions of an unknown acyl-protein thioesterase.

572   PM – plasma membrane, CW – cell wall.

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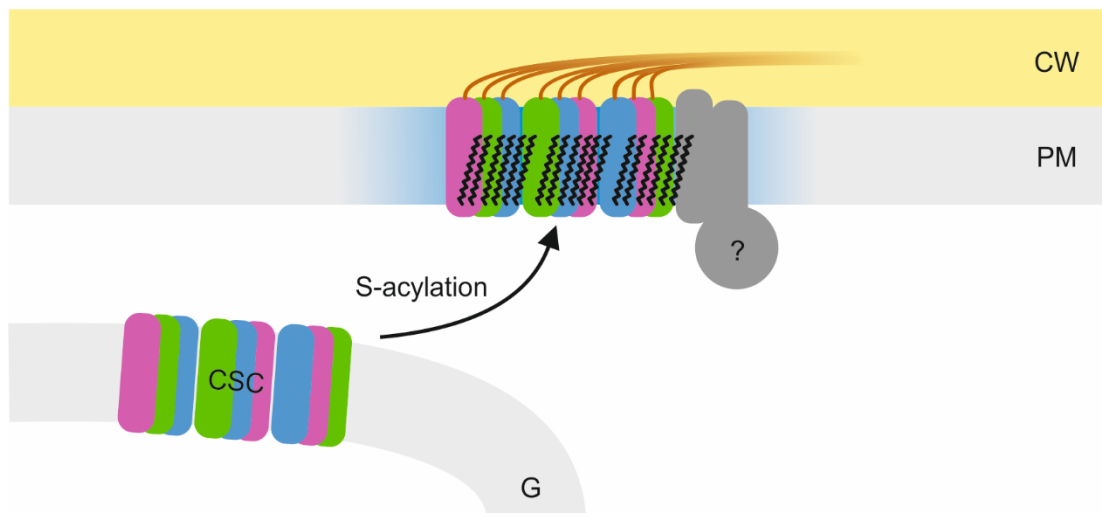576

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Figure 3. Potential roles for S-acylation in cellulose synthase function. Defective S-acylation of one of the three cellulose synthase paralogs (CesA; coloured pink, green and blue) that make up the cellulose synthase 18mer complex (CSC) leads to the CSC remaining in the Golgi (G). The CSC is the most highly S-acylated complex known, as a result the effects of CSC S-acylation is highly likely to influence the lipid composition of the membrane surrounding it. This may create a distinct microdomain (blue shading) to recruit other proteins (dark grey) required by the CSC for correct function. Alternatively this microdomain environment may make it easier for the CSC to move through the membrane during cellulose deposition.

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