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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 responses¹⁻³. Such peptides are
57 generally derived from protein precursors; however, relatively few biogenesis
58 mechanisms have been described^{2,4-9}. 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 metazoans¹⁰⁻¹². 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 interactions¹³⁻¹⁵. 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 bioactivity¹³⁻¹⁵.
66 **PROSCOOPs undergo maturation steps to produce bioactive SCOOPs^{14,16}.**
67 **Cleavage of PROSCOOP12 was reported to be after R43¹⁴. For PROSCOOP10, two**
68 **distinct cleaved peptides (SCOOP10#1 and SCOOP10#2) with hydroxylated prolines**

69 were identified from leaf apoplastic fluids¹⁶. 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^{13-15,17}, 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*)¹³, the SCOOP family has
75 recently been shown to contain up to 28 members^{14,17}. 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 Ca²⁺
78 concentration, and immune gene expression^{13-15,17}. 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^{13,18,19}. 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^{14,15}. 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^{14,15,19-23}. 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^{14,15}. 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^{24,25}.
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 genome¹³.

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

110 23, and then 28 genes^{14,17}, 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)²⁶. 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 activity¹³⁻¹⁵. Interestingly, all of the 30 PROSCOOP

119 transcripts detected in our recent RNA-Seq dataset²⁷ 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)^{14,15,28},

123 ENHANCER OF VASCULAR WILT RESISTANCE 1 (EWR1)^{17,29} and ARACINs³⁰

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

125 have direct antimicrobial activity^{28,30}, 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)³¹.

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 Ca²⁺ 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 SCOOP12¹³⁻¹⁵, 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)¹⁴. 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 receptors³², 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 cleavage¹⁴, 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 SBT3.5³³⁻³⁷. Using
168 Genevestigator³⁸, we observed that among 56 *SBT* genes in *Arabidopsis*³⁷, 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)^{27,39}. Notably, SBT3.3 and SBT3.5 regulate plant immunity
173 and root growth, respectively^{37,40}, similar to PROSCOOP12^{13,19}. Also, the SBT3.5 has
174 a preference for RKLL motif³⁷, 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^{13-15,18,19}. 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 genome⁴¹, 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 activity⁴²⁻⁴⁴). 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 RR¹⁴. 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^{5,49}. SBT3.6, SBT3.7, SBT3.9 and SBT3.10 are SBT3.8 homologues
213 (Extended Data Fig. 8³⁹) 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 gene²⁷. 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-Cas9⁴¹ (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^{octuple}*) 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^{octuple}* is marginally increased compared
258 with Col-0 (Extended Data Fig 15b). We hypothesised that the *sbt3^{octuple}* 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*¹⁵, flg22-induced ROS production was
262 impaired in *sbt3^{octuple}* 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^{21,22}. Induction of the stress marker genes *FRK1* and *CYP81F3*
265 upon ISX treatment was similarly impaired in both *mik2-1* and *sbt3^{octuple}* seedlings (Fig.
266 4c). Moreover, it was recently demonstrated that MIK2 is required for basal resistance
267 against the generalist herbivore *S. littoralis*¹⁹. This resistance – as determined by
268 measuring the larvae fresh weight – was also impaired in *sbt3^{octuple}*, 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^{4,49-52}. However, while MIK2 regulates root growth angle²¹ and basal
278 resistance to *F. oxysporum*^{21,23}, *sbt3^{octuple}* 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^{6,24,25,16}. 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^{14,15,23}, 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^{28,30}, 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^{18,19}.

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.

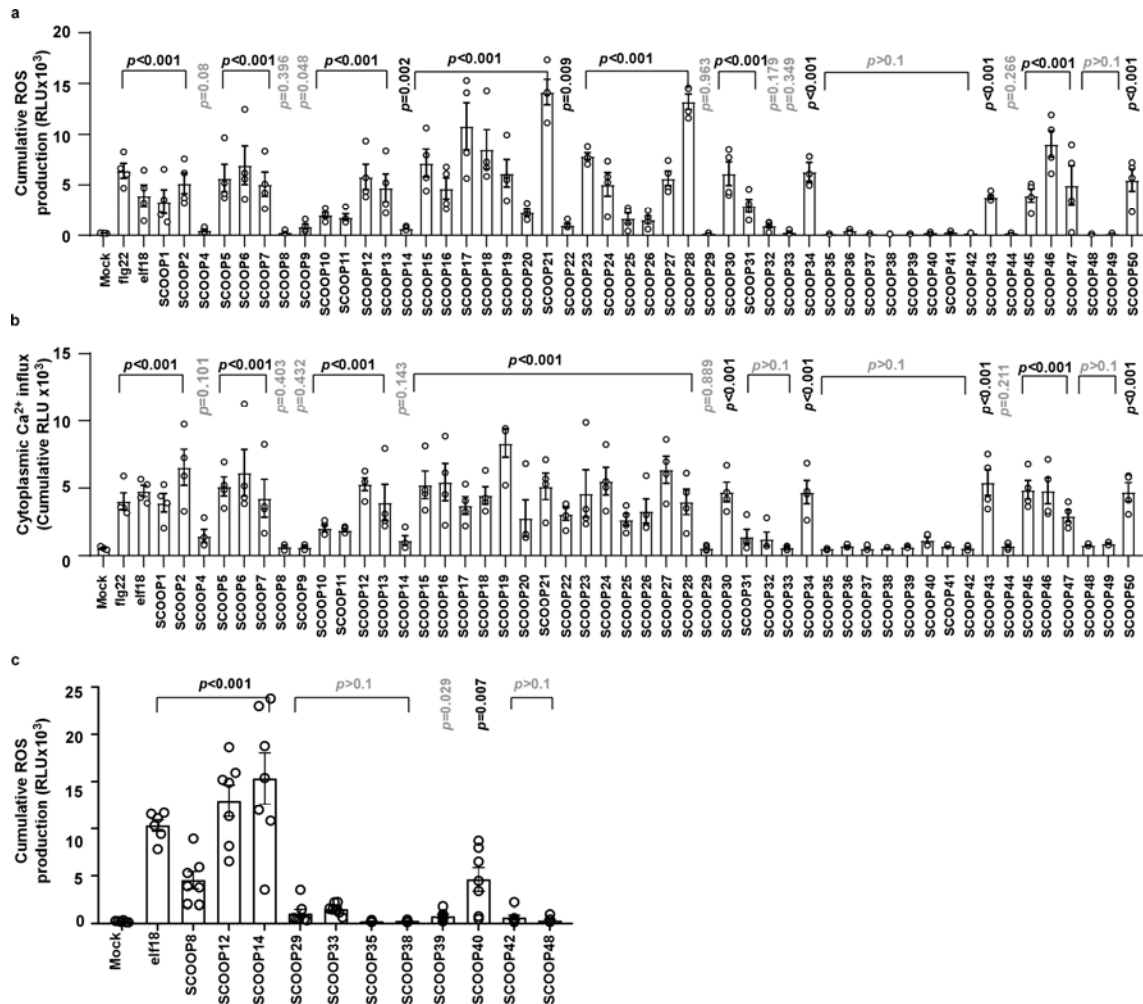
323

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 Ca²⁺ influx in Col-0**

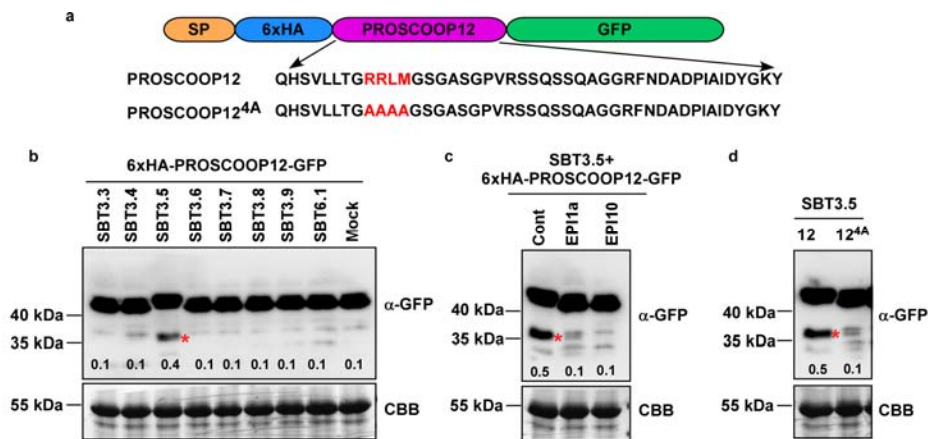
330 (a) Integrated ROS production over 40 min in leaf disks collected from 4-week-old
 331 plants induced in the absence (Mock) or presence of 1 μM 13-mer SCOOP peptides
 332 (n ≥ 8). flg22 and elf18 (100 nM) were used as control to calculate the Z-score to
 333 normalise for variation between plates.

334 (b) Cytoplasmic Ca²⁺ influx measured in Col-0^{AEQ} 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



344

345 **Figure 2 | PROSCOOP12 is cleaved by SBT3.5**

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

349 (b, c and d) Western blot using GFP antibody recognizing the 6xHA-PROSCOOP12-
 350 GFP and truncated SCOOP12-GFP (indicated by the red asterisk) in *Nicotiana*
 351 *benthamiana* leaves after 100 nM flg22 treatment for 2 h. The membrane was stained
 352 with CBB, as a loading control. Numbers on the blots correspond to the ratios of protein
 353 accumulation levels between truncated proteins bands (indicated by the red asterisk)
 354 and full-length protein bands (above the truncated protein bands). (b) Transient co-
 355 expression of 6xHA-PROSCOOP12-GFP with SBT3.3, SBT3.4, SBT3.5, SBT3.6,
 356 SBT3.7, SBT3.8, SBT3.9, SBT6.1 or without SBT (Mock). (c) Transient co-expression
 357 of 6xHA-PROSCOOP12-GFP, SBT3.5 with SBT inhibitors EPI1a/EPI10 or without
 358 SBT inhibitor (Mock). (d) Transient co-expression of SBT3.5 with 6xHA-
 359 PROSCOOP12-GFP (labelled as 12) or 6xHA-PROSCOOP12^{4A}-GFP (labelled as
 360 12^{4A}). All experiments were repeated and analysed three times with similar results.

361

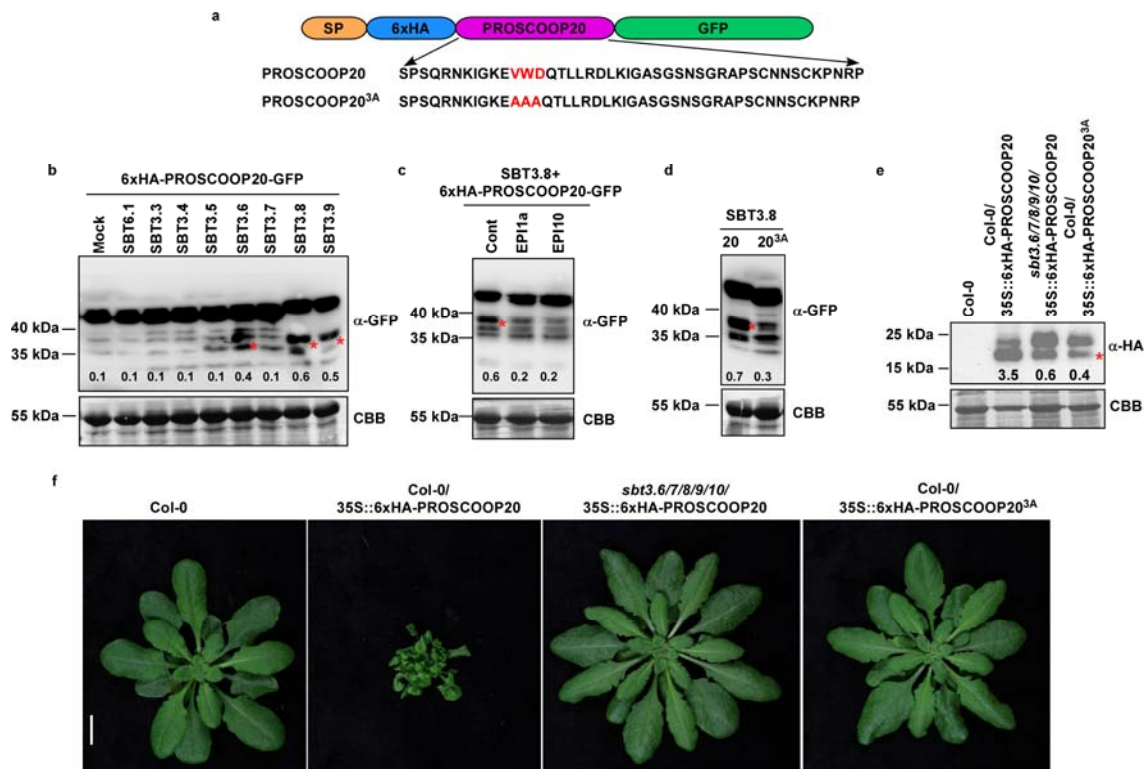


Figure 3 | PROSCOOP20 is cleaved by SBT3.8 and SBT3.9

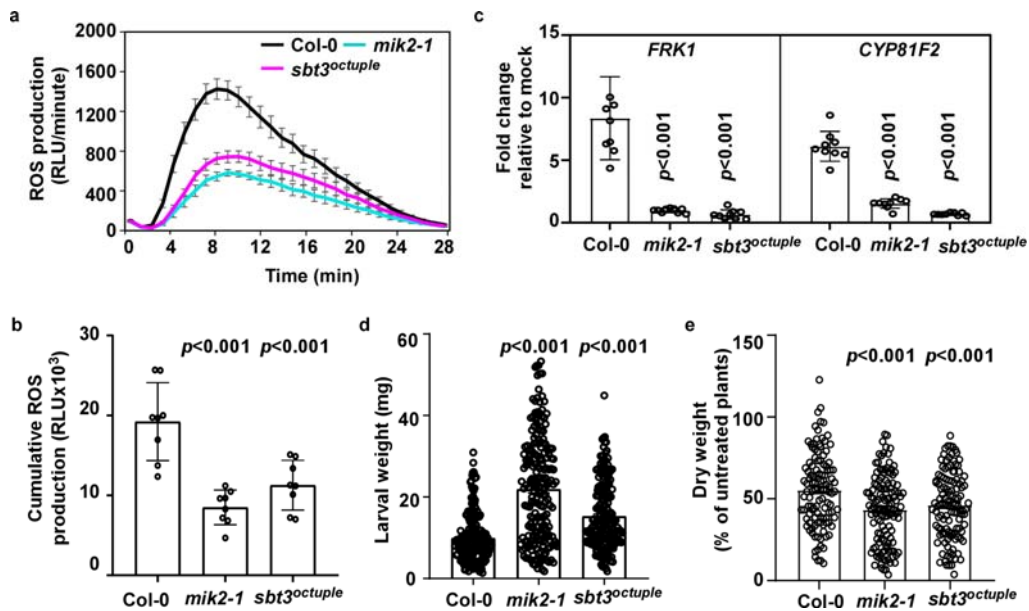
(a) Schematic representation of PROSCOOP20 with native signal peptide (SP), N-terminal 6xHA tag and C-terminal GFP tag. The cleavage motif VWD and mutated residues of VWD/AAA are shown as red characters.

(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^{3A}-GFP (labelled as 20^{3A}). 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^{3A} (labelled as 20^{3A}) 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^{3A} in Col-0 or *sbt3.6/7/8/9/10*. Bar = 1
 388 cm.
 389



390

391 **Figure 4 | *sbt3^{octuple}* phenocopies *mik2***

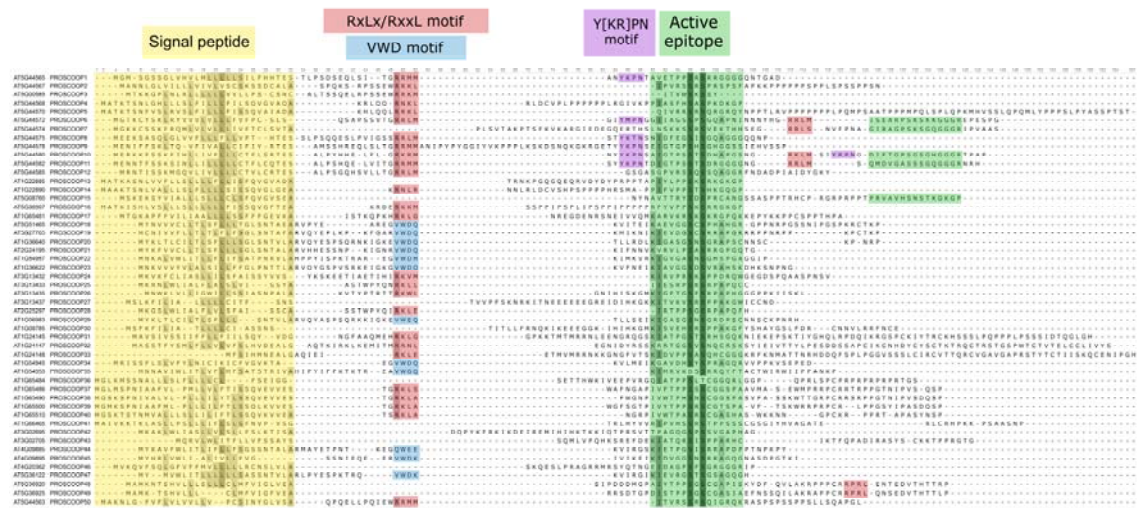
392 (a and b) ROS production in leaf disks collected from 4-week-old *Arabidopsis* plants
 393 induced by 100 nM flg22 (n = 8). (a) Points represent mean. (b) Integrated ROS
 394 production over 30 min.

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^{octuple}*. *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^{octuple}* 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.

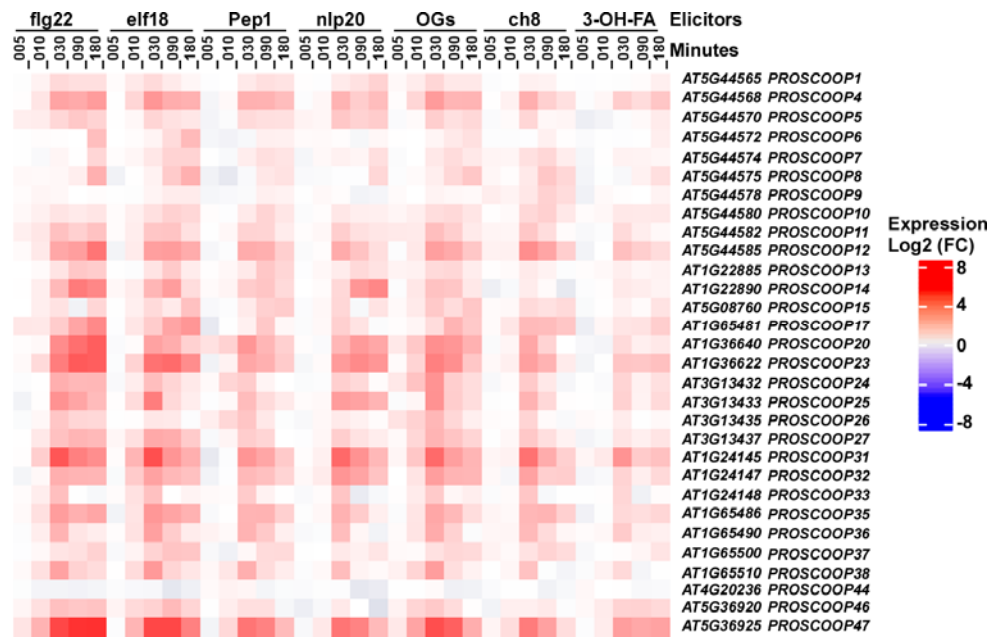


411

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

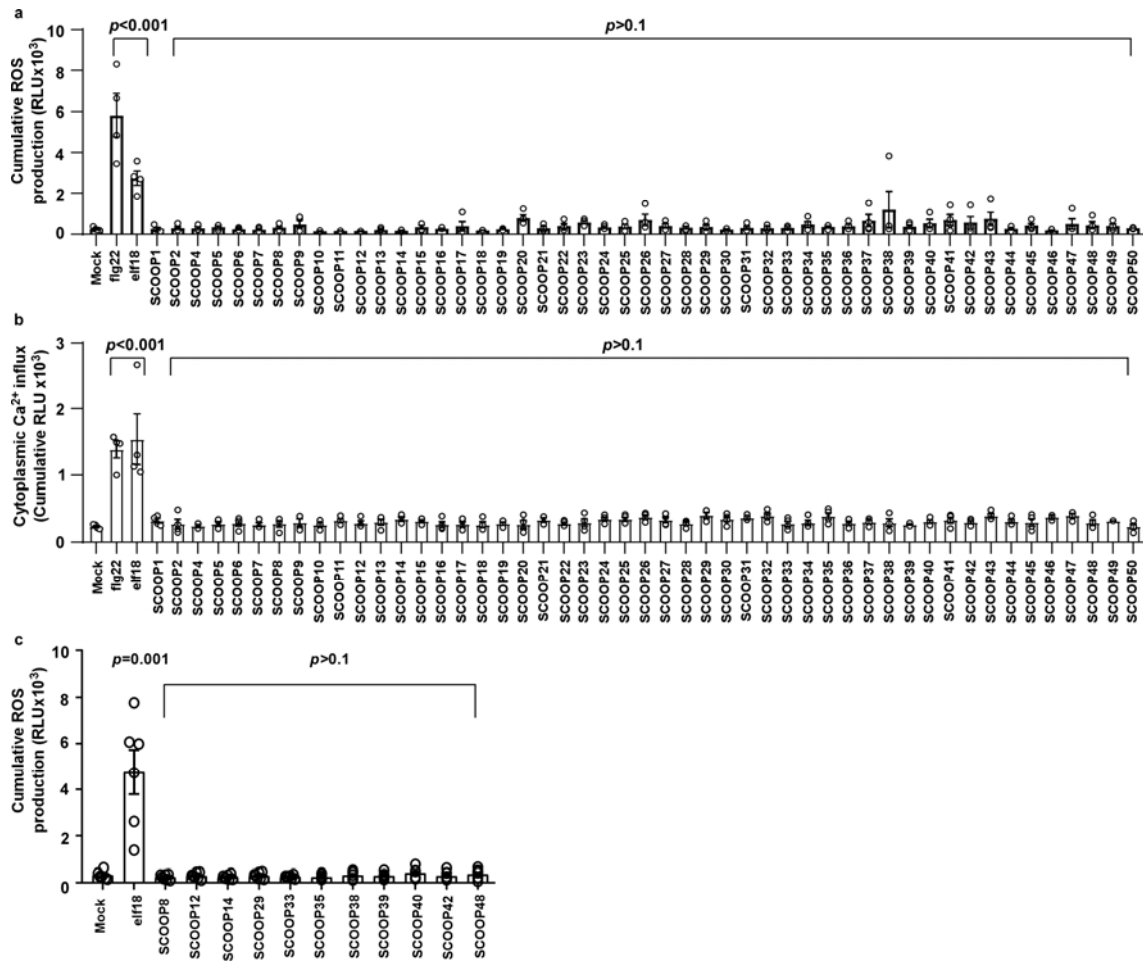


417

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 from²⁷).



422

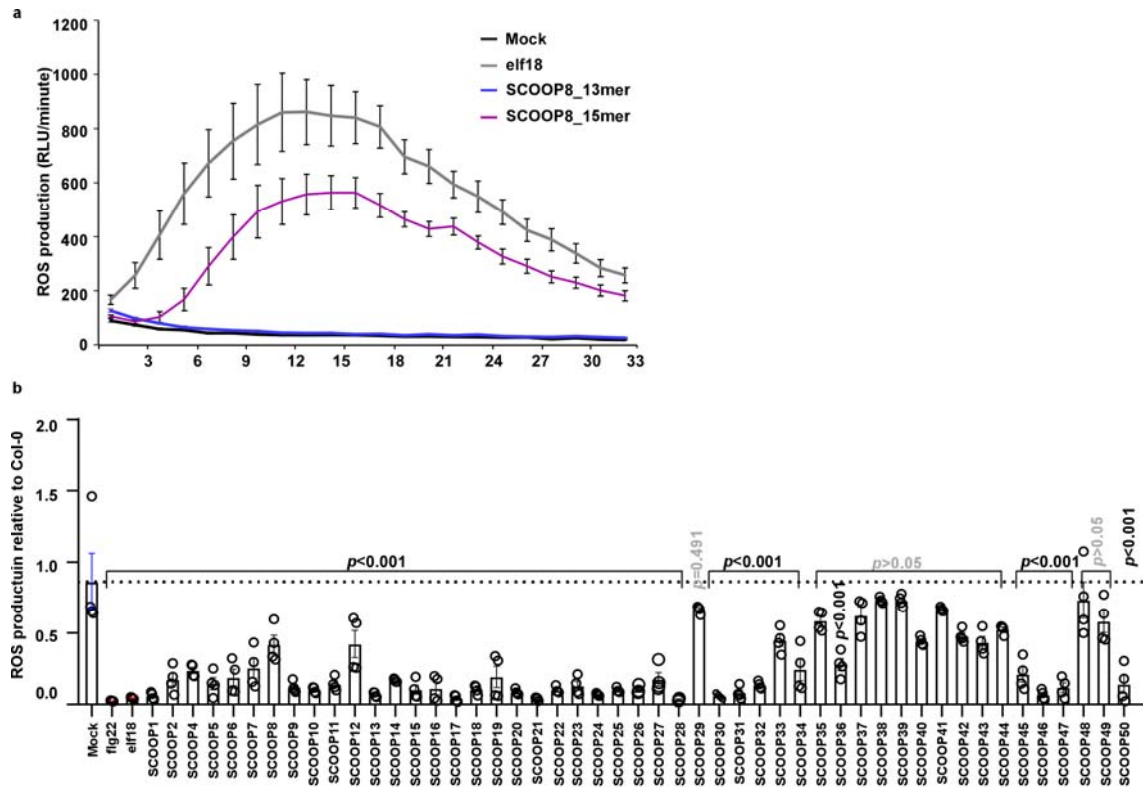
423 **Extended Data Fig. 3 | Divergent SCOOPs do not induce ROS production or Ca²⁺**
 424 **influx in *mik2-1***

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 Ca²⁺ influx measured in *mik2-1^{AEQ}* 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 μ M 13/15-mer SCOOP8 peptides ($n \geq 8$) using 100 nM elf18 as control.

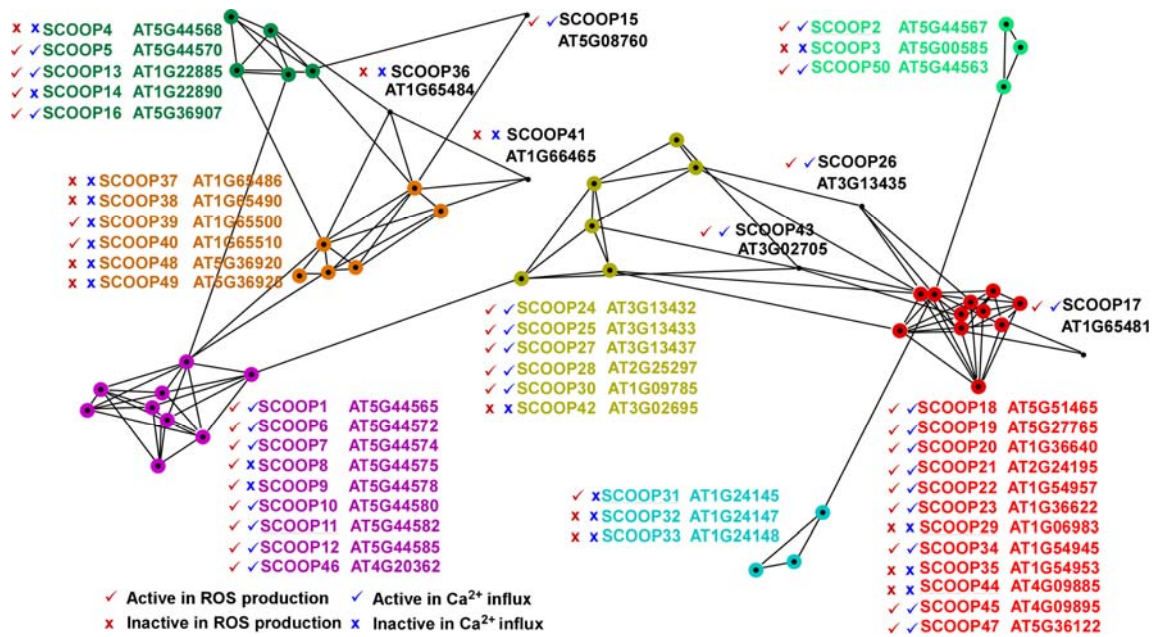
441

(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 μ 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

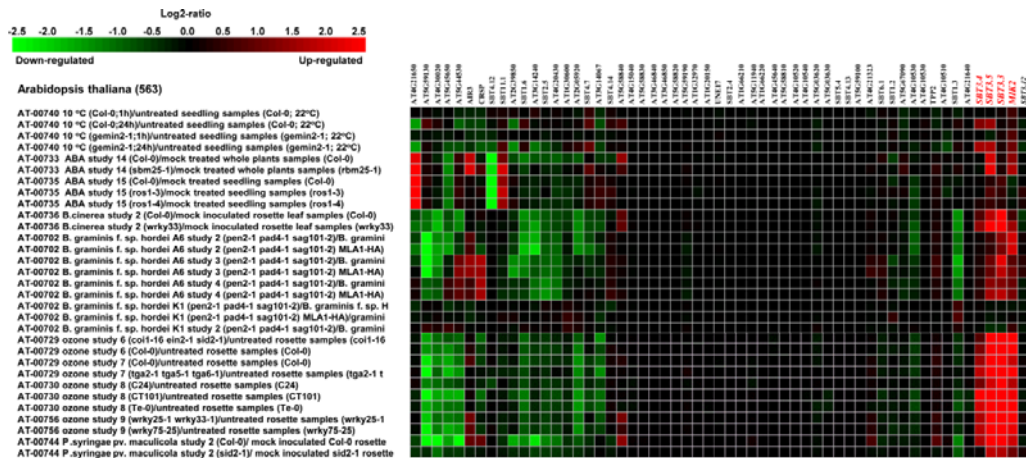


449

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.



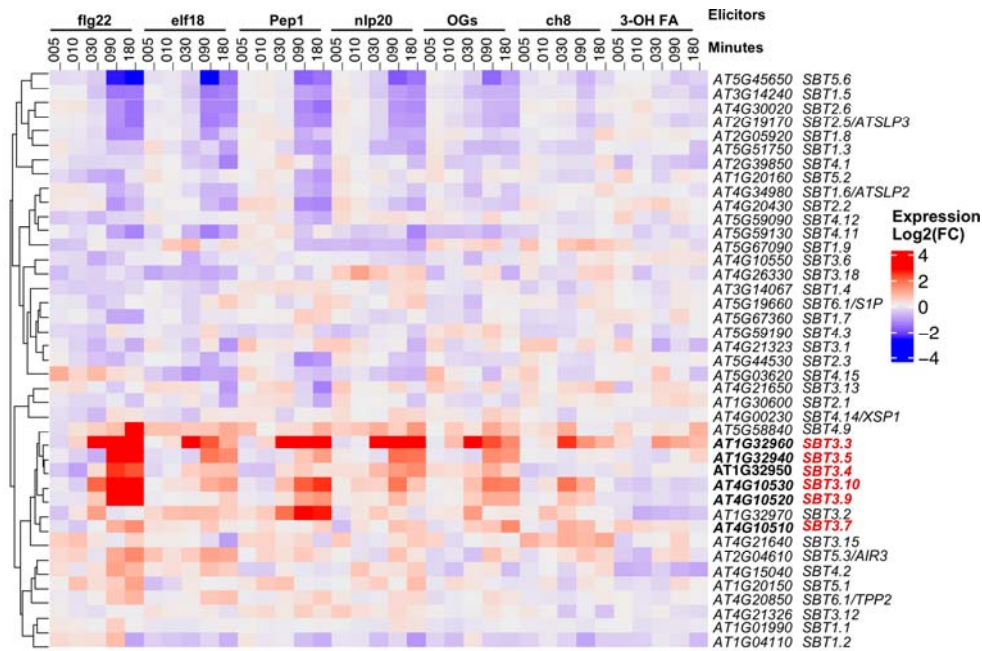
456

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 log₂(FC) expression levels of SBTs in response to stresses (data
 461 obtained from Genevestigator).

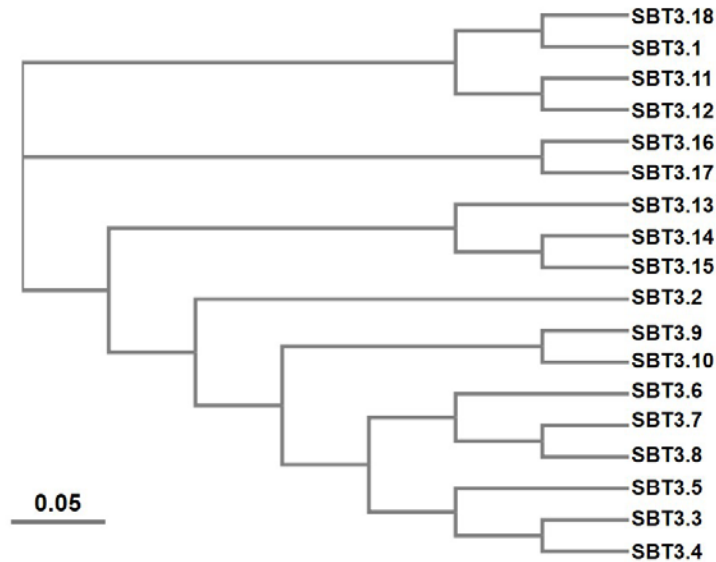
462



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 from²⁷).

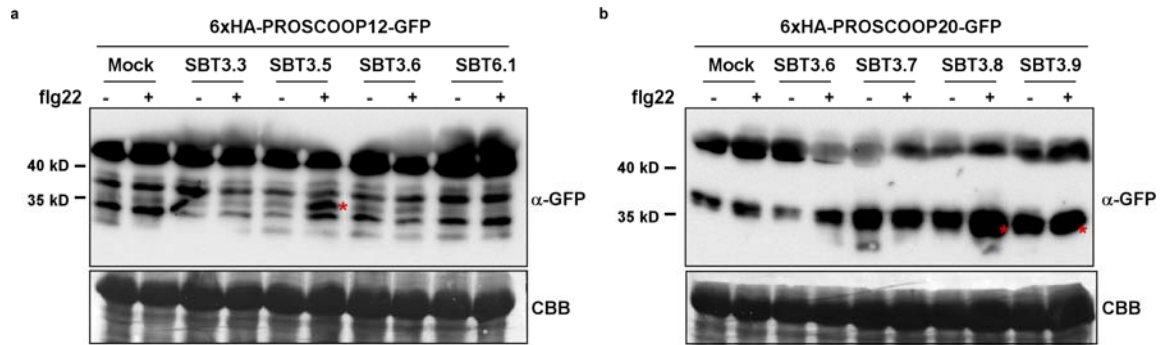


468

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 MEGAX⁵³.

472



473

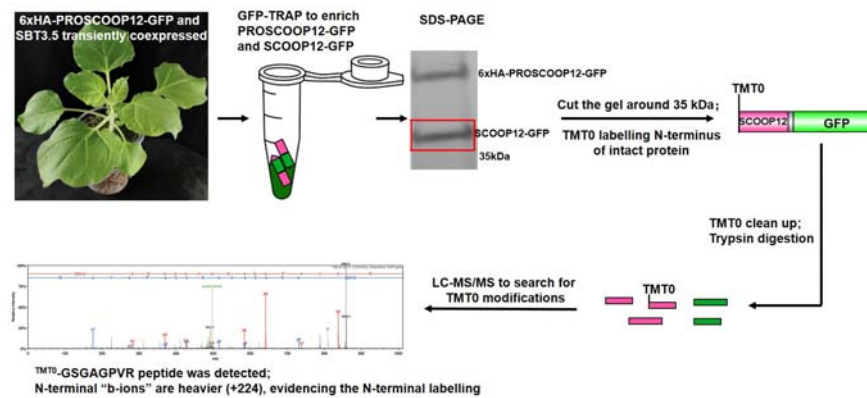
474 **Extended Data Fig. 9 | Cleavage analysis of PROSCOOP12 and PROSCOOP20 by**
 475 **different SBTs**

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.

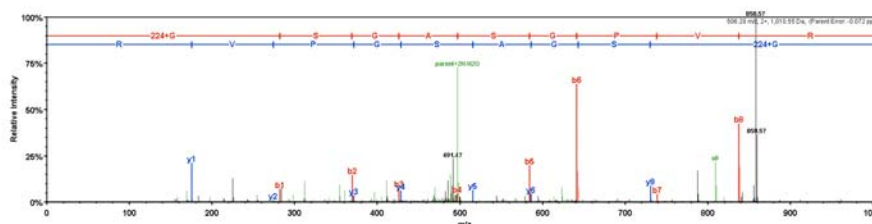
481



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*.**

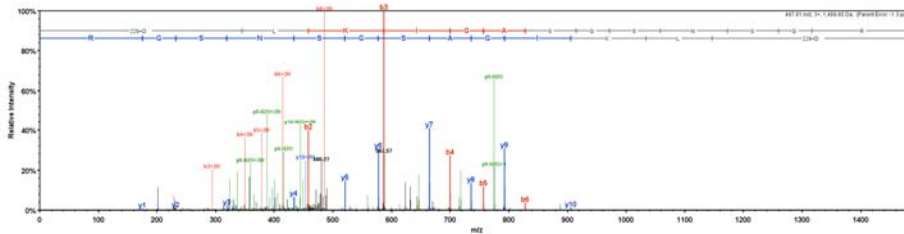


487

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.

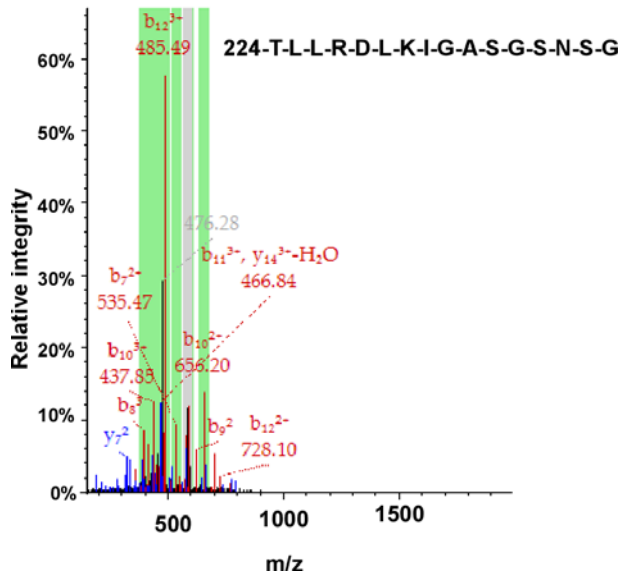
491



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.

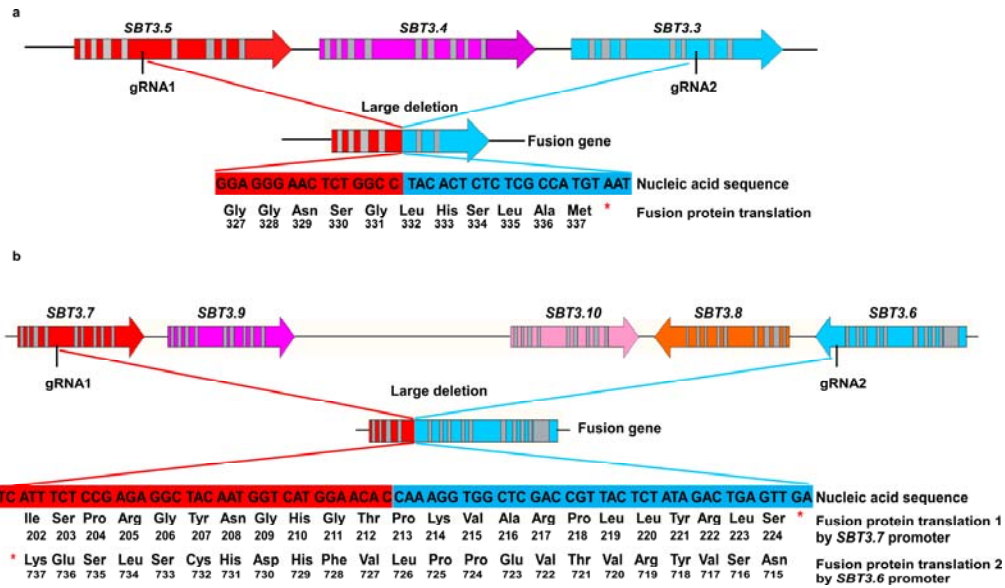


496

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

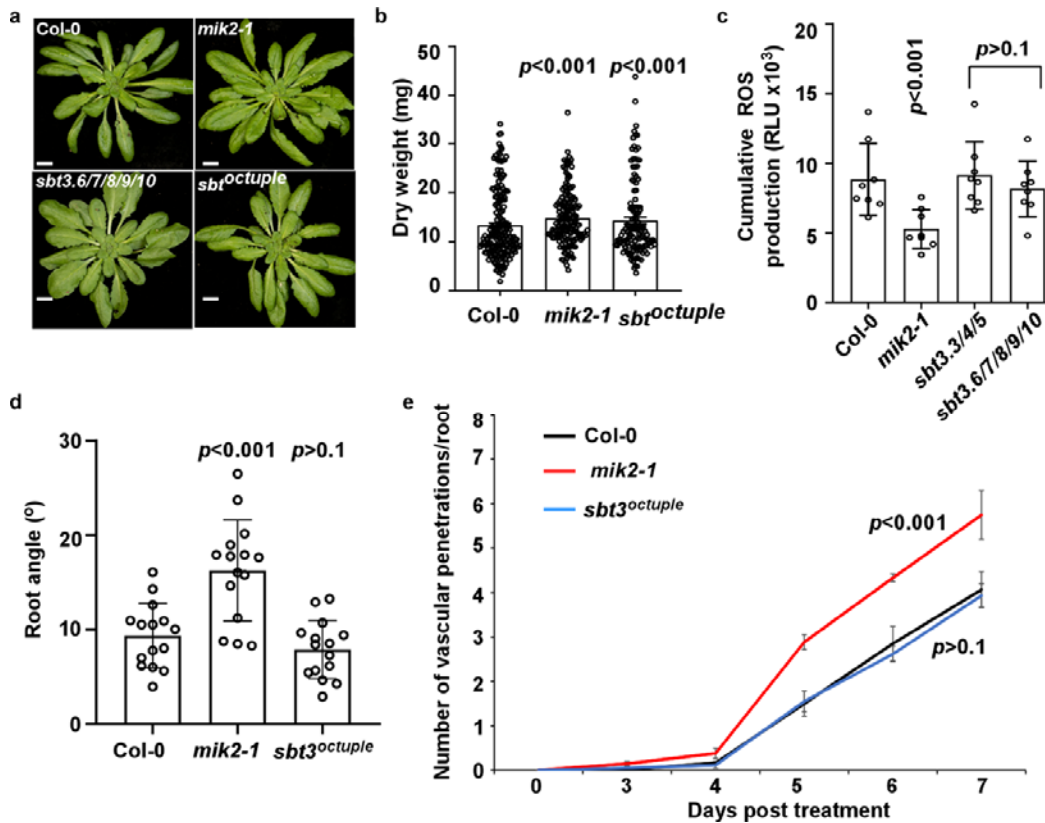
502

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^{octuple}* 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^{15,54}. *mik2-1* and *sbt3.5-1*
544 mutants have been previously described^{21,37}.

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 cloning⁵⁵. pGGZ-35S::SBT3.4
552 and pGGZ-35S::SBT3.9, pGGZ-35S::6xHA-PROSCOOP12-GFP, pGGZ-35S::6xHA-
553 PROSCOOP12^{4A}(RRLM/AAAA)-GFP, pGGZ-35S::6xHA-PROSCOOP20-GFP,
554 pGGZ-35S::6xHA-PROSCOOP20^{3A}(VWD/AAA)-GFP, pGGZ-35S::6xHA-
555 PROSCOOP20, pGGZ-35S::6xHA-PROSCOOP20^{3A}(VWD/AAA).

556 The genomic DNA of *SBT3.4* and *SBT3.9* was amplified by PCR. The CDS of 6xHA-
557 PROSCOOP12, 6xHA-PROSCOOP12^{4A} (RRLM/AAAA), 6xHA-PROSCOOP12 and
558 6xHA-PROSCOOP12^{3A}(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/10⁶. 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^{5,37}. 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 pART7⁵⁶, between the
570 CaMV-35S promoter and terminator. The expression cassette was then transferred
571 into the NotI site of the binary vector pART27⁵⁶.

572 **CRISPR-Cas9 mutagenesis**

573 CRISPR-Cas9 induced mutagenesis was performed as described⁵⁷. 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⁻¹ 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 previously⁵⁸.

593 **Cytoplasmic Ca²⁺ 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 Ca²⁺ 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 similarities⁵⁹. 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 $O.D._{600} = 0.2$ 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 $\mu L \cdot mg^{-1}$ 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 (α -HA-HRP (12013819001, Roche, 1:3000);
623 α -RFP-HRP (sc-9996 HRP, Santa Cruz, 1:5000) and α -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 μL 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^{60,61}. 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 10⁷ 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 previously²¹.

697 **Salt tolerance assays**

698 Salt tolerance assays were performed as previously²¹. 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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