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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Summary

35

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

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 (Russinova *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 *Sl*Eix2, an LRR-RLP

120 recognizing fungal xylanase (Bar *et al.*, 2010). In a BAK1/SERK3-dependent manner, the

121 close homologue *Sl*Eix1 impairs *Sl*Eix2 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). *Sl*SOBIR1 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 *Sl*SOBIR1 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 *Sl*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, *Sl*SOBIR1-GFP, *Sl*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 *Sl*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). *Sl*SOBIR1-HA was

169   obtained by recombining pENTR/D-TOPO®-*Sl*SOBIR1 (Liebrand *et al.*, 2013) to pGWB14,

170 carrying the HA coding sequence (Nakagawa *et al.*, 2007). *Sl*SERK1-Myc and *Sl*SERK3a-

171 Myc were obtained by recombining pENTR/D-TOPO®-*Sl*SERK1 and pDONR201®-

172 *Sl*SERK3a (Liebrand *et al.*, 2013) to pGWB20 carrying the Myc coding sequence (Nakagawa

173 *et al.*, 2007), respectively. pUB::*At*BAK1^{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 OD_{600}=0.25 and OD_{600}=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 $OD_{600} = 0.4$ and GV3101 carrying *TRV-RNA2*

195 containing the respective target sequences at $OD_{600} = 0.2$ 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 *At*BAK1-F (5'-TGG ACT TGC AAA ACT CAT GG-3') and

239   *At*BAK1-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*

244   *al.*, 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 SlSOBIR1 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)). *Sl*SOBIR1-GFP, its close homolog *Sl*SOBIR1-like fused

267   to GFP and also *At*SOBIR1-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-*Sl*SOBIR1 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   *Sl*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-*Sl*SOBIR1 heterodimerization (Fig. 1). Cf-

276   4-*Sl*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 *Sl*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 *Sl*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 *Sl*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, *At*SOBIR1 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   *Sl*SOBIR1-GFP, *Sl*SOBIR1-like-GFP and *At*SOBIR1-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 *At*SOBIR1-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 *Sl*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 *Sl*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 *Sl*SOBIR1. Transient co-expression

324 of Cf-4 and *Sl*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-*Sl*SOBIR1 BiFC heterodimer (Fig. 3b), and in agreement with Avr4-

329 induced co-localization of Cf-4-GFP and *Sl*SOBIR1-mCherry at endosomes (Fig. 3a).

330 Treatment with Avr2 did not trigger re-localization of the plasma membrane-resident

331 reconstituted Cf-4-*Sl*SOBIR1 BiFC heterodimer (Fig. 3b), further supporting ligand-specific

332 internalization of this Cf-4 together with *Sl*SOBIR1. These results indicate that the receptors

333 can be endocytosed as heterodimers, an observation that was previously made for the BRI1-

334 BAK1 complex in *Arabidopsis* protoplasts (Russinova *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

Cf-4 endocytosis requires BAK1/SERK3

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

361

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

372

Cf-4 associates with SERK members in a ligand-depending 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, *Sl*SOBIR1-HA and *Sl*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, *Sl*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, *Sl*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 *Sl*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 *Sl*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 *Sl*SERK3a-Myc, *Sl*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 *Sl*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 *Sl*SERK1 and *Sl*SERK3a is caused by the higher steady state expression levels of
402   *Sl*SERK1-Myc compared to those of *Sl*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 *Sl*SOBIR1 and *Sl*SERK1, which becomes stabilized and/or
406   recruits increased amounts of *Sl*SERK1 upon elicitation with Avr4.
407   We next explored the possibility that ligand-induced recruitment of *Sl*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 *Sl*SOBIR1-HA and either *Sl*SERK1-Myc or *Sl*SERK3a-Myc. Similar to what we

411   observed for activated Cf-4, both *Sl*SERK1-Myc and *Sl*SERK3a-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 *SlSERK1* compromises Cf-4-mediated immunity to *C. fulvum* in tomato (Fradin *et*

437   *al.*, 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 *SlSERK3*, 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 *SlSOBIR1(-like)* on Cf-4-

447 mediated resistance to *C. fulvum* (Liebrand *et al.*, 2013). We furthermore silenced *SlSERK3*

448 together with *SlSERK1*, 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 *Sl*SERK1/3 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 *Sl*SOBIR1 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 *Sl*SOBIR1 represents a PRR complex, in

475 which the Cf-4 ectodomain mediates specific ligand recognition and the *Sl*SOBIR1 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 *Sl*SOBIR1 ((Liebrand *et al.*, 2013); Fig. 5) and induces the interaction of Cf-4 with

489 *Sl*SERK1/3, this could suggest the possibility that a complex consisting of Cf-4, *Sl*SOBIR1

490 and *Sl*SERK3a is formed. In turn, this could result in increased phosphorylation events

491 between the *Sl*SOBIR1 and *Sl*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 *Sl*SERK1 and BAK1/*Sl*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 *Sl*Eix2, because in this case BAK1 was found to negatively

499 regulate this receptor through interaction with its close homologue *Sl*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 *Sl*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

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544

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780

781   **Figure legends**

782

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

796

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

805

Fig. 3 Cf-4 localizes to endosomes in a ligand-dependent manner and together with *Sl*SOBIR1. (**a**) Confocal micrographs show *N. benthamiana* leaf epidermal cells transiently expressing Cf-4-GFP without and with co-expression of ARA6-RFP and *Sl*SOBIR1-mCherry, treated with Avr4 or Avr2 (both at 100µM), as indicated. The left panels show GFP fluorescence, middle left panels show autofluorescence and RFP/mCherry fluorescence, middle right panels depict the overlay images of the two fluorescence signals shown in the left and middle left panels, and the right panels show detail pictures of the dashed squares. Arrowheads point at mobile vesicles positive for Cf-4-GFP and ARA6-RFP and indicate co-localization of Cf-4-GFP with ARA6-RFP and *Sl*SOBIR1-mCherry at endosomes. Images were taken at three dpi and 90 min after elicitation; scale bars = 10 µm. (**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 *Sl*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 *At*BAK1, *At*BAK1-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 *At*BAK1 but not when its kinase-inactive
838   variant *At*BAK1-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 *Sl*SERK3a interacts with Cf-4.** Co-immunoprecipitation from GFP-trap bead pull-
844   downs on *N. benthamiana* co-expressing Cf-4-GFP, *Sl*SOBIR1-HA and *Sl*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 *Sl*SOBIR1-
849   HA and *Sl*SERK3a-Myc, respectively.

850

851 **Fig. 6 *Sl*SERK3a 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*, *SlSERK3, SlSERK1* 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 (100μM) 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 (300µM), as indicated. Images were taken at five dpi and at four days after treatment

887 with the Avrs.

888

889 **Fig. S2 Subcellular localization of Cf-4, *Sl*SOBIR1-like and *At*SOBIR1.** Confocal

890 micrographs show *N. benthamiana* leaf epidermal cells stably expressing Cf-4-GFP and

891 transiently expressing *Sl*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 μm.

895

896 **Fig. S3 *Sl*SOBIR1-like-GFP and *At*SOBIR1-GFP co-localize with endosomal markers.**

897 Confocal micrographs show *N. benthamiana* leaf epidermal cells transiently expressing

898 *Sl*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 μm. (**a**) Co-expression

903 of *Sl*SOBIR1-like-GFP/*At*SOBIR1-GFP with Golgi marker MEMB12-mCherry. (**b**) Co-

904 expression of *Sl*SOBIR1-like-GFP/*At*SOBIR1-GFP with *trans*-Golgi network marker VHA-

905 a1-RFP. (**c**) Co-expression of *Sl*SOBIR1-like-GFP/*At*SOBIR1-GFP with endosome marker

906 RFP-ARA7/RabF2b. (**d**) Co-expression of *Sl*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, *Sl*SOBIR1-GFP or Cf-4-

911 YFPc/*Sl*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 *Sl*SOBIR1 does not interact with FLS2 in BiFC experiments.** (**a**) Confocal
919 micrographs show *N. benthamiana* leaf epidermal cells transiently co-expressing FLS2 and
920 *Sl*SOBIR1 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 μm. (**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 (10μM) 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 μm.

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 μM), 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 μm.

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 (100μM, 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 μm. (**b**)
945 Quantification of Cf-4-GFP-positive vesicles was done with EndoQuant (bars depict means ±
946 2 SE; n = 6; $p < 0.05$; statistical significant differences are indicated by asterisks). Transient
947 co-expression of *At*BAK1, but not its kinase-inactive variant *At*BAK1-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, *At*SOBIR1-Myc and *At*SOBIR1-KD-Myc were revealed with αGFP and

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

953

954   **Fig. S8 Both *Sl*SERK1 and *Sl*SERK3a interact with the Cf-4.** Co-immunoprecipitation

955   from GFP-trap bead pull-downs on *N. benthamiana* co-expressing Cf-4-GFP and *Sl*SOBIR1-

956   HA, and either *Sl*SERK3a-Myc or *Sl*SERK1-Myc. Leaf samples were taken at two dpi, after

957   60 min treatment with Avr2 or Avr4 (100µM), as indicated. Total proteins (Input) and

958   immunoprecipitated proteins (IP) were subjected to SDS/PAGE and blotted. Blots were

959   incubated with αGFP, αHA or αMyc antibodies for the detection of immunoprecipitated Cf-4-

960   GFP and co-purifying *Sl*SOBIR1-HA and *Sl*SERK3a-Myc or *Sl*SERK1-Myc, respectively.

961

962   **Fig. S9 Avr9 induces interaction of Cf-9 with both *Sl*SERK1 and *Sl*SERK3a.** Co-

963   immunoprecipitation from GFP-trap bead pull-downs on *N. benthamiana* co-expressing Cf-9-

964   GFP and *Sl*SOBIR1-HA, and either *Sl*SERK1-Myc or *Sl*SERK3a-Myc. Leaf samples were

965   taken at two dpi, after 60 min treatment with Avr9 or Avr4 (100µM), as indicated. Total

966   proteins (Input) and immunoprecipitated proteins (IP) were subjected to SDS/PAGE and

967   blotted. Blots were incubated with αGFP, αHA or αMyc antibodies for the detection of

968   immunoprecipitated Cf-9-GFP and co-purifying *Sl*SOBIR1-HA and *Sl*SERK1-Myc or

969   *Sl*SERK3a-Myc, respectively.

970

971   **Fig. S10 *Sl*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 (300µM). 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 (α=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 αGFP. 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
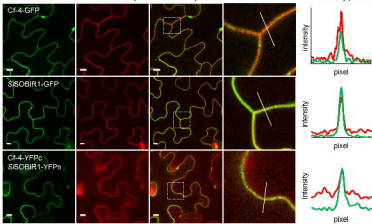
989   *AtBAK1-KD*.

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 µM). 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 µm.

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 µM). 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 µm.

SlSOBIR1-GFP | autofluorescence | overlay | detail

MEMB12-mCherry

VHA-a1-RFP

RFP-ARA7

ARA6-RFP

Cf-4-GFP | autofluorescence

(a)

	Cf-4-GFP		overlay	detail
+ Avr4	autofluorescence			
+ Avr4	ARA6-RFP			
+ Avr2	ARA6-RFP			
+ Avr4	SlSOBIR1-mCherry			

(b)

	Cf-4-YFPc SlSOBIR1-YFPn		overlay	detail
+ Avr4	autofluorescence			
+ Avr4	ARA6-RFP			
+ Avr2	autofluorescence			

Cf-4-GFP

column headers: Cf-4-GFP | detail

Rows (left labels):
- TRV control + Avr4
- TRV::NbSERK3a/b + Avr4
- TRV control + AtCERK1 + Avr4
- TRV::NbSERK3a/b + AtCERK1-KD + Avr4
- TRV control (ARA6-RFP)
- TRV::NbSERK3a/b (ARA6-RFP)

Cf-4-GFP

x-axis: TRV control, TRV::NbSERK3a/b
y-axis: ≥ endosomes/image area

Cf-4-GFP
TRV::NbSERK3a/b

x-axis: AtBAK1, AtBAK1-KD, AtBAK1 + Avr4, AtBAK1-KD + Avr4
y-axis: ≥ endosomes/image area

ARA6-RFP

x-axis: TRV control, TRV::NbSERK3a/b
y-axis: ≥ endosomes/image area

IP: αGFP   Input

Cf-4-αGFP + SlSOBIR1-HA + SlSERK3a-Myc

| kDa | − | flg22 | Avr4 | | − | flg22 | Avr4 |

αGFP   Cf-4-GFP

αGFP (long exposure)

αHA   SlSOBIR1-HA

αHA (long exposure)   SlSOBIR1-HA

αMyc   SlSERK3a-Myc

(a)

TRV::Cf-4	TRV::NbSOBIR1	TRV::NbSERK3a/b	TRV control
full: 6/36	full: 5/36	full: 2/36	full: 27/36
intermediate: 7/36	intermediate: 4/36	intermediate: 8/36	intermediate: 9/36
none: 23/36	none: 27/36	none: 26/36	none: 0/36

(b)

	TRV::			no TRV
SlSERK3a	SlSERK3a SlSERK1	Cf-4	GUS	
(364/36)	(98/31)	(>500/32)	(12/44)	
Cf-4				MM-Cf-0