Journal of General Plant Pathology
https://doi.org/10.1007/s10327-018-0779-2

HOST RESPONSES

A recombinant flagellin fragment, which includes the epitopes flg22 and flgII-28, provides a useful tool to study flagellin-triggered immunity

Serena Ciarroni[1] · Christopher R. Clarke[2,3] · Haijie Liu[2] · Noam Eckshtain-Levi[2] · Angelo Mazzaglia[1] · Giorgio M. Balestra[1] · Boris A. Vinatzer[2]

Received: 13 October 2017 / Accepted: 31 December 2017

Abstract
Plants and animals independently evolved the ability to recognize flagellin (also called FliC), the building block of the bacterial flagellum, as part of their innate immune response. While animals recognize a relatively large region of FliC, most plants recognize one or two short epitopes of FliC: flg22 and flgII-28. However, since most research in plants has focused on flg22 and flgII-28 and not the actual FliC protein, the importance of any FliC region beyond the two epitopes in plant immunity is poorly understood. Here we report cloning, overexpression, and purification of a *Pseudomonas syringae* FliC fragment from amino acid 1 to 143, which includes both FliC epitopes and the adjacent alpha helices. Exposing *Arabidopsis thaliana* leaves to $FliC_{1–143}$ did not reveal any additional FliC recognition capabilities beyond flg22. However, while the kiwifruit species *Actinidia arguta* did not respond to either flg22 or flgII-28, treatment of *A. arguta* leaves with $FliC_{1–143}$ triggered a significant reactive oxygen response, indicating recognition. This result suggests that in some plant species, recognition of FliC requires regions of FliC beyond the two well-known epitopes and that $FliC_{1–143}$ represents a useful tool in the study of plant immunity.

Keywords Flagellin · FliC · Kiwifruit · Reactive oxygen species · PAMP

Introduction

Plants and animals both recognize conserved microbial features known as pathogen- (or microbe) associated molecular patterns (PAMPs or MAMPs) and activate an immune response known as pattern-triggered immunity (PTI) upon such recognition (Katagiri and Tsuda 2010). PTI is sufficient to protect plants from the majority of potential plant diseases. However, this immune response can be overcome by pathogens through delivery of immunity-suppressing effector proteins (Macho and Zipfel 2015) or through mutations in the MAMP leading to altered recognition specificity (reviewed by Vinatzer et al. 2014).

Serena Ciarroni and Christopher R. Clarke contributed equally to this work.

✉ Boris A. Vinatzer
vinatzer@vt.edu

Serena Ciarroni
ciarronis@unitus.it

Christopher R. Clarke
christopher.clarke@ars.usda.gov

Haijie Liu
haijie07@vt.edu

Noam Eckshtain-Levi
noam@vt.edu

Angelo Mazzaglia
angmazza@unitus.it

Giorgio M. Balestra
balestra@unitus.it

[1] Department of Agriculture and Forestry Science (DAFNE), University of Tuscia, Via San Camillo de Lellis snc, Viterbo, Italy

[2] Department of Plant Pathology, Physiology and Weed Science, Virginia Tech, Latham Hall, Blacksburg, VA 24061, USA

[3] Present Address: Agricultural Research Service, USDA, Beltsville, MD 20705, USA

Published online: 23 March 2018

The best-characterized MAMP is bacterial flagellin or FliC, the monomeric component of flagellum. The majority of land plants are able to detect a specific epitope of flagellin known as flg22 (Boller and Felix 2009; Felix et al. 1999). This epitope is located at a bend between the first and second alpha-helix of the flagellin protein (positions 30–51) (Fig. 1) and is specifically sensed by the plant receptor-like kinase (RLK) FLS2 (Gómez-Gómez and Boller 2000). FLS2 directly binds flg22 (Chinchilla et al. 2006), leading to a cascade of signalling events that culminate in immune responses such as defense gene expression (Chinchilla et al. 2007), callose deposition (Gómez-Gómez and Boller 2000), calcium signalling (Boudsocq et al. 2010), and production of reactive oxygen species (ROS) (Felix et al. 1999).

A second region of flagellin, flgII-28 (Cai et al. 2011), is also a MAMP but with recognition specificity limited to a subset of *Solanaceae* (Clarke et al. 2013) that contain the RLK FLS3 (Hind et al. 2016). The flgII-28 epitope encompasses a bend between the second and third alpha-helix of the flagellin protein (positions 84–111) similar to the flg22 epitope (Fig. 1).

Little is known about the role of regions of flagellin beyond flg22 and flgII-28. Intriguingly, we previously found that a mutation in flgII-28 affects growth of *Pseudomonas syringae* in *Arabidopsis thaliana* leaves in an *FLS2*-dependent manner although FLS2 does not recognize flgII-28 and the *A. thaliana* genome does not have an *FLS3* homolog (Clarke et al. 2013; Hind et al. 2016). We hypothesized that the mutation in flgII-28 may alter the overall configuration of FliC, thus affecting accessibility of flg22 by FLS2 or that flgII-28 was possibly recognized in an FLS2-dependent manner in *A. thaliana* but that additional regions of FliC

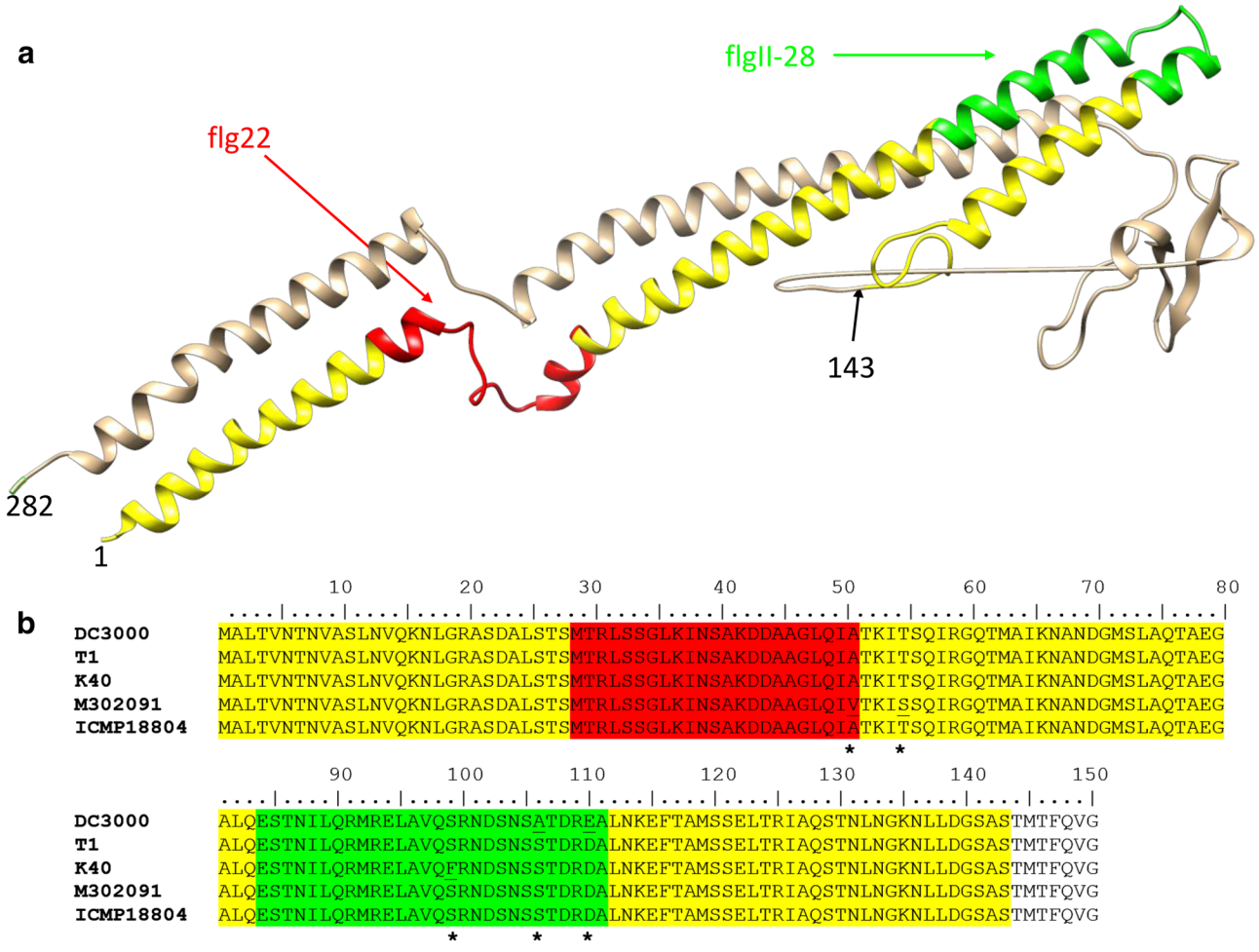

Fig. 1 3D structure and partial sequence alignment of the FliC protein. Highlighted in yellow is the cloned flagellin fragment used in this work, positions 1–143. Epitopes flg22 and flgII-28 are highlighted in red and green, respectively. **a** 3D structure of the entire flagellin based on the DC3000 sequence created by the phyre2 web portal (Kelley et al. 2015) and viewed using UCSF Chimera (https://www.cgl.ucsf.edu/chimera/docs/credits.html). **b** Partial sequence alignment of FliC protein, positions 1–150, *Pseudomonas syringae* pv. *tomato* (*Pto*) strains DC3000, T1 and K40 with *P. syringae* pv. *actinidiae* strain M302091 and *P. syringae* pv. *actinidifoliorum* strain ICMP18804 created by BioEdit software (Hall 1999). Underlined and marked with an asterisk are amino acid polymorphisms

were necessary for this recognition to occur. Some regions besides flg22 and flgII-28 are already known to play a role in flagellin recognition. For example, glycosylation of a region C-terminal of flgII-28 (positions 143, 164, 176, 183, 193, and 201) is necessary for *P. syringae* pv. *tabaci* to cause disease in tobacco, possibly by interfering with recognition of flg22 by FLS2 (Taguchi et al. 2006, 2010).

To further expand our knowledge of the role of the regions outside of flg22 and flgII-28 in flagellin-recognition in plants, here we cloned, overexpressed, and purified the FliC region from position 1–143 ($FliC_{1-143}$) in *Escherichia coli*. Comparison of the ROS response to this fragment with the ROS response triggered by flg22 and flgII-28 confirmed previous results obtained in tomato and *Arabidopsis* and suggests the presence of a flagellin receptor in kiwifruit that was not detectable using the flg22 and flgII-28 epitopes alone.

Materials and methods

Peptides

The flg22 and flgII-28 peptides were purchased from EZBioLab (Carmel, IN, USA) and resuspended in sterile water to make 1 µM stock solutions. Peptides were stored at − 20 °C short-term or − 80 °C long-term.

Plants

Solanum lycopersicum cv. Rio Grande, *Arabidopsis thaliana* ecotype Columbia-0 and the *fls2* mutant (Xiang et al. 2008) were grown with a 16-h photoperiod for 4 weeks in the laboratory on light shelves before use. Two-year-old *Actinidia arguta* cv. Meader plants were maintained in the laboratory under the same conditions until use.

Cloning of the gene fragment encoding $FliC_{1-143}$

Bacterial genomic DNA was extracted using the Accuprep Genomic DNA Extraction Kit (Bioneer, Alameda, CA, USA). The *fliC* gene fragment encoding $FliC_{1-143}$ was amplified using the gene-specific forward primer (5′-AAAAGG TCTCNAGGTATGGCTTTAACAGTAAACACCAAC-3′) and reverse primer (5′-AAAATCTAGATCAGCTGG CGGAACCGTCAAGCAG-3′), which were synthesized by Sigma–Aldrich (St. Louis, MO, USA). PCR reactions were carried out in 50 µL volumes using Iproof Polymerase 2× (Bio-Rad, Hercules, CA, USA) and the following cycler program: 98 °C for 3 min; 35 cycles of 98 °C for 10 s, 58 °C for 30 s, 72 °C for 1 min; 72 °C, 10 min. Amplicons were separated in a 1% agarose gel to confirm presence, size, and purity. Bands were then gel-purified using the AccuPrep gel purification kit (Bioneer). Amplicons and the vector pSUMOstar (LifeSensors, Malvern, PA, USA) were digested with XbaI and BsaI (NEB, Ipswich, MA, USA), respectively, cleaned using the AccuPrep gel purification kit (Bioneer), and ligated using the DNA Ligation Kit "Mighty Mix" (Takara, Mountain View, CA, USA) following the manufacturer's protocol. After ligation, the ligation mix was transformed into *E. coli* CH3-Blue (Bioline, Taunton, MA, USA). Putative transformants growing on LB agar plus carbomycin were checked for the presence of the correct insert by PCR and Sanger sequencing.

Expression and purification of the recombinant $FliC_{1-143}$ protein fragment

For protein expression, confirmed insert-containing pSUMOstar plasmids were used to transform *E. coli* BL21(DE3) (Bioline). Transformed bacteria were grown at 37 °C with shaking in 50 mL with a starting OD_{600} of 0.05 and a final OD_{600} between 0.8 and 1.0. At that point, isopropyl β-D-1-thiogalactopyranoside (IPTG; Bioline) was added at the final concentration of 1 mM, and the cell culture was continued overnight in a 20 °C shaker for protein induction. Bacterial suspensions were then retrieved and cells pelleted by centrifugation at 6000 rpm for 8 min. The supernatant was removed and the pellet resuspended in 1 mL of phosphate buffer, lysozyme was added at final concentration of 0.2 mg/mL (Sigma–Aldrich). The resuspended mixture was incubated on ice at 4 °C overnight. After overnight lysis, the lysate was sonicated three times with 10 s pulses (Misonix, Farmingdale, NY, USA) and the cell debris pelleted by centrifugation at 8000 rpm for 10 min. The supernatant of the clarified lysate was filtered through a 0.45 µm membrane (Merck Millipore, Burlington, MA, USA), then used for metal chromatography.

A His GraviTrap column (GE Healthcare, Chicago, IL, USA) was used to purify the His-tagged fragment from the filtered lysate, according to the manufacturer's instructions by using in sequence buffer solutions characterized by increasing imidazole concentration: 20 mM binding buffer, 50 mM washing buffer, 200 mM elution buffer, 500 mM final elution buffer. Purification was confirmed by 12% protein gel (Mini-PROTEAN TGX, Bio-Rad) electrophoresis. Protein concentration of samples was measured using a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA).

Washing of $FliC_{1-143}$ protein fragment

For avoiding interference with the ROS assay, $FliC_{1-143}$ fragments were washed by ultrafiltration to eliminate as much as possible any imidazole that might remain from the elution buffer after purification. The solution containing $FliC_{1-143}$ was centrifuged in an Amicon Ultra-15 centrifugal filter unit

(Millipore) using the manufacturer's instructions, taking into account the molecular weight of the fragment. The elution buffer was replaced by phosphate buffer. Protein concentration was checked again at the end of the procedure.

ROS assay

Leaf discs were sampled from plants using a #1 cork borer and placed adaxial side up in 200 µL of sterile water in a clear-bottom 96-well plate overnight. One leaf was sampled from each of six plants for each plant–peptide combination. The same leaf was sampled multiple times for each plant–peptide combination in each experiment to account for leaf-to-leaf variability. The ROS reaction mixture containing luminol (34 µg/mL), horseradish peroxidase (20 µg/mL), and the elicitor peptide or $FliC_{1–143}$ (concentration listed in each figure) was prepared and 100 µL added to each well containing a leaf disk. The chemiluminescence produced in each well was measured using a Synergy HT plate reader (Biotek, Winooski, VT, USA) every 3 min for 1 h using the bottom camera at optical sensitivity 225. For more details, see Clarke and Vinatzer (2017). The magnitude of ROS responses is only comparable within an experiment and not between experiments.

Results

$FliC_{1–143}$ of Pto DC3000 and Pto T1 trigger ROS response in tomato

The FliC region from amino acid 1–143 (Fig. 1) was cloned from *P. syringae* pv. *tomato* strain (*Pto*) T1 (Almeida et al. 2008) and from *Pto* strain DC3000 (Buell et al. 2003). The $FliC_{1–143}$ protein fragments were then overexpressed in *E. coli* and purified. After verifying their purity, $FliC_{1–143}$ from *Pto* DC3000 and from *Pto* T1 were used to test their ability to elicit an immune response in tomato leaves using a standard ROS assay (Clarke and Vinatzer 2017). Importantly, both versions of $FliC_{1–143}$ were found to elicit a strong ROS response in a dose-dependent manner (Fig. 2a, b), showing that cloned $FliC_{1–143}$ can be used to study flagellin-triggered

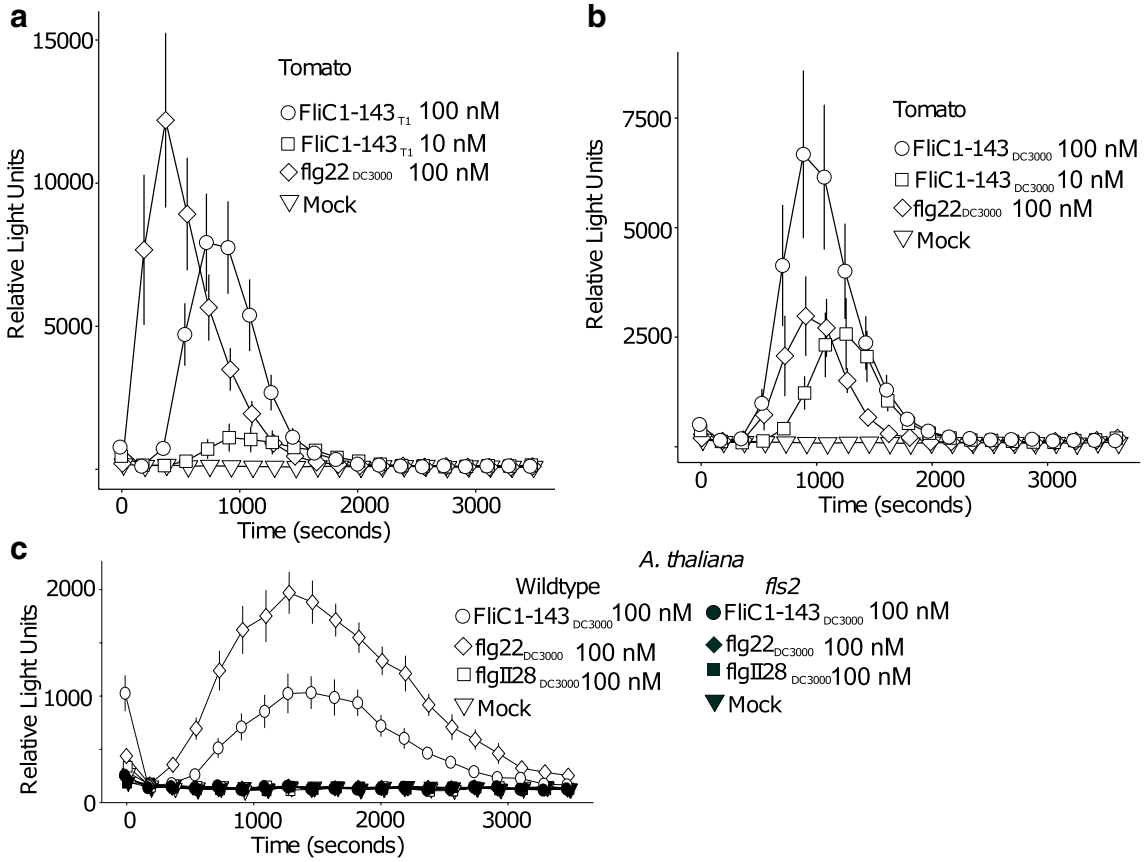

Fig. 2 Recombinant $FliC_{1–143}$ fragments elicit production of ROS in tomato and Arabidopsis. Four-week-old tomato (**a, b**) and *Arabidopsis thaliana* ecotype Columbia (Wildtype), but not *A. thaliana fls2* (**c**), are sensitive to treatment with the $FliC_{1–143}$ fragments using a luminol-based ROS detection method. Error bars represent standard error; $n = 6$ for all plant–peptide combinations in all experiments. Similar results were obtained in three individual experiments

immunity in plant leaves. However, since the flg22 and flgII-28 peptides were synthesized chemically but $FliC_{1-143}$ was purified from *E. coli* (and they thus contain different levels of different contaminants that could affect ROS measurement), the strength of the ROS response triggered by the peptides and $FliC_{1-143}$ should not be compared. Moreover, although the two $FliC_{1-143}$ preparations were washed thoroughly, it is impossible to know if imidazole (which quenches ROS) was eliminated to the same degree in different samples. Therefore, even the strength of the ROS response triggered by the different preparations of $FliC_{1-143}$ should not be compared directly.

Recognition of $FliC_{1-143}$ in *Arabidopsis* is FLS2-dependent

$FliC_{1-143}$ of *Pto* strain DC3000 was also tested in wild-type *FLS2* and mutant *fls2* (Xiang et al. 2008) of *A. thaliana* ecotype Columbia leaves to determine whether $FliC_{1-143}$ could be detected independently of *FLS2*. A strong ROS response was detected in wild-type *FLS2* plants but not in the *fls2* mutant (Fig. 2c). We thus conclude that $FliC_{1-143}$ was detected by *FLS2* in wild-type *A. thaliana* because it contains flg22. Since $FliC_{1-143}$ was not detected in the *fls2* mutant, we can confirm the absence of an *FLS2*-independent flgII-28 receptor in *A. thaliana*.

Kiwifruit recognizes $FliC_{1-143}$ but not the individual epitopes flg22 or flgII-28

Finally, we extended testing of $FliC_{1-143}$ to the kiwifruit species *A. arguta*. We had investigated whether nonsynonymous single nucleotide polymorphisms (SNPs) that distinguish the *fliC* allele of *Pseudomonas syringae* pv. *actinidiae* (*Psa*) from the *fliC* allele of *P. syringae* pv. *actinidifoliorum* (Fig. 1) contribute to the marked difference in virulence on kiwifruit between these two pathovars (Cunty et al. 2015). Surprisingly, neither of the tested *flg22* alleles elicited any ROS burst in kiwifruit leaves (Fig. 3a). Kiwifruit is therefore one of the few land plants that is unresponsive to flg22 treatment (Boller and Felix 2009). Kiwifruit was also unresponsive to treatment with flgII-28 (Fig. 3b), which is expected because this peptide was previously found to elicit an immune response only in a subset of *Solanaceae* (Clarke et al. 2013). However, when kiwifruit leaves were treated with $FliC_{1-143}$ from strain DC3000 and strain T1, both alleles triggered ROS production (Fig. 4). Importantly, $FliC_{1-143}$ from strain T1 is identical to the allele from *P. syringae* pv. *actinidifoliorum*. We can therefore conclude that kiwifruit is sensitive to the $FliC_{1-143}$ fragment despite being blind to the flg22 and flgII-28 epitope sequences contained in it. A control protein consisting in a fragment of the ice nucleation activity (INA) protein of *P. syringae* that was overexpressed and purified in the same way as $FliC_{1-143}$

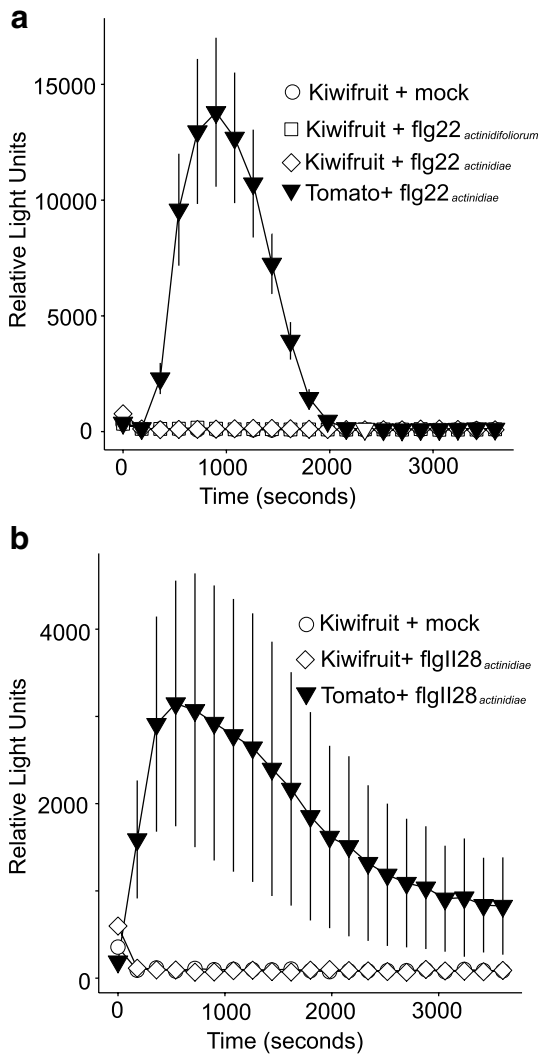

Fig. 3 Neither flg22 nor flgII-28 elicit production of ROS in leaves of 2-year-old kiwifruit cv. Meader. All peptides were applied at 1 µM, and ROS was detected using a luminol-based ROS detection method. Error bars represent standard error; $n = 6$ for all plant–peptide combinations in all experiments. Similar results were obtained in three individual experiments. Since *Pseudomonas syringae* pv. *actinidiae* and *P. syringae* pv. *actinidifoliorum* share the same allele of flgII-28 but differ in flg22, **a** Shows results for flg22 of *P. syringae* pv. *actinidiae* and of *P. syringae* pv. *actinidifoliorum*, while **b** only shows results for flgII-28 of *P. syringae* pv. *actinidiae*

did not trigger any ROS response (Fig. 4). Kiwifruit thus appears to express a flagellin receptor for which neither flg22 nor flgII-28 is sufficient for activation.

Discussion

The protein fragment $FliC_{1-143}$ is an active elicitor of PTI in tomato, *A. thaliana*, and kiwifruit. In *A. thaliana*, recognition was eliminated in *fls2*-knockout plants, indicating that

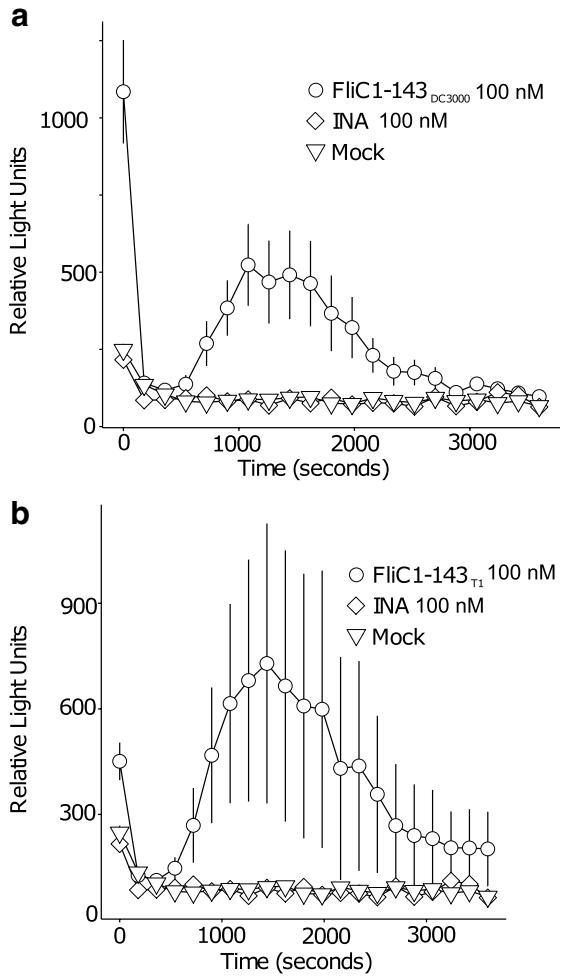

Fig. 4 Recombinant $FliC_{1–143}$ f from DC3000 (**a**) and T1 (**b**) elicit production of ROS in leaves of 2-year-old kiwifruit cv. Meader. All fragments were applied at indicated concentrations, and ROS was measured using a luminol-based ROS detection method. The T1 allele of $FliC_{1–143}$ is identical to the *Pseudomonas syringae* pv. *actinidifoliorum* allele, which differs by two amino acids (positions 51 and 55) from the *P. syringae* pv. *actinidiae* allele. Error bars represent standard error; $n=6$ for all plant-peptide combinations in all experiments. Similar results were obtained in three individual experiments

the recognition is due to the presence of the flg22 epitope in $FliC_{1–143}$. This result confirms that there is no receptor for flgII-28 in *A. thaliana*. However, we still cannot formally exclude that *A. thaliana* has a flgII-28 receptor that depends on *FLS2*. Such a hypothetical receptor could explain the *FLS2*-dependent effect of flgII-28 on *P. syringae* growth observed in *A. thaliana* (Clarke et al. 2013). However, the alternate hypothesis, that allelic variation at the flgII-28 epitope affects accessibility of flg22 by FLS2, seems more likely.

It is possible that other plant families previously identified as unresponsive to flg22 and flgII-28 (Clarke et al. 2013) may also respond to $FliC_{1–143}$, similar to kiwifruit. The kiwifruit receptor may require a region that extends beyond the flg22 epitope, or a novel epitope that is cleaved from $FliC_{1–143}$ in planta, or an even larger region similar to the region required for flagellin recognition by TLR5 in animals (Yoon et al. 2012). To further investigate flagellin recognition in kiwifruit, ROS assays will need to be complemented with additional measurements of the strength of the plant immune response, such as stomatal closure, callose deposition, or plant protection assays (Clarke and Vinatzer 2017), using longer flagellin epitopes and FliC fragments of different lengths, and testing other members of the *Actinidiacea*e family.

In summary, the recombinant $FliC_{1–143}$ fragment confirmed the absence of a flgII-28 receptor in *A. thaliana* and revealed that recognition in kiwifruit requires more than the well-studied flg22 and/or flgII-28 epitopes. Considering that $FliC_{1–143}$ is easily overexpressed and purified from *E. coli*, these results show that $FliC_{1–143}$ is a very useful and practical tool for studying flagellin-triggered immunity in plants. The plasmids expressing $FliC_{1–143}$ are available upon request.

Acknowledgements This work was supported by NSF IOS-0746501 and IOS-1354215 to BAV. BAV was also funded in part by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, US Department of Agriculture. CRC was supported by a National Institute of Food and Agriculture Postdoctoral Research Fellowship (2015-67012-22821). NE-L was supported by BARD, the United States–Israel Binational Agricultural Research and Development Fund, Vadia-BARD Postdoctoral Fellowship Award No. FI-538-15. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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