

1 **Title Page**

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3 **The Proteolytic Landscape of an Arabidopsis Separase-Deficient Mutant Reveals Novel**  
4 **Substrates Associated With Plant Development**

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31 **Abstract**

32 Digestive proteolysis executed by the proteasome plays an important role in plant development.  
33 Yet, the role of limited proteolysis in this process is still obscured due to the absence of studies.  
34 Previously, we showed that limited proteolysis by the caspase-related protease separase  
35 (EXTRA SPINDLE POLES [ESP]) modulates development in plants through the cleavage of  
36 unknown substrates. Here we used a modified version of the positional proteomics method  
37 COmbined FRActional DIagonal Chromatography (COFRADIC) to survey the proteolytic  
38 landscape of wild-type and separase mutant *RADIALLY SWOLLEN 4* (*rsw4*) root tip cells, as  
39 an attempt to identify targets of separase. We have discovered that proteins involved in the  
40 establishment of pH homeostasis and sensing, and lipid signalling in wild-type cells, suggesting  
41 novel potential roles for separase. We also observed significant accumulation of the protease  
42 PRX34 in *rsw4* which negatively impacts growth. Furthermore, we observed an increased  
43 acetylation of N-termini of *rsw4* proteins which usually comprise degrons identified by the  
44 ubiquitin-proteasome system, suggesting that separase intersects with additional proteolytic  
45 networks. Our results hint to potential pathways by which separase could regulate development  
46 suggesting also novel proteolytic functions.

47

## 48 **Introduction**

49 Separase (family C50, clan CD) is an evolutionary conserved cysteine protease participating in  
50 the metaphase-to-anaphase transition and plant development (Liu & Makaroff, 2006; Moschou  
51 & Bozhkov, 2012). In *Arabidopsis thaliana*, separase localizes to microtubules (Moschou *et*  
52 *al.*, 2016a), the plasma membrane and the endosomal compartment *trans*-Golgi Network  
53 (TGN)(Moschou *et al.*, 2013), which acts as a hub for protein vesicle trafficking.

54 Since loss-of-function *Arabidopsis* separase mutants are embryo lethal (Liu &  
55 Makaroff, 2006), the roles of separase beyond cell division are studied by the conditional  
56 temperature-sensitive loss-of-function allele *RADIALLY SWOLLEN 4* (*rsw4*). The *rsw4*  
57 mutation renders separase unstable at 28°C (Wu *et al.*, 2010; Yang *et al.*, 2011; Moschou *et*  
58 *al.*, 2016a). *Rsw4* shows root phenotypes reminiscent of compromised auxin distribution, with  
59 perturbed gravitropism, cell expansion and overall development (Moschou *et al.*, 2013).  
60 Proteolytic activity of separase is indispensable in development since the proteolytic inactive  
61 separase mutant could not rescue *rsw4* defects. Yet, the proteolytic targets of separase remain  
62 obscure.

63 Implementation of positional proteomics is the most direct method for unbiased  
64 identification of protease substrates (Plasman *et al.*, 2013). When sampling complex  
65 proteomes, protease substrates can be identified by exploiting the chemical reactivity of the  
66 alpha-amino groups. Such newly introduced  $\alpha$ -amino groups are typically referred to as neo-  
67 N-termini (neo-Nt). The N-terminal COmbined FRActional DIagonal Chromatography  
68 technology (COFRADIC; (Gevaert *et al.*, 2003) depletes non-Nt-peptides, thereby enriches for  
69 Nt-peptides and further allows the specific identification of neo-Nt through a combination of  
70 stable isotope labelling of neo-Nt, tandem mass spectrometry and bioinformatics.

71 Here we used COFRADIC to identify potential separase substrates that may allow  
72 delineating new signalling pathways relevant to organ growth and development. By contrasting  
73 the proteolytic landscape of wild-type (WT) and *rsw4* we infer potential proteolytic targets  
74 relevant to vesicular trafficking, lipid signaling and root growth control. We succinctly discuss  
75 the most relevant identified proteins and suggest potential molecular mechanisms for further  
76 exploration.

77

## 78 **Results and Discussion**

79 *Inducible depletion of separase phenocopies rsw4 PIN2 polarity defects*

80 A potent readout for vesicular trafficking defects and early events imposed by the absence of  
81 *rsw4* is the mis-localization of the auxin efflux carrier PINFORMED2 (PIN2) in root cortex

82 cells, responsible for auxin flow maintenance (Moschou *et al.*, 2013). Depletion of separase in  
83 *rsw4* causes PIN2 localization switch from the proper rootward to the shootward side of the  
84 plasma membrane in *rsw4* root cortex cells at the restrictive temperature. This PIN2  
85 localization switch correlates with the reduced activity of a GNOM-related vesicular trafficking  
86 pathway in *rsw4*. A functional GNOM-related pathway requires proteolytic cleavage of an as  
87 yet unknown substrate(s) by separase, since the protease-dead allelic variant of separase could  
88 not complement PIN2 localization defects of *rsw4* (Liu & Moschou, 2017).

89 We have established that treatment of *rsw4* seedlings for up to 24 h at the restrictive  
90 temperature (28°C) disrupts separase activity and causes vesicular trafficking defects, but does  
91 not compromise the rate and pattern of cell division and the identity of root cells (Moschou *et al.*  
92 *et al.*, 2013). We argue that this experimental setup enables to study roles of separase beyond its  
93 mitotic role (Moschou *et al.*, 2016b). Note that a temperature of 28°C falls within the  
94 physiological temperature range for Arabidopsis growth (Faden *et al.*, 2016). Yet, to ensure  
95 that the *rsw4*-mediated effect in PIN2 polarity is not due to the temperature shift, we surveyed  
96 PIN2 polarity in dexamethasone inducible RNAi lines of separase (DEX:ESP-RNAi)  
97 expressing PIN2 under its native promoter (Moschou *et al.*, 2013)(**Fig. 1**). We observed PIN2  
98 polarity loss in the DEX:ESP-RNAi lines already 12 h after dexamethasone application. Yet,  
99 no effect was observed in the corresponding WT plants expressing PIN2 after dexamethasone  
100 application (data not shown). These results suggests that the PIN2 localization switch does not  
101 depend on temperature and therefore our experimental setup can be used to infer proteolytic  
102 targets of separase.

103

#### 104 *Systematic characterization of root tip N-terminomes*

105 Proteins are always synthesized with methionine (iMet) as the first Nt residue even when a  
106 non-canonical translation initiation codon (i.e. non-AUG) is used. However, largely depending  
107 on the nature of the second amino acid residue, this first iMet is often cleaved from mature  
108 proteins by the N-terminal Met excision (NME) pathway through the Met aminopeptidase  
109 (MAP)(Bonissone *et al.*, 2013). The N-terminome consists of all peptides that possess mature  
110 protein Nt ( $\pm$ iMet) and neo-Nt that are proxies of *in vivo* proteolytic events.

111 To gain insights into the proteolytic landscape of root cells we analysed the *in vivo* N-  
112 terminomes of root tips from 7-d-old seedlings of WT and *rsw4* plants grown at the restrictive  
113 temperature for 24 h by a modified version of COFRADIC (**Materials and Methods** and **Fig.**  
114 **2A, B**). COFRADIC also enables the identification of sequences spanning the scissile peptidic  
115 bonds (Vandekerckhove *et al.*, 2004; Stes *et al.*, 2014). The root tip proteomes of WT and *rsw4*

116 were labelled with N-hydroxysuccinimide esters of either light (WT) or heavy (*rsw4*) isotopic  
117 variants of butyric acid to mass tag both Nt arising from translation and neo-Nt. The differential  
118 mass tagging of Nt (heavy versus light) enabled us to determine the proteome origin (*rsw4* or  
119 WT, respectively) of each labelled peptide.

120 To identify translational Nt (peptides containing either the iMet or the 2<sup>nd</sup> amino acid  
121 residue of the protein), we filtered-out peptides carrying neo-Nt (peptides containing Nt beyond  
122 the 2<sup>nd</sup> residue). Out of the total number of 6,974 peptides identified (all present in both  
123 genotypes with one exception discussed later), 2,840 possessed translationally yielded Nt  
124 converging to 1,057 proteins (**File S1**). Out of 149 unique proteins retaining iMet, 58 proteins  
125 had as 2<sup>nd</sup> residue glutamate (E), 30 aspartate (D) and 20 lysine (K) (E>D>K>>X) (**Fig. 3A**),  
126 suggesting that residues with sufficiently large gyration radius block MAP activities (Van  
127 Damme *et al.*, 2011; Van Damme *et al.*, 2012). Protein species devoid of iMet showed strong  
128 preference for alanine (A; 29%) and serine (S; 11%) at the 2<sup>nd</sup> position (P1'; P1<sup>iMet</sup>-P1'-P2'-  
129 ...-Pn')(**Fig. 3A**). The functional importance of the 2<sup>nd</sup> residue can be related to the efficiency  
130 of MAP to remove the iMet residue (Shemesh *et al.*, 2010). We thus confirm here that A and  
131 S residues are among the most preferred ones in the P1' position of MAP substrates in root tip  
132 cells (Frottin *et al.*, 2006).

133

134 *Acetylation of native Nt in root tips confirms previous studies and adds to the known plastid*  
135 *types capable of N-terminal acetylation*

136 The exposed Nt of proteins is one of the major determinants of protein stability/half-life and  
137 certain residues, when exposed at the Nt of a protein, act as triggers for degradation and are  
138 therefore called “N-degrons” (Bachmair *et al.*, 1986). Nt-acetylation (Nt-ace) can target  
139 proteins for degradation via a branch of the N-end rule pathway (Hwang *et al.*, 2010). Nt-ace  
140 of nascent polypeptides is carried out by N- $\alpha$ -acetyltransferases (NATs) (reviewed in (Gibbs,  
141 2015). Nt-ace typically occurs co-translationally, and, in contrast to lysine (K) acetylation, it  
142 seems irreversible. Residues that are acetylated following iMet excision can also act as Nt-ace-  
143 degrons when they are not shielded by correct protein folding and/or assembled into oligomeric  
144 complexes. In this way, the acetylation-mediated N-end rule plays important roles in general  
145 protein quality control.

146 Between 70 and 90% of eukaryotic proteins carrying Nt-ace and iMet-ace can be  
147 targeted for degradation when followed by a bulky residue (e.g. tryptophan; W) (Zhang *et al.*,  
148 2015). Out of 2,840 Nt peptides identified 2,067 were *in vivo* acetylated (72%), with 496  
149 carrying iMet-ace (24% of Nt-ace and only 17% of all characterized Nt) (**Fig. 3B**). The majority

150 of experimentally identified Nt-ace (76%) are acetylated after removal of the iMet, displaying  
151 at the 2<sup>nd</sup> position A>S>threonine (T)>>G (**Fig. 3C**). Notably, a similar pattern of acetylation  
152 affinity for the 2<sup>nd</sup> Nt residue has been reported for humans and Arabidopsis leaves (Linster *et*  
153 *al.*, 2015). Interestingly, we identified one neo-Nt carrying acetylation. Manual examination of  
154 this peptide showed that it corresponds to the mature form of the plastid protein CUTA which  
155 carries a peptide leader (Burkhead *et al.*, 2003). This observation is consistent with previous  
156 results demonstrating acetylation of chloroplastic proteins (Rowland *et al.*, 2015), extending  
157 the Nt-ace for plastids, as well.

158

#### 159 *Nt-acetylation is increased in rsw4*

160 The log<sub>2</sub> ratios of light (WT) to heavy (*rsw4*) signals for all identified peptides were calculated  
161 with the Mascot Distiller software. The standard deviation of these values for all the peptides  
162 (according to the Huber-scale estimator (Foyn *et al.*, 2013)) was 0.39 with the median very  
163 close to *zero* (0.09; **File S2**). These data suggest that the proteome of *rsw4* shows specific  
164 changes rather than pleiotropic redistributions and therefore represents an excellent model for  
165 the study of targeted proteomic changes.

166 In WT, 25 acetylated peptides (from 5 proteins) carrying iMet were significantly  
167 enriched (5 proteins; 0.5>log<sub>2</sub>), in contrast to 61 acetylated peptides (20 proteins) enriched in  
168 *rsw4* (**Fig. 3D**). After the removal of the iMet, 1,567 peptides were acetylated of which 105  
169 (19 proteins) were enriched in WT and as many as 359 (20 proteins) enriched in *rsw4*. These  
170 data point to an increased accumulation of proteins carrying Nt-ace in *rsw4* root cells, which  
171 can be a result of either the enhancement of the acetylation pathway or the abrogation of a  
172 proteolytic machinery normally degrading these proteins.

173 In plants, Nt-ace is indispensable since NatA loss-of-function mutant is embryo lethal  
174 (Linster *et al.*, 2015). Furthermore, the Nt-ace of SUPPRESSOR OF NPR1, CONSTITUTIVE  
175 1 (SNC1) is important for plant immunity (Xu *et al.*, 2015). A specific branch of the N-end  
176 rule pathway known as the acetylation N-end rule, targets Nt-ace as degrons and send them for  
177 degradation by the proteasome (Liu & Moschou, 2017) and references therein). In yeast, two  
178 E3 ligases that recognize Nt-ace-degrons were identified: the ER-associated DOA10/TEB4 and  
179 cytosolic NOT4 (Lee *et al.*, 2016). Furthermore, a link between anaphase and protein  
180 acetylation was suggested in yeast (Van Damme *et al.*, 2011). Whether separase crosstalks with  
181 the N-end rule pathway and whether Nt-ace impinges on chromosomal segregation in plants  
182 merits further investigation. It is not surprising however, that separase-mediated limited

183 proteolysis could crosstalk with digestive proteolytic pathways, as it has been recently  
184 discussed (Minina, E.A. *et al.*, 2017).

185

186 *Identification of neo-Nt peptides in root proteomes and thus potential targets of separase*

187 To identify neo-Nt, i.e. the proxies for proteolytic cleavage, we applied the following filtering  
188 criteria: i) positive selection of peptides with butyrylated Nt and ii) exclusion of butyrylated  
189 iMet and 2<sup>nd</sup> residues of the protein. Following filtering, we identified 247 neo-Nt peptides  
190 corresponding to 48 unique proteins (**File S1**). The preferable P1' residue of neo-Nt was S,  
191 followed by T, A, and E (**Fig. 3E**).

192 Next, we focused on two classes of peptides: the significantly enriched neo-Nt peptides  
193 in WT or *rsw4* (based on the labelling used). As the former might also appear because of  
194 differential protein levels in the input proteomes, we scanned the data for the corresponding  
195 precursor protein Nt or other neo-Nt peptides from these precursor proteins. We expected to  
196 deal with three different scenarios of mass spectroscopy results. First, a specific neo-Nt peptide  
197 is present in greater amounts in WT indicating potential separase-mediated (direct or indirect)  
198 proteolytic event. Second, a neo-Nt peptide is present in both samples, but in a significantly  
199 greater amount in *rsw4*. This scenario would indicate that the corresponding protein was  
200 cleaved at the specified site by a protease, which was up/mis-regulated in *rsw4* or that the  
201 protein fragment was less stable in WT. Third, the neo-Nt peptide are in equal amounts in both  
202 samples, indicating that this cleavage event reflects a general metabolic process not affected  
203 by separase depletion.

204 With the exception of the AT1G79320 (METACASPASE6) for which a neo-Nt was  
205 identified only in *rsw4* (removal of a 10 kDa Nt fragment), and may reflect the deregulated  
206 expression of this gene, all other neo-Nt-containing peptides were present in both WT and *rsw4*  
207 (**File S1**). Nine neo-Nt of proteins were preferentially overrepresented in WT, while four in  
208 *rsw4* (**Table 1**). Most of the cleavage events in the identified proteins occurred in coils or  $\alpha$ -  
209 helices (**Fig. 4**), a result consistent with previous findings in non-plant organisms (Timmer *et*  
210 *al.*, 2009).

211 Interestingly, neo-Nt corresponding to the V-ATPase E1, H<sup>+</sup>-ATPases, Annexin 1  
212 (increased in WT) and the SYNTAXIN 71 (SYP71; increased in *rsw4*) were the most striking  
213 proteins relevant to the vesicular trafficking and auxin defects observed in *rsw4*. Annexin 1  
214 and H<sup>+</sup>-ATPases, are plasma membrane proteins (Baucher *et al.*, 2011; Haruta *et al.*, 2015),  
215 while the V-ATPase E1 localizes at the tonoplast and the TGN (Strompen *et al.*, 2005;  
216 Schumacher & Krebs, 2010). Interestingly, separase localizes at the plasma membrane and

217 TGN at least transiently (Moschou *et al.*, 2013). We should note that for the two H<sup>+</sup>-ATPases  
218 (as well as the identified oxygenase) we could not ascribe additional peptides except the  
219 identified neo-Nt and therefore, they could be simply more abundant in WT.

220 In plants, V-ATPase activity acidifies the TGN and the vacuole (Strompen *et al.*, 2005;  
221 Schumacher & Krebs, 2010). In mammalian cells, separase depletion leads to a decrease in  
222 constitutive protein secretion as well as endosomal receptor recycling and degradation (Bacac  
223 *et al.*, 2011). Depletion of separase results in increased vesicular pH mediated by V-ATPase  
224 inhibition. It is thus reasonable to assume that plant separase may also regulate the acidification  
225 of vesicles by modulating the V-ATPase activity.

226 The H<sup>+</sup>-ATPases (AHA) regulate cellular acidification and cell expansion by building  
227 a proton gradient across the plasma membrane. Downregulation of AHA activity occurs after  
228 treatment of plants with the growth inhibitory peptide, RALF, leading to the inhibition of cell  
229 expansion (Haruta *et al.*, 2014). Processing might be a complementary mechanism to control  
230 the activity of AHA proteins. Plants with a hyperactive allele of AHA2 (*ost2*) show abscisic  
231 acid insensitivity and drought sensitivity (Merlot *et al.*, 2007). Furthermore, evidence suggest  
232 a crosstalk between auxin signalling and acidification that involves the activation of AHA in  
233 Arabidopsis roots (Fendrych *et al.*, 2016). A more complete understanding of hprocessing may  
234 control AHA activity will provide new prospects for improving plant growth and drought  
235 tolerance.

236 Annexin 1 (ANN1) is a root-specific annexin regulating root length via sensing pH-  
237 dependent channel activity (Gorecka *et al.*, 2007). In pea (*Pisum sativum*) an ANN1 homologue  
238 can also redistribute in response to gravistimulation (Clark *et al.*, 2000). In maize cells, other  
239 annexins are known to stimulate Ca<sup>2+</sup>-dependent exocytosis (Carroll *et al.*, 1998). In animal  
240 cells, cleavage of ANN1 at the N-terminus by calpain protease mediates the secretory processes  
241 by modifying either requirements for Ca<sup>2+</sup> or ANN1 efficacy in membrane binding, vesicle  
242 aggregation or endocytic processing (Barnes & Gomes, 2002; Sugimoto *et al.*, 2016). Other  
243 annexins, e.g. A2, is a Ca<sup>2+</sup>-, actin-, and lipid-binding protein mediating the formation of lipid  
244 microdomains required for the structural and spatial organization of fusion sites at the plasma  
245 membrane (Gabel *et al.*, 2015). It is tempting to speculate that separase may regulate the  
246 activity of ANN1 by modifying its requirement for Ca<sup>2+</sup>. Interestingly, the Arabidopsis  
247 separase has an EF-hand, responsible for Ca<sup>2+</sup> binding.

248 The identified neo-Nt of the cytoplasmic Acyl-CoA-binding protein 4 (ACBP4) is  
249 preferentially enriched in WT (**Table 1**). ACBP4 is involved in the transport of lipids ( $C \geq 20$ )  
250 to the plasma membrane for the biosynthesis of surface lipids such as wax and cutin (Du *et al.*,



251 2016). Depletion of AtACBP4 in Arabidopsis resulted in decreases in galactolipids and  
252 phospholipids, suggesting its role in membrane lipid biosynthesis, as well as diminished  
253 capability in the generation of signals such as salicylate for induction of systemic acquired  
254 resistance. It is yet unclear what could be the role of ACBP4 processing but may lead to protein  
255 activity modulation. A reduced activity of ACBP4 in *rsw4* could compromise lipid signalling  
256 that could contribute to the observed phenotypes.

257 The product of PROPEP7 (**Table 1**; enriched in WT) is a member of the PROPEP  
258 family of Damage Associated Molecular Pattern (DAMP) elicitor peptides. The cleavage on  
259 the PROPEP7 is interesting, since it corresponds to the predicted cleavage site that will release  
260 the Pep7 elicitor peptide, but has never been experimentally shown (Bartels & Boller, 2015).  
261 Furthermore, this family is believed to have extended functionalities beyond wounding and  
262 immunity, potentially in growth and development (Bartels & Boller, 2015). In fact,  
263 overexpression of PROPEP1 leads to larger root system in Arabidopsis (Huffaker *et al.*, 2006).  
264 Interestingly, the identified cleavage event enriched in *rsw4* on Chaperonin 60 beta may also  
265 be involved in pathogen resistance (**Table 1**). The corresponding mutant, *Arabidopsis lesion*  
266 *initiation 1 (len1)*, develops necrotic lesions which may activate systemic acquired resistance  
267 (Ishikawa *et al.*, 2003), without pathogen attack (Ishikawa, 2005).

268 The peroxidase PRX34 was found at significantly higher levels in *rsw4* (both neo-Nt  
269 and internal peptides). PRX34 is an apoplastic reactive oxygen species (ROS) generator  
270 involved in resistance to pathogens in Arabidopsis (Daudi *et al.*, 2012) and also root  
271 development through a genetic interaction with the mitogen-activated protein kinase 6  
272 (MAPK6) (Han *et al.*, 2015). Interestingly, *PRX34* mutants show larger leaves than WT.  
273 Similarly, oxidases in the apoplast of tobacco plants modulate resistance to pathogens and plant  
274 development in a pathway that involves ROS-MAPKs (Moschou *et al.*, 2008; Moschou *et al.*,  
275 2009; Tisi *et al.*, 2011; Gemes *et al.*, 2016). We assume that it is unlikely that PRX34 is a direct  
276 separase target considering PRX34 localization in the apoplast, but its deregulation is rather an  
277 indirect effect.

278 The Qb-SNARE SYP71 (enriched in *rsw4*) is part of a bigger family of proteins  
279 mediating vesicular fusion (El Kasmi *et al.*, 2013). Together with Qb-SNARE NPSN11 and  
280 VAMP721,722 the SYP71 forms a tetrameric KNOLLE-containing complex. KNOLLE  
281 mediates vesicular trafficking during Arabidopsis cytokinesis and its targeting to the  
282 cytokinetic apparatus known as cell plate is compromised in *rsw4* (Moschou *et al.*, 2013).  
283 Hence, a plausible scenario is that SYP71 is preferentially degraded in *rsw4*, an event which  
284 may impact vesicular fusion. It is highly likely that vesicular fusion is compromised in the

285 absence of separase as it has been shown in Arabidopsis (Moschou *et al.*, 2013) and *C. elegans*  
286 (Bembenek *et al.*, 2010; Mitchell *et al.*, 2014).

287 Finally, SPATULA is a negative (neo-Nt increased in WT), while KLU (neo-Nt  
288 increased in *rsw4*) a positive cell division regulator (Alvarez & Smyth, 1999; Heisler *et al.*,  
289 2001; Anastasiou *et al.*, 2007; Ichihashi *et al.*, 2010; Josse *et al.*, 2011; Makkena & Lamb,  
290 2013). SPATULA is a transcription factor required for specification of carpel and valve tissues  
291 by restricting cell division. The corresponding mutants show enhanced root and leaf growth.  
292 KLU produces an as yet unknown mobile signal with cell division promoting properties. KLU  
293 is involved in generating a mobile growth signal distinct from the classical phytohormones that  
294 defines primordium size.

295

296 *Consensus cleavage sites of substrates enriched in wild-type and thus potential separase*  
297 *targets*

298 Using iceLogo, we inspected the amino acid residues at the prime (P') and nonprime (P)  
299 substrate positions and analysed the frequencies of specific residues after statistical correction  
300 by means of the natural occurrence of amino acids in Arabidopsis proteins (Colaert *et al.*,  
301 2009). Unlike what has been described for kleisin motifs cleaved by separase, our data do not  
302 show specificity of Arabidopsis separase against EXXR (X, any residue) sequence (Sullivan *et al.*  
303 *et al.*, 2004; Lin *et al.*, 2016) (**Fig. 5**). This finding suggests that either not all identified substrates  
304 are direct targets of separase or that separase in plants has evolved broader cleavage specificity.  
305 We should note that so far sequence specificity for separase in non-plants has been deduced  
306 from cleavage sites on only few proteins related to cell division, i.e. SLK19 and kleisins and  
307 may not reflect the general specificity of separases. Noteworthy, the kleisins in plants are a  
308 multimeric gene family (da Costa-Nunes *et al.*, 2006; Ma *et al.*, 2016; Minina, E. A. *et al.*,  
309 2017). Hence, plant separases may have adapted to the expansion of plant kleisins by adjusting  
310 accordingly their substrate recognition repertoire.

311

## 312 **Conclusion**

313 We previously established a role for separase in controlling vesicular trafficking and plant  
314 development (Moschou *et al.*, 2016a). Likewise, *C. elegans* separase is required for secretion  
315 of Rab11-positive vesicles, and in human cells separase controls fusion and acidification of  
316 vesicles (Bembenek *et al.*, 2010; Bacac *et al.*, 2011; Mitchell *et al.*, 2014). Interestingly, fusion  
317 of vacuolar membranes in yeast requires proteasomal degradation of ubiquitinated Ypt7, a  
318 yeast homolog of Rab7 GTPase (Kleijnen *et al.* 2007). Ubiquitination may also regulate

319 membrane fusion events that reassemble fragmented organelles after mitosis in mammalian  
320 cells and vacuole-plasma membrane fusions in plant cells during plant immunity (Meyer &  
321 Popp, 2008). Yet, the role of processing in vesicular trafficking remains unknown. Here, we  
322 show that plant separase may be involved in the regulation of acidification and lipid signalling,  
323 and we assign potential molecular targets for this function. Furthermore, we show that the  
324 proteolytic landscape of *rsw4* mutants involves proteins that modulate resistance to pathogens,  
325 cell expansion and cell division. This finding extends the possible functions of separase,  
326 suggesting potential links of a core component of cell division and development to plant  
327 immune responses.

328

## 329 **Materials and Methods**

### 330 *Plant Material and Growth Conditions*

331 *Arabidopsis thaliana* wild-type (WT) and *rsw4* plants in the Col-0 ecotype background were  
332 grown on vertical plates containing half-strength Murashige and Skoog (MS) medium  
333 supplemented with 1% (w/v) sucrose and 0.7% (w/v) plant agar, at 20°C (permissive  
334 temperature) or 28°C (restrictive temperature), 16/8-h light/dark cycle, and light intensity 150  
335  $\mu\text{E m}^{-2} \text{ s}^{-1}$ . The root swelling phenotype was observed in homozygous *rsw4* plants within 3 d  
336 of incubation at 28°C. The dexamethasone RNAi lines are in Col-0 background and have been  
337 previously described (Moschou *et al.*, 2013).

### 338 *A shortened N-terminal COFRADIC protocol: sample preparation*

339 Seedlings grown on MS plates with nylon filters (0.8  $\mu\text{m}$ ) were harvested and root tips were  
340 collected after manual excision with a blade. Tips were snap frozen in liquid  $\text{N}_2$ , and ground  
341 into a fine powder with a mortar and pestle. Samples were prepared as previously described  
342 (Tsiatsiani *et al.*, 2014). To achieve a total protein content of 1 mg, 0.2 g frozen ground tissue  
343 was re-suspended in 1 mL of buffer containing 1% (w/v) 3-[(3-cholamidopropyl)-  
344 dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% (w/v) deoxycholate, 5 mM  
345 ethylenediaminetetraacetic acid, and 10% glycerol in 50 mM HEPES buffer, pH 7.5, further  
346 containing the suggested amount of protease inhibitors (one tablet/10 mL buffer) according to  
347 the manufacturer's instructions (Roche Applied Science). The sample was centrifuged at  
348 16,000g for 10 min at 4°C, and guanidinium hydrochloride was added to the cleared  
349 supernatant to reach a final concentration of 4 M. Protein concentrations were measured with  
350 the DC protein assay (Bio-Rad), and protein extracts were further modified for N-terminal  
351 COFRADIC analysis as described previously (Staes *et al.*, 2011).

352 Col-0 (WT) primary amines were labelled with the N-hydroxysuccinimide (NHS) ester  
353 of  $^{12}\text{C}_4$ -butyrate and *rsw4* with NHS- $^{13}\text{C}_4$ -butyrate, resulting in a mass difference of  
354 approximately 4 Da between light ( $^{12}\text{C}_4$ ) and heavy ( $^{13}\text{C}_4$ ) labelled peptides. After equal  
355 amounts of the labelled proteomes had been mixed, tryptic digestion generated internal, non-  
356 N-terminal peptides that were removed by strong cation exchange at low pH (Staes *et al.*,  
357 2011). Due to the low amount of input material, the COFRADIC protocol was cut short after  
358 the first reversed phase-high performance liquid chromatography (RP-HPLC) step and the  
359 resulting 15 fractions were subjected immediately for identification by LC-MS/MS.

### 360 *Peptide Identification, and Quantification*

361 Samples were dissolved in 2% ACN, 0.1% TFA and subjected to ESI-MS/MS on a Q Exactive  
362 Orbitrap mass spectrometer operated similarly as previously described with some  
363 modifications (Stes *et al.*, 2014). Peptides were separated on a reverse phase column with a  
364 linear gradient from 98% solvent A' (0.1% formic acid in water) to 40% solvent B' (0.1%  
365 formic acid in water/acetonitrile, 20:80 (v/v) in 127 min at a flow rate of 300 nL/min. MS was  
366 run in data-dependent, positive ionization mode, with the initial MS1 scan (400–2000 m/z;  
367 AGC target of  $3 \times 10^6$  ions; maximum ion injection time of 80 ms) acquired at a resolution  
368 of 70 000 (at 200 m/z), which was followed by up to 10 tandem MS scans of the most abundant  
369 ions at a resolution of 17 500 (at 200 m/z) according the following criteria: AGC target of  $5 \times$   
370  $10^4$  ions; maximum ion injection time of 120 ms; isolation window of 2.0 m/z; fixed first  
371 mass of 140 m/z; underfill ratio of 1.2%; intensity threshold of  $5 \times 10^3$ ; exclusion of  
372 unassigned, 1, 5–8, >8 charged precursors; peptide match preferred; exclude isotopes, on;  
373 dynamic exclusion time, 20 s. From the MS/MS data, Mascot Generic Files (mgf) were created  
374 using the Mascot Distiller software (version 2.5.1.0, Matrix Science). Peak lists were then  
375 searched using the Mascot search engine with the Mascot Daemon interface (version 2.5.1,  
376 Matrix Science). Spectra were searched against the TAIR10 database. Mass tolerance on  
377 precursor ions was set to 10 ppm (with Mascot's C13 option set to 1), and on fragment ions to  
378 20 mmu. The instrument setting was on ESI-QUAD. Endoproteinase semi-Arg C/P was used  
379 with one missed cleavage allowance, rather than trypsin, because cleavage after lysine residues  
380 is abolished by side chain acylation. Variable modifications were set to pyroglutamate  
381 formation of N-terminal glutamine and fixed modifications included methionine oxidation to  
382 its sulfoxide derivative, S-carbamidomethylation of cysteine, and butyrylation ( $^{12}\text{C}_4$  or  $^{13}\text{C}_4$ ) of  
383 the lysine side chain and peptide N-termini. Peptides with a score higher than the MASCOT  
384 identity threshold set at 99% confidence were withheld. The Mascot Distiller Toolbox (version

385 2.5.1.0; Matrix Science) was used to quantify relative peptide abundance and in case of doubt  
386 were manually curated (for example for singletons). MS data have been deposited to the  
387 ProteomeXchange Consortium via the PRIDE (Vizcaino *et al.*, 2016).

388

### 389 **Supplemental Data**

390 **Supplemental File 1.** N-terminome of WT and *rsw4* Arabidopsis root tips.

391 **Supplemental File 2.** Variance distribution of peptides quantifications in WT and *rsw4*.

392

### 393 **Figure Legends**

394 **Figure 1. Temperature does not underpin the rootward-to-shootward PIN2 switch in**  
395 ***rsw4*.**

396 DEX::hpRNAi-ESP plants expressing PIN2::PIN2-GFP were treated for 12 h with 2  $\mu$ M  
397 dexamethasone (in DMSO) and observed using confocal microscopy. The root region and cells  
398 observed (cortex and epidermis) are shown on the left. Representative images of an experiment  
399 replicated three times. Scale bars, 5  $\mu$ m.

400

401 **Figure 2. The COFRADIC pipeline and the experimental setup.**

402 A. The COFRADIC steps are i) labelling: cysteine alkylation and N-trideutero-  
403 acetylation (butylation); ii) trypsin digestion; ii) pyroQ removal and SCX at low pH.

404 B. WT and *rsw4* seedlings were exposed for 12 h at 28°C and their root tips were  
405 harvested, their proteomes extracted and differentially labelled by staple isotopic tags and  
406 analysed by COFRADIC (see also Materials and Methods).

407

408 **Figure 3. Gene ontology (GO) terms of identified root tip N-termini and residue**  
409 **enrichments at position 2 of proteins with or without N-terminal methionine (iMet).**

410 A. Enriched residues of proteins with (top) or without iMet (bottom).

411 B. Distribution (%) of Nt-acetylated peptides lacking iMet (-iMet), with iMet (+iMet) and  
412 peptides that were not Nt-acetylated (with or without iMet; -iMet or +iMet, respectively).

413 C. Acetylation specificity of peptides lacking iMet at the Nt (pooled data from WT and  
414 *rsw4*).

415 D. Acetylation level of peptides in WT and *rsw4* expressed as a ratio between the  
416 acetylated/non-acetylated forms of the same peptides.

417 E. P1'-P6' specificity of limited proteolysis in root tip cells.

418 **Figure 4. Domain architecture (left), cleavage sites and secondary structures (right) of the**  
419 **neo-N-termini enriched in WT (above the dotted red line) or in *rsw4* (below the dotted**  
420 **line).**

421 Domain architecture was defined by SMART (Letunic *et al.*, 2015). Secondary structures  
422 represent predictions defined by YASPIN (Lin *et al.*, 2005). The only proteins identified from  
423 these cleaved in loops are the V-ATPase E1 and PRX34.

424 **Figure 5. Icelogo showing the specificity of cleavage events enriched in WT.**

425

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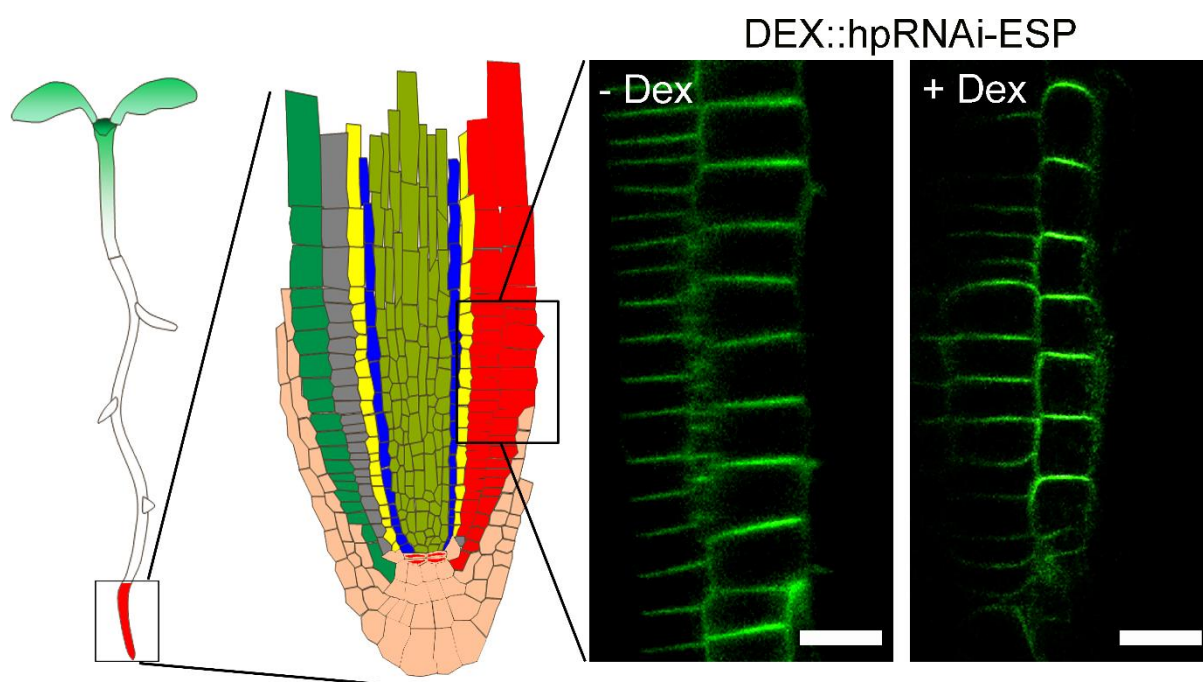
**Table 1.** Preferentially cleaved proteins (ratio >2) in WT and *rsw4* root tips. <sup>1</sup>Only neo-Nt peptides were identified; <sup>2</sup>Non neo-Nt peptides were significantly increased in *rsw4*.

Accession	ID	Sequence	Ratio	Function
<b>Preferentially cleaved in WT</b>				
AT1G35720	Annexin 1	SKAQINATFNR	75,1	Calcium-dependent phospholipid binding
AT5G63400	Adenylate kinase 1	TVTQAEKLDMLKR	15,2	Nucleotide kinase activity
AT4G36930	SPATULA	TTTTTASLIGVHG	10,1	Cell division control
AT3G05420	Acyl-CoA binding protein 4	ATSGPAYPER	13,0	Acyl-CoA binding
AT2G17720	Oxygenase <sup>1</sup>	KSETSSGDEEGER	2,1	Modifies the extensin proteins in root hair cells
AT1G80660	H(+)-ATPase 9 <sup>1</sup>	EAQWAQAQR	2,1	ATPase, P-type, H+ proton pump
AT2G18960	H(+)-ATPase 1 <sup>1</sup>	ELSEIAEQAKR	2,0	ATPase, P-type, H+ proton pump
AT4G11150	V-ATP subunit E1	QDYEKKEKQADV	2,0	ATPase, V1/A1 complex, subunit E
AT5G09978	PROPEP7	SVVSGNVAAR	2,0	Plant immunity
<b>Preferentially cleaved in <i>rsw4</i></b>				
AT1G13710	KLU <sup>1</sup>	VLAALAKR	1031,9	Positive regulation of cell proliferation
AT3G49120	Peroxidase PRX34 <sup>2</sup>	SALVDFDLR	12,8	Unidimensional cell expansion
AT1G55490	Chaperonin 60 beta <sup>1</sup>	IVNDGVTVAR	10,2	SAR, suppression of protein aggregation.
AT3G09740	Syntaxin of plants 71 <sup>1</sup>	ATSDLKNTNVR	2,3	protein targeting to membrane, vesicle docking and fusion

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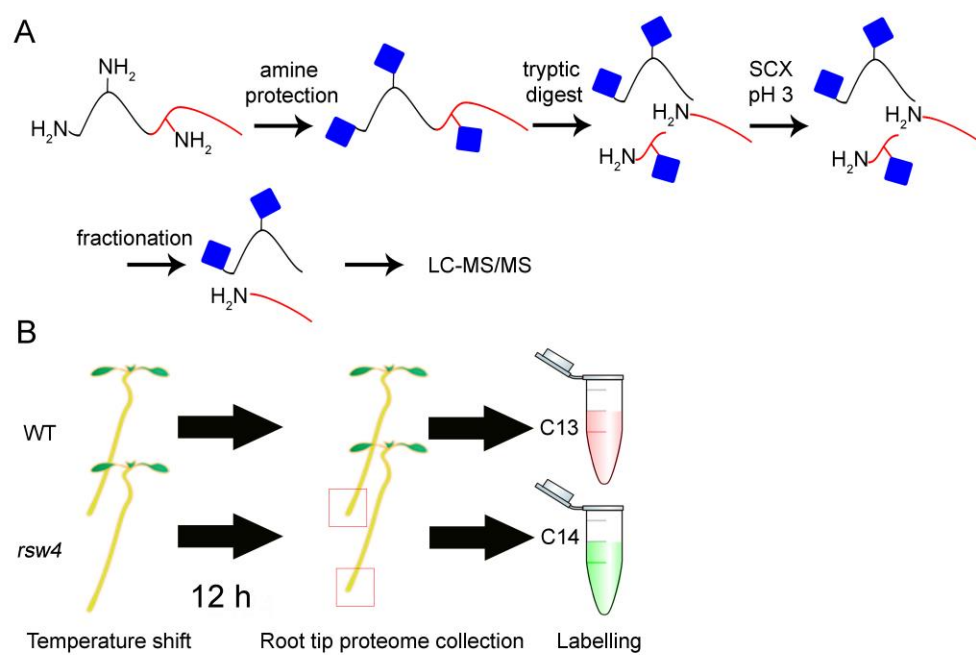


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**Figure 1**

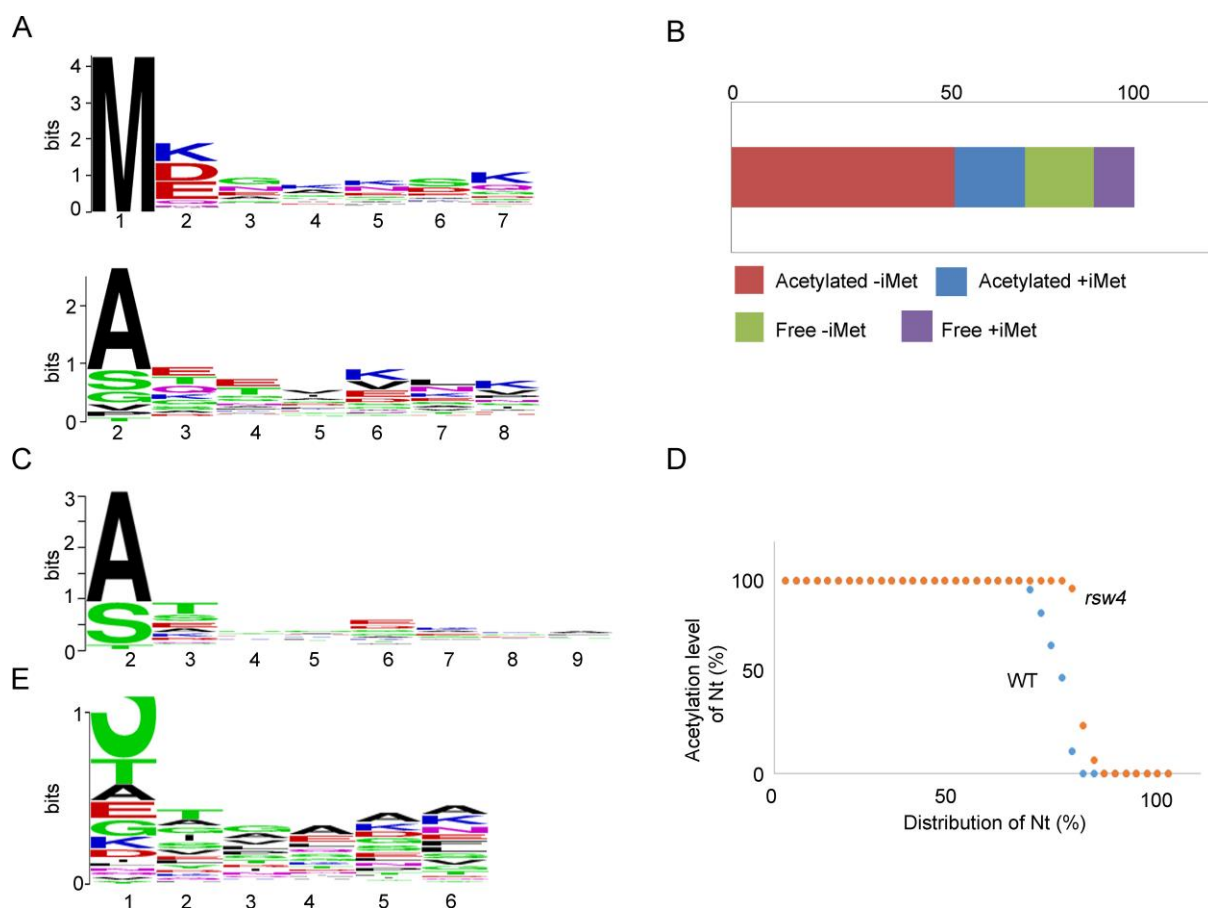


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**Figure 2**



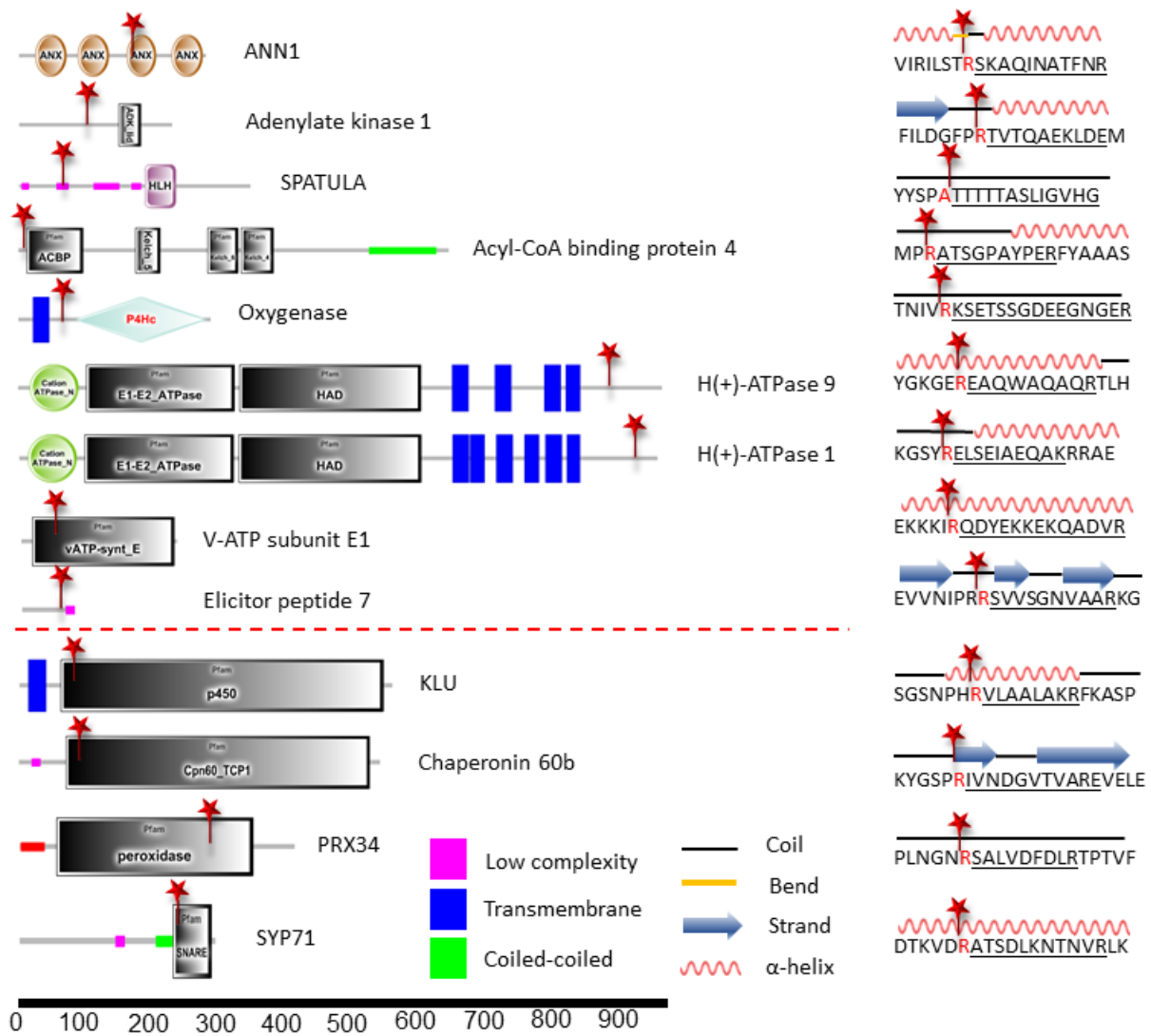
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**Figure 3**

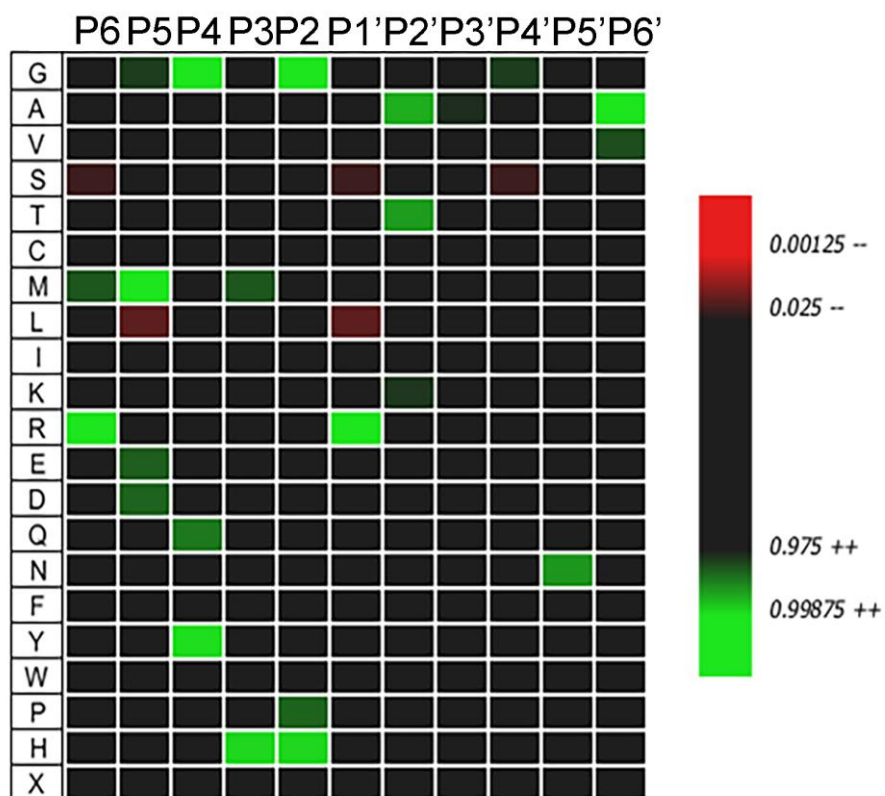
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Figure 4



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Figure 5