

1 **The Cf-4 receptor-like protein associates with the BAK1 receptor-like kinase to initiate**
2 **receptor endocytosis and plant immunity**

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24 **Manuscript information:**

25 Word count:

26 Main body: 5519

27 Introduction: 1180

28 Materials and Methods: 1069

29 Results: 2148

30 Discussion: 1087

31 Acknowledgements 35

32 Figures: 7 (all in colour)

33 Supporting Information: 10 figures, 2 videos

34

35 **Summary**

- 36 • The first layer of plant immunity is activated by cell surface receptor-like kinases (RLKs)
37 and proteins (RLPs) that detect infectious pathogens. Constitutive interaction with the
38 RLK SUPPRESSOR OF BIR1 (SOBIR1) contributes to RLP stability and kinase activity.
39 As RLK activation requires transphosphorylation with a second associated RLK, it
40 remains elusive how RLPs initiate downstream signaling. To address this, we investigated
41 functioning of Cf RLPs that mediate immunity of tomato against *Cladosporium fulvum*.
42 • We employed live-cell imaging and co-immunoprecipitation in tomato and *Nicotiana*
43 *benthamiana* to investigate the requirement of associated kinases for Cf activity and
44 ligand-induced subcellular trafficking of Cf-4.
45 • Upon elicitation with the matching effector ligands Avr4 and Avr9, BRI1-ASSOCIATED
46 KINASE 1 (BAK1) associates with Cf-4 and Cf-9. Furthermore, Cf-4 that interacts with
47 SOBIR1 at the plasma membrane, is recruited to late endosomes after elicitation.
48 Significantly, BAK1 is required for Avr4-triggered endocytosis, effector-triggered
49 defenses in Cf-4 plants and resistance of tomato against *C. fulvum*.
50 • Our observations indicate that RLP-mediated immune signaling and endocytosis require
51 ligand-induced recruitment of BAK1, reminiscent of BAK1 interaction and subcellular
52 fate of the FLAGELLIN SENSING 2 RLK. This reveals that diverse classes of cell
53 surface immune receptors share common requirements for signaling initiation and
54 endocytosis.

55

56

57 Key words:

58 Avr4 effector, BAK1/SERK3, Cf-4 RLP, immunity, receptor endocytosis, SOBIR1,
59 subcellular trafficking

60

61 **Introduction**

62 The innate immune system of higher eukaryotes senses a wide range of pathogens through
63 distinct pattern recognition receptors (PRRs). The engagement of these receptors leads to the
64 activation of signaling pathways resulting in the induction of defense responses. Plants rely
65 on plasma membrane-resident receptor-like kinases (RLKs) and receptor-like proteins (RLPs)
66 as a first layer of the immune system against phytopathogenic microbes, many of them
67 exploiting the extracellular space of plant tissues for their growth (Faulkner & Robatzek,

68 2012). Therefore, decoding the mechanisms underlying the perception of pathogen-derived
69 signals by RLKs and RLPs at the host cell surface and subsequent activation of defense
70 signaling is an important aspect for understanding how plant immunity is initiated. The *Cf*
71 resistance genes encode leucine-rich repeat (LRR)-RLPs conferring immunity to specific
72 races of the pathogenic fungus *Cladosporium fulvum* causing leaf mold disease of tomato
73 (*Solanum lycopersicum*) (Joosten & de Wit, 1999; Rivas & Thomas, 2005; Stergiopoulos &
74 de Wit, 2009). Distinct members of the *Cf* receptor family are activated upon recognition of
75 their matching ligands, which are the so-called avirulence (Avr) proteins that are secreted by
76 the fungus upon invasion of tomato leaves. The founding member of the *Cf* proteins and
77 LRR-RLPs in general is *Cf*-9, which mediates resistance to Avr9-producing strains of *C.*
78 *fulvum*, whereas for example *Cf*-2 and *Cf*-4 recognize Avr2 and Avr4, respectively (Rivas &
79 Thomas, 2005). The *Cf* receptors mediate race-specific immunity to *C. fulvum*, which is
80 typically associated with the hypersensitive response (HR), a form of programmed cell death.

81 Well-characterized PRRs are FLAGELLIN SENSING 2 (FLS2) and ELONGATION
82 FACTOR-TU RECEPTOR (EFR), which are LRR-RLKs initiating broad-spectrum immunity
83 against bacterial infection upon detection of the pathogen-associated molecular patterns
84 (PAMPs) flagellin (*flg22*) and EF-Tu, respectively (Boller & Felix, 2009). To activate
85 immune signaling, FLS2 and EFR form ligand-induced complexes with members of the
86 SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) LRR-RLK family, of which
87 BRI1-ASSOCIATED KINASE 1 (BAK1)/SERK3 plays a prominent role in FLS2-mediated
88 immunity (Heese *et al.*, 2007; Roux *et al.*, 2011). BAK1 was initially found to interact with
89 the LRR-RLK brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1), and
90 is involved in a number of immune pathways and developmental processes, in line with its
91 broader function as a co-receptor (Santiago *et al.*, 2013; Sun *et al.*, 2013; Liebrand *et al.*,
92 2014). All receptors mentioned above are plasma membrane-localized proteins allowing them
93 to detect extracellular microbe-derived ligands. In order to reach their destination after their
94 maturation in the endoplasmic reticulum (ER), they have to be targeted to the plasma
95 membrane by secretory trafficking (Beck *et al.*, 2012a). As part of the immune response,
96 plasma membrane-resident FLS2, when activated by *flg22*, is recruited to ARA7/RabF2b-
97 and ARA6/RabF1-positive endosomes, which are compartments of the late endosomal
98 trafficking pathway (Robatzek *et al.*, 2006; Beck *et al.*, 2012c). This process depends on
99 BAK1/SERK3 (Chinchilla *et al.*, 2007; Beck *et al.*, 2012c), which itself undergoes
100 constitutive endocytosis (Ruscinova *et al.*, 2004).

101 In contrast to RLKs, RLPs lack an intracellular signaling domain and therefore it has

102 been long suggested that these receptors require the interaction with a protein kinase to
103 initiate signaling (Jones *et al.*, 1994; Joosten & de Wit, 1999; Liebrand *et al.*, 2014). For
104 example, the Toll-like receptors (TLRs) of the mammalian innate immune system require
105 interaction with the MyD88 adaptor protein to recruit the kinase IRAK and subsequently
106 activate immune signaling (Moresco *et al.*, 2011; Broz & Monack, 2013). Following the
107 paradigm of the involvement of BAK1/SERK3 in RLK-mediated signaling, there is genetic
108 evidence for a role of BAK1/SERK3 also in plant defense responses mediated by RLPs. Both
109 resistance to the fungal pathogen *Verticillium dahliae*, conferred by the tomato LRR-RLP Ve1
110 and immunity elicited by *Sclerotinia sclerotiorum* through *Arabidopsis thaliana* RLP30, are
111 dependent on BAK1/SERK3 (Fradin *et al.*, 2009; Fradin *et al.*, 2011; Zhang *et al.*, 2013).
112 Furthermore, another SERK family member, SERK1, was also shown to be genetically
113 required for Ve1 and Cf-4 antifungal immunity in *Arabidopsis* and tomato, respectively
114 (Fradin *et al.*, 2011). However, no molecular interaction between these LRR-RLPs and the
115 SERKs has been reported, leaving the possibility that the observed phenotypes are indirect,
116 for example through BAK1/SERK3 function in cell death control or damage-induced
117 immunity (Kemmerling *et al.*, 2007; Gao *et al.*, 2009; Schwessinger *et al.*, 2011; Liu *et al.*,
118 2013; Tintor *et al.*, 2013). In addition to its positive regulatory role, BAK1/SERK3 was also
119 described to negatively regulate immunity in tomato (*Sl*) mediated by *SlEix2*, an LRR-RLP
120 recognizing fungal xylanase (Bar *et al.*, 2010). In a BAK1/SERK3-dependent manner, the
121 close homologue *SlEix1* impairs *SlEix2* endocytosis and HR (Bar *et al.*, 2010).

122 Besides genetic evidence for a role of SERK1, no direct involvement in Cf-mediated
123 immunity of the SERKs has been reported to date. Instead, another LRR-RLK, referred to as
124 SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR), has recently been identified as a
125 critical component in LRR-RLP-mediated immunity (Jehle *et al.*, 2013; Liebrand *et al.*, 2013;
126 Zhang *et al.*, 2013; Zhang *et al.*, 2014). *SlSOBIR1* interacts specifically with RLPs, including
127 the Cf-2, Cf-4 and Cf-9 proteins, and this interaction occurred independently of the presence
128 of their matching Avr ligands. The constitutive association of these LRR-RLPs with
129 *SlSOBIR1* is required for RLP stability and has been proposed to allow activation of a
130 cytoplasmic signaling cascade upon perception of an Avr by the interacting Cf protein
131 (Liebrand *et al.*, 2013; Liebrand *et al.*, 2014). It however remains to be elucidated how
132 ligand-dependent activation of LRR-RLP signaling actually occurs.

133 Colonization of tomato leaf tissue by *C. fulvum* is fully confined to the apoplast and
134 the fungus does not form specialized feeding structures like haustoria. Consistent with this
135 life-style, the Avr proteins secreted by the fungus appear to solely accumulate in the apoplast

136 (Joosten & de Wit, 1999; Thomma *et al.*, 2005; Stergiopoulos & de Wit, 2009; Joosten, 2012).
137 This suggests that the Cf receptors perceive the various secreted Avr proteins of *C. fulvum* at
138 the plasma membrane of the host cells. However, the exact subcellular localization of Cf
139 proteins has remained unclear ever since their first identification (Jones *et al.*, 1994). Initially
140 it was shown that Cf-9 is present both at the ER and at the plasma membrane (Piedras *et al.*,
141 2000). A subsequent study demonstrated a role of a C-terminal dilysine motif of Cf-9 in ER
142 targeting (Benghezal *et al.*, 2000), which however was later found to be dispensable for Cf-9
143 function (van der Hoorn *et al.*, 2001). More recently, ER-resident chaperones were identified
144 as interacting proteins of Cf-4 and were shown to be important for Avr4-triggered immunity
145 (Liebrand *et al.*, 2012), which provided further evidence for ER localization of these RLPs.

146 Here, we have used live-cell imaging of transiently and stably expressed fluorescent
147 protein fusions in *N. benthamiana* to investigate the subcellular localization and dynamics of
148 Cf-4 *in planta*. Our data suggest that Cf-4, together with *S/*SOBIR1, perceives Avr4 at the
149 plasma membrane of the host cells, a process that subsequently induces the association of Cf-
150 4 with BAK1/SERK3, and likewise with SERK1. We furthermore demonstrate a role of these
151 SERK family members in Cf-mediated immunity and show that the Cf-4 receptor undergoes
152 BAK1/SERK3-dependent endocytosis upon Avr4 perception.

153

154 **Materials and Methods**

155 *Plant materials and constructs*

156 *Nicotiana benthamiana* plants were grown under 16 hours of light at 24°C and 45 - 65 %
157 humidity. Various constructs used in this study have been described before (in all cases GFP
158 refers to enhanced (e)GFP); Cf-4-GFP, Cf-9-GFP, *S/*SOBIR1-GFP, *S/*SOBIR1-like-GFP,
159 *At*SOBIR1-GFP, *At*SOBIR1-Myc and *At*SOBIR1^{D489N}-Myc (Liebrand *et al.*, 2012; Liebrand
160 *et al.*, 2013); FLS2-YFPn/c (Frei dit Frey *et al.*, 2012); MEMB12-mCherry (Geldner *et al.*,
161 2009); VHA-a1-RFP, RFP-ARA7 and ARA6-RFP (Dettmer *et al.*, 2006); AtBAK1
162 (Schwessinger *et al.*, 2011). Cf-4-YFPn/c and *S/*SOBIR1-YFPn/c were obtained by PCR-
163 amplifying the coding regions of the respective genes, which were cloned into the pENTR/D-
164 TOPO[®] system (Invitrogen) and subsequently recombined into *pAM-PAT-GWPro*_{35S}
165 (GenBank AY436765). The *35S::ACA8-mCherry* construct was generated by Gateway
166 recombination (Invitrogen) using pDONR201 containing the coding sequences of *ACA8*
167 devoid of stop codon (Frei dit Frey *et al.*, 2012) and a *35S::GW-mCherry* destination vector
168 (provided by R. Panstruga, RWTH Aachen University, Germany). *S/*SOBIR1-HA was
169 obtained by recombining pENTR/D-TOPO[®]-*S/*SOBIR1 (Liebrand *et al.*, 2013) to pGWB14,

170 carrying the HA coding sequence (Nakagawa *et al.*, 2007). *S/SERK1-Myc* and *S/SERK3a-*
171 *Myc* were obtained by recombining pENTR/D-TOPO[®]-*S/SERK1* and pDONR201[®]-
172 *S/SERK3a* (Liebrand *et al.*, 2013) to pGWB20 carrying the Myc coding sequence (Nakagawa
173 *et al.*, 2007), respectively. pUB::*AtBAK1*^{D416N} was obtained by PCR amplification from
174 pGWB14-*BAK1*^{D416N}-HA (Schwessinger *et al.*, 2011) using primers BAK1-TOPO-F (5'-
175 CAC CAT GGA ACG AAG ATT AAT GAT C-3') and BAK1-TOPO-R (5'-TTA TCT TGG
176 ACC CGA GGG GTA TTC-3') introducing a stop codon, directional cloning into pENTR/D-
177 TOPO[®] and subsequent recombination of pENTR/D-TOPO-*BAK1*^{D416N} with pUB-DEST
178 (Grefen *et al.*, 2010) All recombinations were performed using the classical Gateway LR
179 clonase reaction (Invitrogen). The correctness of all constructs was confirmed by sequencing.

180

181 *Transient, stable transformation, and Virus-Induced Gene Silencing (VIGS)*

182 Transient transformation of *N. benthamiana* epidermal cells was done as described before
183 (Choi *et al.*, 2013). Briefly, two-day cultures of *Agrobacterium tumefaciens* GV3101 carrying
184 the respective expression constructs in liquid LB medium supplemented with antibiotics were
185 washed in water prior to *N. benthamiana* leaf infiltrations. For single protein localization and
186 co-localization purposes, final OD₆₀₀=0.25 and OD₆₀₀=0.5, respectively, were used for agro-
187 infiltrations. Microscopy was performed at 2-3 dpi. *N. benthamiana* lines stably expressing
188 Cf-4-GFP were obtained by incubating *N. benthamiana* leaf explants with *Agrobacterium*
189 strain GV3101 carrying plasmid pBIN-KS-35S::Cf-4-GFP (Liebrand *et al.*, 2012). Selection
190 of transformed plants was done as described before (Horsch *et al.*, 1985; Gabriëls *et al.*,
191 2006). Using segregation analysis based on kanamycin resistance, a single locus insertion line
192 was selected. Tobacco Rattle Virus (TRV)-mediated Virus-Induced Gene Silencing (VIGS) in
193 *N. benthamiana* was performed as described before (Liebrand *et al.*, 2012). Briefly, *A.*
194 *tumefaciens* GV3101 carrying *TRV-RNA1* at OD₆₀₀ = 0.4 and GV3101 carrying *TRV-RNA2*
195 containing the respective target sequences at OD₆₀₀ = 0.2 were mixed and co-infiltrated into
196 *N. benthamiana* leaves of two-weeks old plants, and three weeks later leaves were used for
197 further analysis. *TRV* alone was used as a control. The effects on Cf-4-GFP accumulation of
198 VIGS targeting various endogenous *N. benthamiana* genes in combination with co-
199 expression of *A. thaliana* genes were observed via immunoblotting and RT-PCR (Supporting
200 Information Figs. 6c, 9c,d).

201

202 *Bioassay for Avr4-induced immunity*

203 TRV constructs targeting *NbSERK3a/b* (Chaparro-Garcia *et al.*, 2011),

204 TRV2::*NbSOBIR1/NbSOBIR1-like* (Liebrand *et al.*, 2013) and TRV2::*Cf-4* (Gabriëls *et al.*,
205 2006), alongside with TRV2::*GUS* (Tameling & Baulcombe, 2007) controls, were agro-
206 infiltrated into leaves of one-week-old *N. benthamiana:Cf-4-GFP* plants at O.D.600 = 0.5.
207 After three weeks, Avr4 (O.D.600 = 0.03, twice) (Gabriëls *et al.*, 2007), RxD460V (O.D.600
208 = 0.1) (Bendahmane *et al.*, 2002) and BAX (O.D.600 = 0.5) (Lacomme & Santa Cruz, 1999),
209 were agro-infiltrated into mature, fully expanded leaves of the TRV-inoculated plants. HR
210 scores were recorded three days after agro-infiltration. In addition, three-week old
211 *NbSERK3a/b*-silenced leaves were transiently transformed with Cf-4-GFP and 3 days later
212 infiltrated with 300µM Avr4 protein. HR was observed 6 days after Avr4 infiltration. VIGS in
213 tomato, followed by inoculation with conidia of *C. fulvum* race 5-pGPD:*GUS* and subsequent
214 GUS staining and quantification were performed as described before (Liebrand *et al.*, 2013).

215

216 *Immunoblot analysis and co-immunoprecipitation*

217 Immunoblot analysis with anti-GFP and anti-Myc antibodies and pull-down experiments
218 were carried out as previously reported (Liebrand *et al.*, 2012; Liebrand *et al.*, 2013), with the
219 following modifications: Transiently transformed *N. benthamiana* leaves were infiltrated with
220 Avr4, Avr2, or Avr9 proteins or flg22 peptide, at the indicated concentrations, and total
221 proteins were extracted. For detection of the HA-epitope tag the anti-HA-Biotin High
222 Affinity Antibody (clone 3F10; Roche Applied Science) was used. In contrast to the
223 theoretical mass of Cf-4-GFP (app. 123 kDa) Immunoblot analysis revealed a specific band at
224 about 140 kDa in accordance with what was observed in previous studies (Liebrand *et al.*,
225 2012).

226

227 *qRT-PCR analysis*

228 For qRT-PCR, total RNA was isolated from *N. benthamiana:Cf-4-GFP* leaf material, at 2
229 weeks after agro-inoculation with the various VIGS constructs. RNA extraction, cDNA
230 synthesis and qRT-PCR were performed as described (Liebrand *et al.*, 2012). *NbSERK3a/b*
231 expression was investigated using primers rbo16 (5'-TGC GCT GAA GAC CAA CTT GGC
232 T-3') and rbo17 (5'-CTG AAG CTT GCC CAA TGT GTC G-3'). *NbSERK1* expression was
233 investigated using primers rbo20 (5'-ATT GCA CAG TCT GCG T-3') and rbo21 (5'-CGA
234 AGG AAT CTC AAT TTA GTC-3'). Expression of *NbSOBIR1* was investigated using
235 primers to266 and to267 (Liebrand *et al.*, 2013). Expression of endogenous actin was used to
236 calibrate the expression level of the query genes, as previously described (Liebrand *et al.*,
237 2012). qRT-PCRs to determine the expression levels of *AtBAK1* and *AtBAK1^{D416N}* were

238 performed using primers *AtBAK1-F* (5'-TGG ACT TGC AAA ACT CAT GG-3') and
239 *AtBAK1-R* (5'-GAT CAA AAG CCC TTT GTC CA-3').

240

241 *Confocal microscopy and image analysis*

242 Confocal laser microscopy was performed using a Leica SP5 laser point scanning microscope
243 (Leica, Germany) mounted with hybrid detectors (HyD™) as described previously (Beck *et al.*,
244 2012c). Briefly, GFP and RFP/mCherry fluorophores were excited using the 488-nm
245 argon laser and the 561 nm diode, respectively, and fluorescence emission was captured
246 between 500 and 550 nm for GFP and between 580 and 620 nm for RFP/mCherry. For GFP
247 only images, chloroplast autofluorescence was captured between 700 and 800 nm. Sequential
248 scan mode was used for simultaneous imaging of GFP and RFP/mCherry. Abaxial sides of *N.*
249 *benthamiana* leaf discs were imaged. Images were taken using a 63X water immersion
250 objective and processed using the Leica LAS-AF and FIJI (ImageJ) software packages. Spot
251 detection and quantification on confocal micrographs were performed using EndoQuant,
252 which is a modification of EndomembraneQuantifier suitable for standard confocal images
253 (Beck *et al.*, 2012c).

254

255 **Results**

256 *Cf-4 interacts with SISOBIR1 at the plasma membrane*

257 To address the localization and dynamics of the Cf-4 receptor in antifungal immunity, we
258 used functional fluorescently-tagged Cf-4 (Cf-4-GFP) mediating Avr4-triggered HR in *N.*
259 *benthamiana* (Supporting Information Fig. S1; (Liebrand *et al.*, 2012)). We monitored Cf-4-
260 GFP subcellular localization and revealed its presence at the plasma membrane of leaf
261 epidermal cells by co-localizing Cf-4-GFP with the plasma membrane autoinhibitory calcium
262 ATPase ACA8, fused to mCherry (Fig. 1, Supporting Information Fig. S2; (Frei dit Frey *et al.*,
263 2012)). The cell surface localization of this Cf protein is in agreement with the described
264 apoplastic localization of its ligand, Avr4, (Joosten *et al.*, 1994; van den Burg *et al.*, 2006)
265 and the plasma membrane-localization of its constitutive interactor, SOBIR1 (Fig. 2.; (Leslie
266 *et al.*, 2010; Liebrand *et al.*, 2013)). *SISOBIR1-GFP*, its close homolog *SISOBIR1-like* fused
267 to GFP and also *AtSOBIR1-GFP* were all detected at the plasma membrane, co-localizing
268 with ACA8-mCherry, and internal vesicles (Figs. 1, 2, Supporting Information Figs. S2, S3,
269 S4).

270

271 To investigate the subcellular localization of the Cf-4-*SISOBIR1* complex, we used

272 bimolecular fluorescence complementation (BiFC). We observed reconstitution of the YFP
273 molecule by the detection of fluorescence when transiently co-expressing Cf-4 and
274 *S/SOBIR1* that were C-terminally fused to respectively the C- and N-terminal halves of YFP
275 (respectively YFPc and YFPn), indicative of Cf-4-*S/SOBIR1* heterodimerization (Fig. 1). Cf-
276 4-*S/SOBIR1* heterodimerization was found at the plasma membrane, which was confirmed by
277 ACA8-mCherry co-localization (Fig. 1). In this assay, we did not detect positive BiFC when
278 co-expressing FLS2 and *S/SOBIR1* fused to the YFP halves, whereas BiFC did occur when
279 co-expressing FLS2-YFPc and FLS2-YFPn (Supporting Information Fig. S5). These
280 observations indicate homodimerization of FLS2 but no heterodimerization between FLS2
281 and *S/SOBIR1*, which is consistent with previous findings and thus supporting the specificity
282 of BiFC in this system (Frei dit Frey *et al.*, 2012; Sun *et al.*, 2012; Liebrand *et al.*, 2013). All
283 these results show that Cf-4 localizes at the plasma membrane, where this LRR-RLP interacts
284 with the RLK *S/SOBIR1*.

285

286 *SOBIR1* localizes to endosomes

287 In addition to its localization at the plasma membrane, and in accordance to what was shown
288 in previous studies, *AtSOBIR1* was also observed at internal vesicles that showed co-labeling
289 with the endocytic tracer FM4-64, indicative of endosomal localization (Leslie *et al.*, 2010).
290 To examine the identity of these *SOBIR1*-positive vesicles, we transiently co-expressed
291 *S/SOBIR1*-GFP, *S/SOBIR1*-like-GFP and *AtSOBIR1*-GFP with described fluorescent
292 markers of the endomembrane trafficking pathways. These include MEMB12-mCherry for
293 labeling Golgi compartments, VHA-a1-RFP for labeling the *trans*-Golgi network (TGN) and
294 the Rab5 GTPases RFP-ARA7/RabF2b and ARA6/RabF1-RFP for labeling endosomes
295 (Geldner *et al.*, 2009; Beck *et al.*, 2012c). No co-localization between the *SOBIR1*-positive
296 vesicles and MEMB12- and VHA-a1-labeled compartments was detected (Fig. 2, Supporting
297 Information Fig. S3a,b). By contrast, we found clear localization of the *SOBIR1* receptors to
298 RFP-ARA7/RabF2b- and ARA6/RabF1-RFP-positive endosomes (Fig. 2, Supporting
299 Information Fig. S3c,d). These observations are in agreement with the described FM4-64-
300 positive endosomal localization of *AtSOBIR1*-YFP in *Arabidopsis* (Leslie *et al.*, 2010), and
301 suggest that constitutive endocytosis of *SOBIR1* receptors takes place. Furthermore, co-
302 localization with ARA6/RabF1 indicates that the *SOBIR1* receptors might enter the late
303 endosomal pathway (Beck *et al.*, 2012c).

304

305 *Avr4* triggers endocytosis of the Cf-4/*SOBIR1* complex

306 The observation that Cf-4 interacts with *S/SOBIR1* at the plasma membrane, together with
307 the finding that *SOBIR1* receptors are present at endosomes, prompted us to investigate
308 whether Cf-4 is endocytosed when activated by Avr4. To test this, we treated *N. benthamiana*
309 leaves transiently and stably expressing Cf-4-GFP with purified Avr4 and Avr2 proteins, of
310 which the latter is specifically recognized by the Cf-2 receptor. When elicited with Avr4, this
311 triggered internalization of Cf-4-GFP (Fig. 3a, Supporting Information Fig. S6). Co-
312 expression with ARA6/RabF1-RFP showed a strong overlap of this marker with the Avr4-
313 induced Cf-4-GFP-positive vesicles, thereby revealing endosomal localization of activated
314 Cf-4 (Fig. 3, Supporting Information Videos S1, S2). Cf-4-As expected, GFP maintained
315 plasma membrane localization upon control treatment with Avr2, indicating that endocytosis
316 of Cf-4 is ligand-specific and depends on the activation of the Cf-4 receptor (Fig. 3a,
317 Supporting Information Fig. S6). Consistent with *SOBIR1* localizing to endosomes (Fig. 2,
318 Supporting Information Fig. S3), we observed co-localization of Avr4-induced Cf-4-GFP-
319 positive endosomes and endosomal *S/SOBIR1*-mCherry (Fig. 3).

320

321 As both, *SOBIR1* and Cf-4 underwent endocytosis and localized at late endosomal
322 compartments we questioned whether they might get co-internalized. Therefore we
323 investigated whether Cf-4 is endocytosed together with *S/SOBIR1*. Transient co-expression
324 of Cf-4 and *S/SOBIR1*, C-terminally fused to the C- and N-terminal halves of YFP,
325 respectively, results in positive BiFC at the plasma membrane (Fig. 1) and when triggering
326 with Avr4, BiFC was also observed at internal vesicles (Fig. 3b). These vesicles also showed
327 co-localization with ARA6/RabF1-RFP, demonstrating endosomal localization of the
328 reconstituted Cf-4-*S/SOBIR1* BiFC heterodimer (Fig. 3b), and in agreement with Avr4-
329 induced co-localization of Cf-4-GFP and *S/SOBIR1*-mCherry at endosomes (Fig. 3a).
330 Treatment with Avr2 did not trigger re-localization of the plasma membrane-resident
331 reconstituted Cf-4-*S/SOBIR1* BiFC heterodimer (Fig. 3b), further supporting ligand-specific
332 internalization of this Cf-4 together with *S/SOBIR1*. These results indicate that the receptors
333 can be endocytosed as heterodimers, an observation that was previously made for the BIR1-
334 BAK1 complex in *Arabidopsis* protoplasts (Rusinova *et al.*, 2004). In contrast to this finding,
335 we did not observe flg22-induced endocytosis of the FLS2 homodimer as the reconstituted
336 BiFC signal was maintained at the plasma membrane after elicitation (Supporting
337 Information Fig. S5b). Endocytosis of activated FLS2 depends on BAK1/SERK3 (Chinchilla
338 *et al.*, 2007; Beck *et al.*, 2012c), and this may indicate that the reconstituted FLS2 BiFC
339 homodimer could be affected in complex formation with BAK1.

340

341 *Cf-4 endocytosis requires BAK1/SERK3*

342 Because flg22-activated FLS2 traffics via the ARA6/RabF1-positive late endosomal pathway
343 and depends on functional BAK1/SERK3 (Beck *et al.*, 2012c; Choi *et al.*, 2013), this raises
344 the possibility that Avr4-induced endocytosis of Cf-4 also involves BAK1/SERK3. To test
345 this, we applied virus-induced gene silencing (VIGS), using a tobacco rattle virus (TRV)
346 construct targeting the two *NbSERK3* homologues (*NbSERK3a* and *b*) in *N. benthamiana*
347 (Chaparro-Garcia *et al.*, 2011). Monitoring Cf-4-GFP localization in leaves of
348 *TRV::NbSERK3a/b*-inoculated *N. benthamiana* revealed a strongly reduced amount of Avr4-
349 induced Cf-4-GFP-positive endosomes, as compared to the situation in control leaves from
350 plants that had been inoculated with *TRV* only (Fig. 4, Supporting Information Fig. S10b),
351 while Cf-4 protein levels were unaltered (Supporting Information Fig. S10c). By using a
352 heterologous functional complementation approach, Avr4-induced endocytosis of Cf-4-GFP
353 was partially restored when *AtBAK1* was transiently expressed in *NbSERK3a/b*-silenced
354 leaves. No reduction was observed in the amount of ARA6/RabF1-labelled endosomes when
355 *NbSERK3a/b* was silenced (Fig. 4, Supporting Information Fig. S10b,c). Importantly, this
356 suggests that, as is the case for the RLK FLS2, ligand-induced endocytosis of the RLP Cf-4 is
357 BAK1/SERK3-dependent, and that this RLK plays a role in Cf-4 function. This finding is
358 further strengthened by expression of a kinase-inactive *AtBAK1* variant (*AtBAK1-KD*) in
359 *NbSERK3a/b*-silenced leaves that did not restore Cf-4-GFP endocytosis upon triggering with
360 Avr4 (Fig. 4, Supporting Information Fig. S10b,c).

361

362 As a next step, we investigated whether SOBIR1 is required for Avr4-induced Cf-4
363 endocytosis using a similar heterologous functional complementation approach. In agreement
364 with previous findings revealing that Cf-4 abundance is dependent on SOBIR1 (Liebrand *et al.*,
365 2013), Cf-4-GFP levels in *N. benthamiana* were increased by transient expression of
366 *AtSOBIR1-Myc* and its kinase-inactive variant (Supporting Information Fig. S7c).
367 Importantly, transient expression of *AtSOBIR1-Myc* but not kinase-inactive *AtSOBIR1-KD-*
368 *Myc* restored Avr4-induced endocytosis of Cf-4-GFP in *NbSOBIR1-like*-silenced leaves
369 (Supporting Information Fig. S7a,b.). Altogether, these data indicate that the kinase activities
370 of both BAK1 and SOBIR1 are required for endocytosis of Cf-4 upon Avr4 recognition,
371 suggesting the active removal of triggered receptors from the plasma membrane.

372

373 *Cf-4 associates with SERK members in a ligand-dependent manner*

374 We addressed a possible role of BAK1/SERK3 in Cf-4-mediated immunity by co-
375 immunoprecipitation experiments. Cf-4-GFP was purified from *N. benthamiana* leaves
376 transiently co-expressing Cf-4-GFP, *S/SOBIR1*-HA and *S/SERK3a*-Myc, that had either been
377 mock-treated or challenged with Avr4, Avr2 or flg22 at two days after agro-infiltration of the
378 three constructs. While C-terminally tagged BAK1/SERK3 fusion proteins are not signalling
379 competent after flg22 and elf18 trigger during immunity their recruitment behavior to the
380 FLS2 receptor complex is still intact, which suggests suitability for interaction studies with
381 other receptors (Ntoukakis *et al.*, 2011). In all cases, *S/SOBIR1*-HA was detected upon
382 immunoprecipitation of Cf-4-GFP, independent of the treatment conditions (Fig. 5,
383 Supporting Information Fig. S8). This confirms the constitutive interaction between these two
384 receptors as shown by BiFC (Fig. 1) and as was previously reported (Liebrand *et al.*, 2013).
385 By contrast, *S/SERK3a*-Myc was only revealed upon immunoprecipitation of Cf-4-GFP from
386 agro-infiltrated leaves that had first been infiltrated with the matching ligand Avr4, but not
387 when infiltrated with Avr2 or flg22, which was included as control treatment (Fig. 5,
388 Supporting Information Fig. S8). Importantly, this demonstrates that ligand-dependent hetero-
389 complex formation of RLPs as the Cf-4 receptor with *S/SERK3a* takes place, in a way that is
390 similar to what has been shown for RLK-type PRRs (Monaghan & Zipfel, 2012; Liebrand *et*
391 *al.*, 2014).

392

393 Because of the previous genetic evidence for a role of *S/SERK1* in Cf-mediated immunity
394 (Fradin *et al.*, 2011), we examined whether Cf-4 also interacts with this co-receptor by co-
395 immunoprecipitation experiments after transient co-expression of the various receptors in
396 *N. benthamiana* leaves. Unlike what is the case for *S/SERK3a*-Myc, *S/SERK1*-Myc was
397 revealed after immunoprecipitation of Cf-4-GFP, independent of elicitation of Cf-4 by Avr4
398 (Supporting Information Fig. S8). However, the amount of co-immunoprecipitated *S/SERK1*-
399 Myc was strongly enhanced in the Avr4-treated leaves, as compared to the leaves treated with
400 Avr2. We cannot exclude the possibility that this differential pattern between Cf-4 interaction
401 with *S/SERK1* and *S/SERK3a* is caused by the higher steady state expression levels of
402 *S/SERK1*-Myc compared to those of *S/SERK3a*-Myc (Supporting Information Fig. S8; see
403 input). However, following the recent notion of the presence of BRI1-BAK1/SERK3
404 preassembled heterodimers (Bücherl *et al.*, 2013), our data could also indicate that Cf-4 exists
405 in a preformed complex with *S/SOBIR1* and *S/SERK1*, which becomes stabilized and/or
406 recruits increased amounts of *S/SERK1* upon elicitation with Avr4.

407 We next explored the possibility that ligand-induced recruitment of *S/SERK1* and -3

408 could exist as a more general mechanism of Cf receptor complex activation. To this end, we
409 purified Cf-9-GFP from *N. benthamiana* leaves transiently co-expressing this RLP together
410 with *SISOBIR1*-HA and either *SISERK1*-Myc or *SISERK3a*-Myc. Similar to what we
411 observed for activated Cf-4, both *SISERK1*-Myc and *SISERK3a*-Myc were strongly co-
412 immunoprecipitated with Cf-9-GFP when elicited with its matching ligand effector Avr9 but,
413 as expected, not significantly with Avr4 that was included as control treatment (Supporting
414 Information Fig. S9). This supports the idea that additional SOBIR1-dependent RLPs, such as
415 Ve1, RLP30 and ReMax (Fradin *et al.*, 2009; Fradin *et al.*, 2011; Zhang *et al.*, 2013), also
416 recruit members of the SERK family to form active receptor complexes.

417

418 *SERK* members mediate Avr4-triggered immunity

419 Avr4 triggers HR in Cf-4-expressing *N. benthamiana*, which can be diminished by silencing
420 of the gene encoding the Cf-4 receptor itself or *NbSOBIR1*-like ((Liebrand *et al.*, 2013); Fig.
421 6a). To determine whether BAK1/SERK3 is involved in Cf-4-mediated immunity, we
422 examined the Avr4-triggered HR in Cf-4-GFP-expressing *N. benthamiana* leaves (transiently
423 and stably), without and with silencing of *NbSERK3a/b*. We consistently found that the Avr4-
424 triggered HR was significantly reduced in *NbSERK3a/b*-silenced Cf-4-GFP-expressing leaves,
425 as compared to the leaves of *TRV* control plants (Fig. 6a, Supporting Information Fig. S10a).
426 *NbSERK3a/b*-silencing did not generally affect programmed cell death, as this was still
427 induced by the auto-active variant of the nucleotide binding-LRR immune receptor RxD460V
428 and the pro-apoptotic factor BCL2-ASSOCIATED PROTEIN X (BAX) (Lacomme & Santa
429 Cruz, 1999; Bendahmane *et al.*, 2002). The capability of the *NbSERK3a/b*-VIGS construct to
430 knock down the expression of both *NbSERK3a/b* gene homologues was previously shown
431 (Chaparro-Garcia *et al.*, 2011). We further investigated the specificity of *NbSERK3a/b*-
432 silencing within the SERK family and found that also a likely *NbSERK1* homologue was
433 knocked down upon expression of the *NbSERK3a/b*-VIGS construct (Supporting Information
434 Fig. S10b). Thus, it could be that Avr4-triggered HR in *N. benthamiana* requires not only
435 BAK1/SERK3 but also SERK1, an observation that is consistent with the earlier finding that
436 VIGS of *SISERK1* compromises Cf-4-mediated immunity to *C. fulvum* in tomato (Fradin *et al.*,
437 2011).

438

439 To examine whether BAK1/SERK3, in addition to SERK1, is also involved in Cf-4-mediated
440 resistance to Avr4-secreting *C. fulvum* strains, their encoding genes were silenced in Cf-4-
441 expressing tomato plants. A strain secreting Avr4 and expressing the *GUS* reporter gene

442 exhibited strong colonization of tomato leaves lacking the Cf-4 receptor (MM-Cf-0) and
443 when the *Cf-4* gene was silenced (Fig. 6b). Colonization by this fungus was significantly
444 more abundant in Cf-4-expressing tomato leaves silenced for *SISERK3*, as increased numbers
445 of successful colonization attempts were observed compared to the *GUS*-silenced negative
446 control. This phenotype is reminiscent of the effect of silencing of *SISOBIR1(-like)* on Cf-4-
447 mediated resistance to *C. fulvum* (Liebrand *et al.*, 2013). We furthermore silenced *SISERK3*
448 together with *SISERK1*, which also compromised resistance to the pathogen. Taken together,
449 these results show that BAK1/SERK3 is a key positive regulator of full Cf-4-mediated
450 immunity induced upon Avr4 perception.

451

452 **Discussion**

453 The Cf signaling pathway is essential for the immune response of tomato to *C. fulvum* (Rivas
454 & Thomas, 2005; Stergiopoulos & de Wit, 2009). A number of Cf signaling components were
455 identified through genetic and proteomic approaches, but the mechanism by which Cf-4
456 initiates downstream signaling remained unclear (Liebrand *et al.*, 2014). Here, we show that
457 both SERK1 and BAK1/SERK3 are recruited to the Cf-4 receptor in an Avr4-dependent
458 manner, a mechanism we additionally confirmed for Avr9-induced activation of Cf-9.
459 Consistently, we show that Cf-4 requires these RLKs for its function. Our observations imply
460 that Avr4 induces the formation of a complex of Cf-4 and *SISERK1/3* to induce Cf-4
461 signaling. Furthermore, we confirm that Cf-4 interacts with SOBIR1 at the plasma membrane
462 and, more importantly demonstrate ligand-induced SERK3 dependent late endocytic
463 trafficking of the Cf-4 RLP together with *SISOBIR1* as a novel pathway in Cf-mediated
464 downstream events.

465 The requirement of BAK1/SERK3 hints at clear similarities between the Cf-4/9
466 effector receptors and FLS2 MAMP receptor pathways, further evidenced by overlapping
467 transcriptional reprogramming upon activating FLS2- and Cf-mediated immune responses
468 (Navarro *et al.*, 2004). This overlap potentially involves the regulation of downstream
469 responses through similar components which, in addition to the SERK family members,
470 include E3 ligases (PUB12/13 for FLS2 and CMPG1 for Cf-4 immunity; (Gilroy *et al.*, 2011;
471 Lu *et al.*, 2011)) and receptor-like cytoplasmic kinases (BIK1/PBL1 for FLS2, ACIK1 for Cf-
472 4 and CAST AWAY for SOBIR1 (EVR) signaling; (Burr *et al.*, 2011; Monaghan & Zipfel,
473 2012; Liebrand *et al.*, 2014)). Given these similarities, it is conceivable that the constitutive
474 association between the RLP Cf-4 and the RLK *SISOBIR1* represents a PRR complex, in
475 which the Cf-4 ectodomain mediates specific ligand recognition and the *SISOBIR1* kinase

476 domain is regarded as the signaling part. This is in contrast to PRRs exemplified by FLS2, in
477 which both functions are present within the same molecule. Ligand-induced interaction of
478 PRRs with SERK member RLKs is subsequently required to trigger downstream signaling by
479 both RLP- and RLK-type PRRs (Fig. 7).

480

481 Regarding Cf-4 as a PRR is in agreement with the observation that Cf-4 not only recognizes
482 the Avr4 protein secreted by *C. fulvum*, but is also activated by the Avr4 homologue produced
483 by the banana pathogen *Mycosphaerella fijiensis* (Stergiopoulos *et al.*, 2010). Both *C. fulvum*
484 and *M. fijiensis* Avr4 proteins bind to chitin (van den Burg *et al.*, 2003; Stergiopoulos *et al.*,
485 2010) and it was suggested that, reminiscent of PAMP recognition, the chitin-binding motif
486 of Avr4 is the pattern that is recognized through interaction with the LRRs of Cf-4 (Thomma
487 *et al.*, 2011). As elicitation with Avr4 appears to maintain the Cf-4 association with
488 *S/SOBIR1* ((Liebrand *et al.*, 2013); Fig. 5) and induces the interaction of Cf-4 with
489 *S/SERK1/3*, this could suggest the possibility that a complex consisting of Cf-4, *S/SOBIR1*
490 and *S/SERK3a* is formed. In turn, this could result in increased phosphorylation events
491 between the *S/SOBIR1* and *S/SERK1/3* kinases, which then trigger downstream signaling
492 (Fig. 7), similar to what has been proposed for the FLS2-BAK1 model (Schwessinger *et al.*,
493 2011). Furthermore, our data show that *S/SERK1* and BAK1/*S/SERK3* are also recruited to
494 the Cf-9 receptor upon its activation with Avr9, supporting the idea of a general mechanism
495 by which SOBIR1-dependent RLPs form active receptor complexes. Although no molecular
496 interactions have been reported so far, a similar scenario could be proposed for RLP30, which
497 also genetically depends on SOBIR1 and BAK1/SERK3 (Zhang *et al.*, 2013). A role for
498 SERK receptors is less clear for *S/Eix2*, because in this case BAK1 was found to negatively
499 regulate this receptor through interaction with its close homologue *S/Eix1* (Bar *et al.*, 2010).
500 Future studies should determine whether, similar to the trans-phosphorylation of FLS2 by
501 BAK1/SERK3 (Schwessinger *et al.*, 2011), the phosphorylation status of the kinase domain
502 of SOBIR1 also alters upon SERK1/3 recruitment.

503 Receptor-mediated endocytosis is part of the eukaryotic immune response and for
504 example is found for the FLS2 receptor (Husebye *et al.*, 2006; Robatzek *et al.*, 2006; Spallek
505 *et al.*, 2013). An important role of receptor-mediated endocytosis is to control the abundance
506 of receptor (complexes) at the plasma membrane, a process that is well established in animals
507 and involves lysosomal/vacuolar degradation (Lemmon & Schlessinger, 2010). Activated
508 FLS2 traffics into the late endosomal pathway and is a cargo of multivesicular bodies
509 localizing to the lumen of these late endosomes for delivery to the vacuole (Beck *et al.*,

510 2012c; Spallek *et al.*, 2013). This pathway could be responsible for the flg22-induced FLS2
511 degradation, because chemicals affecting endosomal trafficking inhibit the degradation of this
512 receptor (Smith *et al.*, 2014). Our co-localization data strongly suggest that activated Cf-4 is
513 also targeted for vacuolar degradation through the late endosomal pathway, consistent with
514 the observation that Cf-4-GFP protein levels are reduced upon Avr4 elicitation (Supporting
515 Information Fig. S1a). Endosomal sorting for vacuolar degradation requires the transfer of
516 ubiquitin to the plasma membrane cargo and subsequent deubiquitination of the cargo at
517 multivesicular bodies (Beck *et al.*, 2012c). The ubiquitin E3 ligase CMPG1 and the
518 deubiquitinating enzyme UBP12 are known positive and negative regulators of Cf-4- and Cf-
519 9-mediated HR, respectively (Ewan *et al.*, 2011; Gilroy *et al.*, 2011), and given their
520 biochemical function could also be involved in the endocytosis and degradation of Cf
521 proteins.

522 Ligand-induced activation triggers the formation of a hetero-complex consisting of
523 Cf-4 and *S*/SERK1/3. This differs from the situation with TLRs from mammals, which recruit
524 cytoplasmic kinases to initiate signaling, but in plants is reminiscent of the FLS2 pathway
525 that also depends on interaction with the co-receptor BAK1/SERK3 to signal. Endocytic
526 removal of activated PRRs, which is also known for TLR4 (Husebye *et al.*, 2006), might
527 provide a mechanism to regulate receptor presence at the plasma membrane and associated
528 events (Felix *et al.*, 1998; Beck *et al.*, 2012a; Smith *et al.*, 2014). Understanding how plant
529 cells regulate PRR subcellular localization is essential as pathogens are likely to target
530 components of the trafficking pathway to suppress plant defenses, as was recently found for
531 the *Phytophthora infestans* Avr3a effector (Chaparro-Garcia *et al.*) and bacterial HopM1
532 (Nomura *et al.*, 2011). This latter effector of *Pseudomonas syringae* targets a host ADP
533 ribosylation factor guanine nucleotide exchange factor, referred to as *At*MIN7/BEN1,
534 resulting in its breakdown. *At*MIN7/BEN1 is required for PAMP-triggered immunity and
535 regulates endosomal trafficking possibly at the TGN, where HopM1 can also be found
536 (Nomura *et al.*, 2006; Nomura *et al.*, 2011). It remains to be seen whether *C. fulvum* also
537 secretes effectors that enter inside plant cells and are able to alter host subcellular trafficking
538 to promote infection success.

539

540 **Acknowledgements**

541 We thank the members of the Robatzek laboratory for fruitful discussions, Brande Wulff for
542 reading the manuscript and Cyril Zipfel for providing *At*BAK1 constructs. Zeinu Mussa is
543 acknowledged for help with *N. benthamiana* transformations.

544

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779 fungi. *The Plant Cell* **25**(10): 4227-4241.

780

781 **Figure legends**

782

783 **Fig. 1 Cf-4 and *S/SOBIR1* are present at the plasma membrane.** Confocal micrographs
784 show *N. benthamiana* leaf epidermal cells transiently co-expressing the indicated Cf-4 and
785 *S/SOBIR1* fusion proteins and plasma membrane-localized ACA8-mCherry. The first panels
786 show GFP/YFP fluorescence, the second panels show mCherry fluorescence, the third panels
787 depict the overlay images of the two fluorescence signals shown in the first and second
788 panels. Overlay images indicate co-localization of the proteins fused to GFP or YFP and
789 mCherry, as a yellow colour is produced. Dashed squares in these images are shown as detail
790 pictures (magnified in the fourth panels). White lines in the detail pictures indicate the ROIs
791 that correspond to intensity profiles in the last panels. Intensity profiles display grey value of
792 pixels across the ROI in the green and red channels, on a scale of 1-300. Images were taken at
793 three days post infiltration (dpi) for Cf-4-GFP and *S/SOBIR1*-GFP and at two dpi for BiFC of
794 Cf-4, C-terminally fused to the C-terminal half of YFP (YFPc), with *S/SOBIR1* C-terminally
795 fused to the N-terminal half of YFP (YFPn); scale bars = 10 μ m.

796

797 **Fig. 2 *S/SOBIR1* constitutively localizes to endosomes.** Confocal micrographs show *N.*
798 *benthamiana* leaf epidermal cells transiently expressing *S/SOBIR1*-GFP or Cf-4-GFP (left
799 panels), either without or with co-expression of the indicated organelle markers fused to
800 mCherry/RFP (middle, left panels). Co-localization in the overlay images is depicted by the
801 development of a yellow colour (middle, right panels). Dashed squares in these images are
802 shown as detail pictures (magnified in the right panels). Arrowheads in the detail pictures
803 point at mobile vesicles in the upper right panel and indicate *S/SOBIR1* localization at
804 endosomes in the two lower right panels. Images were taken at three dpi; scale bars = 10 μ m.

805

806 **Fig. 3 Cf-4 localizes to endosomes in a ligand-dependent manner and together with**
807 ***S/SOBIR1*.** (a) Confocal micrographs show *N. benthamiana* leaf epidermal cells transiently
808 expressing Cf-4-GFP without and with co-expression of ARA6-RFP and *S/SOBIR1*-mCherry,
809 treated with Avr4 or Avr2 (both at 100 μ M), as indicated. The left panels show GFP
810 fluorescence, middle left panels show autofluorescence and RFP/mCherry fluorescence,
811 middle right panels depict the overlay images of the two fluorescence signals shown in the
812 left and middle left panels, and the right panels show detail pictures of the dashed squares.
813 Arrowheads point at mobile vesicles positive for Cf-4-GFP and ARA6-RFP and indicate co-
814 localization of Cf-4-GFP with ARA6-RFP and *S/SOBIR1*-mCherry at endosomes. Images
815 were taken at three dpi and 90 min after elicitation; scale bars = 10 μ m. (b) Confocal

816 micrographs show *N. benthamiana* leaf epidermal cells transiently expressing Cf-4, C-
817 terminally fused to the C-terminal half of YFP (YFPc) and *S/SOBIR1*, C-terminally fused to
818 the N-terminal half of YFP (YFPn) (left panels), without or with co-expression of ARA6-RFP
819 (middle left panels) and treated with Avr4 or Avr2 (both at 100µM), as indicated. Co-
820 localization between reconstituted YFP and RFP in the overlay images is depicted by the
821 development of a yellow colour (middle, right panels). Dashed squares in these panels are
822 shown as detail pictures (right panels). Arrowheads point at mobile vesicles in the upper right
823 panel and indicate co-localization at endosomes in the middle right panel. Images were taken
824 at three dpi and 90 min after elicitation; scale bars = 10 µm.

825

826 **Fig. 4 Cf-4 endocytosis requires BAK1/SERK3.** Leaves of Cf-4-*N. benthamiana* stable and
827 *N. benthamiana* plants were TRV-silenced for *NbSERK3a/b* and *GUS* as a control for three
828 weeks and subsequently used to transiently express *AtBAK1*, *AtBAK1-KD*, and
829 ARA6/RabF1-RFP as indicated for three days. Confocal micrographs show Cf-4-GFP
830 localisation upon treatment with Avr4 (100µM, left panels) and ARA6/RabF1-RFP-labelled
831 endosomes (left panels), and detail pictures from dashed squares (middle panels).
832 Arrowheads point at Cf-4-GFP- and ARA6/RabF1-RFP-positive vesicles. Quantification of
833 Cf-4-GFP- and ARA6/RabF1-RFP-positive vesicles was done with EndoQuant (right panels,
834 bars depict means ± 2 SE; n = 6; *p* < 0.05; statistical significant differences are indicated by
835 asterisks). Note, much fewer Cf-4-GFP-positive vesicles are observed in *NbSERK3a/b*-
836 silenced leaves compared to the *GUS*-silenced control. The amount of Cf-4-GFP-positive
837 vesicles increased upon transient co-expression of *AtBAK1* but not when its kinase-inactive
838 variant *AtBAK1-KD* was co-expressed. No difference in the amount of ARA6/RabF1-RFP-
839 positive endosomes was observed when *NbSERK3a/b* was silenced. Images were taken 90
840 min after Avr4 elicitation; scale bars = 10 µm. See Supporting information Fig. S10b,c for
841 transcript abundance of silenced genes and protein levels of Cf-4.

842

843 **Fig. 5 *S/SERK3a* interacts with Cf-4.** Co-immunoprecipitation from GFP-trap bead pull-
844 downs on *N. benthamiana* co-expressing Cf-4-GFP, *S/SOBIR1*-HA and *S/SERK3a*-Myc. Leaf
845 samples were taken at two dpi, after 60 min treatments without or with 10µM flg22 or
846 100µM Avr4, as indicated. Total proteins (Input) and immunoprecipitated proteins (IP) were
847 subjected to SDS/PAGE and blotted. Blots were incubated with αGFP, αHA or αMyc
848 antibodies for the detection of immunoprecipitated Cf-4-GFP and co-purifying *S/SOBIR1*-
849 HA and *S/SERK3a*-Myc, respectively.

850

851 **Fig. 6 *SISERK3a* is required for Avr4-triggered HR and immunity against *C. fulvum*.** (a)
852 Images show leaves of transgenic *N. benthamiana* plants stably expressing Cf-4-GFP that had
853 been *TRV*-silenced for either *Cf-4*, *NbSOBIR1*, *NbSERK3a/b* or for *GUS* as control, and were
854 subsequently agro-infiltrated with Avr4 (O.D.600 = 0.03), RxD460V (O.D.600 = 0.1) and
855 BAX (O.D.600 = 0.5) as indicated. Images were taken three days after agro-infiltration. HR
856 is observed as brownish cell death. The numbers below the panels indicate the occurrence of
857 full HR, intermediate or no symptoms out of 36 Avr4 agro-infiltrations that were performed.
858 (b) Images show leaves two weeks after inoculation with an Avr4-secreting, *GUS*-transgenic
859 strain of *C. fulvum* of MM-Cf-0 tomato as a control, and Cf-4 tomato that had been
860 inoculated with recombinant *TRV* constructs targeting *Cf-4*, *SISERK3*, *SISERK1* or *GUS* three
861 weeks earlier. To visualize *C. fulvum* colonization, leaves were stained for GUS activity. The
862 amount of successful colonization attempts (blue spots) versus the total amount of Cf-4
863 leaves that were sampled is indicated between parentheses.

864

865 **Fig. 7 Model of the Cf-4, SOBIR1 and SERK3 subcellular trafficking pathways.**
866 Following its folding and maturation in the endoplasmic reticulum (ER), Cf-4 is
867 predominantly trafficked to the plasma membrane, where it constitutively interacts with
868 SOBIR1, which itself is constitutively endocytosed. Upon colonization of Cf-4 tomato leaves,
869 *C. fulvum* secretes Avr4 into the apoplast, which is recognized by Cf-4 and induces
870 interaction of Cf-4 with BAK1/SERK3. This complex is required for Cf-4-mediated
871 immunity (not shown) and endocytosis into ARA7- and ARA6-positive compartments of the
872 late endosomal pathway, destined for vacuolar degradation. Subcellular localization of
873 markers (ACA8, MEMB12, VHA-a1, ARA6 and ARA7) used in this study is depicted in
874 brown lettering. SV, secretory vesicle; V, vesicle; TGN, *trans*-Golgi network; EE, early
875 endosome; LE, late endosome; MVB, multivesicular body.

876

877 **Supporting Information**

878

879 **Fig. S1 GFP-tagged Cf-4 migrates with the predicted molecular weight and is functional.**
880 (a) Western blot analysis of Cf-4-GFP transiently and stably expressed in *N. benthamiana*,
881 without and with Avr4 (100 μ M) treatment, as indicated. For transient expression, leaf
882 samples were harvested at three days post infiltration (dpi) and Avr4 elicitation was done for
883 90 min. Blots were incubated with anti-GFP antibodies for the detection of Cf-4-GFP. CBB,

884 Coomassie Brilliant Blue staining. **(b)** Images of *N. benthamiana* wild type (WT) leaves (top
885 panel) and leaves transiently expressing Cf-4-GFP (bottom panel), treated with either Avr2 or
886 Avr4 (300 μ M), as indicated. Images were taken at five dpi and at four days after treatment
887 with the Avr's.

888

889 **Fig. S2 Subcellular localization of Cf-4, *S*/SOBIR1-like and *At*SOBIR1.** Confocal
890 micrographs show *N. benthamiana* leaf epidermal cells stably expressing Cf-4-GFP and
891 transiently expressing *S*/SOBIR1-like-GFP and *At*SOBIR1-GFP as indicated (left panels), and
892 co-expressing plasma membrane-localized ACA8-mCherry (middle panels). Overlay images
893 indicate co-localization of the proteins fused to GFP and mCherry, as a yellow colour is
894 produced (right panels). Images were taken at three dpi; scale bars = 10 μ m.

895

896 **Fig. S3 *S*/SOBIR1-like-GFP and *At*SOBIR1-GFP co-localize with endosomal markers.**
897 Confocal micrographs show *N. benthamiana* leaf epidermal cells transiently expressing
898 *S*/SOBIR1-like-GFP or *At*SOBIR1-GFP (left panels), and co-expressed mCherry/RFP-tagged
899 organelle markers (middle, left panels). Overlay images indicate co-localization through
900 generation of a yellow colour (middle, right panels). Dashed boxes indicated in the middle
901 right panels are depicted as detail pictures on the right (right panels). Arrowheads point at co-
902 localizing endosomes. Images were taken at three dpi; scale bars = 10 μ m. **(a)** Co-expression
903 of *S*/SOBIR1-like-GFP/*At*SOBIR1-GFP with Golgi marker MEMB12-mCherry. **(b)** Co-
904 expression of *S*/SOBIR1-like-GFP/*At*SOBIR1-GFP with *trans*-Golgi network marker VHA-
905 a1-RFP. **(c)** Co-expression of *S*/SOBIR1-like-GFP/*At*SOBIR1-GFP with endosome marker
906 RFP-ARA7/RabF2b. **(d)** Co-expression of *S*/SOBIR1-like-GFP/*At*SOBIR1-GFP with late
907 endosome marker ARA6/RabF1-RFP.

908

909 **Fig. S4. Line intensity profiles of Cf-4, SOBIR1 and membrane markers.** Confocal
910 micrographs show transient co-expression of Cf-4-GFP, *S*/SOBIR1-GFP or Cf-4-
911 YFPc/*S*/SOBIR1-YFPn with indicated membrane markers and effector treatments. Left
912 panels correspond to confocal micrographs displayed in **(a)** Fig. 1, **(b)** Fig. 2a, **(c)** Fig. 2b.
913 Dashed squares in these panels are shown as detail pictures (magnified in middle panels).
914 White lines in detail pictures indicate the ROIs that correspond to intensity profiles in the last
915 panels. Intensity profiles display grey value of pixels across the ROI in the green and red
916 channels on a scale of 1-300.

917

918 **Fig. S5 *SISOBIR1* does not interact with FLS2 in BiFC experiments.** (a) Confocal
919 micrographs show *N. benthamiana* leaf epidermal cells transiently co-expressing FLS2 and
920 *SISOBIR1* C-terminally fused to the C- or N-terminal halves of YFP (YFPc and YFPn,
921 respectively), as indicated. Left panels show absence or presence of YFP fluorescence, the
922 middle panels show autofluorescence, and the right panels show the overlay of the
923 fluorescence signals. Images were taken at two dpi, scale bars = 100 μ m. (b) Confocal
924 micrographs of *N. benthamiana* leaf epidermal cells transiently co-expressing FLS2-YFPc
925 and FLS2-YFPn, either without or with flg22 treatment (10 μ M) for 90 min, as indicated. Left
926 panels show absence or presence of YFP fluorescence, the middle panels show
927 autofluorescence, and the right panels show the overlay of the fluorescence signals. Images
928 were taken at two dpi, scale bars = 10 μ m.

929

930 **Fig. S6 Cf-4-GFP endocytosis in stable transgenic plants.** Confocal micrographs of Cf-4-
931 GFP-transgenic *N. benthamiana* plants show leaf epidermal cells expressing Cf-4-GFP, either
932 treated with Avr4 or Avr2 (both at 100 μ M), as indicated. Left panels show GFP fluorescence,
933 middle left panels show autofluorescence, and middle right panels show the overlay of the
934 fluorescence signals shown in the left and middle left panels. Arrowheads in the detail
935 pictures (right panels) point at mobile vesicles (top right panel). Images were taken at 90
936 minutes after elicitation; scale bars = 10 μ m.

937

938 **Fig. S7 Cf-4 endocytosis requires SOBIR1 kinase activity.** Leaves of Cf-4-GFP transgenic
939 *N. benthamiana* plants that had been TRV-silenced for *NbSOBIR1/-like* were used for
940 transient expression of *AtSOBIR1-Myc* and its kinase-inactive variant *AtSOBIR1-KD-Myc*. (a)
941 Confocal micrographs show Cf-4-GFP localisation upon treatment with Avr4 (100 μ M, left
942 panels) and detail pictures from dashed squares (middle panels). Arrowheads point at Cf-4-
943 GFP-positive vesicles. Images were taken at three weeks after inoculation with TRV, at three
944 dpi for transient expression, and at 90 min after elicitation; scale bars = 10 μ m. (b)
945 Quantification of Cf-4-GFP-positive vesicles was done with EndoQuant (bars depict means \pm
946 2 SE; n = 6; $p < 0.05$; statistical significant differences are indicated by asterisks). Transient
947 co-expression of *AtBAK1*, but not its kinase-inactive variant *AtBAK1-KD*, increased Cf-4-
948 GFP-positive vesicles in *NbSOBIR1/-like*-silenced leaves. (c) Immunoblots from extracted
949 total proteins of Cf-4-GFP-transgenic *N. benthamiana* plants that were TRV-silenced for
950 *NbSOBIR1/-like* and subsequently transiently transformed with *AtSOBIR1-Myc* or *AtSOBIR1-*
951 *KD-Myc*. Cf-4-GFP, *AtSOBIR1-Myc* and *AtSOBIR1-KD-Myc* were revealed with α GFP and

952 α Myc antibodies as indicated. Ponceau staining is shown for equal loading.

953

954 **Fig. S8 Both *S/SERK1* and *S/SERK3a* interact with the Cf-4.** Co-immunoprecipitation
955 from GFP-trap bead pull-downs on *N. benthamiana* co-expressing Cf-4-GFP and *S/SOBIR1*-
956 HA, and either *S/SERK3a*-Myc or *S/SERK1*-Myc. Leaf samples were taken at two dpi, after
957 60 min treatment with Avr2 or Avr4 (100 μ M), as indicated. Total proteins (Input) and
958 immunoprecipitated proteins (IP) were subjected to SDS/PAGE and blotted. Blots were
959 incubated with α GFP, α HA or α Myc antibodies for the detection of immunoprecipitated Cf-4-
960 GFP and co-purifying *S/SOBIR1*-HA and *S/SERK3a*-Myc or *S/SERK1*-Myc, respectively.

961

962 **Fig. S9 Avr9 induces interaction of Cf-9 with both *S/SERK1* and *S/SERK3a*.** Co-
963 immunoprecipitation from GFP-trap bead pull-downs on *N. benthamiana* co-expressing Cf-9-
964 GFP and *S/SOBIR1*-HA, and either *S/SERK1*-Myc or *S/SERK3a*-Myc. Leaf samples were
965 taken at two dpi, after 60 min treatment with Avr9 or Avr4 (100 μ M), as indicated. Total
966 proteins (Input) and immunoprecipitated proteins (IP) were subjected to SDS/PAGE and
967 blotted. Blots were incubated with α GFP, α HA or α Myc antibodies for the detection of
968 immunoprecipitated Cf-9-GFP and co-purifying *S/SOBIR1*-HA and *S/SERK1*-Myc or
969 *S/SERK3a*-Myc, respectively.

970

971 **Fig. S10 *S/SERK3* is required for Avr4-induced HR.** (a) Images show *N. benthamiana*
972 leaves from *TRV::NbSERK3a/b*-inoculated plants and *TRV*-inoculated controls, transiently
973 expressing Cf-4-GFP and treated with Avr4 protein (300 μ M). Cf-4-GFP was agro-infiltrated
974 at 3 weeks post inoculation with the recombinant *TRV* constructs and one day later the Avr4
975 protein was infiltrated. Images were taken at six days after Avr4 infiltration. HR is observed
976 as brownish cell death. (b) qRT-PCR analysis showing *NbSERK1*, *NbSERK3a/b* and
977 *NbSOBIR1* expression in *N. benthamiana* inoculated with the indicated *TRV* VIGS constructs.
978 Expression of query genes was normalized to endogenous *NbActin* expression levels. Data
979 presented have been combined from twelve (*NbSOBIR1* expression) or 24 (*NbSERK1* and
980 *NbSERK3* expression) individual qRT-PCRs, based on three independent biological replicates,
981 for each gene of which the expression was studied. Statistical differences ($\alpha=0.05$) between
982 groups were calculated by a one-way ANOVA and different groupings are indicated. Error
983 bars represent the standard deviation. (c) Immunoblots from total protein extracts of Cf-4-
984 GFP-transgenic *N. benthamiana* plants that were *TRV*-silenced for *NbSERK3a/b* and
985 subsequently transiently transformed with *AtBAK1* and *AtBAK1-KD*. Cf-4-GFP was revealed

986 with α GFP. Ponceau staining is shown for equal loading. (d) RT-PCR on cDNA obtained
987 from total RNA extracts of Cf-4-GFP-transgenic *N. benthamiana* plants that were TRV-
988 silenced for *NbSERK3a/b* and subsequently transiently transformed with *AtBAK1* and
989 *AtBAK1-KD*.

990

991 **Video S1. Mobile vesicles in transient Cf-4-GFP expression.** Time series of confocal
992 micrographs of *N. benthamiana* leaf epidermal cells transiently expressing Cf-4-GFP and
993 treated with Avr4 (100 μ M). Images were taken at 3 days post infiltration (dpi) and 1.5 hours
994 after Avr4 treatment. Frame counter is displayed in the top left. Acquisition speed is ~4
995 seconds per frame. Scale bar = 10 μ m.

996

997 **Video S2. Mobile vesicles in stable Cf-4-GFP expression.** Time series of confocal
998 micrographs of *N. benthamiana* leaf epidermal cells stably expressing Cf-4-GFP and treated
999 with Avr4 (100 μ M). Images were taken 1.5 hours after Avr4 treatment. Frame counter is
1000 displayed in the top left. Acquisition speed is ~4 seconds per frame. Scale bar = 10 μ m.

ACA8-mCherry

overlay

detail

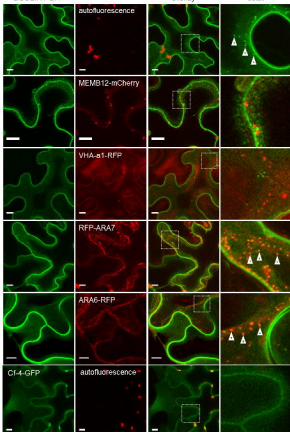
intensity profile

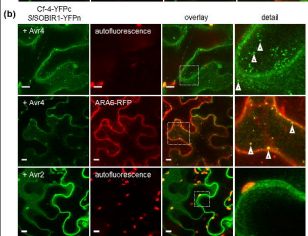
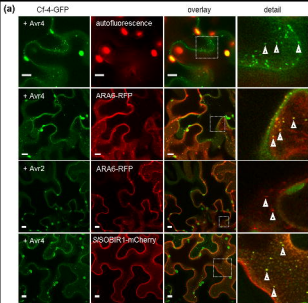


SISOBIR1-GFP

overlay

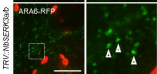
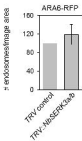
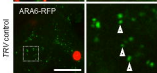
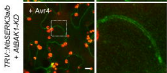
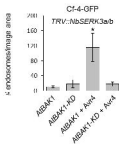
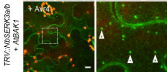
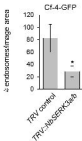
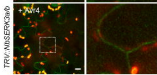
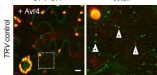
detail





CI-4-GFP

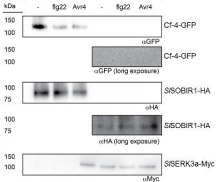
detail



IP: α GFP

Input

CI-4-eGFP + SISOBIR1-HA + SiSERK3a-Myc



(a)TRV::*Cl-4*

TRV::NbSOBIR1

TRV::NbSERK3a/b

TRV control



full: 6/36
intermediate: 7/36
none: 23/36

full: 5/36
intermediate: 4/36
none: 27/36

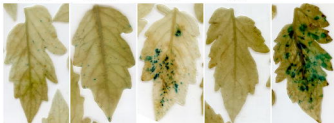
full: 2/36
intermediate: 8/36
none: 26/36

full: 27/36
intermediate: 9/36
none: 0/36

(b)

TRV::

no TRV

*SISERK3a**SISERK3a*
*SISERK1**Cl-4**GUS*

(364/36)

(98/31)

(>500/32)

(12/44)

*Cl-4*MM-*Cl-0*

Fungus

Clostridium botulinum

Apoptosis

Plant Cell

