An EFR-Cf-9 chimera confers enhanced resistance to bacterial pathogens by SOBIR1- and BAK1-dependent recognition of elf18

JINBIN WU1*, IDA-BARBARA RECA2*, FRANCESCO SPINELLI3, DAMIANO LIRONI3, GIULIA DE LORENZO3, PALMIRO POLTRONIERI2, FELICE CERVONE3, MATTHIEU H.A.J. JOOSTEN1, SIMONE FERRARI3,* AND ALEXANDRE BRUTUS4

1Laboratory of Phytopathology, Wageningen University, Droevendaalsesteeg 1, 6708 PB, Wageningen, Netherlands
2CNR-ISPA, via Provinciale Lecce-Monteroni, 73100, Lecce, Italy
3Department of Biology and Biotechnology “Charles Darwin”, Sapienza University of Rome, 00185, Rome, Italy
4DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA

SUMMARY

The transfer of well-studied native and chimeric pattern recognition receptors (PRRs) to susceptible plants is a proven strategy to improve host resistance. In most cases, the ectodomain determines PRR recognition specificity, while the endodomain determines the intensity of the immune response. Here we report the generation and characterization of the chimeric receptor EFR-Cf-9, which carries the ectodomain of the Arabidopsis thaliana EF-Tu receptor (EFR) and the endodomain of the tomato CF-9 resistance protein. Both transient and stable expression of EFR-Cf-9 triggered a robust hypersensitive response (HR) upon elf18 treatment in tobacco. Co-immunoprecipitation and virus-induced gene silencing studies showed that EFR-Cf-9 constitutively interacts with SUPPRESSOR OF BIR1-1 (SOBIR1) co-receptor, and requires both SOBIR1 and kinase-active BRII-ASSOCIATED KINASE1 (BAK1) for its function. Transgenic plants expressing EFR-Cf-9 were more resistant to the (hemibiotrophic bacterial pathogens Pseudomonas syringae pv. tabaci (Pta) 11528 and Pseudomonas syringae pv. tomato DC3000, and mounted an HR in response to high doses of Pta 11528 and P. carotovorum. Taken together, these data indicate that the EFR-Cf-9 chimera is a valuable tool for both investigating the molecular mechanisms responsible for the activation of defence responses by PRRs, and for potential biotechnological use to improve crop disease resistance.

Keywords: BAK1, CF-9, EFR, pattern recognition receptors, plant innate immunity, R genes, SOBIR1.

INTRODUCTION

Plants have evolved an innate immune system that relies on the recognition of potential pathogens by a defined pool of membrane and cytosolic receptors. The first layer of plant immunity comprises plasma membrane pattern recognition receptors (PRRs) that recognize microbe-associated molecular patterns (MAMPs), thereby mounting MAMP-triggered immunity (MTI) (Cook et al., 2015; Couto and Zipfel, 2016; Dangl et al., 2013; Dodds and Rathjen, 2010). Successful pathogens often produce effector proteins capable of suppressing MTI and preventing an effective immune response. As a countermeasure, plants have evolved pathogen strain-specific immune receptors, so-called resistance (R) proteins, which are capable of recognizing particular effectors and subsequently activate effector-triggered immunity (ETI). Although leading to outputs that are qualitatively similar to those of MTI, ETI is generally stronger and faster, and often includes a rapid programmed cell death (PCD) of the host cells at the site of infection, referred to as hypersensitive response (HR) (Chisholm et al., 2006; Jones and Dangl, 2006).

Cell surface PRRs and R proteins include transmembrane (TM)-associated receptor-like kinases (RLKs) and receptor-like proteins (RLPs), of which the latter lack a cytoplasmic kinase domain. Both types of receptors possess an ectodomain for ligand recognition that in many cases is mainly composed of leucine-rich repeats (LRRs) (Boutrot and Zipfel, 2017; Zipfel, 2014). In tomato (Solanum lycopersicum, S), the well-studied RLP Cf-4 triggers a strong HR-associated immunity against the biotrophic pathogenic fungus Cladosporium fulvum secreting the effector Avr4 (Joosten et al., 1997; Thomas et al., 1997). Cf-4 shares identical TM and cytoplasmic domains with CF-9 (Thomas et al., 1997), which confers immunity to C. fulvum secreting the Avr9 effector (van Kan et al., 1991). The RLK SUPPRESSOR OF BIR1-1/EVERSHEDE (SOBIR1/EVR, hereafter referred to as SOBIR1) is required for Cf-4-mediated resistance to C. fulvum (Liebrand et al., 2013) and, through its TM domain, constitutively interacts with Cf-4 (Bi et al., 2016). Increasing evidence suggests that SOBIR1
Elf18, an 18-amino acid N-terminal epitope of the bacterial elongation factor Tu (EF-Tu) (Kunze et al., 2004), is recognized by the Arabidopsis LRR-RLK FLAGELLIN RECEPTOR KINASE 1 (FLS2) (Brutus et al., 2010). WALL-ASSOCIATED KINASE 1 (WAK1) is a receptor of oligogalacturonides (OGs) and triggers defence responses upon perception of this so-called damage-associated molecular pattern (DAMP). Upon OG treatment, a chimera comprising the ectodomain of WAK1 and the EFR kinase domain triggers an EFR-like response (Brutus et al., 2010). On the other hand, a reciprocal EFR-WAK1 chimera recognizes elf18 to induce a stronger OG/WAK1-like oxidative burst response than that triggered by EFR upon perception of elf18 (Brutus et al., 2010). However, the downstream signalling components employed by the transferred PRRs and chimeras are largely unknown, and the signalling pathways that are triggered in the plant from which the PRR originates and those in the recipient plant might not be conserved.

In this work, we aimed to obtain a MAMP-dependent robust resistance response in tobacco, by exploiting the EFR-mediated recognition of the broad-spectrum bacterial MAMP elf18 and the ability of Cf-9 to trigger a fast HR upon activation (Hammond-Kosack et al., 1998; Stergiopoulos and de Wit, 2009). Tobacco SR1 plants transiently expressing the chimera EFR-Cf-9, harbouring the ectodomain of EFR and the TM and cytoplasmic domain of Cf-9 (hereafter referred to as the endodomain), indeed mount a strong elf18-triggered HR. We show that chimeric EFR-Cf-9 protein constitutively interacts with SOBIR1, and requires both SOBIR1 and BAK1 for functionality. Transgenic tobacco plants expressing EFR-Cf-9 activate an HR upon elf18 treatment and show enhanced resistance to the (hemi)biotrophic bacterial pathogens Pta 11528 and Pst DC3000.

RESULTS

EFR-Cf-9 recognizes elf18C to trigger a strong HR in tobacco plants

We previously reported that the ectodomain of EFR (Lys649_{EFR}) is functional in an EFR-WAK1 chimera and perceives elf18, while the ectodomain of FLS2 (Arg806_{FLS2}) is functional in an FLS2-EFR chimera (eJMC in the original article) and perceives flg22 (Brutus et al., 2010). Here, we fused the ectodomains of EFR or FLS2 to constitutively interacts with several RLPs, providing the RLP/SOBI1 complex with a kinase domain to trigger downstream signalling pathways (Albert et al., 2015; Böhm et al., 2014; Domazakis et al., 2018; Gust and Felix, 2014; Hegenaun et al., 2016; Liebrand et al., 2013, 2014; Ma and Borhan, 2015; Wang et al., 2018; Zhang et al., 2013, 2014). An additional RLK, BAK1, the orthologue of the Arabidopsis thaliana (At) BRI-ASSOCIATED RECEPTOR KINASE 1 (BAK1) is recruited to the Cf-4/SOBI1 complex after perception of Avr4 by Cf-4 and is required for Cf-4-mediated resistance (Postma et al., 2016).
the endodomain (Trp811 of Cf-9 (Jones et al., 1994), to obtain EFR-Cf-9 and FLS2-Cf-9 chimeras, respectively (Fig. 1). Both chimeras were in turn fused to eGFP and transiently expressed by agroinfiltration in leaves of tobacco plants stably overexpressing Avr9 (Hammond-Kosack et al., 1998) or transiently co-expressing Avr9. In parallel, EFR-eGFP, FLS2-eGFP and Cf-9-eGFP fusions were agroinfiltrated as controls. Leaf areas transiently expressing eGFP-tagged EFR, EFR-Cf-9, FLS2 and FLS2-Cf-9 did not exhibit any symptom, while leaves co-expressing Cf-9-eGFP and Avr9, as expected, displayed a clear HR (Figs S1 and S2). These results indicate that the chimeras are not responsive to Avr9 and do not per se induce HR-like symptoms. Next, whether EFR-Cf-9 and FLS2-Cf-9 are functional and trigger a ligand-dependent HR-like response was tested. Upon infiltration with 100 nM elf18C, leaf areas transiently expressing EFR-Cf-9/eGFP showed a strong HR, similar to the Cf-9/eGFP/Avr9 control, in two different tobacco cultivars (SR1 and Samsun) (Fig. 2). In contrast, leaf areas transiently expressing FLS2-Cf-9/eGFP did not show visible symptoms upon treatment with 100 µM flg22 (Fig. S2), suggesting that FLS2-Cf-9/eGFP is not functional in tobacco.

Together, these data show that EFR-Cf-9 recognizes elf18C to trigger a strong Cf-9/Avr9-like HR, indicating that while the ectodomain of EFR retained the ability to perceive the elf18C peptide, its fusion to the endodomain of Cf-9 resulted in an output similar to that of the native Cf-9.

**EFR-Cf-9 functionality requires SOBIR1 and BAK1**

It has been reported that the TM domain of SOBIR1 is required for its interaction with Cf-4 (Bi et al., 2016) and that the co-receptors SOBIR1 and BAK1 are both required for the function of Cf-4 (Liebrand et al., 2013; Postma et al., 2016). Cf-9 shares an identical endodomain with Cf-4 (Thomas et al., 1997) and also interacts with SOBIR1 (Liebrand et al., 2013). We therefore investigated whether EFR-Cf-9 also interacts with tomato SOBIR1 (henceforth indicated as S5SOBIR1). EFR-Cf-9-Myc was generated and co-expressed with S5SOBIR1-eGFP in N. benthamiana. In parallel, FLS2-Cf-9 (as the non-functionality of this chimera might be due to a possible lack of interaction with SOBIR1), Cf-4-eGFP, FLS2-eGFP and EFR-eGFP were agroinfiltrated as controls. Immunoprecipitation (IP) of S5SOBIR1 using GFP-Trap beads demonstrated that Cf- was co-precipitated with S5SOBIR1.
(Fig. 3A), which is consistent with our previous finding (Liebrand et al., 2013). In addition, FLS2-Cf-9 and EFR-Cf-9 were also co-purified with $\text{S}S\text{OBIR1}$, indicating that both chimeras constitutively interact with $\text{S}S\text{OBIR1}$, similar to the Cf proteins (Fig. 3A). In reverse co-immunoprecipitation assays (co-IPs), IP of both FLS2-Cf-9-eGFP and EFR-Cf-9-eGFP resulted in the co-purification of $\text{S}S\text{OBIR1}$-Myc (Fig. 3B). In both cases, the FLS2 and EFR wild-type proteins did not interact with $\text{S}S\text{OBIR1}$ (Fig. 3A and B), confirming the specificity of $\text{S}S\text{OBIR1}$ for RLPs (Liebrand et al., 2013, 2014).

Next, we investigated whether EFR-Cf-9 requires $\text{S}S\text{OBIR1}$ and/or BAK1 to trigger an HR upon perception of elf18C. Tobacco Samsun plants were used for silencing $\text{NtSOBIR1}/\text{SOBIR1-like}$ and $\text{NtSERK3a/b}$, orthologues of $\text{SOBIR1}$ and BAK1, respectively (Heese et al., 2007; Liebrand et al., 2013). eGFP-tagged EFR, Cf-9 and EFR-Cf-9 were transiently co-expressed with Avr9 or expressed alone in the silenced plants, followed by treatment with Milli-Q (MQ) water or 100 nM elf18C. Consistent with earlier studies (Liebrand et al., 2013; Postma et al., 2016), silencing of $\text{NtSOBIR1}/\text{SOBIR1-like}$ and of $\text{NtSERK3a/b}$ strongly suppressed the HR triggered by the Cf-9/Avr9 combination, but did not affect the ability of the plant to mount PCD triggered by Rx(D460V) (Fig. 4), which is a constitutively active form of the Rx gene, providing resistance against Potato Virus X (PVX) (Bendahmane et al., 2002). The EFR-Cf-9-dependent elf18C-triggered HR was also severely compromised in the silenced plants, indicating that both $\text{NtSOBIR1}/\text{SOBIR1-like}$ and $\text{NtSERK3a/b}$ are required for EFR-Cf-9 functionality (Fig. 4). Control plants, infected with Tobacco Rattle Virus carrying the $\beta$-glucuronidase (GUS) gene (TRV-GUS), did not show any defect in the EFR-Cf-9-dependent HR (Fig. 4).

Kinase activity of both $\text{SOBIR1}$ and BAK1 is required for the function of Cf-4 (Liebrand et al., 2013; Postma et al., 2016; Van Der Burgh et al., 2018). The AtBAK1-5 mutant contains a point mutation in its kinase domain, which causes this protein to have a slightly lower kinase activity than BAK1 itself (Schwessinger et al., 2011). BAK1-5 has been reported to have a dominant-negative effect on the BAK1-dependent immune response mediated by FLS2 and EFR (Schwessinger et al., 2011). In order to study whether kinase activity of BAK1 is also required for EFR-Cf-9 functionality and whether the kinase-inactive variant AtSOBIR1-RD/N (Bi et al., 2016) displays a dominant-negative effect similar to the hypoactive kinase AtBAK1-5 and kinase-dead AtBAK1-RD/N mutants (Schwessinger et al., 2011), EFR-Cf-9-eGFP was either transiently expressed in combination with AtBAK1-RD/N, AtBAK1-5, AtSOBIR1-RD/N-eGFP or GUS-eGFP, followed by treatment with 100 nM elf18C. The EFR-Cf-9-dependent elf18C-triggered HR was not affected by GUS-eGFP, but was reduced by AtBAK1-RD/N and even more compromised by AtBAK1-5 (Fig. 5), indicating that both BAK1 mutants have a dominant-negative effect on the EFR-Cf-9-triggered HR, possibly because the overexpressed BAK1 mutants compete away the endogenous $\text{NtSERK3a/b}$ from the activated EFR-Cf-9-containing signalling complex. We conclude that the EFR-Cf-9-dependent elf18C-triggered HR depends on the kinase activity of BAK1. Unlike AtBAK1-RD/N and AtBAK1-5, AtSOBIR1-RD/N-eGFP did not affect the HR (Fig. 5). Considering that RLPs constitutively interact with SOBIR1, whereas the recruitment of BAK1 to the signalling complex is ligand-dependent, it is possible that transiently expressed AtSOBIR1-RD/N-eGFP may fail to displace the endogenous $\text{NtSOBIR1}/\text{SOBIR1-like}$ protein that is bound to EFR-Cf-9.

Fig. 3 Both FLS2-Cf-9 and EFR-Cf-9 interact with SOBIR1. (A) eGFP-tagged tomato (S) SOBIR1 was transiently co-expressed with Myc-tagged Cf-4, FLS2, EFR, FLS2-Cf-9 and EFR-Cf-9. (B) Myc-tagged $\text{S}S\text{OBIR1}$ was transiently co-expressed with eGFP-tagged FLS2, EFR, FLS2-Cf-9 and EFR-Cf-9. Agrobacteria driving expression of the various constructs were infiltrated at a final OD$_{600}$ of 1.0. After 2 days, proteins were extracted and immunoprecipitated (IP) using GFP-Trap beads. Proteins were detected by Western blot/immunoblot (WB) using $\alpha$-GFP and $\alpha$-Myc antibodies. Equal loading is indicated by Rubisco band. Experiments were repeated three times and representative images are shown.
Taken together, these data indicate that both SOBIR1 and BAK1 are required for the EFR-Cf-9-dependent elf18C-triggered HR, and that BAK1 has to be kinase-active.

**EFR-Cf-9-transgenic tobacco plants are differentially responsive to elf18 variants**

An untagged version of EFR-Cf-9 was stably expressed in tobacco SR1 plants. Fourteen primary transformants were obtained, of which five had detectable expression of EFR-Cf-9 (Fig. 6A) and were therefore propagated. Two independent lines (K1A and K5A) showing a 3:1 segregation ratio for kanamycin resistance, and therefore likely carrying an insertion in a single locus, were selected for further characterization. Transgenic plants were morphologically identical to the parental plants (Fig. 6B). To verify that the chimera was properly expressed and localized, trypsin digestion of microsomal leaf proteins from wild type (WT) and K1A plants was performed, followed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Six peptides corresponding to the ectodomain of EFR (coverage of 15.49; score of 16.31) were found only in extracts from the transgenic plants (Fig. S3), confirming that EFR-Cf-9 is expressed and is likely membrane-localized. No peptide corresponding to the chimera was found in WT extracts.

Subsequently, the functionality of EFR-Cf-9 in K1A and K5A plants was assessed. Only leaves from both transgenic lines mounted an HR upon elf18C treatment, at concentrations ranging from 1 nM to 10 μM (Fig. 6C). Responsiveness to different elf18 variants was also tested in line K1A, revealing that an HR was triggered also by elf18B and elf18G, though to a lesser extent when compared with elf18C (Fig. S4). This suggests that, although the EFR ectodomain was fused to the endodomain of Cf-9, the structure of the ligand still determines the output. To obtain a more quantitative evaluation of the response mediated by EFR-Cf-9, and to assess whether the presence of EFR-Cf-9 might cause an elevated basal defence response, the expression of the marker genes Avr9/Cf-9 RAPIDLY ELICITED-132 (ACRE-132) (Durrant et al., 2000) and HAIRPIN INDUCED 1 (HIN1) (Gopalan et al., 1996) was analysed. Transcript levels of both genes were similar in MQ water-treated WT and K1A plants, indicating that EFR-Cf-9 does not affect basal defence (Fig. S5). In addition, as expected, WT plants did not respond to any of the elf18 variants tested, whereas they showed a significantly increased expression of ACRE-132 and HIN1 after flg22 elicitation (Fig. S5). In contrast, treatment of K1A plants with elf18C induced a significant increase in transcript levels for ACRE-132 and HIN1, which

| EFR | Cf-9 | EFR-Cf-9 | EFR | Cf-9 | EFR-Cf-9 | EFR | Cf-9 | EFR-Cf-9 |
|-----|------|----------|-----|------|----------|-----|------|----------|
| MQ  |      |          |     |      |          |     |      |          |
| Avr9|      |          |     |      |          |     |      |          |
| elf18C|  TRV: NtSOBIR1/SOBIR1-like | TRV: NtSERK3a/b | TRV: GUS |

**Fig. 4** SOBIR1 and BAK1 are required for the EFR-Cf-9-dependent elf18C-triggered HR. eGFP-tagged EFR, Cf-9 and EFR-Cf-9 were either transiently expressed with Avr9, or expressed alone, in *N. tabacum* Samsun plants silenced for NtSOBIR1/SOBIR1-like, NtSERK3a/b or GUS (n = 4). Agrobacteria driving expression of the constructs were infiltrated at a final OD600 of 1.0, except for the positive control Rx (D460V), which was infiltrated at an OD600 of 0.1. At 2 days post-infiltration, leaves were treated with Milli-Q water (MQ) or 100 nM elf18C. Pictures were taken at 2 days after treatment with elf18C or, in the case of Cf-9-Avr9 interaction, at 12 days after co-infiltration of Cf-9 and Avr9. Experiments were repeated three times with similar results and representative images are shown.
were even higher than in plants treated with flg22 (Fig. S5). Moreover, treatment with elf18B and elf18G also resulted in increased transcript levels for ACRE-132 and HIN1, but to a lesser extent than for elf18C (Fig. S5), which supports our finding that elf18C triggers a stronger HR than the elf18 variants (Fig. S4). On the other hand, WT and K1A plants displayed similar expression levels of both genes in response to flg22 (Fig. S5), indicating that EFR-Cf-9 does not affect the endogenous NtFLS2-mediated response to flg22.

Together, these data indicate that transgenic tobacco plants expressing EFR-Cf-9 are not altered in their basal defence and in their responsiveness to flg22. Moreover, the EFR-Cf-9-transgenic plants respond more efficiently to elf18C than to the different elf18 variants.

**Transgenic tobacco plants expressing EFR-Cf-9 show enhanced resistance to (hemi)biotrophic bacterial pathogens**

To study whether the EFR-Cf-9-mediated immune response eventually leads to resistance against bacterial pathogens, WT and transgenic K1A and K5A plants were inoculated with the (hemi)biotrophic bacterial pathogens *Pta* 11528 and *Pst* DC3000 at a dose of $OD_{600} = 0.002$ (corresponding to $10^4$ CFU/cm$^2$). Compared to WT, a clear reduction in bacterial colonization was found in both transgenic lines inoculated with *Pta* 1152 and, to an even greater extent, *Pst* DC3000 (Fig. 7A and B). To verify that EFR-Cf-9 indeed triggers an HR not only in response to the purified elf18 peptides, but also to bacterial infection, we inoculated different doses ($OD_{600} = 0.002, 0.02$ and $0.2$) of *Pta* 11528 in WT, K1A and K5A plants. Within 48 h, leaf sectors of the transgenic plants inoculated with the highest doses of bacteria ($OD_{600} = 0.02$ and 0.2) displayed HR-like symptoms, whereas WT plants did not show any symptoms with all doses of bacteria (Fig. 8A).

We also tested the susceptibility to the necrotrophic bacterium *Pectobacterium carotovorum* subsp. *carotovorum* strain DSMZ 30169, which is the causal agent of bacterial soft rot. Inoculation with *P. carotovorum* is able to induce an EFR-dependent response in Arabidopsis, indicating the presence of an elf18-like MAMP (Lacombe et al., 2010). Leaves inoculated with *P. carotovorum* at a density of $OD_{600} = 0.02$ (corresponding to about $10^5$ CFU/cm$^2$) showed a ten-fold increase in bacterial count at 24 h, with no significant differences between WT and the transgenic lines (Fig. 7C). Notably, also in this case we observed a rapid and strong HR after inoculation with bacteria in K1A and K5A but not in WT plants (Fig. 8B). This suggests that EF-Tu of *P. carotovorum* is recognized by EFR-Cf-9 and triggers an HR that is not able to restrict colonization by this pathogen.

The fungal pathogen *Botrytis cinerea* does not produce an elf18-like MAMP and its colonization in Arabidopsis is not affected by the lack of EFR (Brutus et al., 2010). Consistently, when *B. cinerea* was inoculated onto WT and transgenic K1A and K5A leaves, no differences in disease severity was observed (Fig. S6), indicating that the ectodomain of EFR-Cf-9 does not recognize any MAMP from *B. cinerea* and that basal defence is not enhanced by the presence of the chimera.

Taken together, these data indicate that pathogen-derived elf18-like MAMPs activate EFR-Cf-9-dependent immunity resulting in an HR, which restricts (hemi)biotrophic bacteria colonization, but does not compromise colonization by necrotrophic bacteria or fungi.

**DISCUSSION**

The chimera EFR-Cf-9 recognizes elf18 to trigger a strong immune response

Interfamily transfer of PRRs is a promising strategy to confer broad-spectrum resistance to pathogens, and it has been successfully employed with EFR (Lacombe et al., 2010; Schwessinger...
et al., 2015), Xa21 (Afroz et al., 2011; Holton et al., 2015; Mendes
et al., 2010; Tripathi et al., 2014) and Ve1 (Song et al., 2017).
Engineering chimeric receptors that combine the properties of
two separate PRRs is another effective strategy to improve host
resistance. Current research on chimeric PRRs has revealed that
the ectodomain determines ligand specificity, while the endodo-
main determines output intensity (Brutus et al., 2010; He et al.,
2000; Holton et al., 2015; Kishimoto et al., 2010; Kouzai et al.,
2013; Schwessinger et al., 2015). Here we generated the chimera
EFR-Cf-9 (Fig. 1), combining the ectodomain of EFR, which pro-
vides broad-spectrum recognition of bacterial pathogens, and
the endodomain of Cf-9, which induces a strong HR-associated
immune response. We demonstrated that, when expressed in
tobacco plants, EFR-Cf-9 recognizes elf18 leading to an HR (Fig.
2). We also showed that EFR-Cf-9 interacts with SOBIR1, similar
to the CF-9 protein itself (Fig. 3), and requires both SOBIR1 and
BAK1 for its function (Fig. 4). In addition, EFR-Cf-9 also recog-
nizes pathogen-derived elf18 or elf18-like MAMPs to activate
immunity (Fig. 7) and triggers HR during bacterial infection (Fig.
8). Moreover, EFR-Cf-9 retains the recognition feature of EFR as
elf18C, compared to elf18 variants with lower EFR-eliciting activ-
ity, triggers a stronger response, (Figs S4 and S5).

In contrast to EFR-Cf-9, FLS2-Cf-9 failed to trigger an HR upon
treatment with flg22 (Fig. S2), although FLS2-Cf-9 also interacts
with SOBIR1 in N. benthamiana (Fig. 3). Although the FLS2 ect-
odomain is functional in FLS2-EFR (Brutus et al., 2010) and the
CF-9 endodomain is functional in EFR-Cf-9 (Fig. 2), it is still pos-
sible that the selected point of junction between the ectodomain
of FLS2 and the endodomain of CF-9 might not be optimal for
functionality of this specific chimera.
Early transduction events mediated by the EFR-Cf-9 chimera are similar to those employed by Cf-9

Although interfamily PRR transfer and expression of chimeric PRRs can provide broad resistance to pathogens (Afroz et al., 2011; Holton et al., 2015; Lacombe et al., 2010; Schoonbeek et al., 2015), the downstream signalling components employed by these receptors are barely known. Indeed, the signalling partners downstream of the perception event are not always conserved in the recipient plant. For example, OsSERK2, which is phylogenetically closely related to AtSERK1 and AtSERK2, is required for the functionality of transgenically expressed EFR in resistance to Xanthomonas oryzae pv. oryzae (Xoo) in rice (Chen et al., 2014). However, in Arabidopsis AtSERK1 and AtSERK2 are not required for EFR function (Roux et al., 2011), indicating that EFR utilizes different SERKs in Arabidopsis and rice. In
addition, specific downstream signalling components may act in an opposite manner in different species. For instance, the Xa21-binding (XB) protein OsXBX24 is an ATPase that negatively affects both Xa21- and EFR-mediated immunity in rice (Chen et al., 2010; Schwessinger et al., 2015), while its orthologue plays a positive role in EFR-mediated immunity in Arabidopsis (Holton et al., 2015). Thus, the components required for functionality of a transferred PRR are not easily predictable.

Ectopic expression of chimeric receptors allows to address the requirement of specific protein domains for physical and functional interaction with partners participating in downstream signalling. For example, both EFR-Xa21 and native Xa21 form a constitutive complex with OsSERK2, indicating that OsSERK2 interaction with Xa21 does not specifically require the Xa21 ectodomain. In Arabidopsis, both Xa21 and EFR-Xa21 interact with BAK1 in a ligand-dependent manner (Schwessinger et al., 2015). In this study, we show that EFR-Cf-9 constitutively interacts with SOBIR1 and requires SOBIR1 for its function (Figs 3 and 4). EFR-Cf-9 is anticipated to also form a complex with BAK1 upon elf18 treatment, as the chimera also requires kinase-active BAK1 for its function (Fig. 5). It should be noted that AtBAK1-5 had a stronger effect than AtBAK1-RD/N in suppressing EFR-Cf-9-mediated HR (Fig. 5). The interaction between AtBAK1 and EFR is kinase activity-independent and AtBAK1-5 has a higher affinity to EFR than WT AtBAK1 (Schwessinger et al., 2011), explaining why AtBAK1-5 is more efficient than AtBAK1-RD/N in suppressing EFR-Cf-9 activity.

SOBIR1 constitutively interacts with Cf-4, which shares an identical endodomain with Cf-9 (Liebrand et al., 2013; Thomas et al., 1997), whereas it does not interact with the RLKs FLS2 and EFR and is not required for RLK-mediated immunity (Gust and Felix, 2014). The external juxtamembrane region of EFR-Cf-9 does not carry the typical stretch of acidic amino acids that are thought to play a role in the interaction of Cf-9 with SOBIR1 (Bi et al., 2016; Gust and Felix, 2014). However, EFR-Cf-9 does interact with SOBIR1, suggesting that the TM of Cf-9, carrying an extensive GxxxGxxxGxxG dimerization motif, is sufficient for interaction with SOBIR1 (Bi et al., 2016). The requirement of the GxxxG motif for the interaction between SOBIR1 and EFR-Cf-9 will be investigated by performing site-directed mutagenesis of this motif in future studies. Together, this indicates that the endodomain, and in particular the TM, of Cf-9 provides the chimera EFR-Cf-9 with the features necessary for Cf-9/Cf-4 signalling, enabling constitutive interaction with SOBIR1 and ligand-dependent BAK1 recruitment to the EFR-Cf-9/SOBIR1 complex, eventually mounting an HR.

Transgenic expression of EFR-Cf-9 affects resistance against bacterial pathogens

In tomato, Cf proteins mediate a strong resistance to the C. fulvum strains carrying the proper avirulence genes (Joosten et al., 1997; Stergiopoulos and de Wit, 2009). It is widely accepted that this resistance is largely based on the HR resulting from the activation of the Cf protein, which leads to localized cell death that restricts pathogen spread. However, the strong resistance mediated by Cf genes has a narrow specificity, and is easily overcome by mutations in the corresponding Avr genes of the pathogen. On the other hand, PRR-mediated immunity triggered upon recognition of MAMPs is effective against a wide range of microbes but is weaker than that of Cf proteins. Hence, the generation of a chimera containing the ectodomain of a PRR and the endodomain of a Cf protein might combine the beneficial features of PRRs and Cf proteins, and enable the chimera to recognize MAMPs and mount an HR, leading to a strong immunity to a wide range of microbes.

EFR-Cf-9 recognizes elf18 to mount an HR (Fig. 2), suggesting that it might also recognize pathogen-derived elf18 to activate immunity. Indeed, transgenic tobacco plants expressing EFR-Cf-9 showed a significantly reduced susceptibility to two (hemibiotrophic bacterial pathogens, Pta 11528 and Pst DC3000 (Fig. 7A and B). In addition, inoculation of a high dose of Pta 11528 causes HR in the transgenic plants (Fig. 8A), indicating that EFR-Cf-9 recognizes both the purified elf18 peptide and the pathogen-derived EF-Tu, and that this recognition effectively restricts bacterial growth. However, compared to Pst DC3000, EFR-Cf-9-mediated resistance to Pta 11528 is less efficient (Fig. 7A and B), suggesting that Pta 11528 might partially suppress the EFR-Cf-9-mediated immune response, as Pta 11528 has been shown to suppress salicylic acid-mediated defence responses (Lee et al., 2013).

Inoculation of the necrotrophic bacterium P. carotovorum in the EFR-Cf-9-transgenic plants also resulted in HR-like symptoms (Fig. 8B), since P. carotovorum carries a form of EF-Tu that is able to activate EFR (Lacombe et al., 2010). However, no significant difference in P. carotovorum growth between WT and transgenic plants was found (Fig. 7C).

CONCLUSION

This work indicates that the EFR-Cf-9 chimera is functional in tobacco plants, since it recognizes elf18 to trigger an HR, which fits our current view on the ectodomain of a PRR determining the ligand specificity, and the endodomain determining the output intensity. Moreover, EFR-Cf-9 constitutively interacts with SOBIR1, and the EFR-Cf-9-mediated HR triggered by elf18 is dependent on SOBIR1 and BAK1, which is reminiscent of our working model for Cf-4/Cf-9 and indicates that the endodomain of Cf-9 confers EFR-Cf-9 the repertoire of Cf-9/Cf-4-like signalling. In addition, transgenic tobacco plants expressing EFR-Cf-9 do not show altered basal defence but are resistant to (hemibiotrophic) bacteria, suggesting a potential biotechnological use of this chimeric receptor to improve crop disease resistance. Furthermore, our
construction of the EFR-Cf-9 chimera reveals that it is possible to customize crop immunity by the generation of chimeric receptors containing features of a varying ligand recognition range and immune response intensity.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

*N. benthamiana*, *N. tabacum* Samsun, *N. tabacum* SR1, *Avr9*-transgenic *N. tabacum* SR1 (Hammond-Kosack et al., 1998), and *EFR-Cf-9*-transgenic *N. tabacum* SR1 plants were grown under 16 h of light at 25 °C, and 8 h of darkness at 21 °C in climate chambers with a relative humidity of 75%.

**Constructs**

The DNA fragments representing the coding sequence (CDS) of the ectodomains of EFR (Lys649EFR) and FLS2 (Arg806FLS2) (Brutus et al., 2010) were fused to the CDS of the TM and cytoplasmic domain of Cf-9 by splicing overlapping extension polymerase chain reaction (SOE-PCR) (Higuchi et al., 1988) to generate DNA fragments encoding EFR-Cf-9 and FLS2-Cf-9, respectively, and cloned into the binary vector pB1121 under the control of the 3SS promoter, or, by Gateway cloning, the fragments were inserted into pK7FWG2.0 to generate 3SS:EFR-Cf-9-eGFP and 3SS:FLS2-Cf-9-eGFP, and inserted into pGW20 to generate 3SS:EFR-Cf-9-10xMyc and 3SS:FLS2-Cf-9-10xMyc.

Construction of SlSOBIR1-eGFP, SlSOBIR1-Myc, Cf-4-Myc, FLS2-Myc and EFR-Myc was reported earlier (Liebrand et al., 2010). The construction of AtSOBIR1-RD/N-eGFP was reported by Bi et al. (2016). AtBAK1-5 (SOLS114) and AtBAK1-RD/N (SOLS106) were previously reported (Schwessinger et al., 2011). Binary vectors carrying 35S:CF9 and 35S:AVR9 in PMOG800 have been described (Van der Hoorn et al., 2000). All plasmids were transferred into *A. tumefaciens* C58C1, carrying the helper plasmid pCH32.

**Generation of transgenic tobacco plants**

Transformation of tobacco plants was performed according to established methods (Horsch et al., 1988). Briefly, *A. tumefaciens* carrying the 35S:EFR-Cf-9 plasmid were suspended in an infection medium composed as follows: Murashige-Skoog (MS) basal medium containing Gamborg’s vitamin mix, 1 mg/L 6-BA, 0.1 mg/L NAA, 200 µM acetosyringone at 25 °C in the dark for 2 days. Infected explants were then transferred to fresh solid MS medium supplemented with vitamin mix, 1 mg/L 6-BA, 0.1 mg/L NAA, 200 µg/mL timentin, 200 µg/mL cefotaxime and 200 µg/mL kanamycin at 25 °C in the light for 30 days for shoot regeneration. Regenerated shoots were transferred to fresh solid MS medium supplemented with vitamin mix, 0.1 mg/L NAA, 200 mg/L timentin, 200 mg/L cefotaxime and 200 mg/L kanamycin at 25 °C in the light for 30 days for root regeneration. From about 1200 co-cultivated leaf explants, 14 primary transformants were obtained and transferred to soil for propagation. Five transformants had detectable transgene expression and were propagated; of these, two (K1A and K5A) showed a 3:1 segregation ratio for kanamycin resistance, and were therefore selected for further characterization.

**Hypersensitive response assays**

The elf18 (elf18D) and flg22 peptides were synthesized by EZBiolab (Carmel, IN, USA). The other elf18 peptides were kind gifts of Cyril Zipfel (The Sainsbury Laboratory, Cambridge, UK). For HR assays in *N. tabacum* SR1 and *N. tabacum* Samsun plants, fully expanded leaves were infiltrated with *Agrobacterium* suspensions at an OD<sub>600</sub> of 1.0, except for Rx (D460V), which was infiltrated with an OD<sub>600</sub> of 0.1 (Liebrand et al., 2013; Postma et al., 2016). MQ water and elf18 and/or flg22 peptides were infiltrated at 2 days post-agroinfiltration (dpi) at the indicated concentrations. Leaves were examined for development of an HR between 2 dpi and 12 dpi. For each treatment, at least three leaves, taken from separate plants, were agroinfiltrated.

**Virus-induced gene silencing (VIGS)**

VIGS plasmids pTRV1-RNA1, pTRV2-PDS, pTRV2-GUS, pTRV2-SlSOBIR1/SOBIR1-like and pTRV2-SlSERK3a/b were described before (Liebrand et al., 2013; Postma et al., 2016). In brief, 2-week-old *N. tabacum* Samsun plants were subjected to VIGS, for which *Agrobacterium* cultures harbouring a pTRV2 plasmid were mixed with pTRV1-RNA1 at a final OD<sub>600</sub> of 0.8. After about three weeks, fully expanded leaves were used for HR assays.

**Co-immunoprecipitation and immunoblot assays**

Co-IPs were performed as described previously (Liebrand et al., 2012). The following antibodies were used: α-GFP-HRP (130-091-833, MACS antibodies, Bergisch Gladbach, Germany), α-cMYC (cMYC9E10, sc-40, Santa Cruz Biotechnology, Heidelberg, Germany), with α-Mouse-HRP (Amersham, GE Healthcare, Eindhoven, Netherlands) as a secondary antibody.

**Pathogenicity assays**

*P. amygdali* pv. *tabaci* (Pta) 11528 and *P. syringae* pv. *tomato* (Pst) DC3000 were grown in 5 mL of low salt Bertani-Luria
(LSBL) medium at 28 °C for 24 h at 200 rpm until OD$_{600}$ was 1.0. Bacteria were centrifuged for 10 min at 2500 × g, the supernatant was discarded and bacteria were rinsed twice with sterile water. Bacteria were suspended in an infiltration medium containing 10 mM MgCl$_2$ at the indicated doses.

P. carotovorum subsp. carotovorum strain DSMZ 30169 was obtained from DSMZ GmbH (Germany). Bacteria were grown in liquid Luria-Bertani (LB) broth at 28 °C for 16 h, centrifuged at 8000 × g for 10 min, and washed with sterile water. Bacteria were then suspended in 50 mM potassium phosphate buffer, pH 7.0 and inoculated at the indicated doses.

Bacteria were syringe-infiltrated into leaf sectors of 4-week-old tobacco plants (six sectors per leaf, three leaves per genotype). Inoculated plants were returned to greenhouse and bacterial count was determined after 0 h and 120 h (for Pta 11528 and Pst DC3000) or 24 h (for P. carotovorum). Briefly, inoculated leaf sectors were sterilized for 1 min in EtOH 70%, washed for 1 min in sterile water, and leaf discs (diameter = 0.4 mm) were cut from each sector. Discs were ground with a pestle in 100 μL of sterile water, then additional 900 μL of water were added; samples were vortexed, and serial dilutions were plated on LB solid medium. Plates were incubated at 28 °C for two days, and colonies were counted for each dilution.

Detached tobacco leaves were inoculated with B. cinerea strain SF1, isolated from cabbage (Ferrari et al., 2003), as previously described (Manfredini et al., 2005).

**Expression analysis of the EFR-Cf-9 chimera and the defence-related genes HIN1 and ACRE-132**

For analysis of the expression of EFR-Cf-9, total RNA was extracted from leaf sectors of 4-week-old plants with Tri-reagent (Sigma) and treated with Turbo-DNase I (Ambion). First-strand cDNA was synthesized using ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA). Expression of EFR-Cf-9 was evaluated by PCR using specific primers (5′-CAAATCTCATCCTGGTTA-3′ and 5′-TCTTTCCTTGCTTTTCATTTTC-3′). The tobacco actin gene Tob66 (accession n. U60491) was amplified using the following primers: 5′-CTGCCCATGTATGGCTATT-3′ and 5′-AGTCTCCAACCTGTGGCAT-3′.

For quantitative analysis of HIN1 and ACRE-132 expression, leaf sectors from 4-week-old plants were infiltrated with water or elicitors at the concentration of 100 nM. After 48 h, infiltrated leaf sectors were collected from three separate plants for each genotype. Total RNA was extracted using NucleoZol reagent (MACHEREY-NAGEL GmbH, Düren, Germany), treated with RQ1 DNase (Promega), and first-strand cDNA was synthesized using ImProm-II Reverse Transcriptase (Promega). Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis was performed using a CFX96 Real-Time System (Bio-Rad), using the GoTaq Real-Time PCR System (Promega). Three technical replicates were performed for each sample, and data were analysed with LinRegPCR, developed at Amsterdam University Medical Centers (AMC) (Ruijter et al., 2009). Expression levels of each gene, relative to EF1a, were determined as previously described (Ferrari et al., 2006), and expressed in arbitrary units. Primer pairs were the following: HIN1, 5′-CTGCAACCATGTAGCTGTC-3′ and 5′-GTGGTGGACAATTGCAAC-3′; ACRE132, 5′-GCTGGCGGTTATCAAGAAT-3′ and 5′-GAAACCATGTAGCTGATT-3′. EF1a, 5′-GCTCCACCTCAGGATGTTT-3′ and 5′-CCACATTTGCACCAAGAAG-3′.

**Mass spectrometry**

Membrane-enriched protein fractions from one leaf of a WT SR1 plant and of a transgenic K1A plant were extracted as previously described (Mattei et al., 2016). Proteins were separated by SDS-PAGE, and lanes were cut into ten slices, that were subjected to in-gel trypsin digestion. Peptides were analysed by LTQ liquid chromatography (LC) Orbitrap MS/MS, and protein identification was performed by MaxQuant platform and Proteome Discoverer as previously described (Mattei et al., 2016).

**ACKNOWLEDGEMENTS**

We would like to thank Cyril Zipfel (The Sainsbury Laboratory, Cambridge, UK) for providing elf18 peptides, Pierre J.G.M. de Wit (Wageningen University and Research, Netherlands) for the plasmids carrying 35S-Cf-9 and 35S-Avr9, and Jonathan Jones (The Sainsbury Laboratory, Cambridge, UK) for providing the tobacco line expressing Avr9. This work was supported by the Institute Pasteur-Fondazione Cenci Bolognetti, by the Ministero dell’Istruzione, dell’Università e della Ricerca, by ERA-Net (ERA-CAPS project “SIPIS”) and by Sapienza Università di Roma (Progetti di Ricerca 2016 and 2017). Co-author JW is supported by a scholarship from the China Scholarship Council (CSC). Co-author I-BR was supported by the SIMISA project from the Ministero dello Sviluppo Economico as CNR-ISPA research fellow. The authors have no conflict of interest to declare.

**REFERENCES**

Afroz, A., Chaudhry, Z., Rashid, U., Ali, G.M., Nazir, F., Iqbal, J. and Khan, M.R. (2011) Enhanced resistance against bacterial wilt in transgenic tomato (Lycopersicon esculentum) lines expressing the Xa21 gene. *Plant Cell Tissue Organ Cult.* 104, 227–237.

Albert, M., Jehle, A.K., Mueller, K., Eisele, C., Lipschis, M. and Felix, G. (2010) Arabidopsis thaliana pattern recognition receptors for bacterial elicitor flagellin can be combined to form functional chimeric receptors. *J. Biol. Chem.* 285, 19035–19042.

Albert, I., Böhm, H., Albert, M., Feiler, C.E., Imkame, J., Wallmeroth, N., Brancato, C., Raaymakers, T.M., Oome, S., Zhang, H., Krol, E., Grefen, C., Gust, A.A., Chai, J., Hedrich, R., Van den Ackerveken, G.
and Nürnberger, T. (2015) An RLP23-SOBIR1-BAK1 complex mediates NLP-triggered immunity. *Nature Plants*, 1, 1514–15148.

Bendahmane, A., Farnham, G., Moffett, P. and Baulcombe, D.C. (2002) Constitutive gain-of-function mutants in a nucleotide binding site-leucine rich repeat protein encoded at the Rx locus of potato. *Plant J.* 32, 195–204.

Bi, G., Liebrand, T.W.H., Bye, R.R., Postma, J., van der Burgh, A.M., Robatzek, S., Xu, X. and Joosten, M.H.A.J. (2016) SOBIR1 requires the GxxGxG dimerization motif in its transmembrane domain to form constitutive complexes with receptor-like proteins. *Mol. Plant Pathol.* 17, 96–107.

Boutrot, F. and Zipfel, C. (2017) Function, discovery, and exploitation of plant pattern recognition receptors for broad-spectrum disease resistance. *Annu. Rev. Phytopathol.* 55, 257–286.

Brutus, A., Sicilía, F., Macone, A., Cervone, F. and De Lorenzo, G. (2010) A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. USA*, 107, 9452–9457.

Böhm, H., Albert, I., Fan, L., Reinhard, A. and Nürnberger, T. (2014) Immune receptor complexes at the plant cell surface. *Curr. Opin. Plant Biol.* 20, 47–54.

Chen, W. and Ow, D.W. (2017) Precise, flexible and affordable gene stacking for crop improvement. *Bioengineered*, 8, 451–456.

Chen, X., Chern, M., Canlas, P.E., Ruan, D., Jiang, C. and Ronald, P.C. (2010) An ATPase promotes autophosphorylation of the pattern recognition receptor XA21 and inhibits XA21-mediated immunity. *Proc. Natl. Acad. Sci. USA*, 107, 8029–8034.

Chen, X., Zuo, S., Schwessinger, B., Chern, M., Canlas, P.E., Ruan, D., Zhou, X., Wang, J., Daudi, A., Petzold, C.J., Heazlewood, J.L. and Ronald, P.C. (2014) An XA21-associated kinase (OsSERK2) regulates immunity mediated by the XA21 and XA3 immune receptors. *Mol. Plant*, 7, 874–892.

Chisholm, S.T., Cooker, G., Day, B. and Staskawicz, B.J. (2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, 124, 803–814.

Cook, D.E., Mesarich, C.H. and Thomma, B.P.H.J. (2015) Understanding plant immunity as a surveillance system to detect invasion. *Annu. Rev. Phytopathol.* 53, 541–563.

Coutu, D. and Zipfel, C. (2016) Regulation of pattern recognition receptor signalling in plants. *Nature Rev. Immunol.* 16, 537–552.

Dangl, J.L., Horvath, D.M. and Staskawicz, B.J. (2013) Pivoting the plant immunity as a surveillance system to detect invasion. *Annu. Rev. Plant Biol.*, 53, 537–552.

De Lorenzo, G., Brutus, A., Savatin, D.V., Sicilía, F. and Cervone, F. (2011) Engineering plant resistance by constructing chimeric receptors that recognize damage-associated molecular patterns (DAMPs). *FEBS Lett.* 585, 1521–1528.

Dodds, P.N. and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature Rev. Genet.* 11, 539–548.

Domażekis, E., Wouters, D., Visser, R.G.F., Kamoun, S., Joosten, M. and Vleeshouwers, V. (2018) The ELR-SOBIR1 Complex Functions as a Two-Component Receptor-Like Kinase to Mount Defense Against Phytophthora infestans. *Mol. Plant-Microbe Interact.* 31, 795–802.

Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E. and Jones, J.D.G. (2000) cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell*, 12, 963–977.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18, 265–276.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G. and Ausbel, F.M. (2003) Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires ED54 and PAD2, but not SID2, ED55 or PAD4. *Plant J.* 19, 203–205.

Ferrari, S., Galletti, R., Vairo, D., Cervone, F. and De Lorenzo, G. (2006) Antisense expression of the Arabidopsis thaliana AtPGIP1 gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 19, 931–936.

Gopalan, S., Wei, W. and He, S.Y. (1996) hpg gene-dependent induction of hint: a plant gene activated rapidly by both harpins and the avrPto gene-mediated signal. *Plant J.* 10, 591–600.

Gust, A.A. and Felix, G. (2014) Receptor like proteins associate with SOBIR1-type of adaptors to form bimolecular receptor kinases. *Curr. Opin. Plant Biol.* 21, 104–111.

Gómez-Gómez, L. and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial eliciator flagellin in *Arabidopsis*. *Mol. Cell*, 5, 1003–1011.

Halpin, C. (2005) Gene stacking in transgenic plants-the challenge for 21st century plant biotechnology. *Plant Biotechnol. J.* 3, 141–155.

Hammond-Kosack, K.E., Tang, S., Harrison, K. and Jones, J.D.G. (1998) The tomato Cf-9 disease resistance gene functions in tobacco and potato to confer responsiveness to the fungal avirulence gene product Avr 9. *Plant Cell*, 10, 1251–1266.

He, Z., Wang, Z., Li, J., Zhu, Q., Lamb, C., Ronald, P. and Chory, J. (2000) Perception of brassinosteroids by the extracellular domain of the receptor kinase BR1. *Science*, 288, 2360–2363.

Heese, A., Dann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C. and Rathjen, J.P. (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. USA*, 104, 12217–12222.

Hegenauer, V., Furst, U., Kaiser, B., Smoker, M., Zipfel, C., Felix, G., Stahl, M. and Albert, M. (2016) Detection of the plant parasite *Cuscuta reflexa* by a tomato cell surface receptor. *Science*, 353, 478–481.

Higuchi, R., Krummel, B. and Saiki, R.K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.*, 16, 7351–7367.

Holton, N., Nebrasov, V., Ronald, P.C. and Zipfel, C. (2015) The phylogenetically-related pattern recognition receptors EFR and XA21 recruit similar immune signaling components in monocots and dicots. *Plant Pathol.* 11, e1004602.

Horsch, R.B., Fraley, R.T., Rogers, S.G., Klee, H.J., Fry, J., Hinchee, M.A. and Shah, D.S. (1988) Agrobacterium-mediated gene transfer to plants; engineering tolerance to glyphosate. *Iowa State Journal of Research*, 62, 487–502.

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J. and Jones, J.D.G. (1994) Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science*, 266, 789–793.

Jones, D.G. and Deng, J.J.L. (2006) The plant immune system. *Nature*, 444, 323–329.

Joosten, M.H.A.J., Vogelsang, R., Cozijnsen, T.J., Verberne, M.C. and de Wit, P.J.G.M. (1997) The biotrophic fungus *Cladosporium fulvum* circumvents Cf-4-mediated resistance by producing unstable AVR4 elicitors. *Plant Cell*, 9, 367–379.

van Kan, J.A.L., van den Ackerveken, G.F.J.M. and de Wit, P.J.G.M. (1991) Cloning and characterization of CDNA of avirulence gene avr9 of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. *Mol. Plant-Microbe Interact.*, 4, 52–59.

Kishimoto, K., Kouzai, Y., Kaku, H., Shibuya, N., Minami, E. and Nishizawa, Y. (2010) Perception of the chitin oligosaccharides contributes to disease resistance to blast fungus *Magnaporthe oryzae* in rice. *Plant J.* 64, 343–354.
Kouzai, Y., Kaku, H., Shibuya, N., Minami, E. and Nishizawa, Y. (2013) Expression of the chimeric receptor between the chitin elicitor receptor CEBiP and the receptor-like protein kinase Pi-d2 leads to enhanced responses to the chitin elicitor and disease resistance against Magnaporthe oryzae in rice. Plant Mol. Biol. 81, 287–295.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. and Felix, G. (2004) The N terminus of bacterial elongation factor Tu elicits innate immune responses in Arabidopsis plants. Plant Cell, 16, 3496–3507.

Lacombe, S., Rougon-Cardoso, A., Sherwood, E., Peeters, N., Dahlbeck, D., van Esse, H.P., Smoker, M., Rallapalli, G., Thomma, B.P.H.J., Staskawicz, B., Jones, J.D.G. and Zipfel, C. (2010) Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. Nature Biotechnol. 28, 365–369.

Lee, S., Yang, D.S., Uppalapati, R.S., Sumner, L.W. and Mysore, K.S. (2013) Suppression of plant defense responses by extracellular metabolites from Pseudomonas syringae pv. tabaci in Nicotiana benthamiana. BMC Plant Biol. 13, 65.

Liebrand, T.W.H., Smit, P., Abd-El-Haliem, A.M., de Jonge, R., Cordewener, J.H.G., America, A.H.P., Sklenar, J., Jones, A.M., Robatzek, S., Thomma, B.P. and Tameling, W.I. (2012) Endoplasmic reticulum-quality control chaperones facilitate the biogenesis of Cf receptor-like proteins involved in pathogen resistance of tomato. Plant Physiol. 159, 1819–1833.

Liebrand, T.W.H., van den Berg, G.C.M., Zhang, Z., Smit, P., Cordewener, J.H.G., America, A.H.P., Sklenar, J., Jones, A.M., Tameling, W.I., Robatzek, S. and Thomma, B.P. (2013) Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. Proc. Natl. Acad. Sci. USA, 110, 10010–10015.

Liebrand, T.W.H., van den Burg, H.A. and Joosten, M.H.A.J. (2014) Two for all: receptor-associated kinases SOBIR1 and BAK1. Trends Plant Sci. 19, 123–132.

Ma, L. and Borhan, M.H. (2015) The receptor-like kinase SOBIR1 interacts with Brassica napus LepR3 and is required for Leptosphaeria maculans AvrLm1-triggered immunity. Front. Plant Sci. 6, 933. https://doi.org/10.3389/fpls.2015.00933.

Manfredini, C., Sicilia, F., Ferrari, S., Pontiggia, D., Salvi, G., Caprari, C., Lorito, M. and De Lorenzio, G. (2005) Polygalacturonase-inhibiting protein 2 of Phaseolus vulgaris inhibits BcpFG1, a polygalacturonase of Botrytis cinerea important for pathogenicity, and protects transgenic plants from infection. Physiol. Mol. Plant Pathol. 67, 108–115.

Mattei, B., Spinelli, F., Pontiggia, D. and De Lorenzio, G. (2016) Comprehensive Analysis of the Membrane Phosphoproteome Regulated by Oligogalacturonides in Arabidopsis thaliana. Front. Plant Sci. 7, 1107. https://doi.org/10.3389/fpls.2016.01107.

Mendes, B., Cardoso, S., Boscariol-Camargo, R., Cruz, R., Mourão Filho, F. and Bergamin Filho, A. (2010) Reduction in susceptibility to Xanthomonas axonopodis pv. citri in transgenic Citrus sinensis expressing the rice Xa21 gene. Plant Pathol. 59, 68–75.

Postma, J., Liebrand, T.W.H., Bi, G., Evrard, A., Bye, R.R., Mbengue, M., Kuhn, H., Joosten, M.H.A.J. and Robatzek, S. (2016) Avr4 promotes Cf-4 receptor-like protein association with the BAK1/SERK3 receptor-like kinase to initiate receptor endocytosis and plant immunity. New Phyto1. 210, 627–642.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F.G., Tor, M., de Vries, S. and Zipfel, C. (2011) The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. Plant Cell, 23, 2440–2455.

Ruijter, J.M., Ramakers, C., Hoogaars, W.H.M., Karlen, Y., Bakker, O., van den Hoff, M.J.B. and Moorman, A.F.M. (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 37, e45.

Schoonbeek, H.J., Wang, H.H., Stefanato, F.L., Craze, M., Bowden, S., Wallington, E., Zipfel, C. and Ridout, C.J. (2015) Arabidopsis EF-Tu receptor enhances bacterial disease resistance in transgenic wheat. New Phytol. 206, 606–613.

Schwessinger, B., Bahar, O., Thomas, N., Holton, N., Nekrasov, V., Ruan, D., Canlas, P.E., Daudi, A., Petzold, C.J., Singan, V.R. and Kuo, R. (2015) Transgenic expression of the dicotyledonous pattern recognition receptor EFR in rice leads to ligand-dependent activation of defense responses. PLoS Pathogens, 11, e1004809.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A. and Zipfel, C. (2011) Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. PLoS Genet. 7, e1002046.

Song, Y., Liu, L., Wang, Y., Valkenburg, D.J., Zhang, X., Zhu, L. and Thomma, B.P. (2017) Transfer of tomato immune receptor Ve1 confers Avell-dependent Verticillium resistance in tobacco and cotton. Plant Biotechnol. J. 16, 638–648.

Stergiopoulos, I. and de Wit, P.J.G.M. (2009) Fungal effector proteins. Annu. Rev. Phytopathol. 47, 233–263.

Thomas, C.M., Jones, D.A., Parniske, M., Harrison, K., Balint-Kurti, P.J., Hatzixanthis, K. and Jones, J.D. (1997) Characterization of the tomato Cf-4 gene for resistance to Cladosporium fulvum identifies sequences that determine recognition specificity in Cf-4 and Cf-9. Plant Cell, 9, 2209–2224.

Thomas, N.C., Oksenberg, N., Liu, F., Caddell, D., Nalyvayko, A., Nguyen, Y., Schwessinger, B. and Ronald, P.C. (2017) The rice Xa21 ectodomain fused to the Arabidopsis EFR cytoplasmic domain confers resistance to Xanthomonas oryzae pv. oryzae. Peer J. 6, e4456.

Tripathi, J.N., Lorenzen, J., Bahar, O., Ronald, P. and Tripathi, L. (2014) Transgenic expression of the rice Xa21 pattern-recognition receptor in banana (Musa sp.) confers resistance to Xanthomonas campestris pv. musacearum. Plant Biotechnol. J. 12, 663–673.

Van Der Burgh, A.M., Postma, J., Robatzek, S. and Joosten, M. (2018) Kinase activity of SOBIR1 and BAK1 is required for immune signalling. Mol. Plant Pathol. 20, 410–422.

Van der Hoorn, R.A., Laurent, F., Roth, R. and De Wit, P.J. (2000) Agroinfiltration is a versatile tool that facilitates comparative analyses of AvrCf-9-induced and AvrCf-4-induced necrosis. Mol. Plant-Microbe Interact. 13, 439–446.

Wang, Y., Xu, Y., Sun, Y., Wang, H., Qi, J., Wan, B., Ye, W., Lin, Y., Shao, Y., Dong, S., Tyler, B.M. and Wang, Y. (2018) Leucine-rich repeat receptor-like gene sequence reveals that Nicotiana RXEG1 regulates glycoside hydrolase 12 MAMP detection. Nat. Commun. 9, 594. https://doi.org/10.1038/s41467-018-03010-8.

Zhang, W., Fraiture, M., Kolb, D., Loffelhardt, B., Desaki, Y., Boutot, F.F.G., Tor, M., Zipfel, C., Gust, A.A. and Brunner, F. (2013) Arabidopsis receptor-like protein30 and receptor-like kinase suppressor of BIR1-1/EVERSHEDE mediates innate immunity to necroptotic fungi. Plant Cell, 25, 4227–4241.

Zhang, L., Kars, I., Essenstam, B., Liebrand, T.W.H., Wagemakers, L., Elberse, J., Tagkalaki, P., Tjoitang, D., van den Ackerveken, G. and van Kan, J.A.L. (2014) Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the Arabidopsis receptor-like protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASE51. Plant Physiol. 164, 352–364.

Zhu, S., Li, Y., Vossen, J.H., Visser, R.G.F. and Jacobsen, E. (2012) Functional stacking of three resistance genes against Phytophthora infestans in potato. Transgenic Res. 21, 89–99.
Zipfel, C. (2014) Plant pattern-recognition receptors. Trends Immunol. 35, 345–351.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D.G., Boller, T. and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell, 125, 749–760.

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

**Fig. S1** EFR-Cf-9 does not trigger an HR in tobacco plants expressing Avr9. eGFP-tagged EFR, Cf-9 and EFR-Cf-9 were transiently expressed in Avr9-transgenic N. tabacum SR1 plants at a final OD_{600} of 1.0. For each construct, at least three leaves, taken from separate plants, were agro-infiltrated. Pictures were taken at three days post-infiltration (dpi). The infiltrated areas are indicated by white dashed lines. Under these conditions, all leaves agroinfiltrated with Cf-9-eGFP, and none of the other samples, showed necrosis of at least half of the infiltrated area. This experiment was repeated three times with similar results. Representative images are shown.

**Fig. S2** FLS2-Cf-9 does not trigger an HR in tobacco plants upon treatment with flg22. eGFP-tagged FLS2, Cf-9 and FLS2-Cf-9 were either expressed alone (first and third column) or transiently co-expressed with Avr9 (second column), or, followed by treatment with Milli-Q (MQ) water (first column) or 100 μM flg22 (third column) after two days. Agrobacteria driving expression of the various constructs were infiltrated at a final OD_{600} of 1.0. Pictures were taken at four days post-infiltration (dpi). The infiltrated areas are indicated by white dashed lines. For each treatment, at least four leaves per genotype, taken from independent plants, were infiltrated. Under these conditions, all leaves infiltrated with Cf-9-eGFP, and none of the other samples, showed necrosis of at least half of the infiltrated area. This experiment was repeated three times with similar results. Representative images are shown.

**Fig. S3** Identification of EFR Cf-9 in microsomal fractions of transgenic tobacco plants. Amino acid sequence of the EFR Cf-9 chimeric receptor. The five peptides identified by mass spectrometry in microsomal protein fractions extracted from transgenic EFR-Cf-9 expressing K1A leaves are underlined. SP, signal peptide; LRR, leucine rich repeat; eJM, external juxtamembrane; TM, transmembrane; iJM, internal juxtamembrane.

**Fig. S4** EFR Cf-9 transgenic plants recognize different elf18 variants. Fully expanded leaves of 4-week-old untransformed wild type (WT) and transgenic tobacco plants expressing EFR Cf-9 (K1A) plants were treated with Milli-Q (MQ) water or with flg22, elf18C, elf18B or elf18G at a concentration of 100 nM. For each peptide, at least four leaves per genotype, taken from independent plants, were infiltrated. Pictures were taken at 40 h post-infiltration. The infiltrated area is indicated by the white dashed line. At this time point, all leaves from K1A plants infiltrated with elf18C, elf18B or elf18C showed necrosis of at least half of the infiltrated area, whereas those infiltrated with flg22 or water, and all leaves from WT plants, failed to display any HR-like symptoms. This experiment was repeated three times with similar results. Representative images are shown.

**Fig. S5** Defence-related genes ACRE-132 and HIN1 are differentially up regulated in transgenic plants expressing EFR-Cf-9 upon treatment with elf18 variants. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT PCR) was used to determine ACRE-132 (A) and HIN1 (B) expression levels in WT and EFR-Cf-9 expressing transgenic K1A plants upon treatment with Milli-Q (MQ) water or with elf18C, elf18B, elf18G and flg22 at a concentration of 100 nM. RNA was extracted from infiltrated leaf sectors obtained from three separate plants for each genotype. Bars represent average ± standard deviation (SD) of three replicates. Different letters indicate statistically significant differences, according to one-way analysis of variance (ANOVA) followed by Tukey’s significance test (P < 0.05). This experiment was repeated twice with similar results.

**Fig. S6** Transgenic plants expressing EFR-Cf-9 show unaltered susceptibility to Botrytis cinerea. Leaves of soil grown 4-week-old wild type (WT) and transgenic plants expressing EFR-Cf-9 (K1A and K5A) were inoculated with B. cinerea spore suspension, and lesion areas were measured at 48 hpi. Bars indicate average lesion area ± standard error (SE) (n > 18). No significant difference was observed between WT and transgenic lines (K1A and K5A), according to Student’s t-test (P > 0.5)