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# Subtilase-mediated biogenesis of the expanded family of SERINE RICH ENDOGENOUS PEPTIDES

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## Abstract

Plant signalling peptides are typically released from larger precursors by proteolytic cleavage to regulate plant growth, development, and stress responses. Recent studies reported the characterization of a divergent family of *Brassicaceae*-specific peptides,

35 SERINE RICH ENDOGENOUS PEPTIDES (SCOOPs), and their perception by the  
36 leucine-rich repeat receptor kinase MALE DISCOVERER 1-INTERACTING  
37 RECEPTOR-LIKE KINASE 2 (MIK2). Here, we reveal that the SCOOP family is highly  
38 expanded, containing at least 50 members in the Columbia-0 reference *Arabidopsis*  
39 *thaliana* genome. Notably, perception of these peptides is strictly MIK2-dependent.  
40 How bioactive SCOOP peptides are produced, and to which extent their perception is  
41 responsible for the multiple physiological roles associated with MIK2 is currently  
42 unclear. Using N-terminomics, we validate the N-terminal cleavage site of  
43 representative PROSCOOPs. The cleavage sites are determined by conserved motifs  
44 upstream of the minimal SCOOP bioactive epitope. We identified subtilases necessary  
45 and sufficient to process PROSCOOP peptides at conserved cleavage motifs.  
46 Mutation of these subtilases, or their recognition motifs, suppressed PROSCOOP  
47 cleavage and associated overexpression phenotypes. Furthermore, we show that  
48 higher-order mutants of these subtilases show phenotypes reminiscent of *mik2-1*,  
49 consistent with impaired PROSCOOP biogenesis, and demonstrating biological  
50 relevance of SCOOP perception by MIK2. Together, this work provides insights into  
51 the molecular mechanisms underlying the functions of the recently identified SCOOP  
52 peptides and their receptor MIK2.

53

## 54 **Introduction**

55 A growing number of plant signalling peptides are being identified and found to play  
56 major roles during growth, development and stress responses<sup>1-3</sup>. Such peptides are  
57 generally derived from protein precursors; however, relatively few biogenesis  
58 mechanisms have been described<sup>2,4-9</sup>. Some plant signalling peptides act as  
59 phyto cytokines, which are secreted **cell-to-cell mobile signals** that regulate plant  
60 immune responses, analogous to cytokines in metazoans<sup>10-12</sup>. SERINE RICH  
61 ENDOGENOUS PEPTIDES (SCOOPs) have recently been reported as a family of  
62 phyto cytokines transcriptionally induced during stress, especially during biotic  
63 interactions<sup>13-15</sup>. SCOOPs are serine-rich, are derived from the C-terminus of divergent  
64 secreted PROSCOOPs, and are characterised by the presence of a 13-15 amino acid  
65 **conserved** epitope that includes an 'SxS' motif which is essential for bioactivity<sup>13-15</sup>.  
66 **PROSCOOPs undergo maturation steps to produce bioactive SCOOPs<sup>14,16</sup>.**  
67 **Cleavage of PROSCOOP12 was reported to be after R43<sup>14</sup>. For PROSCOOP10, two**  
68 **distinct cleaved peptides (SCOOP10#1 and SCOOP10#2) with hydroxylated prolines**

69 were identified from leaf apoplastic fluids<sup>16</sup>. The exact length of cleaved SCOOPs  
70 produced *in planta* and the proteases involved are however unknown. Also, given that  
71 synthetic unmodified 13- or 15-mer SCOOP peptides are bioactive<sup>13-15,17</sup>, the exact  
72 role of potential posttranslational modifications of SCOOPs *in planta* is still unclear.  
73 While initially reported as a 14-member family in the genome of the Col-0 ecotype of  
74 *Arabidopsis thaliana* (later referred to as *Arabidopsis*)<sup>13</sup>, the SCOOP family has  
75 recently been shown to contain up to 28 members<sup>14,17</sup>. Active SCOOP peptides induce  
76 cellular outputs typical of pattern-triggered immunity such as the production of  
77 apoplastic reactive oxygen species (ROS), rapid increase in cytosolic Ca<sup>2+</sup>  
78 concentration, and immune gene expression<sup>13-15,17</sup>. Amongst SCOOPs, SCOOP12 –  
79 the founding member of the family – has been studied in more depth and was shown  
80 genetically to regulate immunity to diverse pathogens and pests, as well as root growth  
81 and ROS homeostasis<sup>13,18,19</sup>. SCOOPs are perceived by the *Arabidopsis* leucine-rich  
82 repeat receptor kinase MALE DISCOVERER 1-INTERACTING RECEPTOR LIKE  
83 KINASE 2 (MIK2) and recruit the BRASSINOSTEROID INSENSITIVE 1-  
84 ASSOCIATED KINASE 1(BAK1) as co-receptor<sup>14,15</sup>. Interestingly, MIK2 is involved in  
85 multiple diverse aspects of plant biology, such as responses to the cellulose  
86 biosynthesis inhibitor isoxaben (ISX), root growth angle, salt stress tolerance,  
87 resistance to the root vascular fungal pathogen *Fusarium oxysporum* 5176 and the  
88 generalist herbivore *Spodoptera littoralis*, as well as responsiveness to immune  
89 elicitors<sup>14,15,19-23</sup>. Although MIK2 is the sole SCOOP receptor, to which extent the  
90 multiple functions of MIK2 are all depending on SCOOP perception is still unknown.  
91 Like most signalling peptides, SCOOPs undergo proteolytic processing to generate an  
92 active mature peptide *in planta*; however, little is known mechanistically about this  
93 process<sup>14,15</sup>. Many signalling peptides are cleaved by the subtilisin-like serine  
94 proteinases, which have been shown to cleave signalling peptides in a sequence- and  
95 modification-specific manner to generate bioactive peptides<sup>24,25</sup>.  
96 Here we report a comprehensive annotation of putative PROSCOOP genes in the  
97 *Arabidopsis* Col-0 genome revealing the existence of 50 putative SCOOP peptides, of  
98 which at least 36 exhibited MIK2-dependent biological activity, making the SCOOPs  
99 one of the largest families of signalling peptides identified in flowering plants. Based  
100 upon co-expression during biotic stress, we identify several proteases from subtilase  
101 subgroup 3 transcriptionally responsive to the same environmental stimuli as MIK2,  
102 including biotic elicitor treatment, which promote PROSCOOP cleavage and are

103 required for PROSCOOP activity *in planta*. Finally, we show that higher order subtilase  
104 mutants phenocopy *mik2* mutants suggesting impaired SCOOP signalling.

105

## 106 **Results and discussion**

### 107 **Identification of PROSCOOP genes within the *A. thaliana* Col-0 genome**

108 Originally, 14 PROSCOOP genes were identified in the *Arabidopsis* Col-0 genome<sup>13</sup>.

109 Recently, tolerating more divergence in the core motif, this number was expanded to

110 23, and then 28 genes<sup>14,17</sup>, highlighting the need for a revised bioinformatic analysis of

111 the PROSCOOP family. We comprehensively re-evaluated the PROSCOOP repertoire

112 through iterative searches using BLASTP/TBLASTN and the MOTIF ALIGNMNET

113 AND SEARCH TOOL (part of the MEME suite)<sup>26</sup>. Using this approach, we identified

114 22 additional putative PROSCOOPs, which we named PROSCOOP29-

115 PROSCOOP50 according to their location within the genome (Extended Data Fig. 1;

116 Extended Data Tables 1 and 2). Despite having low amino acid sequence similarity, all

117 predicted SCOOP peptides have the conserved 'SxS' motif (Extended Data Fig.1)

118 previously shown to be required for activity<sup>13-15</sup>. Interestingly, all of the 30 PROSCOOP

119 transcripts detected in our recent RNA-Seq dataset<sup>27</sup> are up-regulated upon elicitor

120 treatment (Extended Data Fig. 2), consistent with their possible function as

121 phyto cytokines. Interestingly, some SCOOPs correspond to previously annotated

122 peptides, such as SECRETED TRANSMEMBRANE PEPTIDES (STMPs)<sup>14,15,28</sup>,

123 ENHANCER OF VASCULAR WILT RESISTANCE 1 (EWR1)<sup>17,29</sup> and ARACINs<sup>30</sup>

124 (Extended Data Table 2). Notably, some of these peptides were previously shown to

125 have direct antimicrobial activity<sup>28,30</sup>, suggesting that PROSCOOPs are analogous to

126 metazoan host-defence peptides (HDPs) encoding dual activities as both

127 immunomodulatory phyto cytokines and anti-microbial peptides (AMPs)<sup>31</sup>.

128 To test whether the newly identified SCOOPs are bioactive, we synthesized all of the

129 corresponding 13-mer peptides, and found that many of them induced ROS production

130 and triggered cytosolic Ca<sup>2+</sup> fluxes when applied exogenously to Col-0 plants (Fig. 1a,

131 b). Importantly, these responses were abolished in *mik2-1* (Extended Data Fig. 3a and

132 b). Notably, while a 13-amino-acid peptide is defined at the minimal active epitope for

133 SCOOP12<sup>13-15</sup>, the length of a minimal active epitope appears to vary within the family.

134 For example, the 13-mer derived from SCOOP8 is inactive, but a 15-mer that includes

135 two additional N-terminal amino acids is active (Extended Data Fig. 4a)<sup>14</sup>. Thus, for a

136 selection of inactive 13-mer peptides, we synthesized the corresponding 15-mers and

137 tested their activity. Several such 15-mer peptides (e.g. SCOOP14 and SCOOP40)  
138 could induce MIK2-dependent ROS production (Fig. 1c and Extended Data Fig. 3d). In  
139 our assays, a few SCOOPs (e.g. SCOOP29, SCOOP33, SCOOP35, SCOOP38,  
140 SCOOP42, SCOOP48) however remained inactive (either as 13-mer or 15-mer) (Fig.  
141 1 and Extended Data Fig. 5), warranting further investigation of these SCOOPs in the  
142 future.

143 Overall, our data demonstrate that the *Arabidopsis* SCOOP family contains at least 50  
144 members, and that all tested active synthetic SCOOP peptides induce MIK2-  
145 dependent responses (Extended Data Fig. 5). In addition, all tested active SCOOPs  
146 induce BAK1-dependent ROS production (Extended Data Figure 4b), indicating that  
147 SCOOPs induce MIK2-BAK1 complex formation. These findings thus define the  
148 SCOOP family as one of the largest families of plant signalling peptides currently  
149 known. Strikingly, while other large signalling peptide families, such as  
150 CLAVATA3/ENDOSPERM SURROUNDING REGION-RELATED (CLE) peptides, are  
151 perceived by several phylogenetically related receptors<sup>32</sup>, the perception of all active  
152 SCOOP peptides is mediated by a single receptor, MIK2, making this ligand-receptor  
153 pair unique within the plant kingdom. Future structural work is required to define the  
154 molecular basis of SCOOP binding by MIK2. Some of the synthesized SCOOPs are  
155 inactive (Extended Data Fig. 5), possibly because the length and/or posttranslational  
156 modifications of peptides are important for activity. Some SCOOPs might also be  
157 perceived by another receptor that was lost in Col-0. It is also possible that the  
158 SCOOPs could possess other bioactivities, for example direct antimicrobial activity, or  
159 they may have become pseudogenized. These are interesting questions to pursue in  
160 future work.

161

### 162 PROSCOOP12 is cleaved by SBT3.5

163 Whilst PROSCOOPs have been shown to undergo proteolytic cleavage<sup>14</sup>, the  
164 mechanisms by which bioactive SCOOPs are released from PROSCOOPs is still  
165 unknown. From the PROSCOOPs sequence alignment (Extended Data Fig. 1), we  
166 noticed that most PROSCOOPs contain a 'RXLx/RxxL' motif that is recognized and  
167 cleaved by some subtilases (SBTs) including SBT6.1 and SBT3.5<sup>33-37</sup>. Using  
168 Genevestigator<sup>38</sup>, we observed that among 56 *SBT* genes in *Arabidopsis*<sup>37</sup>, SBT3.3,  
169 SBT3.4 and SBT3.5 are transcriptionally up-regulated with MIK2 under different  
170 conditions (Extended Data Fig. 6). These phylogenetically related *SBT* genes were

171 also amongst the most up-regulated in response to elicitor treatments (Extended Data  
172 Fig. 7; Extended Data Fig. 8)<sup>27,39</sup>. Notably, SBT3.3 and SBT3.5 regulate plant immunity  
173 and root growth, respectively<sup>37,40</sup>, similar to PROSCOOP12<sup>13,19</sup>. Also, the SBT3.5 has  
174 a preference for RKLL motif<sup>37</sup>, which is similar to a motif found in many PROSCOOP  
175 sequences (Extended Data Fig. 1). To test if related SBT3 proteases can cleave  
176 PROSCOOPs at such a motif, we focused on PROSCOOP12, the best-characterised  
177 PROSCOOP with strong activity<sup>13-15,18,19</sup>. We chose to test cleavage using  
178 *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana* which does  
179 not contain SBT3 or PROSCOOP members in its genome<sup>41</sup>, thus enabling gain-of-  
180 function experiments. As a probe for proteolytic cleavage, we inserted a 6xHA tag  
181 between the predicted native signal peptide and PROSCOOP12 coding sequence  
182 fused to an in-frame C-terminal GFP tag (SP-6xHA-PROSCOOP12-GFP; Fig. 2a). We  
183 then co-expressed this construct with untagged SBT3s (since the presence of a tag  
184 could affect SBT protease activity<sup>42-44</sup>). Notably, expression of SBT3.5 resulted in the  
185 increased accumulation of a lower molecular weight protein band under flg22 treatment  
186 (Fig. 2b and Extended Data Fig. 9a; indicated by a red asterisk) at a size corresponding  
187 to SCOOP12-GFP. However, it is not clear whether SBT3.3 and SBT 3.4 could cleave  
188 PROSCOOP12 or not in our assays. It could be that SBT3.3 and SBT3.4 cleavage  
189 activity is too weak to be detected in this assay. The basal level of the cleaved  
190 SCOOP12-GFP products could be caused by endogenous SBTs or other proteases in  
191 *Nicotiana benthamiana*.

192 To test the dependency upon SBT3.5's enzymatic activity, we made use of the specific  
193 subtilase inhibitors, EXTRACELLULAR PROTEINASE INHIBITOR 1a and 10 (EPI1a  
194 and EPI10), normally produced by the plant pathogen *Phytophthora infestans* as  
195 virulence effectors, which have been used previously to assess SBT's functions<sup>4,6,45-  
196 48</sup>. As expected, SBT3.5-induced accumulation of the PROSCOOP12 cleavage  
197 product was reduced when EPI1a and EPI10 were co-expressed (Fig. 2c). We next  
198 sought to establish the importance of the putative subtilase cleavage motif present in  
199 PROSCOOP sequences. Mutation of RRLM into AAAA strongly reduced cleavage of  
200 PROSCOOP12 by SBT3.5 (Fig. 2d). A previous study showed that PROSCOOP12 is  
201 processed at RRLM motif but cleaved downstream of RR<sup>14</sup>. Using mass-spectrometry  
202 (MS) coupled with N-terminal labelling (Extended Data Fig. 10), we identified the  
203 GSGAGPVR peptide as the N-terminus of the SCOOP12-GFP cleavage product  
204 (Extended Data Fig. 11), suggesting the cleavage site is after the RRLM motif *in planta*.



205 Together, these data indicate SBT3.5 can cleave PROSCOOP12 to release active  
206 SCOOP12.

207

### 208 **PROSCOOP20 is cleaved by SBT3.6, SBT3.8 and SBT3.9**

209 In addition to PROSCOOPs containing a 'RxLx/RxxL' motif, we noticed a subgroup of  
210 PROSCOOPs that lack the 'RxLx/RxxL' motif but contain a 'VWD' motif (Extended  
211 Data Fig. 1), a previously reported cleavage motif for the aspartate-dependent  
212 subtilase SBT3.8<sup>5,49</sup>. SBT3.6, SBT3.7, SBT3.9 and SBT3.10 are SBT3.8 homologues  
213 (Extended Data Fig. 8<sup>39</sup>) and SBT3.7, SBT3.9 and SBT3.10 are highly up-regulated by  
214 elicitors treatment (Extended Data Fig. 7). We hypothesized that PROSCOOPs  
215 containing the VWD motif could be cleaved by SBT3.8 or its paralogs. To test this, we  
216 selected PROSCOOP20, which we recently identified as a 'core immunity response'  
217 gene<sup>27</sup>. Using a similar approach, we co-expressed SP-6xHA-PROSCOOP20-GFP  
218 (Fig. 3a) with different SBT3s. We found that expression of SBT3.6, SBT3.8 and  
219 SBT3.9 led to an increased accumulation of a lower molecular weight protein band,  
220 indicative of SCOOP20-GFP (Fig. 3b and Extended Data Fig. 9b; indicated by the red  
221 asterisk).

222 Notably, expression of SBT3.3, 3.4, 3.5 or 3.7 had no apparent effect on  
223 PROSCOOP20 cleavage (Fig. 3b), while expression of SBT3.3, 3.4, 3.6, 3.7, 3.8 or  
224 3.9 did not affect PROSCOOP12 cleavage, underlining the specificity of distinct  
225 PROSCOOPs cleavage by the different subtilases identified (Fig. 2b). The well-  
226 characterised subtilase SBT6.1/S1P did not seem to affect either PROSCOOP12 or  
227 PROSCOOP20 cleavage (Fig. 2b and 3b). The ability of the tested SBTs to induce  
228 PROSCOOP cleavage thus seems to correlate with the presence of distinct potential  
229 cleavage motifs (RRLM for PROSCOOP12, VWD for PROSCOOP20).

230 The increased accumulation of the cleaved band was reduced by co-expression with  
231 the inhibitors EPI1a and EPI10 (Fig. 3c). Similarly, mutation of the predicted 'VWD'  
232 motif into 'AAA' reduced PROSCOOP20 cleavage by SBT3.8 (Fig. 3d). These data  
233 suggest PROSCOOP20 cleavage is dependent on SBT3.8 activity.

234 Using N-terminal labelling-coupled MS (Extended Data Fig. 10), DLKIGASGSNSG  
235 peptide was detected (Extended Data Fig. 12), while TLLRDLKIGASGSNSG  
236 (Extended Data Fig. 13) was only detected once from three replicates, suggesting  
237 there could be another cleavage site after VWD motif to produce mature SCOOP20.



238 We next sought to genetically characterise the role of SBT3.8 in *Arabidopsis*. We were  
239 concerned about genetic redundancy as several SBT3.8 paralogs were able to  
240 promote PROSCOOP20 cleavage *in planta*, so decided to generate a higher-order  
241 *sbt3.6/7/8/9/10* mutant using CRISPR-Cas9<sup>41</sup> (Extended Data Fig. 14). This was  
242 facilitated by the tandem genomic arrangement of related SBT3s (Extended Data Fig.  
243 6). Notably, while overexpression of PROSCOOP20 in Col-0 caused a dwarf rosette  
244 phenotype, loss of SBT3.6/7/8/9/10 or the processing motif VWD (which was mutated  
245 into AAA) suppressed this phenotype (Fig. 3f, e). Together, these data indicate that  
246 SBT3.8 and potentially related subtilases mediate the cleavage of PROSCOOP20  
247 around the VWD motif, which is required for SCOOP20-induced signalling.

248

#### 249 **Loss of SBT3s partially phenocopies the loss of MIK2**

250 Our study revealed that the SCOOP family is much larger than previously predicted,  
251 which renders the genetic characterization of these peptides challenging. As we could  
252 demonstrate that different sub-group 3 subtilases are involved in SCOOP cleavage,  
253 we generated an octuple mutant for SBT3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9 and 3.10  
254 (*sbt3<sup>octuple</sup>*) by crossing independent *sbt3.3/4/5* and *sbt3.6/7/8/9/10* mutants generated  
255 using CRISPR-Cas9 (Extended Data Fig. 14). The *sbt* higher order mutants didn't  
256 show any macroscopic growth and developmental phenotype (Extended Data Fig.  
257 15a), however, the dry weight of *mik2-1* and *sbt<sup>octuple</sup>* is marginally increased compared  
258 with Col-0 (Extended Data Fig 15b). We hypothesised that the *sbt3<sup>octuple</sup>* mutant is  
259 impaired in PROSCOOP processing, and thus provides a genetic background to test  
260 for the requirement of SCOOP recognition in different MIK2-dependent processes.

261 Notably, similar to what observed in *mik2-1*<sup>15</sup>, flg22-induced ROS production was  
262 impaired in *sbt3<sup>octuple</sup>* plants (Fig. 4a, b), but not in *sbt3.3/4/5* or *sbt3.6/7/8/9/10*  
263 (Extended Data Fig. 15c). MIK2 is also involved in the induction of stress responses  
264 upon ISX treatment<sup>21,22</sup>. Induction of the stress marker genes *FRK1* and *CYP81F3*  
265 upon ISX treatment was similarly impaired in both *mik2-1* and *sbt3<sup>octuple</sup>* seedlings (Fig.  
266 4c). Moreover, it was recently demonstrated that MIK2 is required for basal resistance  
267 against the generalist herbivore *S. littoralis*<sup>19</sup>. This resistance – as determined by  
268 measuring the larvae fresh weight – was also impaired in *sbt3<sup>octuple</sup>*, albeit to an  
269 intermediate level compared to *mik2-1* plants (Fig. 4d).

270 These data suggest that the control of elicitor-induced ROS production, ISX-induced  
271 stress responses and basal resistance to *S. littoralis* all require SBT3 function, and

272 thus by extension potential PROSCOOP processing and subsequent SCOOP  
273 perception by MIK2. However, it must be noted that these subtilases have other targets  
274 *in planta* which may contribute to the phenotypes observed. For example, SBT3.8 also  
275 cleaves phytosulfokine (PSK) and CLE-LIKE/GOVERN/ROOT GROWTH FACTOR  
276 (CLEL/GLV/RGF) peptides, which have reported roles in the regulation of immune  
277 signalling<sup>4,49-52</sup>. However, while MIK2 regulates root growth angle<sup>21</sup> and basal  
278 resistance to *F. oxysporum*<sup>21,23</sup>, *sbt3<sup>octuple</sup>* plants behaved as wild-type Col-0 plants in  
279 these assays (Extended Data Fig. 15d, e), which could be due to non-cleaved  
280 PROSCOOPs and the generation of bioactive SCOOPs from some PROSCOOP  
281 precursors via subtilases beyond those characterised in our study. Notably, the recent  
282 identification of native SCOOP10 peptides in leaf apoplastic fluids suggests that the  
283 cleavage of PROSCOOP10, which lacks 'RxLx/RxxL' and 'VWD' motifs, could be  
284 dependent upon Y[KR]PN motif reported as cleavage site of subtilases SBT4.12,  
285 SBT4.13 and SBT5.2<sup>6,24,25,16</sup>. Furthermore, it is possible that some of the phenotypes  
286 observed in the *mik2* mutant may be independent of its function as the SCOOP  
287 receptor. Moreover, the strong susceptibility of *mik2* mutant plants to *F. oxysporum*  
288 (*Fo*), which is not phenocopied by the loss of SBT3s, suggests that resistance against  
289 this fungus may be due to the direct perception of *Fo*-derived elicitors (potentially  
290 exhibiting SCOOP-like sequences) by MIK2<sup>14,15,23</sup>, which would not necessarily require  
291 proteolytic cleavage by a plant subtilase. The exact *Fo*-derived elicitor(s) recognised  
292 by MIK2 during infection however remain to be identified.

293

294 Collectively, our study provides additional information about the recently identified  
295 family of SCOOP peptides, their post-translational processing and biological roles.  
296 Notably, as several SCOOPs have been previously proposed as antimicrobial  
297 peptides<sup>28,30</sup>, it will be interesting to understand the evolutionary and biochemical basis  
298 of the dual activity of these SCOOPs. This work also provides a starting point to dissect  
299 the multiple functions of MIK2. Notably, it will be interesting in the future to identify  
300 which specific SCOOPs mediate these functions, as recently demonstrated for  
301 SCOOP12 in the context of herbivory and root growth<sup>18,19</sup>.

302

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315

### 316 **Author contributions**

317 H.Y., J.R. and C.Z. conceived and designed the experiments. H.Y., J.R., X.K., J.S.,  
318 S.A., G.S.A., E.S., M.C.G., N.G.B., L.T.V.C. and K.W.B. generated materials,  
319 performed experiments, and/or analyzed the data. P.R., C.S.R., A.S., C.T., J.P.R.,  
320 F.M., A.S., J.R. and C.Z. supervised the project. A.S. contributed conceptually to the  
321 study and also provided materials. H.Y., J.R. and C.Z. wrote the manuscript with input  
322 from all authors.

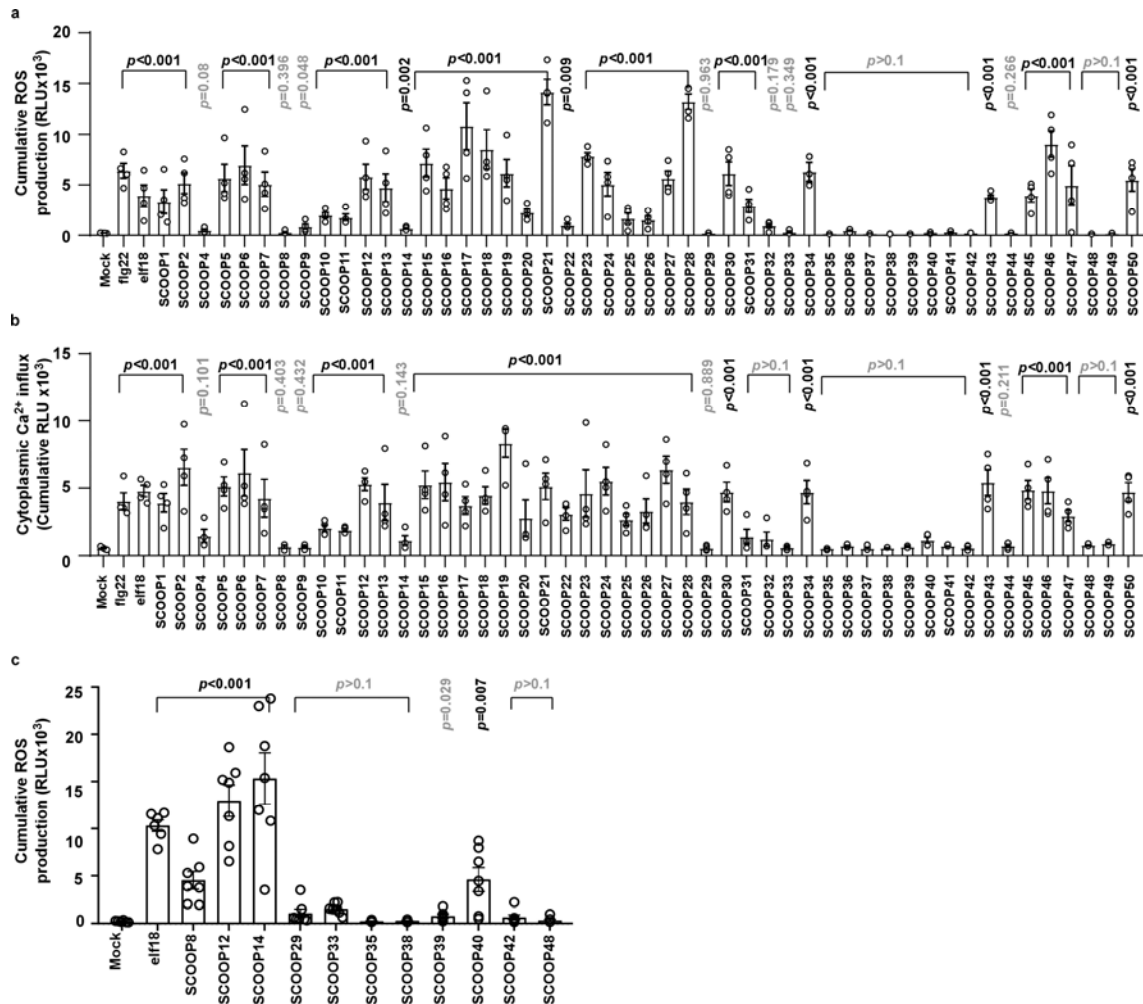
323

### 324 **Declaration of interests**

325 The authors declare no competing interests.

326

### 327 **Figure and Table legends**



328

329 **Figure 1 | Divergent SCOOPs induce ROS production and Ca<sup>2+</sup> influx in Col-0**

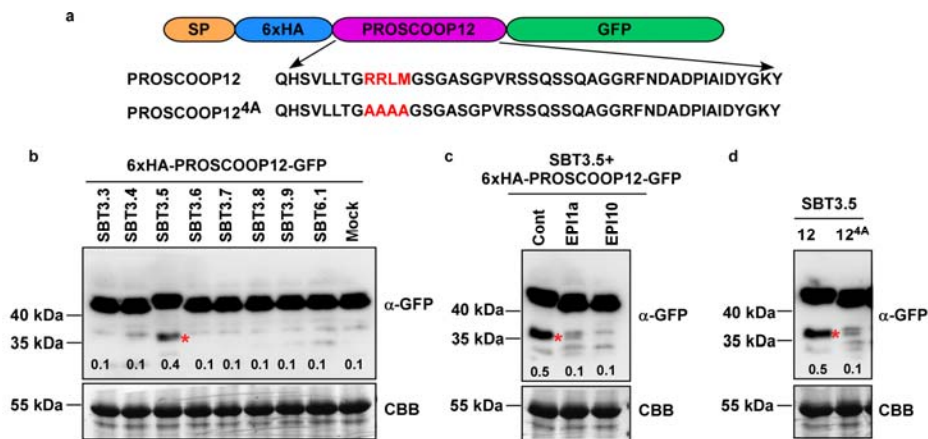
330 (a) Integrated ROS production over 40 min in leaf disks collected from 4-week-old  
 331 plants induced in the absence (Mock) or presence of 1 μM 13-mer SCOOP peptides  
 332 (n ≥ 8). flg22 and elf18 (100 nM) were used as control to calculate the Z-score to  
 333 normalise for variation between plates.

334 (b) Cytoplasmic Ca<sup>2+</sup> influx measured in Col-0<sup>AEQ</sup> seedlings induced in the absence  
 335 (Mock) or presence of 1 μM 13-mer SCOOP peptides (n=4) using 100 nM flg22 and  
 336 elf18 as control.

337 (c) Integrated ROS production over 40 min in leaf disks collected from 4-week-old  
 338 plants induced in the absence (Mock) or presence of 1 μM 15-mer SCOOPs (n ≥ 8)  
 339 using 100 nM elf18 as control.

340 Error bars represent SD; P-values indicate significance relative to the mock in a two-  
 341 tailed T-test. All experiments were repeated and analysed three times with similar  
 342 results. ROS, reactive oxygen species.

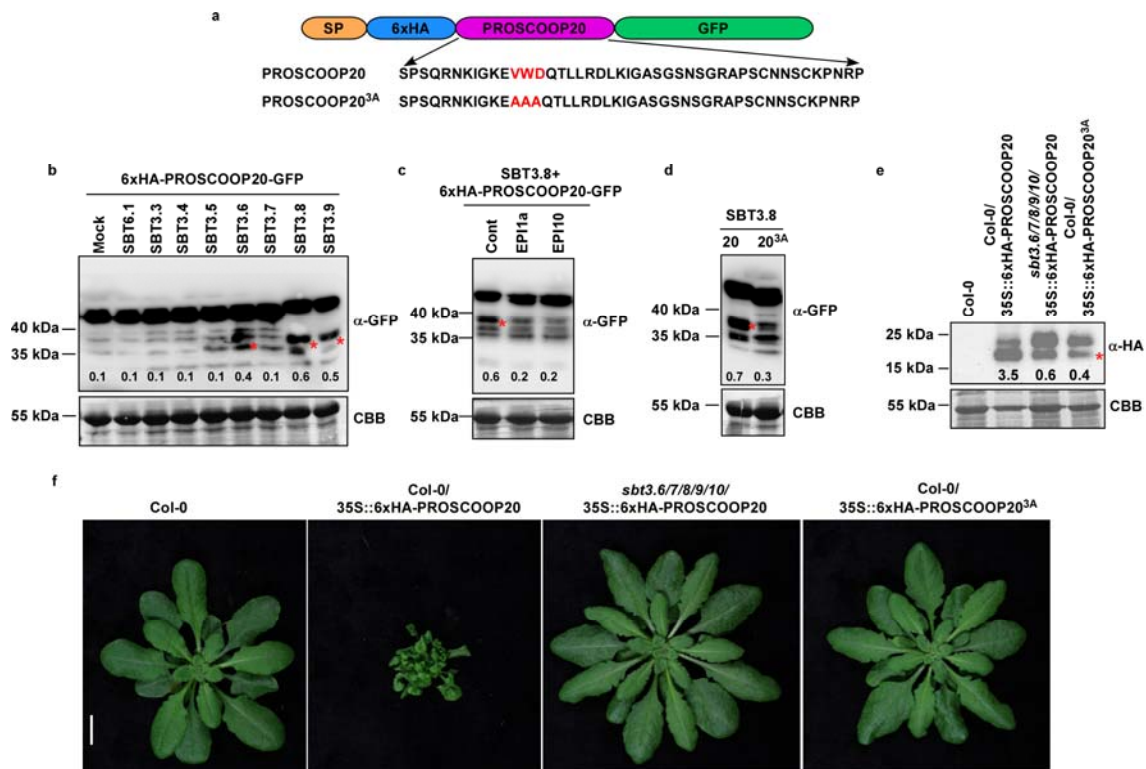
343



## Figure 2 | PROSCOOP12 is cleaved by SBT3.5

(a) Schematic representation of PROSCOOP12 with native signal peptide (SP), N-terminal 6xHA tag and C-terminal GFP tag. The cleavage motif RRLM and mutated residues of RRLM/AAAA are shown as red characters.

(b, c and d) Western blot using GFP antibody recognizing the 6xHA-PROSCOOP12-GFP and truncated SCOOP12-GFP (indicated by the red asterisk) in *Nicotiana benthamiana* leaves after 100 nM flg22 treatment for 2 h. The membrane was stained with CBB, as a loading control. Numbers on the blots correspond to the ratios of protein accumulation levels between truncated proteins bands (indicated by the red asterisk) and full-length protein bands (above the truncated protein bands). (b) Transient co-expression of 6xHA-PROSCOOP12-GFP with SBT3.3, SBT3.4, SBT3.5, SBT3.6, SBT3.7, SBT3.8, SBT3.9, SBT6.1 or without SBT (Mock). (c) Transient co-expression of 6xHA-PROSCOOP12-GFP, SBT3.5 with SBT inhibitors EPI1a/EPI10 or without SBT inhibitor (Mock). (d) Transient co-expression of SBT3.5 with 6xHA-PROSCOOP12-GFP (labelled as 12) or 6xHA-PROSCOOP12<sup>4A</sup>-GFP (labelled as 12<sup>4A</sup>). All experiments were repeated and analysed three times with similar results.



**Figure 3 | PROSCOOP20 is cleaved by SBT3.8 and SBT3.9**

(a) Schematic representation of PROSCOOP20 with native signal peptide (SP), N-terminal 6xHA tag and C-terminal GFP tag. The cleavage motif VWD and mutated residues of VWD/AAA are shown as red characters.

(b, c and d) Western blot using GFP antibody recognizing the 6xHA-PROSCOOP20-GFP and truncated SCOOP20-GFP (indicated by the red asterisk) in *Nicotiana benthamiana* leaves after 100 nM flg22 treatment for 2 h. The membrane was stained with CBB, as a loading control. Numbers on the blots correspond to the ratios of protein accumulation levels between truncated proteins bands (indicated by the red asterisk) and full-length protein bands (above the truncated proteins bands).

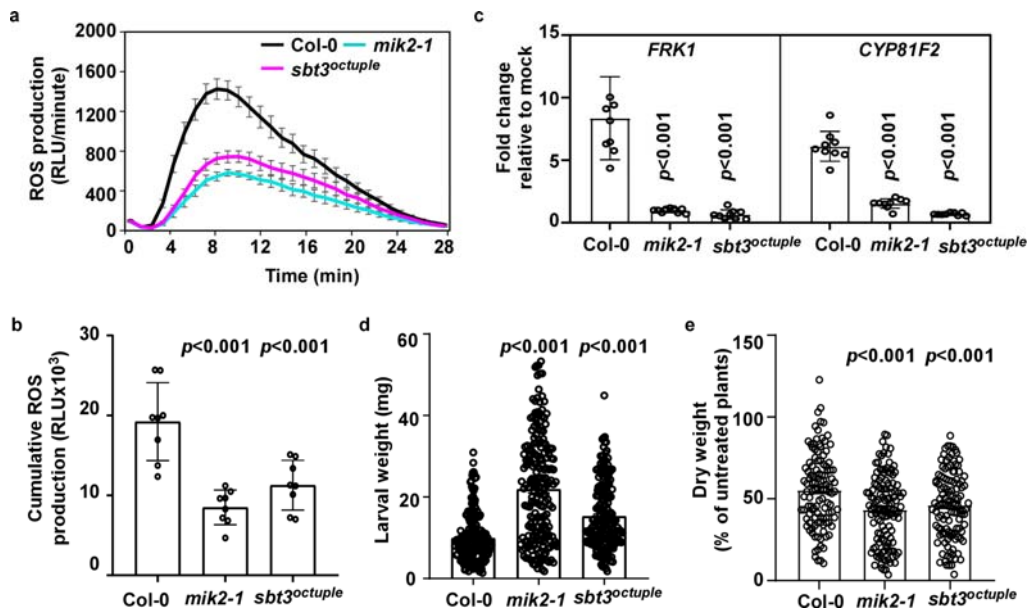
(b) Transient co-expression of 6xHA-PROSCOOP20-GFP with or without SBT3.3, SBT3.4, SBT3.5, SBT3.6, SBT3.7, SBT3.8, SBT3.9, SBT6.1 (Mock). (c) Transient co-expression of 6xHA-PROSCOOP-GFP, SBT3.8 with SBT inhibitors EPI1a/EPI10 or without (Mock). (d) Transient co-expression of SBT3.8 with 6xHA-PROSCOOP20-GFP (labelled as 20) or 6xHA-PROSCOOP20<sup>3A</sup>-GFP (labelled as 20<sup>3A</sup>). All experiments were repeated and analysed three times with similar results.

(e) Western blot using HA antibody recognizing the 6xHA-PROSCOOP20 in stable transgenic *Arabidopsis* seedlings expressing 35S::6xHA-PROSCOOP20 (labelled as 20) or 35S::6xHA-PROSCOOP20<sup>3A</sup> (labelled as 20<sup>3A</sup>) in Col-0 or *sbt3.6/7/8/9/10*. The



382 membrane was stained with CBB, as a loading control. #1: line 1, #2, line 2. Numbers  
 383 on the blots are the ratios of protein accumulation levels between truncated protein  
 384 bands (indicated by the red asterisk) and full-length protein bands (above the truncated  
 385 protein bands).

386 (f) Phenotype of stable transgenic *Arabidopsis* seedlings expressing 35S::6xHA-  
 387 PROSCOOP20 or 35S::6xHA-PROSCOOP20<sup>3A</sup> in Col-0 or *sbt3.6/7/8/9/10*. Bar = 1  
 388 cm.  
 389



390

391 **Figure 4 | *sbt3<sup>octuple</sup>* phenocopies *mik2***

392 (a and b) ROS production in leaf disks collected from 4-week-old *Arabidopsis* plants  
 393 induced by 100 nM flg22 (n = 8). (a) Points represent mean. (b) Integrated ROS  
 394 production over 30 min.

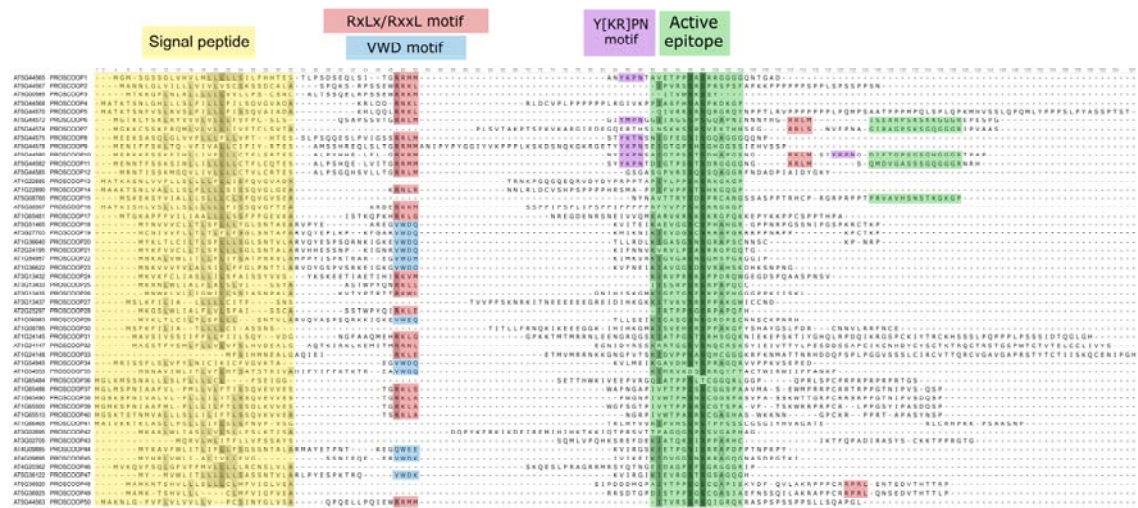
395 (c) Immune marker genes expression in 13-day-old *Arabidopsis* seedlings determined  
 396 by qRT-PCR. Seedlings were treated with 0.6 μM ISX or Mock. Expression of *FRK1*  
 397 and *CYP81F2* was normalized relative to *Actin7* expression values. Depicted is the  
 398 fold change in expression relative to mock treatment.

399 (d) Insect performance of *Spodoptera littoralis* on Col-0, *mik2-1* and *sbt3<sup>octuple</sup>*. *S.*  
 400 *littoralis* larvae were feeding on 5-week-old plants for 12 days. Data represents the  
 401 mean ± SD of three independent experiments. P-values indicate significance relative  
 402 to Col-0. (Mann-Whitney-U-Test). Number of individual larvae measured: Col-0 n=215,  
 403 *mik2-1* n=204 and *sbt3<sup>octuple</sup>* n=211.



404 (e) Dry weight of NaCl-treated plants is expressed as percentage of the ratio to the dry  
 405 weight of untreated plants. One week after germination, plants were transferred to pots  
 406 with soil saturated with or without 75 mM of NaCl in demineralized water. After 3 weeks  
 407 of treatment the rosettes were cut, and dry weight was determined.

408 (a-c, e) Error bars represent SD; P-values indicate significance relative to Col-0 in a  
 409 two-tailed T-test. All experiments were repeated and analysed three times with similar  
 410 results.

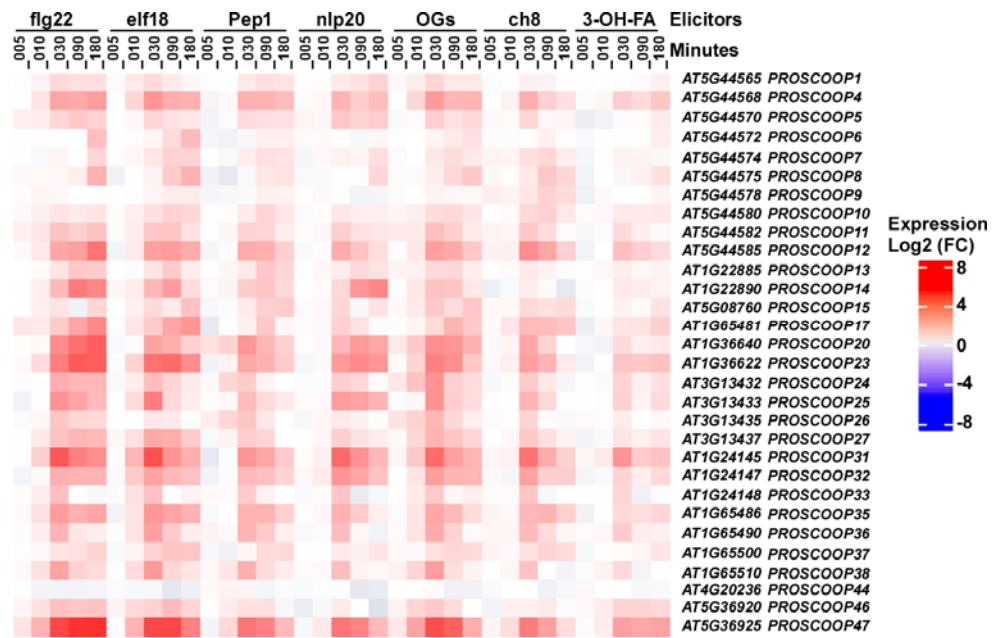


411

412 **Extended Data Fig. 1 | Sequence alignment of *Arabidopsis* PROSCOOPs**

413 Signal peptide, variable regions containing the predicted cleavage motifs  
 414 RxLx/RxxL/VWD, and the active epitope containing the conserved motif SxS are  
 415 indicated by coloured boxes.

416

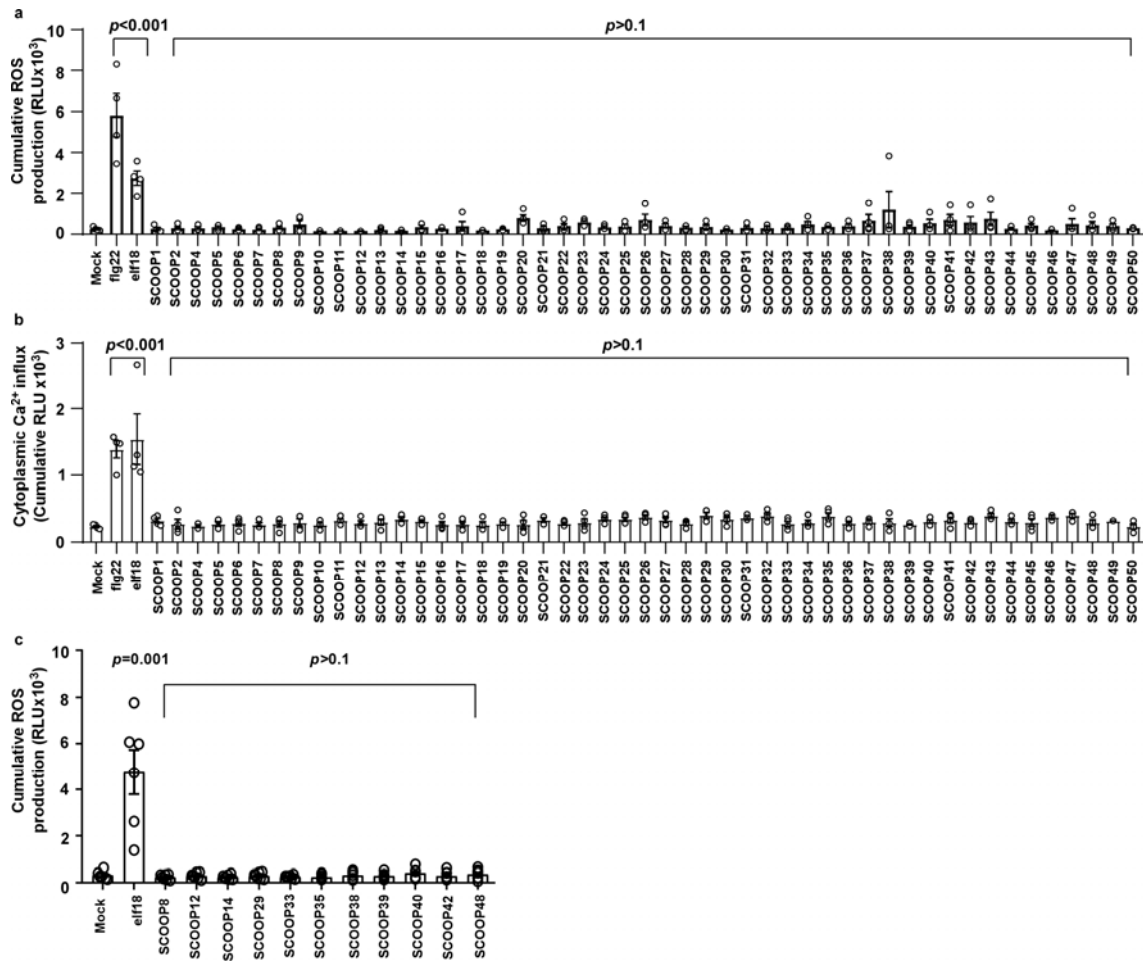


417

418 **Extended Data Fig. 2 | All *PROSCOOPs* identified by RNA-Seq are up-regulated**  
 419 **upon elicitor treatment**

420 Heat map showing  $\log_2(\text{FC})$  expression levels of *PROSCOOPs* identified by RNA-Seq

421 in response to a range of elicitors (data obtained from<sup>27</sup>).



422

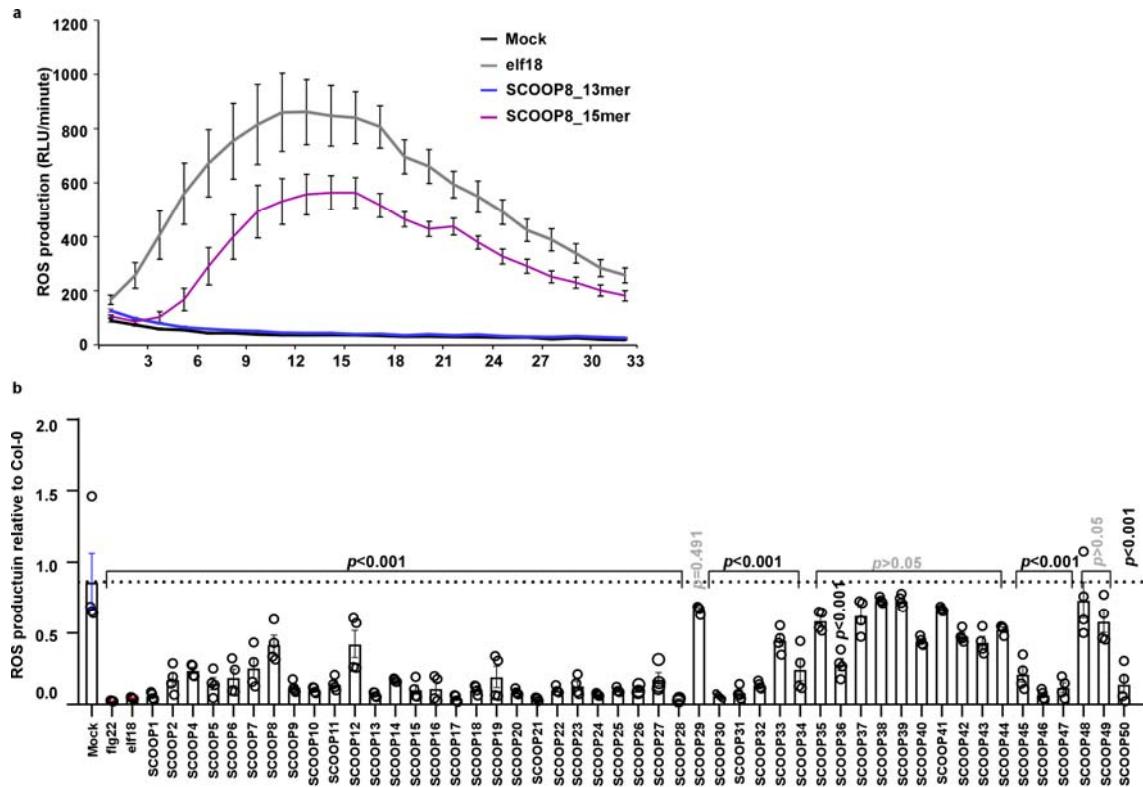
423 **Extended Data Fig. 3 | Divergent SCOOPs do not induce ROS production or Ca<sup>2+</sup>**  
 424 **influx in *mik2-1***

425 (a) Integrated ROS production over 40 min in leaf disks collected from 4-week-old  
 426 *Arabidopsis mik2-1* plants induced in the absence (Mock) or presence of 1 μM 13-mer  
 427 SCOOPs peptides (n ≥ 8) using 100 nM flg22 and elf18 as control.

428 (b) Cytoplasmic Ca<sup>2+</sup> influx measured in *mik2-1<sup>AEQ</sup>* seedlings induced in the absence  
 429 (Mock) or presence of 1 μM 13-mer SCOOPs peptides (n ≥ 8) using 100 nM flg22 and  
 430 elf18 as control. (c) Integrated ROS production over 40 min in leaf disks collected from

431 4-week-old *Arabidopsis mik2-1* plants induced in the absence (Mock) or presence of  
 432 **15-mer** SCOOPs (n ≥ 8) using 100 nM elf18 as control.

433 Error bars represent SD; P-values indicate significance relative to the mock in a Two-  
 434 tailed T-test. All experiments were repeated and analysed three times with similar  
 435 results.



436

437

**Extended Data Fig. 4 | Additional analysis of SCOOP-induced responses**

438

(a) Integrated ROS production over 40 min in leaf disks collected from 4-week-old *Arabidopsis* plants induced in the absence (Mock) or presence of 1  $\mu$ M 13/15-mer SCOOP8 peptides ( $n \geq 8$ ) using 100 nM elf18 as control.

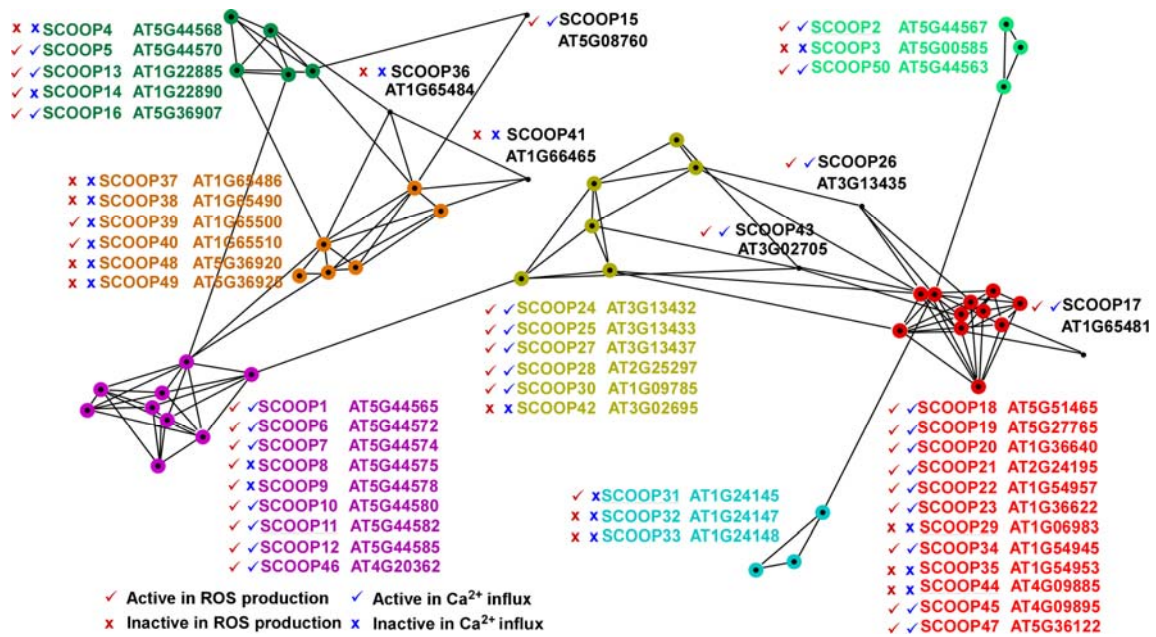
441

(b) Integrated ROS production over 40 min in leaf disks collected from 4-week-old *Arabidopsis bak1-5 bkk1* plants relative to Col-0 induced in the absence (Mock) or presence of 1  $\mu$ M 13-mer SCOOPs peptides ( $n \geq 8$ ) using 100 nM flg22 and elf18 as control.

445

Error bars represent SD; P-values indicate significance relative to the Mock in a Two-tailed T-test. All experiments were repeated and analysed three times with similar results.

448

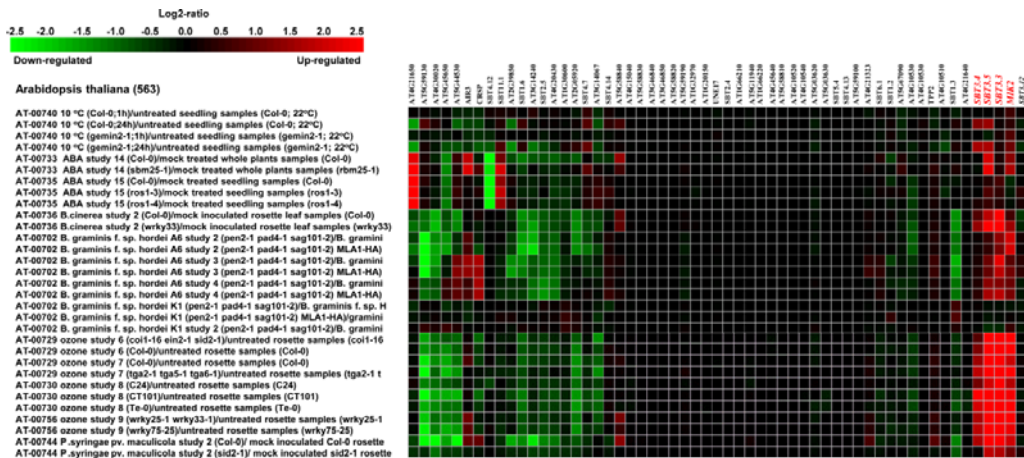


449

450 **Extended Data Fig. 5 | Similarity relationships inside the PROSCOOP family and**  
 451 **summary of SCOOPs activity in Col-0**

452 CLANS clustering based on all-against-all pairwise sequence similarities resulted in 7  
 453 groups (highlighted with different colours) and 6 singletons. P-values lower than 1.E-2  
 454 and 1.E-5 are represented by grey and black edges respectively.

455 Coloured asterisk indicates effects of SCOOP peptides.



456

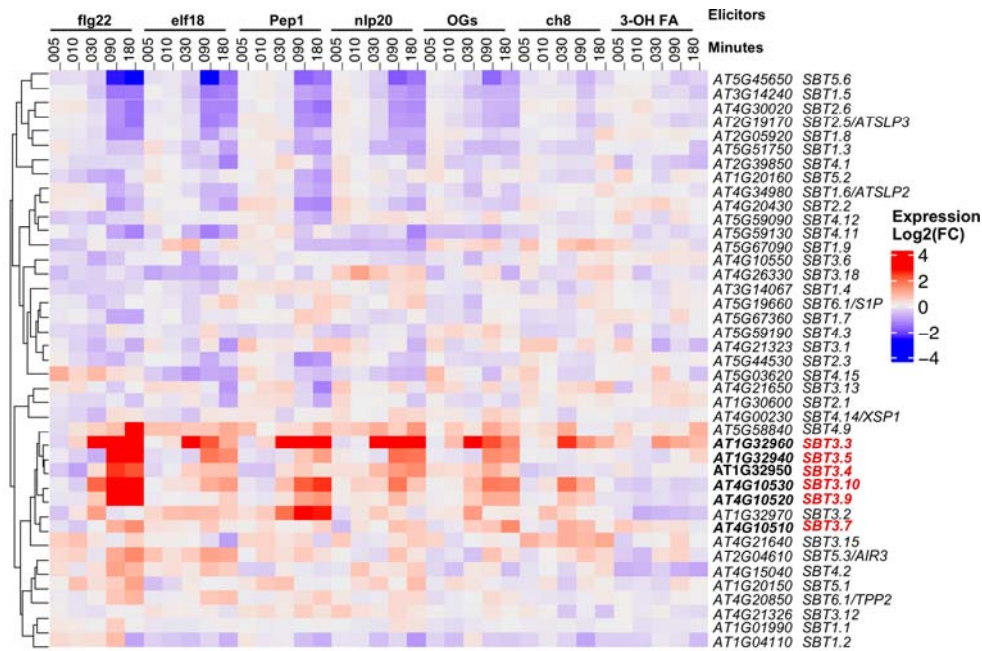
457

458 **Extended Data Fig. 6 | SBT3.3/3.4/3.5 are co-regulated with MIK2 in response to**  
 459 **different stresses**

460 Heat map showing log<sub>2</sub>(FC) expression levels of SBTs in response to stresses (data  
 461 obtained from Genevestigator).

462

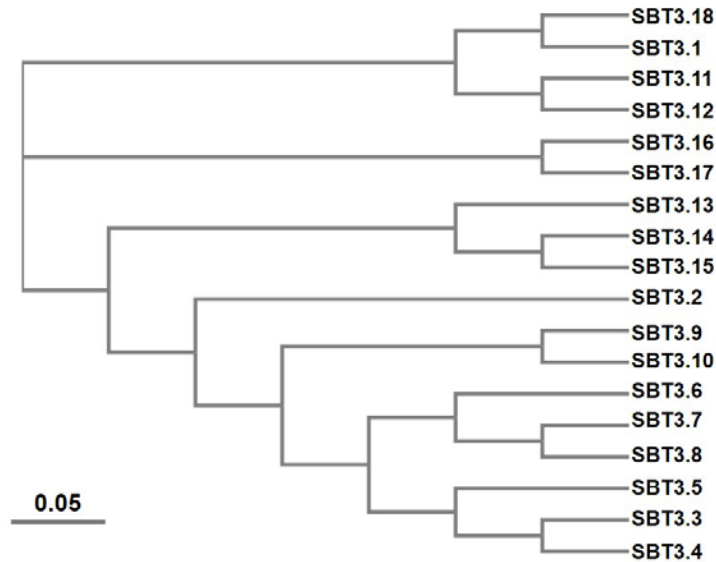




463

464 **Extended Data Fig. 7 | Transcriptional regulation of Arabidopsis *SBT* genes by**  
 465 **elicitors**

466 Heat map showing  $\log_2(\text{FC})$  expression levels of *SBT* genes in response to a range of  
 467 elicitors (data obtained from<sup>27</sup>).

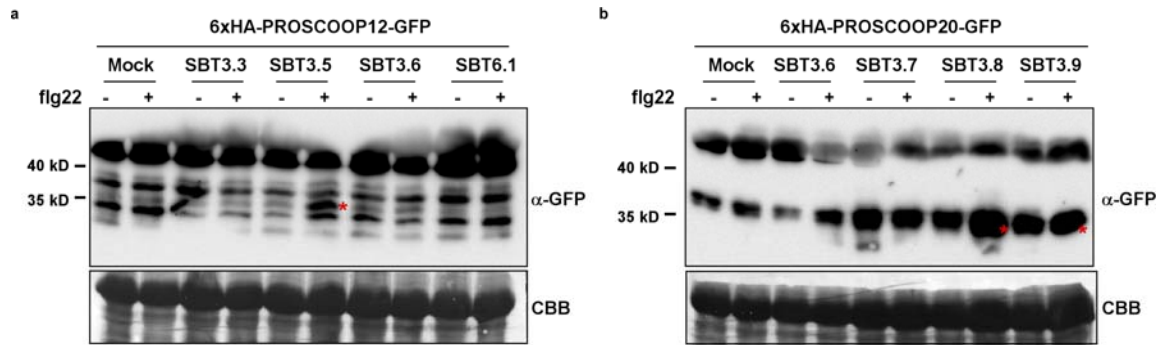


468

469 **Extended Data Fig. 8 | Phylogeny of Arabidopsis *SBT3* subgroup**

470 Phylogeny of the full-length amino acid sequences of *SBT3* was inferred using the  
 471 Maximum-likelihood method and JTT matrix-based model conducted in MEGAX<sup>53</sup>.

472



473

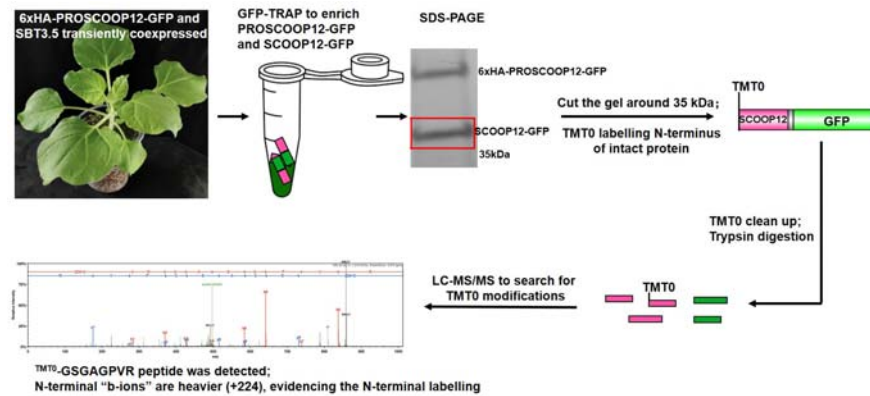
474 **Extended Data Fig. 9 | Cleavage analysis of PROSCOOP12 and PROSCOOP20 by**  
 475 **different SBTs**

476 (a) Related to Fig. 2 b, indicating flg22-induced SBT3.5-mediated PROSCOOP12  
 477 cleavage.

478 (b) Related to Fig. 3 b, indicating flg22-induced SBT3.6/SBT3.7/SBT3.8-mediated  
 479 PROSCOOP20 cleavage.

480 Red asterisks indicate the cleaved SCOOP-GFP bands.

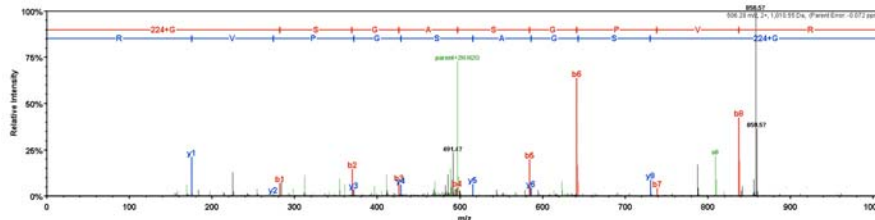
481



482

483 **Extended Data Fig. 10 | The diagram of TMT-labelling coupled MS**

484 **The working flow of TMT-labelling coupled MS to identify SBT3.5-mediated 6xHA-**  
 485 **PROSCOOP12-GFP cleavage sites and SBT3.6-mediated 6xHA-PROSCOOP20-**  
 486 **GFP cleavage sites in *Nicotiana benthamiana*.**



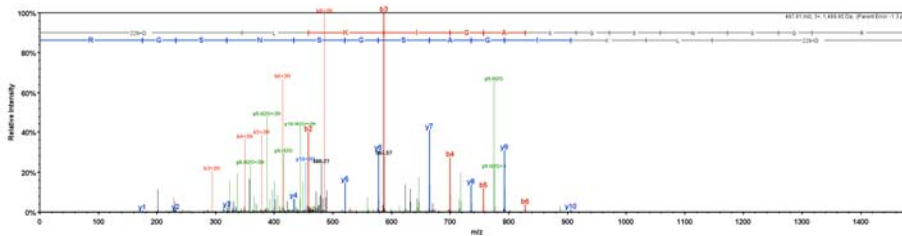
487

488 **Extended Data Fig. 11 | Mass spectrometry of TMT labelling peptide**



489 TMT-GSGAGPVR peptide was detected by N-terminal labelling-coupled mass  
490 spectrometry with three independent experiments.

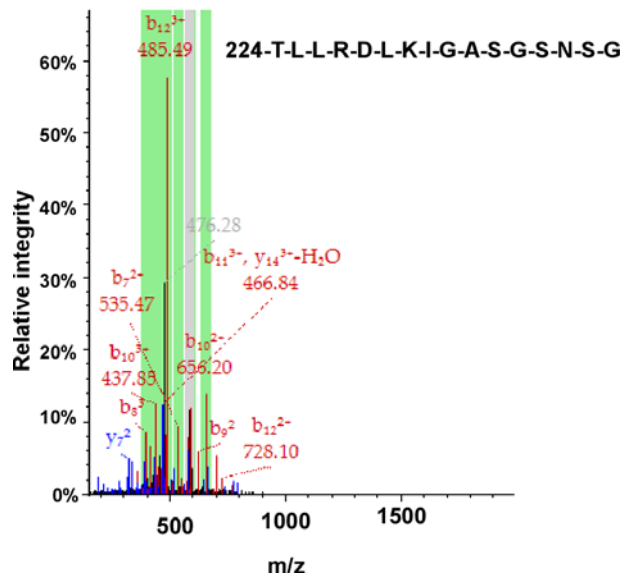
491



492

493 **Extended Data Fig. 12 | Mass spectrometry of TMT labelling peptide**

494 TMT-DLKIGASGSNSG peptide was detected by N-terminal labelling-coupled mass  
495 spectrometry with three independent experiments.

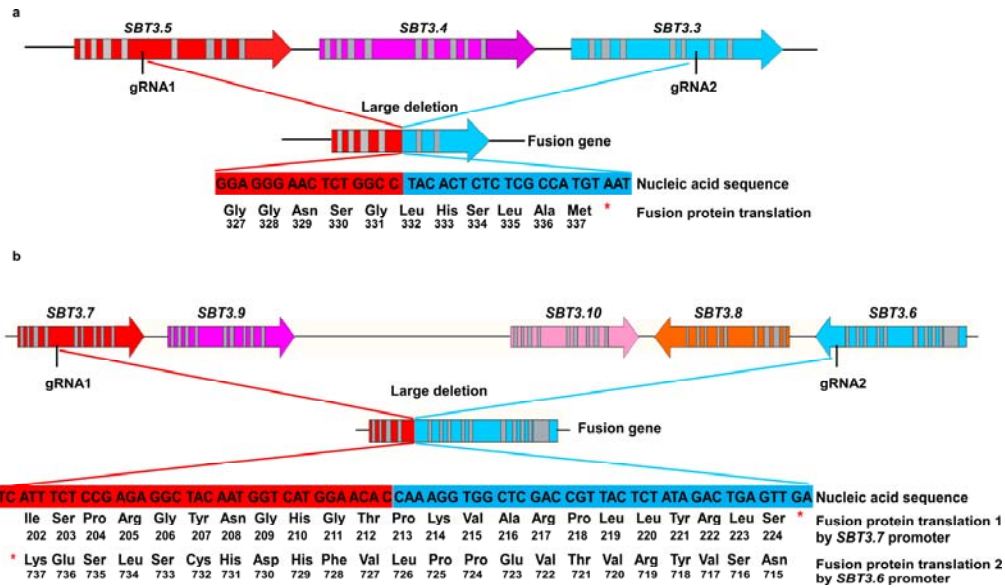


496

497 **Extended Data Fig. 13 | Mass spectrometry of TMT labelling peptide**

498 TMT-TLLRDLKIGASGSNSG peptide was detected by N-terminal labelling-coupled  
499 mass spectrometry with only one time from three repeats.

500



501

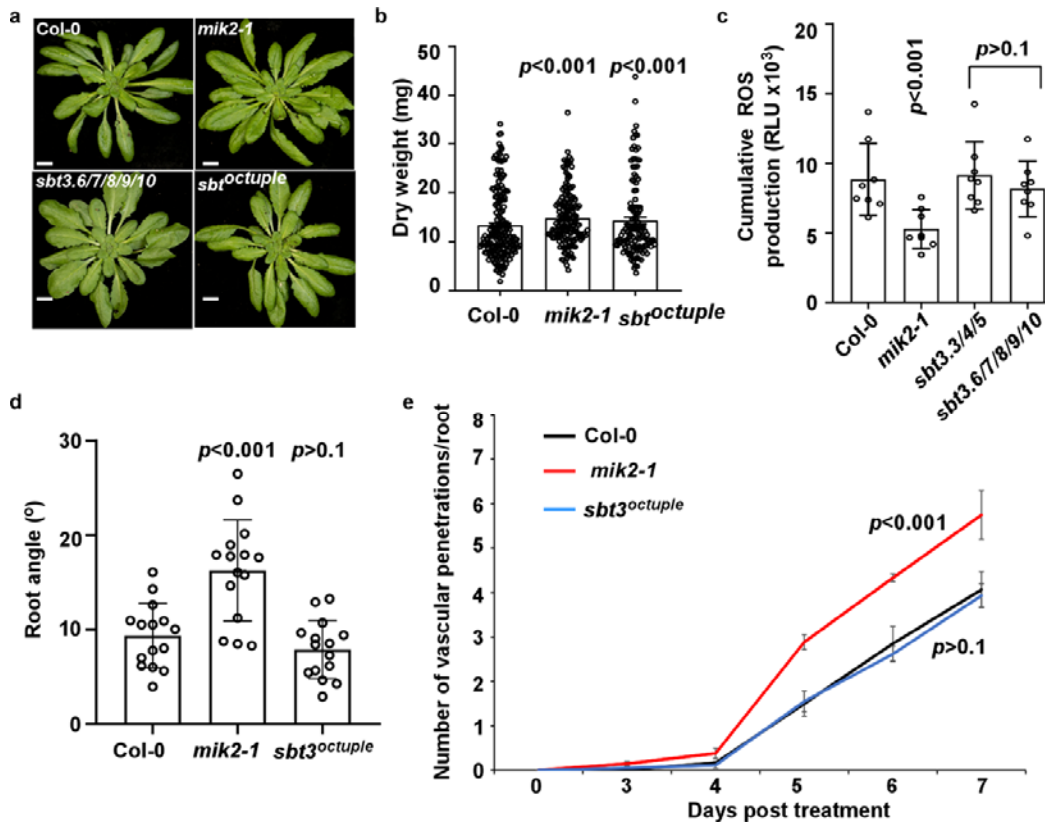
502

**Extended Data Fig. 14 | CRISPR deletes the majority of *SBT3.3/3.4/3.5* and *SBT3.6/3.7/3.8/3.9/3.10* genomic region**

503

504 (a) Schematic view of the large genomic deletion of clusters *SBT3.3/3.4/3.5*. The fusion  
 505 protein may be transcribed, consisting of the first 331 amino acids of *SBT3.5* with an  
 506 out-of-frame fusion with *SBT3.3* leading to an early stop. Primers F1/R1 and F2/R2  
 507 used for the genotyping and sequence (Extended Data Table 3).

508 (b) Schematic view of the large genomic deletion of clusters *SBT3.6/3.7/3.8/3.9/3.10*.  
 509 One fusion protein driven by the promoter *SBT3.7* may be transcribed, consisting of  
 510 212 amino acid of *SBT3.7*, fused to 12 nonsense amino acids from *SBT3.6* genomic  
 511 region. Another fusion protein driven by the promoter *SBT3.6* may be transcribed,  
 512 consisting of 726 amino acids of *SBT3.6*, fused to 11 nonsense amino acids from  
 513 *SBT3.7* genomic region. Primers F3/R3 and F4/R4 used for the genotyping and  
 514 sequence (Extended Data Table 3).



515

516 **Extended Data Fig. 15 | Phenotypic analysis of *sbt* higher-order mutants**

517 (a) Phenotype of 5-week-old plants. Bar=1cm.

518 (b) Dry weight of 4-week-old plants. One week after germination, plants were  
 519 transferred to pots with soil watered from below in demineralized water. After 3 weeks  
 520 the rosettes were cut, and dry weight was determined.

521 (c) Integrated ROS production over 30 min in leaf disks collected from 4-week-old  
 522 Arabidopsis plants induced by 100 nM flg22 application (n=8).

523 (d) Quantification of the root angle of 9-day-old seedlings grown in an upright position  
 524 on MS agar medium (n=15).

525 (e) Cumulative Arabidopsis root vascular penetration by Fo5176 in WT (Col-0), *mik2-*  
 526 *1*, and *sbt3<sup>octuple</sup>* plants at different days post transfer (dpt) to plate-containing spores.  
 527 Means ± SEM; N = 3 replicates, each one containing 20 plants. Statistical significance  
 528 calculated via repeated measures two-way ANOVA with Tukey post-hoc test (p value  
 529 ≤ 0.05 (genotype), p value ≤ 0.05 (time), p value ≤ 0.05 (genotype). P values are  
 530 indicated in the graph with respect to WT at any dpt.

531 Error bars represent S.D; P-values indicate significance relative to Col-0 in a Two-  
 532 tailed T-test. All experiments were repeated and analysed three times with similar  
 533 results.

534

## 535 **Material and Methods**

### 536 **Plant material and growth conditions**

537 *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as wild-type control. Plants  
538 for ROS burst assays were grown in individual pots at 21 °C with a 10-h photoperiod.  
539 Seeds grown on plates were surface-sterilized using chlorine gas for 5–6 h, and sown  
540 on Murashige and Skoog (MS) media supplemented with vitamins, 1 % sucrose, and  
541 0.8 % agar and stratified at 4 °C for 2–3 days. *Nicotiana benthamiana* plants were  
542 grown on peat-based media at 24 °C, with 16-h photoperiod.

543 Aequorin lines of *Arabidopsis* were described previously<sup>15,54</sup>. *mik2-1* and *sbt3.5-1*  
544 mutants have been previously described<sup>21,37</sup>.

### 545 **Synthetic peptides**

546 All synthetic peptides were ordered at >80 % purity (physiological assays) or >95 %  
547 purity (biochemical assays) (EZBiolabs). Sequences of all peptides can be found in  
548 Supplementary Table 1. The gene models from which the peptide sequences were  
549 extracted are listed in Supplementary Table 2.

### 550 **Molecular cloning**

551 These constructs below were generated by Greengate cloning<sup>55</sup>. pGGZ-35S::SBT3.4  
552 and pGGZ-35S::SBT3.9, pGGZ-35S::6xHA-PROSCOOP12-GFP, pGGZ-35S::6xHA-  
553 PROSCOOP12<sup>4A</sup>(RRLM/AAAA)-GFP, pGGZ-35S::6xHA-PROSCOOP20-GFP,  
554 pGGZ-35S::6xHA-PROSCOOP20<sup>3A</sup>(VWD/AAA)-GFP, pGGZ-35S::6xHA-  
555 PROSCOOP20, pGGZ-35S::6xHA-PROSCOOP20<sup>3A</sup>(VWD/AAA).

556 The genomic DNA of *SBT3.4* and *SBT3.9* was amplified by PCR. The CDS of 6xHA-  
557 PROSCOOP12, 6xHA-PROSCOOP12<sup>4A</sup> (RRLM/AAAA), 6xHA-PROSCOOP12 and  
558 6xHA-PROSCOOP12<sup>3A</sup>(VWD/AAAA) were synthesized. Clones were verified by  
559 Sanger sequencing.

560 pGreen0229-pPROSCOOP12::EPI1a/10 was generated by replacing the IDA  
561 promoter from the construct pGreen0229-pIDA::EPI1a/10<sup>6</sup>. 1500 bp upstream of the  
562 start codon of the PROSCOOP12 gene were amplified by PCR using the primer (Table  
563 S3). The PCR products and pGreen0229-pIDA::EPI1a/10 were digested with NotI and  
564 PstI. Then the PCR product was cloned into the respective restriction sites of  
565 pGreen0229 upstream of the EPI1a or EPI10 constructs.

566 Expression constructs pART27-35S::SBT3.5 and pART7-35S::SBT3.8 have been  
567 described previously<sup>5,37</sup>. Expression constructs for SBT3.3 and SBT3.7 were

568 generated accordingly. Briefly, the open reading frames of SBT3.3 and SBT3.7 were  
569 amplified by PCR and cloned into the multiple cloning site of pART7<sup>56</sup>, between the  
570 CaMV-35S promoter and terminator. The expression cassette was then transferred  
571 into the NotI site of the binary vector pART27<sup>56</sup>.

### 572 **CRISPR-Cas9 mutagenesis**

573 CRISPR-Cas9 induced mutagenesis was performed as described<sup>57</sup>. The *RPS5a*  
574 promoter drove Cas9 expression and FASTred selection was used for positive and  
575 negative selection. Primers used to generate the vectors can be found in **Extended**  
576 **Data Table 3**. Mutants were screened by PCR genotyping and confirmed by Sanger  
577 sequencing. Primers used for genotyping and sequencing can be found in **Extended**  
578 **Data Table 3**.

### 579 **ROS measurement**

580 Leaf disks were harvested from 4-week-old *Arabidopsis* plants using a 4-mm diameter  
581 biopsy punch (Integra™ Miltex™) and placed into white 96-well-plates (655075,  
582 Greiner Bio-One) containing 100 µL water. Leaf disks were rested overnight. Prior to  
583 ROS measurement, the water was removed and replaced with ROS assay solution  
584 (100 µM Luminol (123072, Merck), 20 µg mL<sup>-1</sup> horseradish peroxidase (P6782, Merck))  
585 with or without elicitors. Immediately after light emission was measured from the plate  
586 using a HIGH-RESOLUTION PHOTON COUNTING SYSTEM (HRPCS218, Photech)  
587 equipped with a 20 mm F1.8 EX DG ASPHERICAL RF WIDE LENS (Sigma Corp). For  
588 assays were not all treatments could be conducted on one plate a Z-score was  
589 calculated. Internal controls of mock and 100 nM elf18 were included on each plate  
590  $Z(\text{standard score}) = \frac{x (\text{integrated ROS production for one leaf disk}) - \mu (\text{Mean}$   
591  $\text{integrated ROS production for mock treatment})}{\sigma (\text{standard deviation of integrated}$   
592  $\text{ROS production for mock treatment})}$  as performed previously<sup>58</sup>.

### 593 **Cytoplasmic Ca<sup>2+</sup> measurement**

594 Seedlings were initially grown on 1/2 MS plated for 3 days before being transferred to  
595 96-well plates (655075, Greiner Bio-One) in 100 µL liquid MS for 5 days. The evening  
596 before Ca<sup>2+</sup> measurements the liquid MS was replaced with 100 µL 20 µM  
597 coelenterazine (EC14031, Carbosynth) and the seedlings incubated in the dark  
598 overnight. The following morning the coelenterazine solution was replaced with 100 µL  
599 water and rested for a minimum of 30 min in the dark. Readings were taken in a  
600 VARIOSKAN™ MUTIPLATE READER (ThermoFisher) before and after adding 50 µL

601 of 3 x concentrated elicitor solution or Mock. Readings were normalised to the average  
602 RLU value before elicitor addition ( $L_0$ ).

### 603 **CLANs clustering of PROSCOOP family**

604 The 50 PROSCOOP sequences have been analysed and clustered using the CLANS  
605 software based on all-against-all BLASTP pairwise sequence similarities<sup>59</sup>. P-value  
606 cut-off has been set to 1.E-2 for edge definition and the Convex method (stdev cut-off  
607 0.5, minimum of 3 sequences per cluster) has been applied for clustering.

### 608 **Transient expression in *Nicotiana benthamiana***

609 *Agrobacterium tumefaciens* strain GV3101 transformed with the appropriate construct  
610 were grown overnight in L-media and spun-down. The bacteria were resuspended in  
611 10 mM  $MgCl_2$  and adjusted to O.D.<sub>600</sub> = 0.2 prior to infiltration into the youngest fully  
612 expanded leaves of 3-week-old plants. Two days later, leaf tissues were collected,  
613 treated in liquid MS medium by 100 nM flg22 for 2 hours and flash-frozen in liquid  
614 nitrogen.

### 615 **Protein extraction and western blot**

616 *N. benthamiana* leaf tissues were flash-frozen in liquid nitrogen. Plant tissue was  
617 ground in liquid nitrogen prior to boiling in 2× loading sample buffer (4 % SDS, 20 %  
618 glycerol, 10 % 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris-HCl;  
619 (10  $\mu$ L.mg<sup>-1</sup> tissue)) for 10 min at 95 °C. The samples were then spun at 13,000 × g  
620 for 5 min prior to loading and running on SDS-PAGE gels of an appropriate  
621 concentration. Proteins were transferred onto PVDF membrane (ThermoFisher) prior  
622 to incubation with appropriate antibodies ( $\alpha$ -HA-HRP (12013819001, Roche, 1:3000);  
623  $\alpha$ -RFP-HRP (sc-9996 HRP, Santa Cruz, 1:5000) and  $\alpha$ -GFP-HRP (A-0545, Merck;  
624 1:50000). Western blots were imaged with a LAS 4000 IMAGEQUANT SYSTEM (GE  
625 Healthcare). Staining of the blotted membrane with Coomassie brilliant blue was used  
626 to confirm loading.

### 627 **GFP-TRAP enrichment**

628 After protein extraction from *N. benthamiana* leaf tissues, the 6xHA-  
629 PROSCOOP12/20-GFP and SCOOP12/20-GFP was enriched by GFP-TRAP.  
630 Proteins were extracted using the extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl,  
631 2.5 mM EDTA, 10 % Glycerol, 1 % IGEPAL, 5 mM DTT, 1 % Plant Protease Inhibitor  
632 (P9599, Sigma)) (v/v). Proteins were solubilised at 4 °C with gentle agitation for 30 min  
633 before filtering through miracloth. The filtrate was centrifuged at 15000 × g for 20 min  
634 at 4 °C. An input sample was taken. To each 15 mL of protein extract 40  $\mu$ L of GFP-

635 TRAP AGAROSE BEADS (50 % slurry, ChromoTek) washed in extraction buffer were  
636 added and incubated with gentle agitation for 4 h at 4 °C. Beads were harvested by  
637 centrifugation at 1500 x g for 2 min and washed 3 times in extraction buffer. 50 µL of  
638 1.5 x elution buffer (NuPage) were added and incubated at 90 °C for 10 min. The  
639 samples were then spun at 13,000 × g for 5 min prior to loading and running on SDS-  
640 PAGE gels of an appropriate concentration. Samples were then cut from the SDS-  
641 PAGE gel for subsequent MS analysis.

#### 642 **N-terminal labelling and mass spectrometry**

643 Proteins separated by SDS-PAGE were excised, destained with 25% Acetonitrile,  
644 reduced by 10mM DTT 55 °C for 30min, and carbamidomethylated with 40mM  
645 chloroacetamide. The gel was washed with copious exchanges of 50mM TEAB and  
646 dehydrated with 100% acetonitrile. Protein free N-termini were labelled with 100µg  
647 TMT reagent (ThermoFisher) dissolved in 20µL 100% acetonitrile and directly added  
648 to the gel followed by 80 µL of 50mM TEAB (ThermoFisher) buffer. The reaction left at  
649 room temperature for 90min was subsequently quenched with 5% hydroxylamine; after  
650 this, all proteoforms with accessible N-termini should be N-terminally TMT-labelled.

651 The reagent leftovers were extracted by 50mM ammonium bicarbonate buffer and the  
652 gel dehydrated with 100% acetonitrile. Proteins were in-gel digested by 100ng of  
653 trypsin (ThermoFisher), peptides extracted with 25% Acetonitrile, freeze-dried and  
654 measured by Orbitrap Eclipse (ThermoFisher Scientific) LC-MS system with a data-  
655 dependent acquisition MS method.

656 The data were processed with a common proteomic pipeline consisting of MS Convert  
657 program to generate peak-lists followed by peptide sequence matching on Mascot  
658 (Matrix Science Ltd.) server. The individual samples were compared and tandem mass  
659 spectra visualized in Scaffold (Proteome Software Inc.).

#### 660 ***RNA extraction and qRT-PCR***

661 Two 5-day-old seedlings were transferred into transparent 24-well plates (Greiner Bio-  
662 One) containing 1 mL liquid MS media, sealed with porous tape and grown for a further  
663 7 days. The liquid MS media was taken out and 1 mL fresh liquid MS medium  
664 containing 600 nM isoxaben (ISX) (Sigma-Aldrich, St. Louis, MO, USA) or Mock was  
665 added, and seedlings were harvested after 9 h treatment. All seedlings were ground in  
666 liquid nitrogen. Total RNA was extracted using plant total RNA mini kit (FavorPrep)  
667 according to the manufacturer's instructions and DNase treatment was performed  
668 using the RNeasy kit (Qiagen). RNA was quantified with a Nanodrop



669 spectrophotometer (Thermo Fischer Scientific). qRT-PCR was performed on cDNA  
670 synthesised using the RevertAid first strand cDNA synthesis kit (Thermo Fisher  
671 Scientific) according to the manufacturer's instructions. cDNA was amplified by  
672 quantitative PCR using PowerUP SYBR Green Master mix (Thermo Fischer Scientific)  
673 running on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fischer  
674 Scientific).

#### 675 **Insect performance**

676 *Spodoptera littoralis* (Egyptian cotton worm) eggs were obtained from Syngenta (Stein  
677 AG; Switzerland). For hatching, *S. littoralis* eggs were incubated for 48 h at 28 °C. For  
678 measurements of insect performance, 60–80 freshly hatched *S. littoralis* larvae were  
679 placed on 11 5-week-old plants per genotype in transparent plexiglass boxes. *S.*  
680 *littoralis* larvae were allowed to feed on those plants for 12 days and individual larval  
681 weights were determined subsequently on a high precision balance (Mettler-Toledo;  
682 XP205DR, Switzerland).

#### 683 ***Fusarium oxysporum* 5176 infection assay**

684 Plants were infected on plates as previously described<sup>60,61</sup>. Briefly, seeds were  
685 germinated on Whatman filter paper strips in ½ MS+0.9% agar. Eight-day-old seedling  
686 were transferred to a mock or infection plate. The infection plates were prepared by  
687 spreading 100 µL 1 x 10<sup>7</sup> pSIX1:GFP spores / mL on the MS+0.9% bactoagar surface.  
688 Vascular penetration sites were recorded from 3 to 7 days post transfer (dpt) to spore-  
689 containing plates, determined when strong and linear signals were visible under a  
690 Leica M205 FCA fluorescent stereo microscope equipped with a long pass GFP filter  
691 (ET GFP LP; Excitation nm: ET480/40x; Emission nm: ET510 LP).

#### 692 **Root skewing assay**

693 Seeds were sown directly on 1/2 MS agar square plates and stratified for 2 days at  
694 4 °C. Plates were transferred to 22 °C under a 16-h photoperiod, in an upright position  
695 for 9 d. The root angle was measured by the ImageJ software, as performed  
696 previously<sup>21</sup>.

#### 697 **Salt tolerance assays**

698 Salt tolerance assays were performed as previously<sup>21</sup>. Plants were grown in pots under  
699 an 11-h photoperiod, at 22 degrees and 70% humidity. One week after germination,  
700 plants were transferred to pots which were saturated with 4 L of either 0 or 75 mM of  
701 NaCl solution. During the experiment, all plants were watered with demineralized water  
702 from below. After 3 weeks of treatment, plants were cut off and dried in an oven at 70

703 degrees for 1 week to determine dry weight. Plants were randomised over trays using  
704 a randomized block design. Randomisation was similar for each treatment. The  
705 experiment was repeated three times with similar results

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