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## Progress towards Understanding Protein S-acylation: Prospective in Plants

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# 1        **Progress towards Understanding Protein S-acylation: Prospective in Plants**

2  
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6  
7        S-acylation, also known as S-palmitoylation or palmitoylation, is a reversible post-  
8        translational lipid modification in which long chain fatty acid, usually the 16-carbon  
9        palmitate, covalently attaches to a cysteine residue(s) throughout the protein via a  
10       thioester bond. It is involved in an array of important biological processes during  
11       growth and development, reproduction and stress responses in plant. S-acylation is a  
12       ubiquitous mechanism in eukaryotes catalyzed by a family of enzymes called Protein  
13       S-Acyl Transferases (PATs). Since the discovery of the first PAT in yeast in 2002  
14       research in S-acylation has accelerated in the mammalian system and followed by in  
15       plant. However, it is still a difficult field to study due to the large number of PATs and  
16       even larger number of putative S-acylated substrate proteins they modify in each  
17       genome. This is coupled with drawbacks in the techniques used to study S-acylation,  
18       leading to the slower progress in this field compared to protein phosphorylation, for  
19       example. In this review we will summarize the discoveries made so far based on  
20       knowledge learnt from the characterization of protein S-acyltransferases and the S-  
21       acylated proteins, the interaction mechanisms between PAT and its specific substrate  
22       protein(s) in yeast and mammals. Research in protein S-acylation and PATs in plants  
23       will also be covered although this area is currently less well studied in yeast and  
24       mammalian systems.

25  
26       **Keywords:** lipid modification, S-acylation, PATs, substrate recognition and specificity,  
27       yeast, mammalian, plants

## 31 INTRODUCTION

32 Lipid modification is a common mechanism in organisms, in which a fatty acid attaches  
33 to specific amino acid residues, leading to increased hydrophobicity of proteins which  
34 aids their anchoring to membranes or specific lipid rafts (Levental et al., 2010). The  
35 three most commonly known lipid modifications are N-myristoylation, prenylation and  
36 S-acylation (Figure 1). N-myristoylation is an irreversible, co-translational protein  
37 modification in which 14-carbon myristoyl group is covalently attached to N-terminal  
38 glycine residue via an amide bond (Martin et al., 2011). Prenylation is a post-  
39 translational lipid modification which involves the transfer of either a 15-carbon  
40 farnesyl or a 20-carbon geranyl-geranyl moiety to CaaX C-terminal cysteine of the  
41 target protein. S-acylation, more commonly known as S-palmitoylation, is a post-  
42 translational lipid modification in which a long chain fatty acid, usually the 16-carbon  
43 palmitate, covalently attaches to the specific cysteine residue(s) throughout the protein  
44 via a thioester bond (Resh, 2006; Greaves and Chamberlain, 2011).

45 It is noteworthy that three types of protein palmitoylation are found so far,  
46 including S-palmitoylation, N-palmitoylation and O-palmitoylation. While S-  
47 palmitoylation can occur at any Cys residues along the protein sequence in which the  
48 palmitate is reversibly attached via thioester bond as shown in Figure 1, N-  
49 palmitoylation is a stable lipid modification at the N-terminal residue (very often Cys)  
50 through amide linkage. A small group of secreted proteins have been identified as N-  
51 palmitoylated proteins, including the epidermal growth factor (EGF) like ligand ‘Spitz’  
52 and Hedgehog family members in *Drosophila* and mammals (Pepinsky et al., 1998;  
53 Chamoun et al., 2001; Miura et al., 2006; Buglino and Resh, 2012). Since N-  
54 palmitoylation can be easily converted by S-palmitoyl migration, it is still not very clear  
55 whether N-palmitoylation is an independent enzyme-catalyzed reaction or just from S-  
56 to N-palmitoyl transfer (Ji et al., 2016). Less frequently, palmitoyl group can also be  
57 linked to a serine residue through ester bond via the so-called O-palmitoylation. The  
58 identified O-palmitoylated targets so far include Wnt/Wg proteins and the peptide

59 hormone preghrelin (Takada et al., 2006; Yang et al., 2008). Although palmitate is  
60 thought to be the most common fatty acid found to be attached to S-palmitoylated  
61 proteins recent studies proved that other acyl groups such as stearate (C18:0) or oleate  
62 (C18:1) are also accepted in S-palmitoylation. Therefore, S-acylation is a more  
63 representative term than palmitoylation (Jones et al., 1997; Sorek et al., 2007; Hurst  
64 and Hemsley, 2015). In contrast to other lipid modification, such as myristoylation,  
65 prenylation, N-palmitoylation or O-palmitoylation, S-acylation is a unique  
66 posttranslational modification in that it is usually reversible (Fukata et al., 2010). As  
67 such it is important for cellular protein sorting, vesicle trafficking, activation state  
68 control, protein stability, membrane microdomain partitioning of protein and protein  
69 complex assembly (Baekkeskov and Kanaani, 2009; Greaves and Chamberlain, 2007;  
70 Charollais and Van Der Goot, 2009; Hemsley, 2009; Hemsley et al., 2013).

71 Some other lipid modifications, such as Glycosylphosphatidylinositol (GPI) and  
72 glycosylinositolphosphorylceramide (GIPC) anchors can link the whole glycolipids to  
73 the protein instead of the simple fatty acid or polyisoprene group (Hemsley, 2015). GPI  
74 and GIPC anchors modify proteins at the lumen side instead of in the cytosol as do the  
75 other three lipid modifications (Ganesan and Levental, 2015). Lipid modifications  
76 which are only found in specific proteins, such as cholesterol addition at the C-terminal  
77 glycine of proteins have also been reported (Buglino and Resh, 2012). All these lipid  
78 modifications are widely present in mammals and plants (except for N- or O-  
79 palmitoylation which is only found in mammals so far) and all play important roles  
80 during growth and development through the modification of an array of proteins.

81 Although all lipid modifications can facilitate the attachment of proteins to  
82 membranes, modification with palmitoyl groups provide more affinity, about 10 times  
83 stronger than myristoyl groups and 100 times than farnesyl groups (Silvius and Heureux,  
84 1994; Hemsley, 2009).

85

## 86 S-ACYLATION

87 S-acylation can occur both on soluble and transmembrane proteins (Roth et al., 2006;  
88 Blaskovic et al., 2013). S-acylation of soluble proteins allows their association with  
89 membranes, trafficking, regulation and signalling (Roth et al., 2006; Blaskovic et al.,  
90 2013). For example, a constitutive de/re-acylation of H- and N- small Rat sarcoma (Ras)  
91 drives their subcellular localization from plasma membrane (PM) to Golgi which  
92 initiates RAS activation (Rocks et al., 2005). Although the direct mechanism of S-  
93 acylation on transmembrane proteins is not very clear it is thought that it plays multiple  
94 roles in altering signalling capacity (Merrick et al., 2011), reducing activity (Huang et  
95 al., 2010), trafficking modification (Abrami et al., 2008; Flannery et al., 2010) and  
96 changing stability of these proteins (Maeda et al., 2010; Abrami et al., 2006; Blaskovic  
97 et al., 2013). For example, S-acylation of transmembrane proteins, such as death  
98 receptor 4 (Oh et al., 2012),  $\beta$ -secretase BACE1 (Motoki et al., 2012), cannabinoid  
99 receptor (Oddi et al., 2012) and influenza virus M2 protein (Thaa et al., 2011), can  
100 promote their association with membrane lipid rafts. However, for some peripheral  
101 membrane proteins such as transferrin receptor and caveolin, their palmitoylation sites  
102 are localized to non-raft domains, therefore palmitoylation is not necessary for their raft  
103 localization (Alvarez et al., 1990; Dietzen et al., 1995; Charollais and Van Der Goot,  
104 2009). In the case of the tumor endothelial marker 8 (TEM8) palmitoylation was  
105 actually found to negatively regulate its raft association (Abrami et al., 2006).

## 106 S-acylation in Yeast

107 A proteomic method using the acyl-biotinyl exchange (ABE) chemistry combining with  
108 the traditional [ $^3\text{H}$ ] palmitate in vivo labeling protocol identified 48 S-acylated proteins  
109 that span a wide range of cellular functions in *Saccharomyces cerevisiae* (Roth et al.,  
110 2006). These include a large number of SNAREs (soluble N-ethylmaleimide-sensitive  
111 fusion protein-attachment protein receptor) that are involved in vesicle fusion.  
112 Redundant SNAREs, such as plasma membrane (PM) localized synaptobrevin  
113 homologs Snc1 and Snc2, were first identified to be S-acylated proteins in 1995 (Couve

114 et al., 1995), and subsequently confirmed independently (Valdez-Taubas and Pelham,  
115 2005; Roth et al., 2006). Ykt6 is another commonly known S-acylated SNARE. It  
116 requires both C-terminal prenylation and palmitoylation to target to the membrane,  
117 which is different from all other single transmembrane domain (TMD) containing  
118 SNAREs (Fukasawa et al., 2004). Tlg1 lacking S-acylation undergoes ubiquitination,  
119 implying S-acylation can protect proteins from degradation (Valdez-Taubas and Pelham,  
120 2005). Other SNAREs that have been confirmed to be S-acylated are Sso1, Sso2, Vam3,  
121 Tlg2 and Syn8 (Valdez-Taubas and Pelham, 2005; Roth et al., 2006). S-acylation is also  
122 very common in many important signalling proteins, such as the heterotrimeric G  
123 protein alpha and gamma subunits Gpa1 (Song and Dohlman, 1996; Song et al., 1996),  
124 Gpa2 (Harashima and Heitman, 2005) and Gy (Ste18, Hirschman and Jenness, 1999);  
125 small monomeric G proteins (GTPases) such as Rho1, Rho2 (Roth et al., 2006), Rho3  
126 (Zhang et al., 2013), Ras1 and Ras2 (Deschenes et al., 1990; Bartels et al., 1999;  
127 Mitchell et al., 2012). A recent study shows that the pathogenesis, morphogenesis and  
128 sexual differentiation of an encapsulated yeast *Cryptococcus neoformans* is achieved  
129 through the important roles that S-acylation plays in modulating the localization of  
130 Ras1 (Nichols et al., 2015). Interestingly, all of these signaling proteins acquire  
131 prenylation or myristoylation before S-acylation occurs (Roth et al., 2006).

132 In addition, many amino acid permeases (AAP) were proved to be S-acylated (Roth  
133 et al., 2006). For example, the yeast type I casein kinases, Yck1, Yck2, and Yck3, which  
134 play important roles in cellular morphology, bud emergence and endocytosis of mating  
135 pheromone receptor, are membrane localized via S-acylation for function (Roth et al.,  
136 2006; Roth et al., 2011). ENV7 (late endosome and vacuole interface) encodes a protein  
137 kinase that plays important roles in vacuole morphology, and its proper membrane  
138 localization and function relies on S-acylation of the N-terminal triple cysteines motif  
139 (C<sup>13</sup>C<sup>14</sup>C<sup>15</sup>) (Manandhar et al., 2013 and 2014; Cocca, 2014). S-acylation of telomere-  
140 binding protein Rif1 anchored it to the inner nuclear membrane, which influences its  
141 role in heterochromatin dynamics (Park et al., 2011). Mutagenesis of cysteine in  
142 different positions of Arsenite permease Acr3p can cause its completely or partially

143 dysfunction as a low affinity As(III)/H<sup>+</sup> and Sb(III)/H<sup>+</sup> antiporter, and Cys90 which  
 144 localizes in the cytosolic loop but in close proximity to transmembrane regions has the  
 145 high possibility to be S-acylated (Maciaszczyk-Dziubinska et al., 2013). It was also  
 146 reported that S-acylation is necessary for the export of chitin synthase Chs3 from ER  
 147 (Lam et al., 2006). The information described in this section is summarized in **Table 1**.

148 **TABLE 1| Individually confirmed S-acylated proteins in yeast**

Groups	Specific proteins	References
SNAREs	Snc1/2, Ykt6, Tlg1/2, Sso1/2, Vam3, Syn8	Couve et al., 1995; Valdez-Taubas and Pelham, 2005; Roth et al., 2006; Fukasawa et al., 2004
G proteins	Gpa1/2, Ste18, Rho1/2/3, Ras1/2	Song and Dohlman, 1996; Song et al., 1996; Hirschman and Jenness, 1999; Harashima and Heitman, 2005; Roth et al., 2006; Zhang et al., 2013; Deschenes et al., 1990; Bartels et al., 1999; Mitchell et al., 2012; Nichols et al., 2015
AAPs	Tat1/2, Gnp1, Sam3, Hip1, Bap2, Agp1, Gap1	Roth et al., 2006
Protein kinases	Yck1/2/3, Env7	Roth et al., 2006; Roth et al., 2011; Cocca, 2014
Other proteins	Rif1, Acr3p, Chs3	Park et al., 2011; Maciaszczyk-Dziubinska et al., 2013; Lam et al., 2006

149 **S-acylation in Mammals**

150 Following study of S-acylation in yeast research that has extended to mammalian  
 151 systems considerable knowledge has been gained in recent years, revealing the  
 152 involvement of protein S-acylation in the regulation of growth, development, and  
 153 cancer and disease status. For example, a global rat neural palmitoyl-proteome



154 characterized almost 300 S-acylated proteins, again with the ABE method adapted from  
155 the yeast study (Kang et al., 2008). Similarly 331 S-acylated proteins were identified  
156 from human prostate cancer cells (Yang et al., 2010), 57 from human B lymphoid cells  
157 (Ivaldi et al., 2012) and 150 from endothelial cells (Marin et al., 2012). By bio-  
158 orthogonal labeling of S-acylated proteins with 17-octadecynoic acid (ODYA) about  
159 125 and over 400 S-acylated proteins were identified from human Jurkat T-cells and  
160 mouse T-cell hybridoma cells, respectively (Martin and Cravatt, 2009; Martin et al.,  
161 2012).

162 It is worth noting that proteins that have been proved to be S-acylated in yeast, their  
163 homologous proteins in mammals tend to be also S-acylated. For instance, many human  
164 SNAREs were proved to be also S-acylated (Greaves et al., 2010), S-acylation of  $\alpha$   
165 subunits of G proteins is necessary for their membrane localization and function  
166 (Wedegaertner et al., 1993; Grassie et al., 1994; Ponimaskin et al., 1998; Ponimaskin et  
167 al., 2000). However, G-protein  $\gamma$  subunits have not been reported to be S-acylated in  
168 mammals. Many G-protein-coupled receptors (GPCRs) (Blaskovic et al., 2013) and  
169 Ras GTPase (Rocks et al., 2005) are also S-acylated. Mitochondrial targeting of a  
170 microphage protein phospholipid scramblase 3 (Plscr3) is dependent on its S-acylation  
171 (Merrick et al., 2011).

172 Some S-acylated proteins in mammals can also make themselves avoid degradation  
173 by attaching a palmitate molecule. For instance, LRP6 (lipoprotein-receptor-related  
174 protein 6) is S-acylated and the removal of acyl group leads to destabilization or  
175 ubiquitination (Abrami et al., 2008). Similarly, the palmitoylation of TEM8 (Abrami et  
176 al., 2006), CCR5 (chemokine and HIV receptor) (Percherancier et al., 2001) and  
177 Rhodopsins (Maeda et al., 2010) prevents the degradation of these proteins. It was also  
178 reported that for some other proteins, their degradation depends on the S-acylation. For  
179 example, a cancer-promoting protein CDCP1 (CUB domain-containing protein 1) is  
180 degraded upon S-acylation, leading to a decrease of ovarian cancer cell migration  
181 (Adams et al., 2015). Therefore, it seems that S-acylation can play opposite roles in

182 protein degradation.

183 Many signalling proteins involved in keeping T-cell homeostasis are S-acylated,  
184 such as T-cell co-receptors CD4 and CD8, tyrosine kinases Lck and Fyn, and adaptor  
185 proteins LAT (linker for activation of T cells) and Cbp/PAG (Bijlmakers, 2009; Hundt  
186 et al., 2009; Akimzhanov and Boehning, 2015). S-acylation of Lck at both Cys3 and  
187 Cys5, which are redundant for the function of Lck, is essential for propagating T-cell  
188 receptor signaling and releasing apoptotic calcium (Akimzhanov and Boehning, 2015).  
189 Similarly, LAT is also a dual (Cys26 and Cys29) S-acylated protein which is required  
190 for T cell development and activation. However, S-acylation of Cys26 alone is enough  
191 for its PM localization and proper function (Hundt et al., 2009).

192 S-acylation of synaptic proteins is important for synaptic plasticity, and the key S-  
193 acylated synaptic proteins include postsynaptic density protein PSD-95,  $\delta$ -catenin,  
194 gephyrin, A-kinase anchoring protein AKAP79 and 150, the small GTPase Cdc42. Lack  
195 of S-acylation of these proteins lead to impaired performance on learning and memory  
196 tasks (Brigidi et al., 2015). Huntington's disease is a neurodegenerative disorder caused  
197 by mutation in the gene encoding the S-acylated Huntingtin (HTT) (Butland et al.,  
198 2014). Defects in S-acylation can also cause mental problems such as schizophrenia  
199 and X-linked mental retardation (XLMR), however, the specific S-acylated target  
200 proteins involved in this process have not been isolated (Mukai et al., 2004; Raymond  
201 et al., 2007). Alzheimer's disease (AD) is a neurodegenerative dementia which accounts  
202 for 60% to 70% of cases of dementia. Many studies have demonstrated that S-acylation  
203 plays very important roles in the pathogenesis of AD, and the related S-acylated  
204 proteins include  $\beta$ - and  $\gamma$ -secretase enzymes, and the major APP (amyloid precursor  
205 protein) cleaving enzyme BACE1, which are S-acylated at four sites (Benjannet et al  
206 2001; Hornemann, 2015).

207 Autophagic protein microtubule-associated protein 1 light chain-3B (LC3B) is a  
208 positive regulator of chronic obstructive pulmonary diseases such as emphysema.  
209 LC3B is associated with the extrinsic apoptotic factor Fas, and their interaction is

210 mediated by caveolin-1 (Cav-1). Interestingly, both Fas and Cav-1 are S-acylated  
211 proteins (Chen et al., 2010). S-acylation of the bone developmental regulator membrane  
212 type1-metalloprotease (MT1-MMP) is a key modulator of bone homeostasis (Song et  
213 al., 2014). Goltz syndrome, caused by loss of function of the S-acylated protein  
214 Porcupine (Galli et al., 2007; Hornemann, 2015), is an X-linked dominant form of  
215 ectodermal dysplasia, which is primarily characterized by skin manifestations as  
216 atrophic and hypoplastic areas and results in osseous defects and dental anomalies later  
217 (Wang et al., 2007).

218 An increasing number of reports indicate that S-acylation is involved in cancer. For  
219 instance, Ras is a negative regulator of cell proliferation, and S-acylation of Ras  
220 maintains its steady state plasma membrane localization which is essential for  
221 transduction of extracellular proliferative signals (Rocks et al., 2005; Schmick et al.,  
222 2015). S-acylation of the neurotensin receptor 1 (NTSR-1), a key mediator in breast,  
223 pancreas, prostate, colon and lung cancers, is essential for its localization and efficient  
224 signaling (Heakal et al., 2011). The induction of apoptosis is an efficient way to stop  
225 tumor development, many proteins involved in apoptosis are S-acylated including FasL  
226 (Fas Ligand) (Guardiola-Serrano et al., 2010), FasR (Fas receptor) (Chakrabandhu et  
227 al., 2007), DR4 (a receptor of the tumor necrosis factor-related apoptosis-inducing  
228 ligand) ( Rossin et al., 2009), DCC (deleted in colorectal cancer) (Furne et al., 2006),  
229 UNC5H (Maisse et al., 2008) and BAX (BCL-2-associated X) (Fröhlich et al., 2014).  
230 The spread of cancer cells from their original site to other parts of the body is through  
231 metastasis. It was reported that S-acylation of metastasis-associated proteins  
232 KAT1/CD82, CD9 and CD151 is essential for their function of suppressing metastasis  
233 or inhibiting tumor cell adhesion and migration (Zhou et al., 2004; Termini et al., 2014;  
234 Hemler, 2014). Integrin  $\beta 4$  (ITG $\beta 4$ ) can interact with growth factor receptors and  
235 enhance invasive potential of cancer cells (Soung and Chung, 2011). This is helped by  
236 the S-acylation of ITG $\beta 4$  which is required for its lipid raft localization in the membrane  
237 and signaling activity. The level of ITG $\beta 4$  S-acylation is correlated with the invasive  
238 potential of breast cancer cells (Coleman et al., 2015). Another S-acylated protein

239 related to breast cancer is CD44 which negatively regulates cell migration (Xie et al.,  
 240 2009). Endothelial nitric oxide synthase (eNOS), which localizes through S-acylation  
 241 to the Golgi complex and PM cholesterol-rich microdomains, promotes angiogenesis  
 242 and tumorigenesis (Fernandez-Hernand et al., 2006; Wei et al., 2011). **Table 2** lists the  
 243 identified S-acylated proteins in mammals described in this section.

244 **TABLE 2 | S-acylated proteins individually verified in mammalian cells**

Groups	Examples	References
SNAREs	SNAP23, SNAP25, SNAP25b	Greaves and Chamberlain, 2010; Greaves et al., 2010
G Proteins	Go1 $\alpha$ , G $\alpha$ <sub>12</sub> , G $\alpha$ <sub>13</sub> , GPCRs, GTPase	Wedegaertner et al., 1993; Grassie et al., 1994; Ponimaskin et al., 1998; Ponimaskin et al., 2000; Rocks et al., 2005
T-cell specific proteins	CD4/8, Lck, Fyn, LAT, Cbp/PAG	Bijlmakers, 2009; Hundt et al., 2009; Akimzhanov and Boehning, 2015
B-cell specific proteins	CD20/23	Ivaldi et al., 2012
Synaptic proteins	PSD-95, $\delta$ -catenin, gephyrin, AKAP79/150, Cdc42, HTT, $\beta$ - and $\gamma$ - secretases, BACE1	Brigidi et al., 2015; Butland et al., 2014; Benjannet et al 2001; Hornemann, 2015
Cancer related proteins	CDCP1, Ras, NTSR-1, FasL, FasR, DR4, DCC, UNC5H, BAX, CD82/9/151/44, ITG $\beta$ 4, Enos	Adams et al., 2015; Schmick et al., 2015; Heakal et al., 2011; Guardiola-Serrano et al., 2010; Chakrabandhu et al., 2007; Rossin et al., 2009; Furne et al., 2006; Maisee et al., 2008; Fröhlich et al., 2014; Zhou et al., 2004; Termini et al., 2014; Hemler, 2014; Soung and Chung, 2011; Coleman et al., 2015; Xie et al., 2009; Fernandez-Hernand et al., 2006; Wei et al., 2011

Other Proteins	Plscr3, LRP6, Fas, Cav-1, MT1-MMP; Porcupine, TEM8, CCR5	Merrick et al., 2011; Abrami et al., 2008; Abrami et al., 2006; Percherancier et al., 2001; Chen et al., 2010; Song et al., 2014; Galli et al., 2007
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245

246 The above studies clearly demonstrate that S-acylation is involved in a wide range  
 247 of human diseases including mal-development, infectious diseases, autoimmune  
 248 diseases, neuropsychiatric disorders, dermatosis, osteoporosis and cancer (Ivaldi et al.,  
 249 2012; Chavda et al., 2014; Hornemann, 2015; Yeste-velasco et al., 2015).  
 250 Understanding S-acylation will provide invaluable information to the insight of disease  
 251 processes which in turn will aid the development of drugs to control and target these  
 252 various diseases. Therefore, the relationship between protein S-acylation and disease in  
 253 human becomes a hot research topic in the medical field in recent years.

#### 254 **S-acylation in Plants**

255 Our understanding of plant S-acylation is rudimentary and the limited knowledge  
 256 comes mainly from targeted studies on the functional characterization of individual  
 257 proteins that happen to be S-acylated, including, mainly heterotrimeric G protein and  
 258 some small monomeric G-proteins. For instance, the  $\alpha$  subunit GPA1 and  $\gamma$  subunit  
 259 AGG2 of plant heterotrimeric G protein are S-acylated. GPA1 has dual lipid  
 260 modification with a myristoylation site at the G2 position and an adjacent S-acylation  
 261 site at the C5 position, ensuring its localization to the PM (Adjobo-Hermans et al.,  
 262 2006). Apart from promoting PM localization, S-acylation of GPA1 may also stabilise  
 263 the newly formed heterotrimer. AGG2 is S-acylated at Golgi before delivered to the PM,  
 264 and its membrane localization is dependent on its prenylation and S-acylation (Zeng et  
 265 al., 2007). Therefore, S-acylation may act as a membrane targeting signal and restricts  
 266 AGG2 shuttle in and out of PM (Zeng et al., 2007; Hemsley, 2009). Some small  
 267 GTPases are also known to be S-acylated. For instance, S-acylation of AtROP6 is

268 responsible for its activation and inactivation cycles (Sorek et al., 2007). AtROP9 and  
269 AtROP10, which are involved in ABA signalling, contain 3 and 2 S-acylation sites  
270 respectively (Lavy et al., 2002; Zheng et al., 2002; Hemsley, 2009). For AtRABF1  
271 (ARA6), both S-acylation and myristoylation are essential for its prevacuolar  
272 compartment localization (Ueda et al., 2001).

273 Proteins involved in  $Ca^{2+}$  signalling such as calcineurin B-Like proteins AtCBL1,  
274 AtCBL2, AtCBL3 and AtCBL6 in Arabidopsis (Batistic et al., 2008; Batistic et al.,  
275 2012); calcium dependent protein kinases OsCPK2 in rice (Martin and Busconi, 2000);  
276 LeCPK1 in tomato (Leclercq et al., 2005); MtCPK3 in *Medicago truncatula*  
277 (Gargantini et al., 2006) and StCDPK1 in *Solanum tuberosum* (Raices et al., 2003) were  
278 reported to be S-acylated. AtCBL1 is a dually lipid modified protein, in which  
279 myristoylation targets it to the endoplasmic reticulum (ER), but the trafficking from ER  
280 to PM and subsequent PM anchoring depends on S-acylation (Batistic et al., 2008).

281 Other S-acylated proteins are the pathogenesis related proteins such as RPM1  
282 interacting protein 4 (RIN4) and leucine-rich repeat receptor like kinase (FLS2) (Kim  
283 et al., 2005; Hemsley et al., 2013; Running, 2014; Boyle et al., 2016); NDR1/HIN1-  
284 like (NHL) stress response proteins (Hemsley et al., 2013; Hurst and Hemsley, 2015);  
285 POLTERGEIST (POL) and POLTERGEIST LIKE 1 (PLL1) (their PM localization is  
286 dependent on both myristoylation and S-acylation at their N-termini) (Gagne and Clark,  
287 2010); the Lost In Pollen tube guidance 1 (LIP1) and 2 (LIP2), mutations of their S-  
288 acylation sites abolished PM localization. Although individual knockout mutant of  
289 LIP1 and LIP2 did not have any defects the double mutant can cause sterility due to  
290 loss of pollen tube guidance (Liu et al., 2013). S-acylation of remorin proteins, a group  
291 of well-known plasma membrane marker proteins, contribute to their subcellular  
292 localization (Konrad et al., 2014). Very recently, Kumar and his coworkers confirmed  
293 that a number of the catalytic subunits of cellulose synthase complex (CSC) in  
294 Arabidopsis are S-acylated. These include the cellulose synthase A 1 (CESA1), CESA4,  
295 CESA6, CESA7 and CESA8 where up to 6 S-acylation sites were in each of these

296 proteins (Kumar et al., 2016). SGN1, a receptor-like cytoplasmic kinase (RLCK),  
 297 localizes in a strictly polar fashion to the endodermal outer plasma membrane, and this  
 298 is dependent on the S-acylation of N-termini (Alassimone et al., 2016) (See summary  
 299 in **Table 3**).

300 **TABLE 3 | S-acylated proteins individually identified in plants**

<b>Groups</b>	<b>Examples</b>	<b>References</b>
SNAREs	AtSYP71, AtSYP122, AtNPSN11	Hemsley et al., 2013
G-proteins	AtGPA1, AtAGG2, AtROP6/9/10, AtRABF1	Ueda et al., 2001; Lavy et al., 2002; Zheng et al., 2002; Adjobo-Hermans et al., 2006; Sorek et al., 2007; Zeng et al., 2007
Proteins in Ca <sup>2+</sup> signalling	AtCBL1/2/3/6, OsCPK2, LeCPK1, MtCPK3, StCDPK1	Martin and Busconi, 2000; Raices et al., 2003; Leclercq et al., 2005; Gargantini et al., 2006; Batistic et al., 2008; Batistic et al., 2012;
Cellulose Synthase complex	AtCESA1, AtCESA4, AtCESA6, AtCESA7, AtCESA8	Kumar et al., 2016
Others	RIN4, FLS2, POL, PLL1, LIP1, LIP2, remorins, SGN1	Kim et al., 2005; Gagne and Clark, 2010; Hemsley et al., 2013; Running, 2014; Liu et al., 2013; Konrad et al., 2014; Alassimone et al., 2016

301

302 On a proteomic level Hemsley and coworkers identified about 600 putative S-  
 303 acylated proteins from Arabidopsis using a biotin switch isobaric tagging for relative  
 304 and absolute quantification (Hemsley et al., 2013). These proteins are involved in many  
 305 processes across plant growth, development and stress responses, including the

306 mitogen-activated protein kinases (MAPKs), leucine-rich repeat receptor-like kinases  
307 (LRR-RLKs) and RLK superfamily members, integral membrane transporters,  
308 ATPases, SNAREs and others. Similarly, about 450 S-acylated proteins were identified  
309 from Poplar cell suspension very recently. Except for the commonly known  
310 intracellular trafficking related proteins such as protein kinases, SNAREs, band 7  
311 family proteins and tetraspanins, some cell wall related proteins were also found to be  
312 S-acylated (Srivastava et al., 2016). These results greatly expand the range of functions  
313 of protein S-acylation involves in plants, demonstrating the important roles of protein  
314 S-acylation in plant growth, development and stress signaling.

### 315 **S-acylation in Other Organisms**

316 S-acylated proteins were also identified from other organisms. For example, more than  
317 400 putative S-acylated proteins were isolated from the most severe human malaria  
318 causing parasite *Plasmodium falciparum*, involved in almost all the stages of its life  
319 cycle (Hodson et al., 2015). A number of S-acylated proteins are localized in the inner  
320 membrane complex (IMC). IMC is a membranous two layered structure located  
321 underneath the plasma membrane, which IMC plays central roles in host cell invasion  
322 and cytokinesis in *Plasmodium falciparum* (Cavalier-Smith, 1993; Wetzel et al., 2014).  
323 In another parasite *Toxoplasma gondii*, S-acylated proteins were also proved to be  
324 involved in many physiological processes including motility, invasion and division  
325 (Frenal et al., 2010; Beck et al., 2010; Frenal et al., 2014). In *Aspergillus fumigatus*,  
326 one of the most common species that cause the invasive aspergillosis in individuals  
327 with an immunodeficiency, Ras pathway signaling is its critical virulence determinant  
328 and the properly localized and activated Ras is dependent on a series of posttranslational  
329 lipid modification including S-acylation (Abdallah and Fortwendel, 2005). Study also  
330 showed that S-acylation is essential for spermatogenesis of *Caenorhabditis elegans*  
331 (Gleason et al., 2006).

332 S-acylation not only occurs on proteins synthesized in eukaryotic cells but also for  
333 proteins secreted by prokaryotic bacteria and viruses and subsequently S-acylated by



334 their eukaryotic hosts. For example, *Legionella* and other bacterial pathogens can  
335 secrete effectors that mimic the substrates of host lipid transferases, which can help  
336 them target the proper host membranes after S-acylation and other lipid modifications  
337 (Ivanov and Roy, 2013). A group of cysteine protease type III effectors secreted by the  
338 plant pathogen *Pseudomonas syringae*, rely on their S-acylation by the host cells to be  
339 targeted to plasma membrane and activated (Downen et al., 2009). S-acylation can also  
340 take place on viral proteins and in fact the first reported S-acylated protein was a  
341 glycoprotein from *Vesicular stomatitis* virus (Schmidt and Schlesinger, 1979; Hurst and  
342 Hemsley, 2015). Another viral S-acylated protein is the hemagglutinin of Influenza  
343 virus. S-acylation of its all three cysteine residues by the host cell S-acylation  
344 machinery is essential for the replication and infection of the virus (Zurcher et al., 1994;  
345 Wagner et al., 2005; Brett et al., 2014).

#### 346 **PROTEIN S-ACYL TRANSFERASES (PATS)**

347 While spontaneous palmitoylation does occur on some proteins in the cells (i.e.  
348 Bizzozero et al., 2000; Kummel et al., 2006; Kostiuk et al., 2008) it is generally  
349 accepted that S-acylation is an enzymatic process catalyzed by a family of proteins, the  
350 Protein S-Acyl Transferases (PATs for short). This is because research on PATs was  
351 much delayed compared to that on the S-acylated proteins. The first PAT, Akr1 was  
352 identified from *Saccharomyces cerevisiae* in 2002 which is 20 years later than S-  
353 acylation of protein first reported (Roth et al., 2002; Schmidt and Schlesinger, 1979;  
354 Bartels et al., 1999). Since then, the significance of this enzyme family has been  
355 gradually recognized by studies carried out by an increasing number of researchers in  
356 this field, leading to the great enrichment of our knowledge of PATs, particularly in  
357 yeast and mammals.

#### 358 **The Structure and Functional Domains of PATs**

359 Compared to the numbers of enzymes that catalyze the N-myristoylation or prenylation,  
360 there are much more DHHC-containing PATs existing in eukaryotes. In contrast to the

361 cytoplasmic catalyzing enzymes for S-prenylation and N-myristoylation, PATs are  
362 transmembrane proteins with 4-6 TMDs and cytosolic N- and C- termini (Hemsley et  
363 al., 2013). Most importantly, PATs also have a highly conserved catalytic Asp-His-His-  
364 Cys Cysteine Rich Domain (DHHC-CRD) of ~50 amino acids (Roth, et al., 2002). This  
365 domain was proposed as Cx2Cx9HCx2Cx4DHHCx5Cx4Nx3F (Mitchell et al., 2006),  
366 usually residing on the cytoplasmic face of membranes between transmembrane  
367 domains (TMD) 2 and 3 of PATs (Gottlieb et al., 2015). It was reported that mutation  
368 of cysteine in DHHC domain inhibits both acyl intermediate formation and acyl chain  
369 transfer activity of PATs (Mitchell et al., 2006; Gottlieb et al., 2015). Indeed, when  
370 cysteine residue in the DHHC motif of AtPAT24, AtPAT10 and AtPAT14 of Arabidopsis  
371 was mutated to alanine or serine, all 3 AtPATs lost their PAT activities (Hemsley et al.,  
372 2005; Qi et al., 2013; Li et al., 2015). The DHHC-CRD domain in Swf1 cannot be  
373 replaced by those from Pfa3, Pfa4 or Erf2, and similar results were also found for Pfa3,  
374 the DHHC-CRD of which cannot be replaced by that of Swf1 or Erf2. The  
375 irreplaceability of DHHC-CRD demonstrates interaction between this domain and  
376 other regions is required for proper PAT function (Gonzalez Montoro et al., 2011).  
377 Although the acyl intermediate happened on the cysteine residue in the DHHC motif  
378 study on human DHHC3 showed that mutation of other conserved cysteines in the CRD  
379 also decreased its activity (Gottlieb et al., 2015). In addition, cysteine residues within a  
380 novel CCX<sub>7-13</sub>C(S/T) motif downstream of the conserved DHHC-CRD of human PATs  
381 DHHC5, DHHC6 and DHHC8 were also proved to be S-acylated (Yang et al., 2010).  
382 Therefore, it seems that cysteine residues in the DHHC-CRD as well as other motifs  
383 play joint roles in of PATs auto-acylation and subsequent transfer of the fatty acid to  
384 their substrate proteins. It is also interesting to note that many residues in the DHHC-  
385 CRD domain are reported to determine substrate specificity of a PAT, such as A145 and  
386 K148 in Swf1 (Gonzalez Montoro et al., 2011).

387 Some PATs also have an N-terminal ankyrin repeat (AR) domain. Usually two AR  
388 containing DHHC PATs are found in per genome, such as in mammals (Fukata et al.,  
389 2004), yeast (Roth et al., 2006), fly (Bannan et al., 2008), apicomplexan parasite (Frenal

390 et al., 2013), nematode (Edmonds and Morgan, 2014) and plants (Yuan et al, 2013). It  
391 is thought that AR can help these PAT to recognize its specific targets for S-acylation  
392 (Lemonidis et al., 2015). However, other functions of AR that are independent from S-  
393 acylation were also found in some such PATs (Harada et al., 2003; Yang and Cynader,  
394 2011; Hemsley and Grierson, 2011). For example, AR is essential for Akr1 to interact  
395 with G $\beta$  $\gamma$  dimer and to suppress cell cycle arrest induced by  $\beta$  subunit, and this process  
396 does not require Akr1 being a functional PAT (Hemsley and Grierson, 2011).

397 Both the N- and C-termini of all PATs characterized so far are highly variable and  
398 cytosolic. The highly variability of N- and C- terminal domains are believed to be  
399 essential for substrate specificity of PATs, even though there have no experimental  
400 evidence to support this at present (Huang et al., 2009; Greaves et al., 2010; Gonzalez  
401 Montoro et al., 2011). Many PATs also have a conserved aspartate-proline-glycine  
402 (DPG) motif close to the second TMD, and a threonine-threonine-asparagine-glutamate  
403 (TTxE) motif adjacent to the last TMD. Both of them are cytosolic but their roles in the  
404 function of PATs are still waiting to be explored. Another important region that contains  
405 16-amino acids and is conserved in 70% eukaryotic PATs is the PaCCT motif  
406 (Palmitoyltransferase Conserved C-Terminus). Absence of the PaCCT motif abolished  
407 the function of Pfa3 in yeast; the tyrosine residue within this motif of Swf1 is essential  
408 for its PAT activity towards Tlg1 (Gonzalez- Montoro et al., 2009).

409 The general structure and the functional/conserved domains of PATs is illustrated  
410 in **Figure 2**.

#### 411 **DHHC Proteins Are Commonly Found in Eukaryotes**

412 Since the first DHHC containing protein, Akr1 was found and proved to be a PAT from  
413 yeast in 2002, significant advances have been made in understanding of DHHC protein  
414 family in yeast, mammals, worm and plants. So far, 6 of the 7 yeast DHHC proteins  
415 have been confirmed to be PATs, and they are Akr1 (Roth et al., 2002; Lobo et al., 2002),  
416 Erf2 (Valdez-Taudas and Pelham, 2005), Swf1 (Smotryst et al., 2005), Pfa3 (Hou et al.,

417 2009), Pfa4 (Smotrys et al., 2005) and Pfa5 (Roth et al., 2006). Akr2 is highly  
418 homologous to Akr1 with a typical DHHC-CRD and two ARs, however, *akr2* mutant  
419 did not show any remarkable phenotype and there is no direct evidence to show whether  
420 it has PAT catalytic activity or not (Kihara et al., 2005; Linder and Deschenes, 2007).

421 Among the 22 human DHHC proteins (DHHC1-22), 17 were proved to have PAT  
422 activities excluding DHHC4, 11, 13, 19 and 22 (Ohno et al., 2012). One more DHHC  
423 protein (DHHC23) was found in mice but its homologue cannot be found in human  
424 (Ohno et al., 2006; Greaves and Chamberlain, 2010). *Caenorhabditis elegans* has 15  
425 DHHC-PATs, but so far only 1, SPE10 (spermatogenesis 10), was characterized in some  
426 detail and showed that it is essential for membranous organelles to deliver fibrous  
427 bodies to the spermatid (Gleason et al., 2006).

428 Plant genomes also possess various numbers of DHHC containing protein  
429 sequences. A recent survey from 31 plant species with complete genomes including  
430 Arabidopsis, identified 804 DHHC proteins. The numbers of DHHC proteins were  
431 variable in different species from 6 in *volvox carteri* to 52 in *Panicum virgatum*.  
432 Expression pattern of DHHC proteins in *Zea mays* and their response to phytohormones  
433 and abiotic stress showed that these DHHC proteins may play important roles in plant  
434 growth and development as well as stress responses (Yuan et al., 2013). Arabidopsis  
435 has 24 DHHC-containing proteins, named as ATPAT1-24 (Hemsley et al, 2005; Batistic,  
436 2012). According to their phylogenetic relationship they are divided into 3 main groups,  
437 where group A has the most members including AtPATs 1 to 9, group B consists AtPATs  
438 11-16, group C is made of AtPATs 18 to 22, whilst AtPAT10, 17, 23 and 24 do not  
439 belong to any groups (Batistic, 2012). All these putative PATs have 4 TMDs except for  
440 AtPAT15 and AtPAT17 where 3 and 6 TMDs are found respectively.

441 Similar to what was found in yeast and mammals, Arabidopsis genome also has 2  
442 ankyrin repeats containing PATs, AtPAT23 and AtPAT24 that are highly homologous.  
443 Being the first PAT identified in higher plant, AtPAT24 was confirmed to be an S-acyl  
444 transferase because it not only auto-acylated but also rescued the yeast PAT Akr1

445 knockout mutant *akr1* for its morphological and temperature sensitive defects. AtPAT24  
446 can also restore the correct localization of one of the Akrl palmitoylating proteins, the  
447 yeast casein kinase 2 (Yck2). The AtPAT24 loss-of-function mutant *tip1* exhibits  
448 defects in cell size control, pollen tube and root hair growth, as well as cell polarity  
449 (Hemsley et al., 2005). Following this, the biological functions of 3 other AtPATs have  
450 also been characterized in some detail recently. For example, three T-DNA insertion  
451 mutant alleles were identified for AtPAT10 and all showed pleiotropic defects,  
452 including cell expansion, cell division, vascular patterning, fertility and salt stress in  
453 *Arabidopsis* (Qi et al., 2013; Zhou et al., 2013). Single mutants of *atpat13* and *atpat14*  
454 are semi-dwarf and show precocious leaf senescence, and the double mutant *atpat13*  
455 *atpat14* has even stronger phenotype than each of their parent single mutant plants (Lai  
456 et al., 2015; Li et al., 2015).

457 DHHC containing proteins were also identified from other organisms. For example, 22  
458 such proteins were found in *Drosophila* (Bannan et al., 2008), 18 in *Toxoplasma gondii*,  
459 17 in *Neospora caninum*, 12 in *Trypanosome brucei*, 12 in *Plasmodium falciparum*, 11  
460 in *Plasmodium berghei*, 10 in *Cryptosporidium* species, 9 in *Theileria parva*, 8 in  
461 *Babesia bovis* and 6 in *Eimeria tenella* (Fréchal et al., 2013). It was reported that  
462 TgDHHC7 from *Toxoplasma gondii* is essential for rhoptry organelles localization and  
463 parasite invasion (Fréchal et al., 2013).

#### 464 **Expression Pattern and Subcellular Localization of PATs**

465 The spatial and temporal expression patterns of genes are very important for their  
466 cellular functions. However, only very limited information available for the expression  
467 patterns of PAT family proteins so far. It was shown that the humans DHHC1, 3-10, 12-  
468 14, 16-18 and 20-22, are ubiquitously expressed in different tissues. DHHC19 had very  
469 high expression level in testis with weak expression in thymus and small intestine while  
470 DHHC11 was only expressed in testis. In addition, only very low level of *DHHC2*  
471 transcript was present in kidney and testis, and similar low levels of *DHHC15* transcript  
472 were found in heart, brain, lung, kidney, thymus, and small intestine (Ohno et al., 2006).

473 The expression patterns of DHHC proteins in *Drosophila* were also analyzed.  
474 Among them, CG1407, CG5620, CG6017, CG6627 and CG17257 exhibited maternal  
475 expression and were enriched in neural tissues, with transcripts of all of them except  
476 for CG1407 also detected in larval brains. Some DHHC proteins were only expressed  
477 in testis, such as CG4483, CG4956, CG13029, CG17075, CG17195-17198, CG17287  
478 and CG18810, and others expressed only in ovary, such as CG5880, CG6017 and  
479 CG34449 (Bannan et al., 2008).

480 Study of the expression patterns of PATs in parasite also showed that most of them  
481 have ubiquitous expression with a few being more tissue or developmental stage  
482 specific. For instance, TgDHHC18 is specially expressed in bradyzoites, TgDHHC10  
483 at oocyst stage, PfDHHC6 and PfDHHC10 at gametocyte stage (Frenal et al., 2013;  
484 Lopez-Barragan et al., 2011).

485 In *Arabidopsis*, 19 of the 24 *AtPATs* expressed in a broad and constant pattern with  
486 transcripts detected in most tissues at all different developmental stages in *Arabidopsis*.  
487 However, *AtPAT1*, 2, 3, 11 and 21 had relatively low expression levels than other *AtPATs*,  
488 and *AtPAT2* and 3 also exhibited stronger expression in pollen (Batistič, 2012; Yuan et  
489 al., 2013). In *Oryza sativa*, 26 of the 30 *OsPATs* can express in more than one type of  
490 tissue, among which *OsPAT29* was only expressed during germination stage, *OsPAT21*  
491 and *OsPAT26* were only expressed in the internode and stamen respectively. The  
492 transcripts of *OsPAT13* and *OsPAT28* were barely detectable in the tissues examined  
493 (Yuan et al., 2013). In *Zea mays* 28 of 38 *ZmPATs* have extensive expression in different  
494 developmental stages and tissues with *ZmPAT13* and *ZmPAT22* having higher  
495 expression in anther (Yuan et al., 2013). However, in *Glycine max*, specific, rather than  
496 broad expression patterns were found where 7 *GmPATs* were specifically expressed in  
497 the flowering stage and the transcripts of the remaining 11 *GmPATs* were detected in all  
498 developmental stages except for flowering stage (Yuan et al., 2013).

499 Therefore, it is clear that most PATs exhibit a broad expression pattern in different  
500 developmental stages and tissues with a few stage or tissue specific PATs in all

501 organisms reported. This suggests that most PATs are involved in a broad range of  
502 functions in a given organism, such as Arabidopsis.

503 PATs distributed in the entire endomembrane system in the cell and this locality  
504 nature of PATs may determine the specific set of proteins they modify. For example, the  
505 plasma membrane-localized Pfa5 in yeast and DHHC5, 20 and 21 in human are the  
506 PATs involved in S-acylation mediated signal transduction of PM localized  
507 heterotrimeric G protein alpha subunit G $\alpha$ , the  $\beta$ 2-adrenergic receptor and endothelial  
508 nitric oxide synthase (Mumby et al., 1994; Loisel et al., 1996; Robinson et al., 1995;  
509 Ohno et al., 2006). The ER- and Golgi-localized DHHC proteins may be responsible  
510 for palmitoylation of de novo synthesized proteins during the processes of membrane  
511 localization and delivery to other organelles (Ohno et al., 2006). The tonoplast-localized  
512 Pfa3 in yeast palmitoylates Vac8p for its vacuolar membrane targeting (Hou et al., 2005;  
513 Smotrys et al., 2005). Therefore, to determine the subcellular localization of individual  
514 PAT is very important in order to understand its function by identifying the substrate  
515 protein(s) it modifies and signaling pathways it is involved.

516 Although PATs are found in all endomembrane systems in the eukaryotic cell they  
517 have different preference in different species as to which endomembrane compartment  
518 they are localized. For instance, in yeast, 3 PATs, Swf1, Pfa4 and Erf2 are localized at  
519 ER; Akr1 and Akr2 are localized at Golgi; while Pfa3 and Pfa5 are localized at vacuole  
520 and plasma membrane respectively (Valdez-Taubas and Pelham, 2005; Ohno et al.,  
521 2006). In human, 8 PATs are localized at ER (DHHC1, 6, 10, 11, 13, 14, 16, 19), 7 at  
522 Golgi (DHHC3, 4, 7, 8, 15, 17, 18), 2 at PM (DHHC5 and 20), 4 at both ER and Golgi  
523 (DHHC2, 9, 12 and 22), and 1 at both Golgi and PM (DHHC21) (Ohno et al., 2006).

524 Similar to the mammalian PATs, DHHC-PATs in Drosophila are also mainly  
525 localized at ER (14: CG4483, CG4676, CG4956, CG5196, CG5620, CG6627,  
526 CG10344, CG13029, CG17075, CG17195, CG17196, CG17197, CG17198 and  
527 CG17287) and Golgi (6: CG5880, CG6017, CG6618, CG8314, CG17257 and  
528 CG18810). The only exception is CG1407 which is localized at PM (Bannan et al.,

529 2008). In Apicomplexan, such as *Toxoplasma gondii*, TgDHHCs are not only localized  
530 on the common organelles such as Golgi (TgDHHC1, 5, 6, 9, 11, 12, 15 and 17), ER  
531 (TgDHHC3, 8 and 16), PM (TgDHHC4 and 13), but also the Apicomplexan-specific  
532 organelles such as IMC (TgDHHC2 and 14) and rhoptries (TgDHHC7) (Fréchal et al.,  
533 2013).

534 In some contrast to the subcellular localization described above, studies carried  
535 out on transiently expressed in tobacco leaves of the 24 Arabidopsis PATs show that 9  
536 of them are localized on PM, including AtPAT04-09, 12, 19 and 21. Therefore, it was  
537 proposed that PM is the main site for S-acylation in Arabidopsis plant (Batistic, 2012).  
538 This is different from mammalian PATs where most of them are localized in Golgi and  
539 therefore Golgi is thought to be the major S-acylation machinery (Ohno et al., 2006;  
540 Batistic, 2012). It is also interesting to note that many AtPATs that are localized on ER  
541 and PM are also associated with vesicles around them. For example, AtPAT3, 15, 17  
542 and 18 are localized at ER as well as on the vesicles associated with them; AtPAT13,  
543 20 and 22 at PM and also vesicles (Batistic, 2012). AtPAT10, 14, 16, 23 and 24 are  
544 mainly localized at Golgi, and the Golgi-localization of AtPAT10 and AtPAT14 were  
545 further confirmed in stably transformed Arabidopsis plants (Qi et al, 2013; Li et al,  
546 2015). While the main residence of AtPAT01 and AtPAT02 are the endosomal  
547 compartments AtPAT10 and AtPAT11 were found on the tonoplast (Batistic, 2012; Qi  
548 et al, 2013; Zhou et al, 2013). It is noteworthy that a few Arabidopsis PATs have dual  
549 subcellular localizations, such as AtPAT10 (Golgi and tonoplast) and AtPAT13/20/22  
550 (PM and vesicles) (Batistic, 2012; Qi et al., 2013). Similar observations were also made  
551 with some mammalian PATs (Valdez-Taubas and Pelham, 2005; Ohno et al., 2006).  
552 However, the significance of this dual-localization nature of PATs is currently unknown.

553 Little is known about how the PAT proteins achieve their respective localization in  
554 the cell. A recent study show that the lysine-based sorting signals KXX and KKXX are  
555 present in the mammalian DHHC4 and DHHC6, respectively, and it is these motifs that  
556 restrict their localization to the ER (Gorleku et al., 2011). It is also revealed that the C-



557 terminal 68 amino acids of the mammalian DHHC2 play an important role to define its  
558 subcellular localization to the ER and Golgi (Fukata et al., 2013). However, there is  
559 currently no information available on how plant PAT are targeted to individual  
560 membranes in the cell.

### 561 **The Identified PAT/Substrate Pairs**

562 As an enzyme PAT carries out its function mainly through substrate protein(s) it S-  
563 acylates. Therefore, to understand how PATs operate it is important to identify the  
564 target proteins they modify. However, to match an individual PAT and its S-acylated  
565 substrate proteins has proved to be a very difficult task so far. This is because: 1. The  
566 number of potential S-acylated proteins far exceed the number of their modifying PATs.  
567 For example, there are 7 PATs in yeast, however, ~50 S-acylated proteins were  
568 identified by a proteomic approach (Roth et al., 2006). Similarly, much more S-acylated  
569 proteins were isolated than the number of PATs present in mammals and Arabidopsis  
570 (Martin and Cravatt, 2009; Hemsley et al., 2013). Therefore, it seems most likely that  
571 at least some if not all PATs can S-acylate multiple substrate proteins, i.e., PATs do not  
572 have strict substrate specificity; 2. Many substrate proteins can be modified by more  
573 than one PATs. For instance, in yeast, the S-acylation of Vac8 is only partially reduced  
574 in the yeast PAT knock-out strain *pfa3*, thus it is most likely that Vac8 is S-acylated by  
575 Pfa3 as well as one another or other PATs (Smotrys et al., 2005). Similarly, Ras2 S-  
576 acylation is only partially suppressed in the absence of Erf2 hence other PATs are also  
577 capable to S-acylate Ras2 (Roth et al., 2006; Gonzalez Montoro et al., 2011). Therefore,  
578 these PATs have specific yet overlapping substrate specificity. For some peripheral  
579 membrane proteins in mammalian cells, their S-acylation is devoid of specificity  
580 altogether (Rocks et al., 2010). However, reports show that some PATs do have their  
581 preferentially modified proteins. For example, Swf1 in yeast prefers to function on  
582 transmembrane proteins that have cysteines close to TMDs (Roth et al., 2006). In  
583 human, integrin  $\alpha 4\beta 6$  is strictly modified by DHHC3 (Sharma et al., 2012); 3. No  
584 consensus sequences in S-acylated proteins have been found. Although many S-

585 acylated proteins have been identified and some of them are S-acylated by the same  
586 PAT, there are no consensus sequences characterised in these proteins (Gonzalez  
587 Montoro et al., 2011).

588 In yeast, each of the five PATs have been mapped to one or more substrate proteins.  
589 However, the total number of these individual substrate proteins are still far less than  
590 ~50 S-acylated proteins identified (Roth et al, 2006). For example, Akr1 S-acylates  
591 casein kinases Yck1, Yck2 and Yck3 (Roth et al., 2002). It also S-acylates sphingosine  
592 kinase Lcb4 because 60-80% of reduction in S-acylation of Lcb4 was found in *akr1*  
593 mutant yeast (Kihara et al., 2005). Other proteins that are also S-acylated by Akr1 are  
594 Meh1, Sna4 and the unknown function proteins such as Ypl199c, Ykl047w and  
595 Ypl236c (Roth et al., 2006). Therefore, Akr1 alone can S-acylate at least 9 substrate  
596 proteins in yeast. It was noted that Akr1 prefers hydrophilic proteins that tether to  
597 membranes solely through N- or C-terminal palmitoyl modifications (Roth et al., 2006).  
598 Erf2 is responsible for the S-acylation of Ras and other signalling proteins such as Rho2,  
599 Rho3, Gpa1, Gpa2 and Ste18, all of which are heterolipidated (Bartels et al., 1999;  
600 Lobo et al., 2002; Roth et al., 2006; Zhang et al., 2013). Swf1 tends to S-acylate proteins  
601 that have juxta-TMD mapping cysteines, such as SNAREs (Valdez-Taubas and Pelham,  
602 2005), mannosyltransferases including Mnn1, Mnn10 and Mnn11 and prion induction  
603 protein Pin2 (Roth et al., 2006). Pfa4 is devoted to the palmitoylation of a group of  
604 Amino Acid Permeases (AAPs). AAPs is a family of plasma membrane transporters  
605 with 12 TMDs and a conserved C-terminal Phe-Trp-Cys palmitoylation site.  
606 Experiments in *Cryptococcus neoformans* showed that Pfa4 is also responsible for PM  
607 localization of Ras1 via palmitoylation (Merino et al., 2014). On the other hand, one  
608 substrate protein can be palmitoylated by multiple PATs. For example, S-acylation of  
609 Gpa2 is mediated by both Pfa5 and Erf2 (Roth et al., 2006; Zhang et al., 2013; Greaves  
610 and Chamberlain, 2011); Meh1 was S-acylated by Pfa3 and Akr1 (Greaves and  
611 Chamberlain, 2011); and an unknown protein Yg1108 was S-acylated equally by Erf2  
612 and Pfa4 (Greaves and Chamberlain, 2011). However, so far the substrates of Akr2 has  
613 not been identified. Therefore, it is clear that both PATs and their substrate proteins are

614 highly redundant in yeast. A summary of PATs and their substrate proteins in yeast is  
 615 shown in **Table 4**.

616 **TABLE 4 | Substrates of Yeast PATs**

<b>PATs</b>	<b>Substrates</b>	<b>references</b>
<b>Akr1</b>	Lcb4, Yck1, Yck2, Yck3, Meh1, Sna4, Ypl199c, Ykl047w , Ypl236c, Vac8	Roth et al., 2002; Babu et al., 2004; Kihara et al., 2005; Roth et al., 2006; Roth et al., 2011
<b>Erf2</b> <b>(shr5)</b>	Ras1, Ras2, Rho2, Rho3, Gpa1, Gpa2, Ste18, Ycp4, Psr1, Yg1108	Bartels et al., 1999; Lobo et al., 2002; Ohno et al., 2006; Roth et al., 2006; Greaves and Chamberlain, 2011; Zhang et al., 2013;
<b>Swf1</b>	Many SNAREs, Mnn1, Mnn10 , Mnn11, Pin2	Valdez-Taubas and Pelham, 2005; Roth et al., 2006
<b>Pfa3</b>	Vac8, Meh1	Hou et al., 2005; Smotrys et al., 2005; Roth et al., 2006
<b>Pfa4</b>	APPs, Lcb4, Ras1, Yg1108	Ohno et al., 2006; Roth et al., 2006; Greaves and Chamberlain, 2011; Nichols et al., 2015
<b>Pfa5</b>	Gpa2	Greaves and Chamberlain, 2011

617

618 Many substrate proteins of mammalian PATs have also been identified in recent  
 619 years. These include GTP-binding proteins, cytoskeletal proteins, enzymes,  
 620 neurotransmitter receptors and synaptic scaffolding proteins (**Table 5**). Similar to what  
 621 is found in yeast, some proteins can be modified by more than one PAT and most PATs  
 622 can modify multiple proteins such as DHHC2, 3, 5, 7, 8, 13, 15, 17 and 21 (**Table 5**).  
 623 For instance, PSD-95, a protein that scaffolds receptors and signaling enzymes at the  
 624 postsynapse (Topinka and Brecht, 1998) can be S-acylated by DHHC2, 3, 7, 8, 15 and  
 625 17 (Fukata et al., 2004; Fukata et al., 2006; Greaves and Chamberlain, 2011; Butland  
 626 et al., 2014). SNAP-25, a t-SNARE protein that regulates neurotransmitter release, is  
 627 the substrate of DHHC2, 3, 7, 8, 15 and 17 (Greaves et al., 2009). S-acylation of a  
 628 tyrosine kinase Fyn is mediated by DHHC2, 3, 7, 15, 20 and 21 (Mill et al., 2009). All  
 629 these mentioned PAT/substrates and other pairs are listed in **Table 5**. On the other hand,  
 630 one PAT can palmitoylate multiple substrate proteins. For example, DHHC2

631 palmitoylates cytoskeleton-associated protein 4 (CKAP4) and AKAP79/150 (Chavda et  
632 al., 2014; Keith et al., 2012); DHHC3 does integrin  $\alpha6\beta4$ , Calmodulin-dependent  
633 protein kinase isoform 1 $\gamma$  (CaMKI $\gamma$ ), NMDA receptor subunits 2A and 2B (NR2A/B)  
634 and DR4 (Sharma et al., 2012; Takemoto-Kimura et al., 2007; Hayashi, et al., 2009;  
635 Yeste-Velasco et al., 2015); the S-acylation of Grip1b,  $\delta$ -catenin, Flotillin-2,  
636 somatostatin receptor 5 and Ankyrin-G is carried out by DHHC5 (Brigidi et al., 2015).  
637 In the same fashion many other proteins are also palmitoylated by specific PATs (**Table**  
638 **5**). Importantly, some DHHC proteins have been indicated to be involved in certain  
639 diseases, such as DHHC8 in schizophrenia, DHHC9 and 15 in X-linked mental  
640 retardation, DHHC17 in Huntington's disease and many PATs are involved in different  
641 types of cancer including DHHC2, 3, 7, 9, 11, 14, 17, 20 and 21 (Chavda et al., 2014;  
642 Yeste-Velasco et al., 2015). However, for some of them, their specific substrate proteins  
643 have not been identified.

644 **TABLE 5 | Mammalian PATs and their (regulated) target proteins**

<b>PAT</b>	<b>Targets</b>	<b>References</b>
<b>DHHC2</b>	PSD-95, CKAP4, SNAP23/25, eNOS, Fyn, NDE1, NDEL1, CD9/151, ABCA1, AKAP79/150	Fukata et al., 2004; Huang et al., 2004; Fernandez-Hernando et al., 2006; Sharma et al., 2008; Zhang et al., 2008; Shmueli et al., 2009; Singaraja et al., 2009; Greaves et al., 2010; Chavda et al., 2014
<b>DHHC3 (GODZ)</b>	PSD-95, SNAP23/25/25b, G $\alpha$ , CSP, Integrin $\alpha6\beta4$ , GABA $\gamma$ 2, eNOS, GluR1/2, GAD65, STREX, Fyn, BACE1, NDE1, NDEL1, NCAM140, CaMKI $\gamma$ , NR2A/B, DR4, PI4KII	Fukata et al., 2004; Hayashi et al., 2005; Fang et al., 2006; Fernandez-Hernando et al., 2006; Takemoto-Kimura et al., 2007; Greaves et al., 2008; Huang et al., 2009; Mill et al., 2009; Shmueli et al., 2009; Tsutsumi et al., 2009; Vetrivel et al., 2009; Greaves et al., 2010; Tian et al., 2010; Yeste-Velasco et al., 2015
<b>DHHC4</b>	BACE1	Vetrivel et al., 2009
<b>DHHC5</b>	Grip1b, $\delta$ -catenin, Flotillin-2, somatostatin receptor 5, Ankyrin-G, STREX	Tian et al., 2010; Kokkola et al., 2011; Li et al., 2012; Thomas et al., 2012; Brigidi et al., 2014; Brigidi et al., 2015
<b>DHHC6</b>	Chaperone calnexin	Lakkaraju et al., 2012
<b>DHHC7</b>	PSD-95, G $\alpha$ , CSP, Fyn, eNOS, SNAP25/23/25b, GABA $\gamma$ 2, STREX,	Fukata et al., 2004; Fang et al., 2006; Fernandez-Hernando et al., 2006; Fukata et al., 2006; Greaves et al., 2008; McCormick et al., 2008; Ponimaskin et

	BACE1, NDE1, NDEL1, NCAM140, sortillin, PDE10A2, CD9, ER, PR, AR, PI4KII	al., 2008; Greaves et al., 2009; Shmueli et al., 2009; Tsutsumi et al., 2009; Vetrivel et al., 2009; Charych et al., 2010; Greaves et al., 2010; Tian et al., 2010; Ohno et al., 2012
<b>DHHC8</b>	eNOS, SNAP25, paralemmin-1, GAD65, PSD95, PSD93	Fernandez-Hernando et al., 2006; Mukai et al., 2008; Huang et al., 2009
<b>DHHC9</b>	H- and N-Ras, STREX	Swarthout et al., 2005; Tian et al., 2010
<b>DHHC12</b>	ABCA1	Singaraja et al., 2009; Chavda et al., 2014
<b>DHHC13</b> <b>(HIP14L)</b>	MT1-MMP, HTT, GAD65	Huang et al., 2009; Saleem et al., 2010; Song et al., 2014
<b>DHHC15</b>	PSD95, GAP43, SNAP25b, CSP, GABA <sub>A</sub> γ2, Fyn, BACE1, CD151, CI-MPR, sortillin	Fukata et al., 2004; Fang et al., 2006; Greaves et al., 2008; McCormick et al., 2008; Sharma et al., 2008; Mill et al., 2009; Vetrivel et al., 2009; Greaves et al., 2010
<b>DHHC17</b> <b>(HIP14)</b>	PSD95, CLIP3, CSP, GAD65, GAP43, GLUR1/2, GPM6A, HTT, JNK3, Lck, SNAP25/23/25b, STREX, SYT1, SPRED1/3, Ras	Fukata et al., 2004; Greaves et al., 2008; Huang et al., 2009; Mill et al., 2009; Greaves et al., 2010; Tian et al., 2010; Ohno et al., 2012; Butland et al., 2014
<b>DHHC18</b>	H- and N-Ras, Lck	Fukata et al., 2004
<b>DHHC19</b>	R-Ras, PDE10A2	Charych et al., 2010; Baumgart et al., 2010
<b>DHHC20</b>	Fyn, BACE1, ABCA1	Mill et al., 2009; Singaraja et al., 2009; Vetrivel et al., 2009
<b>DHHC21</b>	PECAM1, SOD1, Lck, eNOS, Fyn, ABCA1, ER, PR, AR	Fernandez-Hernando et al., 2006; Takemoto-Kimura et al., 2007; Mill et al., 2009; Vetrivel et al., 2009; Antinone et al., 2013; Akimzhanov and Boehning, 2015; Yeste-Velasco et al., 2015

645

646 Very little information is available for PAT/substrate pairs in other organisms. The  
647 only PAT/substrate pair characterized was in *Plasmodium falciparum* where PfDHHC1,  
648 an apicomplexan-specific and inner membrane complex-localized PAT, has identical  
649 expression pattern to two S-acylated proteins PfISP1 and PfISP3 (Wetzel et al., 2014).

650 In plant, the only putative PAT/substrates pairing identified is ATPAT10/AtCBL2,  
651 3, 6. This was achieved by transient expression of AtCBL2, AtCBL3 and AtCBL6 in  
652 Arabidopsis protoplast, showing that the tonoplast localization of AtCBLs is lost in  
653 protoplast prepared from AtPAT10 loss-of-function mutant (Zhou et al., 2013).

654 Therefore, although many hundreds of S-acylated proteins, including putative ones  
655 isolated by large proteomic approaches were identified from different species at present,  
656 there are many more to come in the future due to the readily available proteomics  
657 facilities in large institutions. A framework for characterizing PAT/substrate selectivity  
658 is urgently required to set out to match individual PATs and their S-acylated substrate  
659 proteins in order to understand the mechanism of S-acylation in individual organism  
660 and in general.

661

## 662 **DE-S-ACYLATION**

663 Similar to phosphorylation and ubiquitination, S-acylation process is reversible, which  
664 makes it a very important lipid modification of proteins (Hemsley, 2009). S-acylation  
665 turnover by de-S-acylation, can be constitutive or stimulated (Smotrys and Linder,  
666 2004). Ras proteins were the first proteins to be reported to have dynamic S-acylation  
667 with different H-Ras has different de-S-acylation rates (Baker et al., 2003). S-acylation/  
668 de-S-acylation of Fyn, a member of the Src kinase family, happens with a half-life of  
669 1.5-2 hours (Wolven, et al., 1997; Zeidman et al., 2009). The de-S-acylation of G $\alpha$   
670 subunits is stimulated by the activation of G-protein-coupled receptors (Mumby et al.,  
671 1994; Linder and Deschenes, 2007). De-S-acylation of PSD-95 is enhanced by neuronal  
672 activity (El-Husseini et al., 2002).

673 At present only four protein thioesterases have been identified to catalyze the de-  
674 S-acylation process, including acyl protein thioesterases 1 (APT1) and 2 (APT2),  
675 palmitoyl thioesterases 1 (PPT1) and 2 (PPT2) (Tomatis et al 2010; Hornemann, 2015).

676 These enzymes carry out the de-S-acylation step in which the palmitate or other long  
677 chain fatty acids are removed from the S-acylated proteins (Linder and Deschenes,  
678 2007). APT1 was first found in rat liver as a lysophospholipase and its substrates  
679 include Ras, G $\alpha$  subunit, RGS4, SNAP-23 and eNOS (Toyoda et al., 1999; Akimzhanov  
680 and Boehning, 2015). APT2 was reported to de-S-acylate the growth-associated protein  
681 43 (Tomatis et al., 2010). PPT1 is a soluble lipase that is localized in lysosomes and it  
682 is responsible for the degradation of S-acylated proteins (Linder and Deschenes, 2007;  
683 Chavda et al., 2014). The loss-of-function of PPT1 resulted in severe infantile neuronal  
684 ceroid lipofuscinosis (Vesa et al., 1995). PPT2 has very limited acyl protein thioesterase  
685 activity, which prefers de-S-acylating short-chain lipid substrate. Interestingly, study  
686 has shown that it is up-regulated in obesity (Burger et al., 2012; Fox et al., 2012).

687 It is surprising that only four thioesterases have been identified so far yet many  
688 hundreds of S-acylated proteins were isolated from different genomes. The  
689 explanations for this could be: 1) thioesterases are broad specificity enzymes, each of  
690 which can de-S-acylate a wide range of substrates; 2) not all S-acylated proteins  
691 undergo de-S-acylation; 3) of course, it could be because many more thioesterases have  
692 not been found (Chavda et al., 2014). There currently no protein thioesterases have been  
693 identified from plant.

694

## 695 **MECHANISM OF PROTEIN S-ACYLATION**

696 It is well recognized that DHHC proteins transfer acyl group via a two-step catalytic  
697 mechanism in which the enzyme first modifies itself with palmitate (or other long chain  
698 fatty acids) in a process termed autoacylation. The enzyme then transfers the acyl group  
699 from itself onto its substrate proteins. However, the number and location of the S-  
700 acylated cysteines of a given PAT in the autoacylated intermediate is unknown. It is  
701 well accepted that the cysteine in the DHHC motif is the auto-S-acylation site because  
702 mutation in this residue results in loss of auto-acylation of many characterized PATs  
703 from yeast (Montoro et al., 2011), mammals (Ohno et al., 2012) and Arabidopsis

704 (Hemsley et al., 2005; Qi et al., 2013). However, cysteines in other positions of PATs  
705 such as the CRD and other domains may also be autoacylated (Gottlieb et al., 2015).  
706 For instance, DHHC3 has 6 auto-S-acylation sites where 5 in the CRD, including Cys-  
707 132, Cys-133, Cys-146, Cys-157 and Cys-163, 1 in the N-terminal domain (Cys-24)  
708 (Gottlieb et al., 2015).

## 709 **Techniques Used for Prediction and Confirmation of S-acylated Cysteines in** 710 **Proteins**

711 There is no consensus for sequences in the S-acylated proteins despite that many such  
712 proteins have been isolated through proteomics approach or individually confirmed via  
713 radioactive labelling or/and mutation studies. Nevertheless, it is noted that: 1) in some  
714 S-acylated soluble proteins the cysteine residues that are S-acylated are frequently  
715 surrounded by basic or hydrophobic amino acids, such as GAP-43 (Liu et al., 1993) at  
716 N-terminal motif, Yck2 (Roth et al., 2002) at C-terminal motif and SNAP-25b (Lane  
717 and Liu, 1997) at cysteine string motif (Smotrys and Linder, 2004); 2) in other S-  
718 acylated soluble proteins the Cys residue is located near the prenylated or myristoylated  
719 residues, resulting in the so-called dual lipidation. These proteins include the  
720 Arabidopsis  $\alpha$  and  $\gamma$  subunits of heterotrimeric G protein (Adjobo-Hermans et al., 2006;  
721 Zeng et al., 2007), small GTPases (Roth et al., 2006; Zhang et al., 2013; Deschenes et  
722 al., 1990; Bartels et al., 1999); 3) for transmembrane proteins, the Cys residues are often  
723 situated in the cytoplasmic regions of membrane-spanning regions (Roth et al., 2006;  
724 Ohno et al., 2012). For instance, the S-acylation of C261-263 triplet in death receptor  
725 4 (DR4) (Rossin et al., 2009) and C474 in  $\beta$ -secretase BACE1 (Motoki et al., 2012)  
726 promotes their association with lipid raft.

727 Based on the above information several software packages have been developed to  
728 predict the S-acylated cysteines, such as a clustering and scoring strategy known as  
729 CSS-Palm (Zhou et al., 2006), which has been updated to the latest version CSS-Palm  
730 4.0 (freely available at <http://csspalm.biocuckoo.org/>), incremental feature selection  
731 (IFS)-Palm (Hu et al., 2011), weight, amino acid composition and position specific



732 scoring (WAP)-Palm (Shi et al., 2013) and PalmPred (Kumari et al., 2014). All of them  
733 are on-line so that one can input the protein sequence of interest to predict the possibility  
734 of its S-acylation and where the Cys residues are located within the sequence. The  
735 prediction data from these platforms can then be confirmed experimentally. These  
736 techniques include:

737 1. PAT inhibitors. The palmitate analog, 2-bromopalmitate (2-BP) is the most  
738 commonly used inhibitor of S-acylation, which inhibits palmitoylation in cells and PAT  
739 activity of DHHC proteins *in vitro* (Webb et al., 2000; Fukata et al., 2004; Jennings et  
740 al., 2009). However, it lacks specificity and can also inhibit myristoylation and reduce  
741 de-acylation through inhibiting activities of acyl-protein thioesterases (Webb et al.,  
742 2000; Pedro et al., 2013). Tunicamycin and cerulenin are also used to inhibit S-acylation,  
743 but similar effect was found as 2-BP (Patterson and Skene, 1995; Lawrence et al., 1999).  
744 Recently, a compound, 2-(2-hydroxy-5-nitro-benzylidene)-benzo[*b*]thiophen-3-one,  
745 was shown to have more specificity, but it does not have selectivity for specific PAT,  
746 i.e., it inhibits activities of all PATs (Jennings et al., 2009), which means it still cannot  
747 be used to study the function of individual PAT. Therefore, it is clear that results  
748 obtained from these inhibitors should be further validated by mutational or biochemical  
749 analysis.

750 2. Mutational analysis to change the potential S-acylated Cysteine to alanine or  
751 serine. Both alanine and serine were frequently used to replace the cysteine to achieve  
752 similar results (Hemsley et al., 2005; Qi et al., 2013; Li et al., 2015). Cysteine and serine  
753 have very similar structure, when the cysteine is mutated to serine it can maintain the  
754 size and the properties of the putative S-acylated protein, in this case, serine is a better  
755 substitution for cysteine. However, compare to alanine, serine is more hydrophilic than  
756 cysteine and might also cause unwanted side chain effects (Nagano et al., 1999). In this  
757 specific study, both alanine and serine as the substitutions for cysteine are accepted so  
758 far. This is followed by comparing the effect on the differences of functions or the  
759 subcellular localizations to native protein. If a difference was found the proteins were

760 most likely S-acylated at the cysteine residues that were mutated.

761 3. Biochemical assays to analyze the attachment of fatty acids of the individual  
762 proteins. This includes: 1) traditionally feed with tritiated fatty acids followed by  
763 exposures to X-ray film (Lavy et al., 2002); 2) azido-alkyne CLICK-chemistry (Martin  
764 & Cravatt, 2009); 3) Acyl-exchange, or Biotin-switch assay (Wan et al., 2007; Hemsley  
765 et al., 2008); and 4) direct resin immobilisation (Forrester et al., 2010).

766 4. Direct detection of the S-acyl group by gas chromatography–mass spectrometry  
767 (GC-MS) analysis. The identification of lipid groups attached to proteins can help to  
768 understand the biophysical properties of the protein. This method has been successfully  
769 used to demonstrate S-acylation of CBL1 and CBL2, which are attached by palmitate  
770 and/or stearate (Batistic et al., 2008; Batistic et al., 2012).

771

## 772 **SPECIFICITIES OF PAT-SUBSTRATE INTERACTION**

773 Although it is generally accepted that PATs are lacking specificity towards their  
774 substrate proteins and vice versa studies in yeast showed that some PATs do exhibit  
775 preference to some substrate protein(s) compared than others. For instance, Akr1  
776 prefers to S-acylate soluble proteins at their N- or C-terminus, such as Ypl236c S-  
777 acylated at N-terminal cysteine and Yck1 S-acylated at C-terminal cysteine. Erf2 and  
778 Pfa5 show preference for pre-lipidated substrates, such as prenylated Ras1 and Ras2,  
779 myristoylated Gpa1 and Gpa2. Swf1 and Pfa4, on the other hand, prefer single and  
780 multiple transmembrane proteins such as SNAREs and AAPs (Roth et al., 2006; Ohno  
781 et al., 2012). Similar conclusions were made from studies of mammalian DHHC  
782 proteins where it was found that DHHC3, 7, 8 and 14-17 had high activities towards  
783 soluble proteins, while DHHC2, 20 and 21 were highly active to integral membrane  
784 proteins (Ohno et al., 2012). However, it was also noted that most DHHC proteins in  
785 both yeast and mammals had overlap activity to modify pre-lipidated substrates, such

786 as 4 yeast PATs and 16 mammalian PATs can all S-acylate the myristoylated Gpa2  
787 (Ohno et al., 2012).

788 The question here is how PATs and their substrates recognize each other? To  
789 address this, studies were carried out on some PATs and their S-acylated proteins in  
790 mammalian system. It was reported that the AR domain of the two ankyrin repeat  
791 containing PATs DHHC13 and 17 in mammals can act as substrate-recruiting signal and  
792 recognizes the [VIAP][VIT]XXQP motif that is shared between some S-acylated  
793 proteins including SNAP25/23, CSP, HTT and CLIP3 (Lemonidis et al., 2015). Fusing  
794 the AR domain of DHHC17 to the N terminus of DHHC3 that lacks an AR domain, can  
795 make DHHC3 a PAT for HTT which also supports the notion that the AR domain  
796 contributes to the substrate specificity of DHHC17 for HTT (Huang et al., 2009).  
797 DHHC3 and 7 interact with GABA<sub>A</sub>γ2 through a 14-amino acid cysteine rich domain  
798 (Fang et al., 2006). DHHC7 has two splicing isoforms, the longer one has additional  
799 111bp compared to the shorter one, which might possess its tissue-specific function  
800 since it expresses specifically in placenta, lung, liver, thymus and small intestine (Ohno  
801 et al., 2006). The recognition and S-acylation of PSD95 by DHHC17 depend on the N-  
802 terminal 13 amino acids of PSD-95 (Huang et al., 2009). The cysteine rich 'CCPCC'  
803 motif of PI4KII is required for its S-acylation by DHHC3 and DHHC7 (Lu et al., 2012).  
804 Subtle changes in the S-acylation domains of proteins can alter their PAT specificity,  
805 which were proved from SNAP23/25 (Greaves et al., 2010). For instance, a SNAP25  
806 mutant which lacks a proline located 25 residues downstream of the S-acylated domain  
807 can only be modified by DHHC3 but not DHHC17 (Greaves et al., 2009). Therefore,  
808 specific domains in PATs and their substrate proteins are required for recognition and  
809 S-acylation to occur.

810 Subcellular localizations of PATs can have a profound effect on the type of proteins  
811 it can S-acylate (Greaves and Chamberlain, 2011). A transmembrane protein might only  
812 have access to be S-acylated by the PATs localized on the same membrane. For example,  
813 the PM-localized Gpa1 is S-acylated by the PM-localized Pfa5 in yeast (Ohno et al.,

814 2006); tonoplast-localization of AtCBL2 and AtCBL3 is via S-acylation carried out by  
815 AtPAT10 which is also localized on tonoplast in Arabidopsis (Zhou et al., 2013).

816 For S-acylation of a protein to occur its prior membrane attachment via TMD,  
817 another lipid modification or protein-protein interaction is often acquired (Hemsley,  
818 2015). For instance, TEM8 localizes at PM with one TMD, S-acylation of which  
819 negatively regulate its raft association (Abrami et al., 2006). Some proteins require  
820 another lipid modification such as myristoylation to target to certain membrane first  
821 before the S-acylation can take place. AtCBL1 is one of these proteins where  
822 myristoylation targets it on the ER, then the unknown ER-localized PAT S-acylates the  
823 myristoylated AtCBL1. This dual-lipidated AtCBL1 can subsequently be trafficked to  
824 the PM (Batistič et al., 2008). It was reported that the N-terminal 12-amino acid peptide  
825 of AtCBLs is sufficient to mediate the dual lipid modification and target to PM or  
826 tonoplast (Batistic et al., 2008). Therefore, the localization of a specific PAT for a given  
827 S-acylated substrate protein relies on where this protein is localized after the first lipid  
828 modification.

### 829 **Important Molecules That Are Involved In S-Acylation**

830 Special molecules have either positive or negative effect on S-acylation of certain  
831 proteins. These molecules could be another protein, hormone, ions or protein inhibitors.  
832 For instance, although most DHHC-PATs in mammals can catalyse S-acylation  
833 independently DHHC9 needs a Golgi-localized protein GCP16 to specially  
834 palmitoylate H- and N-Ras (Swarthout et al., 2005). Ykt6, which possibly works as a  
835 co-factor of Pfa3 enhanced the S-acylation and vacuole localization of Vac8 (Dietrich  
836 et al., 2004; Hou et al., 2005; Meiringer and Ungermann, 2006). S-acylation and  
837 localization of Ras protein is catalysed by the Erf2p-Erf4p complex in yeast (Zhao et  
838 al., 2002). Zinc ion is tightly bound to the cysteine rich domain of the DHHC3, which  
839 is essential for its structural integrity and PAT activity (Gottlieb et al., 2015).  
840 Selenoprotein K (SelK), an 11-kDa endoplasmic reticulum protein of unknown function  
841 (Shchedrina et al, 2011) is required for the S-acylation of both IP3R (inositol-1, 4, 5-

842 triphosphate receptor) and CD36 (Fredericks and Hoffmann, 2015). S-acylation of  
843 PI4KII is cholesterol-dependent (Lu et al., 2012). Some compounds might have  
844 negative effect to specific S-acylation, such as curcumin can prevent S-acylation of  
845 integrin  $\beta$ 4 by DHHC3 in breast cancer cells (Coleman et al., 2015). Understanding the  
846 involvement of these molecules in S-acylation could provide important information in  
847 designing and developing new drugs to target the disease and cancer-related S-acylation  
848 machinery.

849

## 850 **CONCLUSION AND FUTURE PERSPECTIVES**

851 Ever since the discovery of the first S-acyltransferase, Akr1 from yeast in 2002, which  
852 lead to the realization of protein S-acylation being an enzymatic process rather than a  
853 simultaneous addition of a long chain fatty acid to proteins, research on S-acylation of  
854 proteins has accelerated in a remarkable speed in the past decade. Yeast, as a simple  
855 unicellular model eukaryote, has been the first choice for researchers to study S-  
856 acylation. The knowledge learnt from the yeast system has then been applied in guiding  
857 similar studies in other organisms. As such the important roles of protein S-acylation in  
858 growth and development, especially in different human diseases, such as cancers, have  
859 hence attracted much attention and become a hot area of research in recent years.

860 Although progress has been made toward understanding various aspects of protein  
861 palmitoylation the corresponding research in plants is trailing behind that in yeast and  
862 mammals. Judging from the wide arrays of S-acylated proteins identified recently from  
863 Arabidopsis and Poplar by proteomic studies it is clear that S-acylation plays variable  
864 and important roles in plant growth, development and environmental adaption  
865 (Hemsley et al, 2013; Srivastava et al., 2016). The knowledge learnt and methodologies  
866 developed from yeast and mammals will no doubt provide important clues and  
867 necessary tools for us to conduct more efficient research on S-acylation in plants in the  
868 coming years. Specifically, 1. the roles of the remaining 21 AtPATs in Arabidopsis will  
869 need to be characterized. PATs from other plant species, such as poplar, especially with

870 its S-acylated proteins being isolated recently, will also need to be characterized to see  
871 if plant PATs share functional similarity, this will further validate the data obtained from  
872 Arabidopsis PATs so far; 2. To match individual PATs with their S-acylated substrate  
873 proteins in Arabidopsis and poplar. At present, the only plant PAT with tentative mapped  
874 substrate proteins is the Arabidopsis AtPAT10 where it was found that the tonoplast  
875 localization of transiently expressed CBL2,3,6 were lost in the protoplast prepared from  
876 leaf cells of AtPAT10 loss-of-function mutant plant (Zhou et al, 2013). This indicates  
877 that AtPAT10 functions in calcium signaling and salt stress through the actions of these  
878 CBLs. Similar approaches could be used to map other PAT/substrate(s) pairs in  
879 Arabidopsis and poplar. This will provide further insights to substrate specificity of  
880 PATs and molecular mechanisms how PATs function in plants; 3. De-palmitoylation  
881 enzymes. S-acylation of proteins is a reversible process where S-acylation is catalyzed  
882 by PATs and De-palmitoylation is by acyl protein thioesterases. While 4 such enzymes  
883 have been identified and characterized from mammals none from plant. Therefore,  
884 research in this area is paramount.

885

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1604

Provisional

1605 **FIGURE LEGENDS**

1606

1607 **FIGURE 1** | Formulae of N-myristoylation, S-acylation and prenylation. For N-  
1608 myristoylation, a 14-carbon myristoyl group is covalently attached by an amide bond  
1609 to the alpha-amino group of an N-terminal glycine (G, in red); S-acylation is the  
1610 attachment of a 16-carbon palmitate to cysteine residue (C, in red) via thioester bond;  
1611 and Prenylation makes a 15-carbon farnesyl link to the CaaX cysteine residue in C-  
1612 termini.

1613

1614 **FIGURE 2** | Topology structure and conserved domains of PATs. Most PATs have 4  
1615 transmembrane domains (TMDs, blue columns) and their N- and C-termini are in the  
1616 cytoplasm. A highly conserved catalytic DHHC-CRD (aspartate-histidine-histidine-  
1617 cysteine cysteine rich domain) resides between the 2nd- and 3rd-TMDs. The majority  
1618 of PATs also have the DPG (aspartate-proline-glycine), TTxE (threonine-threonine-any  
1619 amino acid-glutamic acid) and PaCCT (Palmitoyltransferase Conserved C-Terminus)  
1620 domains, and all of them are cytosolic.

Provisional



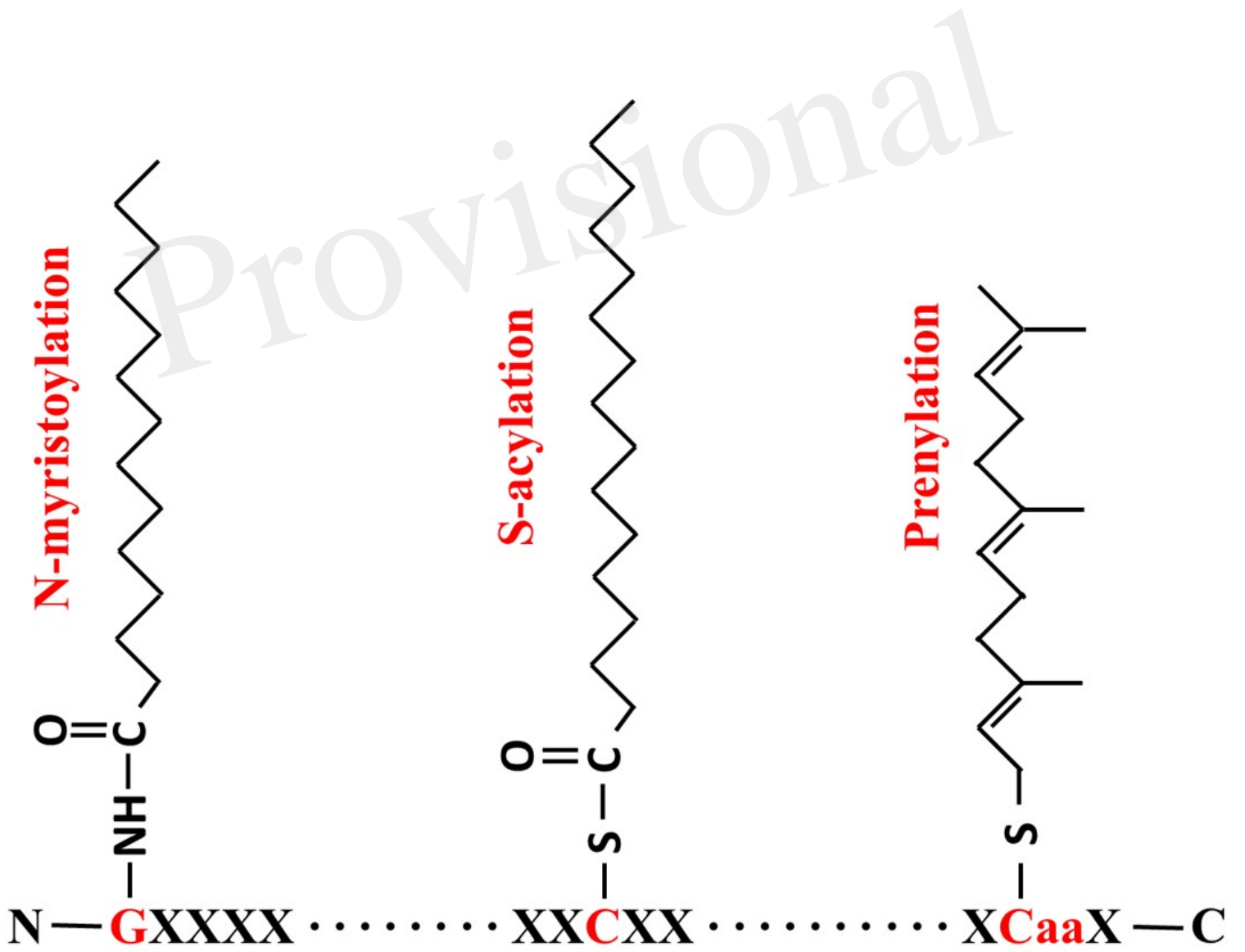


Figure 02.JPEG

