Brain, Behavior, and Immunity xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Extracellular self-DNA as a damage-associated molecular pattern (DAMP) that triggers self-specific immunity induction in plants

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ARTICLE INFO

Article history:
Received 3 July 2017
Received in revised form 4 October 2017
Accepted 13 October 2017
Available online xxxx

Keywords:
Damaged-self recognition
DAMP
Defence signalling
eDNA
MAPK signalling
ROS

ABSTRACT

Mammals sense self or non-self extracellular or extranuclear DNA fragments (hereinafter collectively termed eDNA) as indicators of injury or infection and respond with immunity. We hypothesised that eDNA acts as a damage-associated molecular pattern (DAMP) also in plants and that it contributes to self versus non-self discrimination. Treating plants and suspension-cultured cells of common bean (*Phaseolus vulgaris*) with fragmented self eDNA (obtained from other plants of the same species) induced early, immunity-related signalling responses such as H_2O_2 generation and MAPK activation, decreased the infection by a bacterial pathogen (*Pseudomonas syringae*) and increased an indirect defence to herbivores (extrafloral nectar secretion). By contrast, non-self DNA (obtained from lima bean, *Phaseolus lunatus*, and *Acacia farnesiana*) had significantly lower or no detectable effects. Only fragments below a size of 700 bp were active, and treating the eDNA preparation DNAse abolished its inducing effects, whereas treatment with RNAse or proteinase had no detectable effect. These findings indicate that DNA fragments, rather than small RNAs, single nucleotides or proteins, accounted for the observed effects. We suggest that eDNA functions a DAMP in plants and that plants discriminate self from non-self at a species-specific level. The immune systems of plants and mammals share multiple central elements, but further work will be required to understand the mechanisms and the selective benefits of an immunity response that is triggered by eDNA in a species-specific manner.

1. Introduction

Multicellular organisms suffer different types of cellular damage that may, or may not, include infectious processes. Janeway's classical model states that the immune system evolved to distinguish the infectious non-self from the non-infectious self (Janeway et al., 2001). However, in most environments, injury to the outer layers of an organism (the skin or gut epithelia in the case of mammals, the epidermis of leaves and roots in the case of plants) inevitably leads to infection. Moreover, responses such as wound sealing and tissue repair are also required in non-infected injured tissues and, in most cases, they are independent of the exact nature of the harming agent. Thus, multicellular organisms require an endogenous signalling pathway that enables them to perceive injury and mount adequate local and systemic responses (Heil and Land 2014). The danger model holds that the onset of a successful immune response depends on the detection of 'danger' or 'damage'-associated molecular patterns (DAMPs): endogenous indicators of injury (Land et al., 1994; Matzinger 2002, 1994). During injury, tissue disruption and the resulting de-compartmentalization of cells lead to the release of intra-cellular molecules into the extracellular space and to the fragmentation of macromolecules (Heil and Land, 2014). All these molecules potentially can be perceived by the surrounding, intact cells as DAMPs that trigger 'damaged-self recognition': an induction of immunity in damaged organisms that is independent of exogenous molecules such as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Heil, 2009; Heil and Land, 2014).

In mammals, well-studied DAMPs include high-mobility group box proteins (HMGBs), extracellular ATP, or extracellular and cytosolic DNA fragments (Garg et al., 2015; Vénéreau et al., 2015). For the sake of simplicity, hereinafter we employ the term 'eDNA' collectively for extracellular and extranuclear (i.e., cytosolic) DNA. Whereas eDNA molecules of nuclear and mitochondrial origin are considered DAMPs (Toussaint et al., 2017), bacterial and viral DNA molecules are considered MAMPs or PAMPs (Altfeld and Gale, 2015; Dempsey and Bowie, 2015; Jounai et al., 2013; Kaczmarek et al., 2013; Tang et al., 2012; Wang et al., 2016; Wu and Chen, 2014). However, it remains matter of discussion whether mitochondrial DNA is perceived as DAMP or rather as

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https://doi.org/10.1016/j.bbi.2017.10.010
0889-1591/

a MAMP when it appears outside of cells (Zhang et al., 2010). This situation is paralleled by fructans, plant storage polysaccharides that have been suggested to act as DAMPs when they appear in the apoplast, but that might also be of bacterial or fungal origin and then represent MAMPs (Versluys et al., 2017). Nevertheless, mammalian cells sense DAMPs as well as MAMPs via a range of receptor-dependent and -independent pathways that involve, among others, toll-like receptors (TLRs), purinergic receptors, DNA-dependent activator of IFN-regulatory factors (DAI), interferon regulatory factor (IRF), or the NACHT, LRR and PYD domains-containing protein 3 (NLPR3) inflammasome (Di Virgilio et al., 2017; Lupfer and Anand, 2016) Magna and Pisetsky, 2016; Schlee and Hartmann, 2016; Takahashi et al., 2017; Takaoka et al., 2007). In fact, mammalian immune cells sense eDNA independently of whether it has been released from dying host cells or produced, e.g., by retroviral reverse transcriptase (Altfeld and Gale, 2015; Gallucci and Maffei, 2017; Kato et al., 2017). The activation of these sensors triggers immunity-related responses like mitogen-activated protein kinase (MAPK) signalling, the formation of reactive oxygen species (ROS), the synthesis of interferons (IFNs) and multiple other signalling processes that lead to inflammation, the maturation of dendritic cells to antigen-presenting cells and, ultimately, to active innate and adaptive immune response (Land, 2015).

Research into the mechanisms that enable the mammalian immune system to discriminate "self from non-self" in the sensing of nucleic acids has mainly focused on the differentiation of host (self) versus viral or microbial (non-self) eDNA (Schlee and Hartmann, 2016). For plants, by contrast, recent studies revealed a surprising level of specificity at which DAMPs of different taxonomic origin trigger immunity. For example, treating intact leaves of common bean (*Phaseolus vulgaris*) with leaf homogenate – which arguably contains a complex blend of DAMPs – induced various immunity-related responses, but only when using homogenate prepared from conspecific leaves (Duran-Flores and Heil, 2014). Even the application of homogenate from the closely related lima bean (*Phaseolus lunatus*) led to a significantly reduced response (Duran-Flores and Heil, 2014). However, it remains unknown which ones of all the molecules that are released from damaged tissue account for this surprising specificity in the plant immune response.

Based on the central role of eDNA in the mammalian immune system and recent anecdotal evidence for an equivalent function in plants (summarized in Gallucci and Maffei, 2017; Gust et al., 2017),we hypothesized that eDNA is a particularly promising candidate of a DAMP that could contribute to the species-specificity in plant damaged-self recognition; mainly for the following reasons. First, delocalized self nucleic acids — such as extranuclear DNA or extracellular RNA — are well-known DAMPs in mammals, "because they are reliable indicators of cellular damage" (Desmet and Ishii, 2012). Upon its recognition, eDNA triggers the generation of ROS, downstream MAPK signalling cascades, the release of cytokines, inflammation and other immunity-related responses (Altfeld and Gale, 2015; Anders and Schaefer, 2014; Dempsey and Bowie, 2015; Heil and Land, 2014; Jounai et al., 2013; Kaczmarek et al., 2013; Patel et al., 2011; Tang et al., 2012; Wang et al., 2016). Second, eDNA has been suggested to act in plant immunity (Duran-Flores and Heil, 2015; Gallucci and Maffei, 2017; Gust et al., 2017; Hawes et al., 2011) because it was reported as an indicator of bacterial infection in *Arabidopsis thaliana* (Yakushiji et al., 2009), as an inducer of immunity to fungal infections in pea roots (*Pisum sativum*) (Wen et al., 2009) and, most recently, as a trigger of Ca^{2+} signalling and membrane depolarization in lima bean and maize (*Zea mays*) (Barbero et al., 2016). Third, the effects of eDNA can depend on the taxonomic distance between the source and the receiver: the application of non-self eDNA from lima bean or an insect did not result in membrane depolarization in maize (Barbero et al., 2016) and the inhibitory effect of eDNA on the growth of organisms in different phyla (Mazzoleni et al., 2015a,b, Mazzoleni et al., 2014) showed taxonomic specificity: eDNA of *Lepidium sativum* inhibited the root growth of Arabidopsis in a dosage-dependent manner, but 'self eDNA' prepared from Arabidopsis had a much stronger effect (Mazzoleni et al., 2015a). Based on the above-mentioned reports, we reasoned that self eDNA might contribute to the taxonomic specificity in plant damaged-self recognition (Duran-Flores and Heil, 2015).

In the present study, we aimed at investigating whether eDNA can cause the same species-specific responses in bean as they had been observed after the application of leaf homogenates. We used *P. vulgaris* as the receiver species and applied fragmented self-eDNA, prepared from different individuals but the same cultivar as the receiver, as well as non-self eDNA, which was prepared from *P. lunatus* and *Acacia farnesiana* (*A. farnesiana* is a member of the Fabaceae family but does not belong to the same subfamily as bean). We quantified the generation of ROS and the activation of MAPKs as two early, general responses to stress and the secretion of extrafloral nectar (EFN) and the infection by a bacterial phytopathogen as two indicators of the phenotypic components of the plant immune system. The secretion of EFN is a widespread, inducible plant response to herbivory. EFN attracts ants, predators, parasitoids and other natural enemies of the herbivores to the plant, thereby serving as a means of 'natural biological control' (see Heil, 2015 for a recent overview). Putative effects of RNA or proteins on the observed responses were excluded using nucleases and proteinases, respectively. Based on our results, we suggest that eDNA is likely to represent a DAMP that contributes to the specificity in plant damaged-self recognition.

2. Material and methods

2.1. Biological material

For all experiments in plants, four-week-old common bean plants were used as receivers (*Phaseolus vulgaris*, Negro San Luis variety; seeds were obtained from the national germplasm collection at INIFAP, Celaya, GTO, México). The plants were grown under greenhouse conditions and natural light (average day-time temperature, 28 °C; night-time temperature, 20 °C), watered on Mondays, Wednesdays and Fridays, and fertilized weekly with a commercial fertilizer (Ferviafol 20-30-10®, Agroquímicos Rivas S. A. de C.V., Celaya, GTO, México). Lima bean (*Phaseolus lunatus*) seeds were collected from a wild population 5-km west of Puerto Escondido, in the state of Oaxaca in Southern Mexico (~15°55′ N and 097°09′ W), and cultivated under greenhouse conditions. Before cultivation, the seeds were surface-sterilized with 70% ethanol for 1 min and with a 20% hypochlorite solution for 10 min and then washed five times with sterile water. Wild *Acacia farnesiana was* collected from the area around CINVESTAV - Irapuato, in the state of Guanajuato in Central Mexico (~20°72′ N and 101°33′W). The bacterial phytopathogen (rifampicin-resistant Pseudomonas syringae pv. *syringae* strain 61) was provided by Dr. Choong-Min Ryu (KRIBB, Daejeon, South Korea).

2.2. Suspension cells

Surface-sterilized common bean seeds were germinated under sterile conditions in solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with a pH of 5.8 and 3% sucrose. After seven days, the apical meristem or root was cut 3 mm from the tip. These tips were transferred to solid MS medium with a pH of 5.8 that was enriched with 0.5 mg L^{-1} of indoleacetic acid (IAA) and 5

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mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) (both from Sigma-Aldrich) and then incubated for 4 weeks in a growth room at 25 °C and a light:dark regime of 16 h: 8 h to enable the undifferentiated cells (callus) to proliferate. After that time, the callus was transferred to a 250 mL flask with 50 mL of liquid MS medium enriched with 0.5 mg L^{-1} of IAA and 5 mg L^{-1} of 2,4-D and then incubated on a shaking tray (160 rpm) under the same conditions. A suspension culture of cells was obtained 4 weeks after the callus was transferred to the liquid medium and maintained under a light:dark regime of 16 h:8 h at a constant 25 °C. The cells were continuously subcultured every 2 weeks, transferring 2 mL of culture to a new flask with MS liquid medium and then used for experiments 7 days after subculturing.

2.3. Extraction and fragmentation of DNA

The DNA was extracted based on a method reported by Dellaporta et al. (1983). Leaves of common bean, lima bean or acacia were ground in a mortar with liquid nitrogen, weighed and then placed in 50 mL tubes (5 g in each tube). A total of 20 mL of Dellaporta buffer (100 mM Tris-HCl pH 8, 5 mM EDTA pH 8, 50 mM NaCl and 10 mM β-mercaptoethanol) were added to each tube and then shaken for a few seconds on a vortex shaker. Next, the tubes were heated to 65 °C for 10 min in a water bath before adding 6.6 mL of 5 M potassium acetate and placing the tubes on ice. After 30 min on ice, the tubes were centrifuged at 12000*g* for 20 min: the supernatant was separated, transferred to a new 50 mL tube and centrifuged one more time; the supernatant was then separated and collected in a new 50 mL tube. Next, 20 mL of precooled isopropanol were added to the supernatant, which was then kept at −20 °C for 1 h. The tubes were then centrifuged at 12000*g* for 20 min, the supernatant was discarded and the pellet was dried for 5 min before adding 5 mL of 70% ethanol to the tube and shaking. The tubes were centrifuged at 12000*g* for 10 min, the supernatant was discarded again and the pellet was dried for 5 min and then suspended in 1 mL of sterile distilled water and purified using a Maxi DNA purification Kit (Qiagen). The DNA was quantified using a NanoDrop 2000 spectrometer (Thermo Scientific) and then fragmented by sonication to obtain fragments of less than 1000 bp using an ultrasonic processor (Misonix XL2020). A solution of 500 μg mL^{-1} of DNA was prepared with sterile distilled water and sonicated for 6 min at a power level of 5.5 with a 1 s pulse 'On' and a 1 s pulse 'Off'. The successful fragmentation of DNA was verified on a 3% agarose gel using ethidium bromide. The DNA from common bean was used 'self eDNA'; the DNA from lima bean or acacia was used as 'non-self eDNA'.

2.4. Effect of eDNA on the primary root growth of germinated seeds

In order to confirm whether previous observations made by Mazzoleni et al., (2015a,b) also applied to common bean, surface-sterilized common bean seeds (n = 9 per treatment) were germinated in 9-cm Petri dishes on sterile filter paper imbibed with 5 mL of different concentrations (0, 2, 20, 50, 100, 150, 200 or 250 μg mL^{-1}) of self eDNA fragments in sterile water. Sterile distilled water with 0 μg mL^{-1} of eDNA were used as the control treatment. Petri dishes were placed in a growth room at 25 °C with 16 h of light and 8 h of darkness. The primary root length was measured with a flexible tape after four days. The self eDNA and the non-self eDNA effect were compared using surface-sterilized common bean seeds that germinated in Petri dishes on sterile filter paper imbibed with 5 mL of 200 μg mL^{-1} of self eDNA or non-self eDNA (n = 3 seeds per treatment). The Petri dishes were placed in the growth room and the primary root length was measured after four days.

2.5. Effect of eDNA on the accumulation of the ROS (H_2O_2)

To determine whether eDNA activates early immunity responses, common bean plants were treated with 200 μg mL^{-1} of self eDNA or non-self eDNA fragments in 0.05% (v v^{-1}) Tween 20. Groups of nine plants were used for each treatment. Plants treated with 0.05% Tween 20 were used as controls. The solution of eDNA or Tween was applied with a micropipette on both sides of three randomly selected leaves until the surface was completely wet. Two hours after the treatment, 10 discs of 1-cm diameter were punched out of each leaf. The leaf discs from the same plant were placed in a 2 mL tube, weighed and suspended in 1-mL of Milli-Q water. This suspension was continuously stirred for 10 min and then centrifuged at 12 000*g* for 15 min. Next, 10 μL of the supernatant were mixed with 90 μL of the substrate solution containing ferrous iron and xylenol orange (Hydrogen Peroxide Assay Kit, National Diagnostics, Atlanta, GA, USA). Blanks were prepared using Milli-Q water instead of the sample. The mixture was incubated for 30 min at room temperature and the absorbance was measured at 560 nm in a microplate reader (Synergy 2, BioTek Instruments Inc., Winooski, VT, USA) and compared to a calibration curve obtained using H_2O_2 at concentrations of 0–250 nmol mL^{-1}.

2.6. Effect of eDNA on the activation of MAPKs in leaves and suspension cell cultures

In order to determine whether MAPKs respond to eDNA and to define the time of maximum activation, the activation of MAPKs was assessed at different time points (1, 3, 5, 10, 15, 30, 60 and 120 min) after self eDNA had been applied to the leaves. Three plants were used per time point and three randomly selected leaves per plant were treated with 200 μg mL^{-1} of self eDNA fragments in 0.05% (v v^{-1}) Tween 20. The solution of eDNA or Tween was applied with a micropipette on both sides of the leaves until the surface was completely wet. Plants that had been mechanically damaged with a needle were used as positive controls (Duran-Flores and Heil, 2014), and plants without any mechanical damage and plants treated with 0.05% Tween 20 solution as negative controls (n = 3 for each of the three controls). At the end of each of the treatment times, three treated leaves per plant were excised, pooled and placed in liquid nitrogen to determine the activation of MAPKs based on established methods (Stratmann and Ryan, 1997; Stratmann et al., 2000). The pooled leaves were pulverized in liquid nitrogen before placing 100 mg of the pulverized leaves in 2 mL tubes with 1 mL of extraction buffer [50 mM Hepes-KOH (pH 7.6)], 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 20% (v v^{-1}) glycerol, 1 mM Na_3VO_4, 1 mM NaF, 0.5% PVP, 2 mM DTT, 1 mM PMSF and one complete proteinase inhibitor mix tablet (Roche) per 50 mL). The tubes were then vortexed, followed by centrifugation at 12000*g*. The supernatant was used for the MAPK assays. To assess the effect of each time period on the suspension cell cultures, 1 mL of cell culture suspension (1×10^{-8} cells mL^{-1}) was transferred to a 24 multiwell plate and shaken at 160 rpm on an orbital shaker at room temperature. After 1 h of equilibration, 100 μL of 2200 μg mL^{-1} of self eDNA were added to a final concentration of 200 μg mL^{-1} of self eDNA. Cells treated with 0.1 mL of sterile water were used as controls. After 1, 3, 5, 10, 15, 30, 60 or 120 min of treatment, the cells were mixed with 1 mL of the extraction buffer and frozen in liquid nitrogen. Next, 2 mL of the suspension culture were transferred to 2 mL tubes, cells were sonicated twice for 20 s (Ultrasonic Processor Misonix XL2020) and centrifuged at 13 000*g*. The supernatant was used for the MAPK assays. In order to compare the effect of self to non-self eDNA in plants and cells in suspension culture, all three types of eDNA were used at 200 μg mL^{-1} and the activation of MAPK was tested 30 min after treatments.

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In order to define the size range of the eDNA fragments that activate the MAPKs, eDNA fragments of common bean were separated in a 3% agarose gel, stained using ethidium bromide, and the regions containing fragments of 700–1000 bp, 350–700 bp and <350 bp were excised from the gel on a UV transilluminator. The DNA fragments were extracted from the gel and purified using a DNA purification kit (Qiagen). Next, 1 mL of each suspension cell culture (1×10^{-8} cells mL^{-1}) was treated with 0.1 mL of 2200 μg mL^{-1} of these eDNA fragments. Cells treated with 0.1 mL of sterile water were used as controls. After 30 min of treatment, the cells were mixed with extraction buffer and the supernatant was obtained as mentioned above and used for the MAPK assays. This experiment was only performed using suspension cell cultures because a larger quantity of DNA would have been needed to perform this experiment using entire plants.

The protein concentration in the supernatant was determined using a protein assay kit (Bio-Rad) with BSA (Bio-Rad) as the standard and MAPKs were tested by performing immunoblotting. For immunoblotting, the proteins were separated using SDS-PAGE and then transferred for 30 min to a 0.2 μm PVDF membrane (Trans-Blot Turbo Mini PVDF transfer pack: Bio-Rad) in a Trans-Blot Turbo Transfer System (Bio-Rad). After transfer, the membrane was blocked in 5% BSA TBS-Tween 20 (0.1%) overnight at 4 °C and shaken using a labquake with 30 reversals min^{-1}. The membrane was then incubated for 3 h with anti-pMAPK (anti-p42/p44) as the primary antibody (Cell-Signalling) at 1:2500 in blocking solution, washed five times with 0.1% TBS-Tween 20 [1 M Tris-HCl (pH 7.5), 150 mM NaCl, 1% v v^{-1} Tween 20] and incubated with a secondary antibody (anti-rabbit IgG coupled to alkaline phosphatase, Sigma-Aldrich) at 1:20,000 for 1 h at room temperature. The membrane was washed five times with TBS-Tween 20 (0.1%), and 1 mL of Lumi-Phos Plus AP chemiluminescent substrate (Lumigen) was poured onto the membrane for the detection of phosphorylated MAPKs in an imaging system (Bio-Rad).

2.7. Confirming eDNA as the active principle

In order to confirm that the effects observed were due to eDNA and not caused by impurities such as small RNAs or proteins, fragments of common bean DNA of less than 1000 bp were treated with DNase 1 (Invitrogen), RNase A (Invitrogen) or proteinase K (Thermo Fisher Scientific) or combination of these, according to product manual. The enzymes in the eDNA solution were deactivated before the use of it according to product manual. The activity of the nucleases was confirmed on a 3% agarose gel. Plants were treated with a solution of 200 μg mL^{-1} of nuclease- or proteinase-treated fragments in 0.05% (v v^{-1}) Tween 20 ($n = 9$ per treatment). The solution was applied with a micropipette on both sides of three randomly selected leaves of each plant until the surface was completely wet. Leaves treated with 0.05% Tween 20 were used as negative controls and leaves treated with eDNA fragments without nuclease were used as positive controls. Putative direct effects of the enzymes were tested by applied enzyme solutions to leaves. Two hours after the treatment, 10 discs of 1 cm in diameter were punched out of each leaf and H_2O_2 was quantified as indicated above ("Effect of eDNA on H_2O_2 accumulation"). Further plants were treated in the same manner and after 30 min of treatment, the leaves were excised and frozen in liquid nitrogen and used for the MAPK activation test as indicated above ("Effect of eDNA on the activation of MAPKs in plants and suspension cells culture").

2.8. Effect of eDNA on EFN secretion

In order to determine whether eDNA activates a late immunity-response in common bean, we quantified the EFN secreted by plants treated with eDNA. At 9:00 am the plant organs called stipules or extrafloral nectaries (that secreted the EFN) of 24 plants were washed with distilled water until there was no trace of EFN. After 1 h, the four youngest leaves of each plant were treated with 50 μg mL^{-1} of self eDNA or non-self eDNA fragments of less than 1000 bp in 0.05% Tween 20, applied with a 1-mL micropipette until both surfaces of the leaves were completely wet. Plants treated with 0.05% Tween 20 were used as controls. After 24 h, the EFN was quantified on extrafloral nectaries of each of the four youngest leaves. To quantify EFN, 10 μL of distilled water were applied to each of the four leaf nectaries using a micropipette by expelling and sucking up the water five times. The percentage of soluble solids in the EFN was measured using a portable refractometer (ATAGO®), and the total volume was measured directly from the refractometer with a graduated microcapillary tube. Next, the leaves were cut, oven-dried at 60 °C for 72 h, and weighed. The amount of EFN was reported as mg of soluble solids per g of leaf dry mass (Heil et al., 2000, 2001). To confirm the eDNA effect on EFN secretion, DNA fragments of common bean were treated with DNAse 1, RNAse A, proteinase K or combination of these, and applied to the four youngest leaves of each of six plants. Putative direct effects of the enzymes were tested by applied enzyme solutions to leaves. Plants treated with 0.05% Tween were used as controls. After 24 h, the EFN present in each of the four youngest leaves was quantified.

2.9. Effect of eDNA on immunity against phytopathogen

In order to test for induced immunity to a pathogenic bacterium, solutions of 200 μg mL^{-1} of self or non-self eDNA fragments in 0.05% (v v^{-1}) Tween 20 (control: pure Tween solution), were applied with a micropipette to both sides of the leaves of common bean plants until the surface was completely wet (seven plants per treatment). Five minutes after the treatment, the plants were inoculated by spraying 10 mL per plant with a suspension of *Pseudomonas syringae* (at 1×10^7 cells mL^{-1}, determined as optical density = 0.06 at 600 nm 5 in a GENESYS™ 20 spectrophotometer; Thermo Fisher Scientific Inc, NY, NY, USA). Seven plants were used per treatment, all the infection levels were quantified eight days after inoculation in one randomly selected leaf per plant. Leaf material was weighed and ground in a mortar with approximately 500 μL of sterile distilled water. The resulting liquid was decanted and completed to to 1.5 mL with sterile distilled water. Dilutions 1:10, 1:100 and 1:1000 were prepared from each sample and 20 μL of each dilution were plated on KB medium (B medium as described by (King et al., 1954) with rifampicin (100 μg mL^{-1}; Sigma Aldrich). After two days, bacterial colonies were counted to express infection rates as the colony forming units (CFUs) of *P. syringae* per g of fresh leaf mass. Putative direct effects of the eDNA solutions on *P. syringae* were tested by plating 100 μL of each of the eDNA or of the control treatment (Tween20® at 0.05%, n = 5 repetitions) on Petri dishes with KB medium with rifampicin. After 5 min, 20 μL of a 1:10 1:100, 1:1 000 or 1: 10,000 v/v dilution of 1×10^7 cells mL^{-1} *P. syringae* suspension were spread on the same plates. A group of n = 5 plates for each type of eDNA and the control treatments were left without inoculation. The colony forming units (CFU) in each Petri dish were counted two days later.

3. Results

Self eDNA inhibited the growth of the primary root (Fig. 1A) of common bean seedlings in a dosage-dependent manner. A significant inhibition was observed at a concentration of 50 μL mL^{-1} of self eDNA, but higher concentrations had a stronger effect (Fig. 1B). Based on these results, we selected the concentration of

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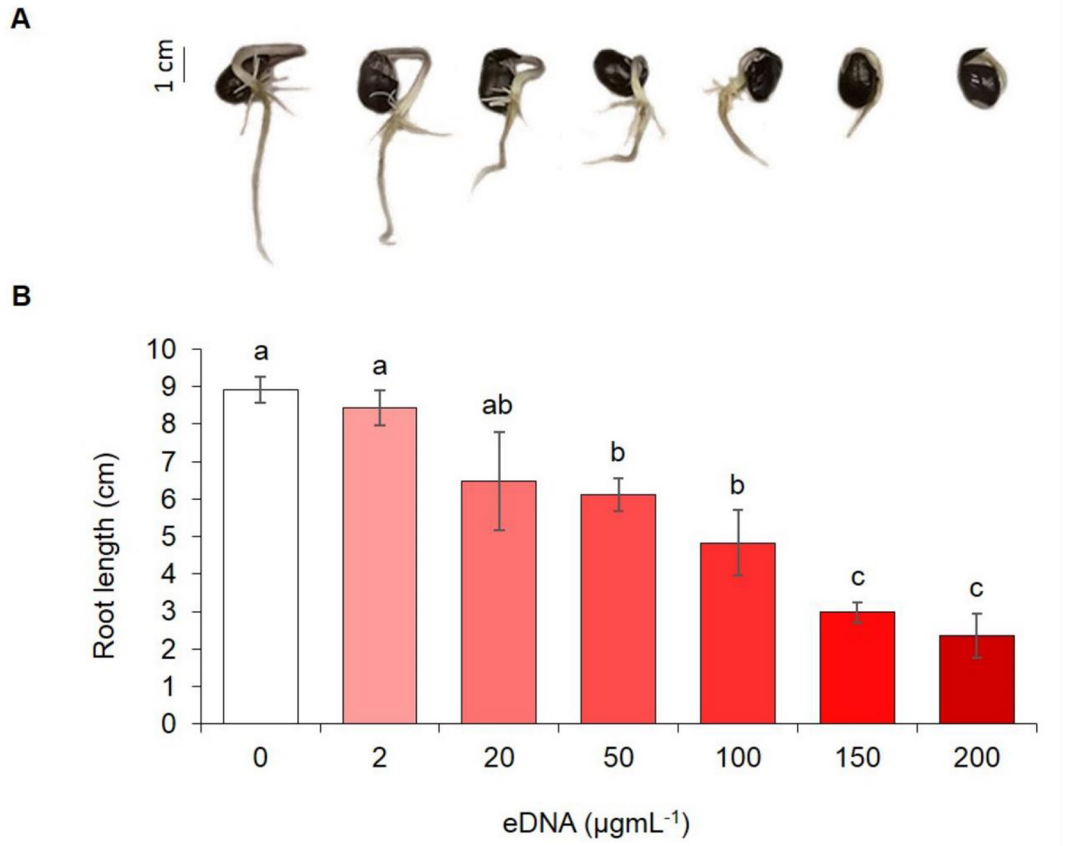

Fig. 1. Extracellular self-DNA (eDNA) inhibits root growth in a concentration-dependent manner. (A) The length of the primary root of common bean (*Phaseolus vulgaris*) seedlings after four days in germination medium containing different concentrations of self eDNA is depicted in (B) as mean ± SE. As the concentration of eDNA increases, the bars are depicted in a more intense red colour; the white bar represents the control (0 μg mL^{-1} of eDNA). Different letters above bars indicate significant differences among treatments (univariate ANOVA and *post hoc* Tukey test: $p < .05$, $n = 9$).

200 μg mL^{-1} for use in the subsequent experiments. The observed effect shows taxonomic specificity: self eDNA inhibited root growth most strongly, non-self eDNA from lima bean caused a weaker, but still significant effect, whereas non-self eDNA from acacia did not significantly inhibit the growth of the primary root (Fig. 2A and B).

The effect of eDNA on the formation of H_2O_2 in the leaves of common bean also showed taxonomic specificity. Self eDNA caused a significant (ca. three-fold) increase in H_2O_2, whereas non-self eDNA caused no statistically significant effect, in spite of a tendency towards enhanced H_2O_2 levels in response to the application of lima bean eDNA (Fig. 2C). The activation of MAPKs after mechanical damage to leaves was detectable at 1 min and strongest at 15 min, whereas the response to self eDNA was slightly slower (detectable at 5 min and strongest at 30 min, see Fig. 3A). The application of self eDNA to common bean cells in suspension culture revealed a similar temporal pattern (peaking at 30 min) with an overall stronger activation of MAPKs (Fig. 3A). Again, MAPKs responded to eDNA in a species-specific way. Self eDNA caused strongest activation of MAPKs (quantified at 30 min after the application of eDNA), non-self eDNA from lima bean caused a weaker, but detectable response, whereas we detected no response to non-self eDNA from acacia (Fig. 3B).

We used nucleases and a proteinase to control for putative effects of small RNAs or proteins, respectively, in the eDNA preparation and followed the activation of MAPKs (Fig.4A) and the formation of ROS (Fig.4B) in common bean plants. Whereas the treatment with RNAse or proteinase did not detectably affect the activation of MAPKs and the formation of H_2O_2 by self eDNA, no effects could be detected when the self eDNA had been treated with DNAse before its application (Fig.4B). When we used deactivated enzymes, no changes to the inducing effects were observed (data not shown).

We observed a significant induction of EFN in plants treated with self eDNA, but not in plants treated with non-self eDNA (Fig. 5A). Treating the self eDNA with RNAse or proteinase did not reduce its inducing effect on EFN secretion, whereas EFN secretion was not significantly induced by self eDNA that had been treated with DNAse (Fig. 5B).

Plants that were treated with either self or non-self eDNA exhibited significantly lower infection rates by *P. syringae* phytopathogen (Fig. 6). Nevertheless, infection rates in leaves treated with self eDNA were significantly lower than in leaves treated with non-self eDNA (Fig. 6). When we tested for putative direct effects of the eDNA solutions against *P. syringae,* no effect was observed (Data not shown).

Finally, we used cells in suspension culture to investigate the range of fragment sizes of eDNA that are active. An activation of MAPKs could be observed in response to fragments with lengths ranging from 350 to 700 bp and shorter than 350 bp, and the effect was quantitatively comparable to the effect observed after the application of the complete eDNA preparation (fragment sizes <1000 bp). By contrast, no detectable activation of MAPKs was detected after the application of fragments with lengths of 700–1000 bp (Fig. 7).

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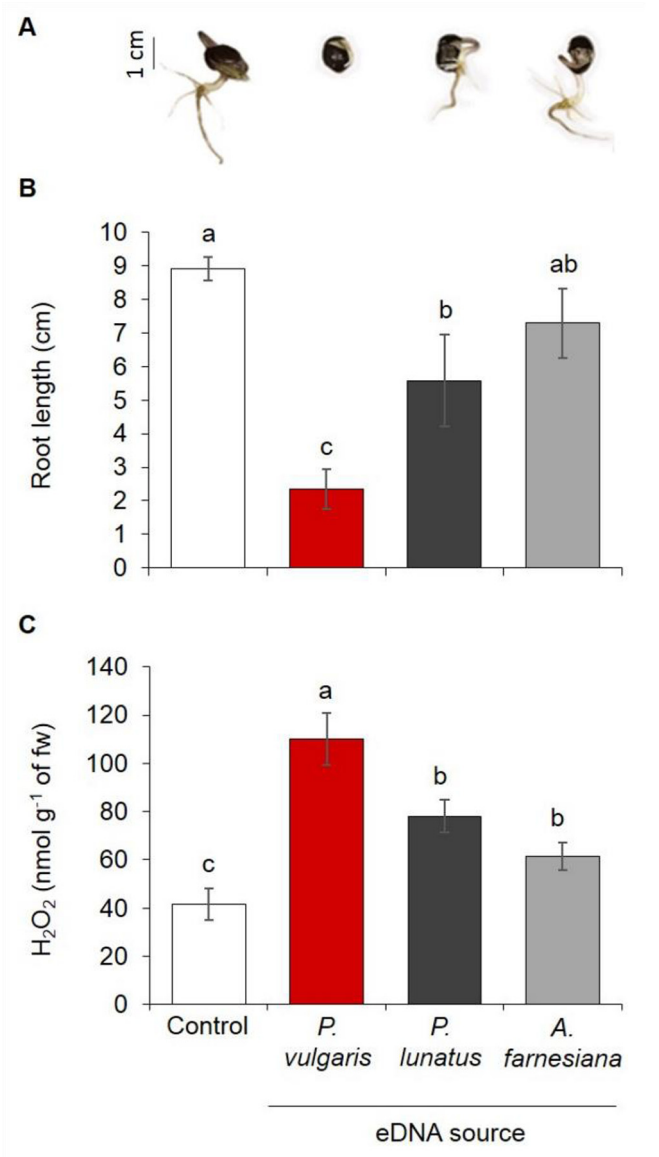

Fig. 2. Root growth and H_2O_2 generation are differently affected by self and non-self eDNA. (A) The length of the primary root of common bean (*Phaseolus vulgaris, P. vulgaris*) seedlings after four days in germination medium containing 200 μg mL^{-1} of eDNA is depicted in (B) as mean ± SE. (C) The concentration of H_2O_2 in nanomole per gram fresh mass 2 h after applying 200 μg mL^{-1} of eDNA is depicted as mean ± SE. White bars bar represent the control (0 μg mL^{-1} of eDNA), red bars represent self eDNA, grey bars represent non-self eDNA (from *Phaseolus lunatus* or *Acacia farnesiana*). Different letters above bars indicate significant differences among treatments (univariate ANOVA and *post hoc* Tukey test: $p < .05$, $n = 9$).

4. Discussion

4.1. Confirming eDNA as a DAMP in plants

In this study, we asked whether eDNA can act as a DAMP in plants and whether eDNA might contribute to self versus non-self discrimination during plant damaged-self recognition. Fragmented self eDNA induced four immunity-related traits in common bean in patterns that were similar to the reported effects of leaf homogenates (Duran-Flores and Heil 2014). All tested immunity-related traits responded more strongly to self eDNA than to non-self eDNA. For example, non-self eDNA from an acacia caused only a minor formation of ROS; non-self eDNA from lima bean had much weaker effects than self eDNA on the formation of ROS and the activation of MAPKs, self eDNA reduced infection by the bacterial pathogen significantly more than non-self eDNA (although all three types of eDNA strongly reduced the infection by the bacterium), and no type of non-self eDNA had a detectable effect on the secretion of EFN. Similarly, a growth-inhibition effect of eDNA that was reported in earlier studies (Mazzoleni et al., 2015a,b), depended on the taxonomic distance to the receiver.

It remains an open question whether growth inhibition by eDNA is causally related to its effect on immunity (Duran-Flores and Heil, 2015; Veresoglou et al., 2015). However, immunity-related responses in plants are often associated with a transient inhibition of growth (Yakushiji et al., 2009), because limited resources are allocated to immunity which in consequence are not available for further growth (Heil and Baldwin, 2002; Walters and Heil, 2007). In principle, this trade-off between growth and immunity in plants is equivalent to sickness behaviour: the reduction in many behavioural activities that is frequently shown by infected or heavily injured mammals, including humans. Furthermore, our findings complement a recent report on the depolarization of membranes and the influx of Ca^{2+} that was triggered by self eDNA in maize and lima bean (Barbero et al., 2016). In summary, our results support a role of eDNA as a DAMP in plants and are consistent with the hypothesis that eDNA can contribute to the species-specific discrimination of self versus non-self.

Differential effects of self eDNA versus non-self eDNA are frequently reported. For example, others compared eDNA from plants versus animals, bacteria or fungi (Mazzoleni et al., 2015b), eDNA from a bacterium versus a fish (Yakushiji et al., 2009), from salmon versus a mammal (Barton et al., 2006), from monocots versus dicots (Barbero et al., 2016), from species of the same plant family but different genera (Mazzoleni et al., 2015a,b) and, of course, the effects of bacterial or viral non-self eDNA versus mammalian self eDNA (McGlasson et al., 2017; Meller et al., 2015). However, we are not aware of a study that compared the immune responses to eDNA from two species in the same genus and conclude that our study reveals a higher taxonomic specificity in a response to eDNA than previously reported. It remains an open question whether this lack of reports indicates that mammalian cells respond less specifically to eDNA than plant cells or rather, that this possibility has never been considered. Testing the effects of non-human, mammalian DNA on human cells (e.g., comparing eDNA from monkeys to eDNA from humans) or similar scenarios seemingly was out of the scope of the immunological sciences. Therefore, our findings might have relevance for the research into the human immune system. For example, herring testis DNA, interferon-stimulatory DNA, or poly(dA:dT) are established tools to study receptors of retroviral double-stranded DNA (Altfeld and Gale, 2015; Gao et al., 2013). If mammalian cells possessed an as-yet overlooked species-specific response to eDNA, DNA fragments of non-viral origin would be insufficient to reveal the complete set of agonists that can interact with the mammalian eDNA sensors.

4.2. Caveats and open questions

Our observation of differential effects of eDNA preparations from closely related plant species (the *Phaseolus lunatus* genome is assumed to share ca. 98% of sequences with *Phaseolus vulgaris*; A. Herrera Estrella, pers. comm.) opens several questions. In general terms, the discrimination of self versus non-self nucleic acids has been suggested as a prerequisite to avoid auto-immunity (Barton et al., 2006). In this scenario, however, one would expect reduced responses to self eDNA, whereas we found the opposite effects. Moreover, we observed an induction of resistance to a bacterial pathogen as well as of extrafloral nectar, although these responses depend on two different signalling pathways that usually inhibit each other. In the following, we discuss three major

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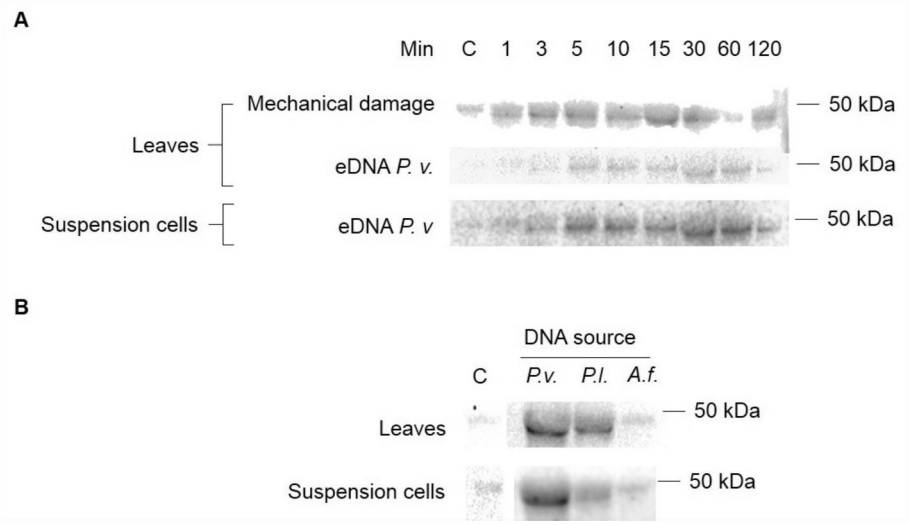

Fig. 3. Extracellular self-DNA (eDNA) activates mitogen-activated protein kinases (MAPKs). (A) The activation of MAPKs in leaves or suspension cells of common bean (*Phaseolus vulgaris*, *P. v.*) was tested at different times after treatment with 200 μg mL^{-1} of self-eDNA or mechanical damage (only leaves). Intact leaves and suspension cells treated with water served as controls (C). (B) The activation of MAPKs in leaves or suspension cells was tested 30 min after treatment with 200 μg mL^{-1} of self-eDNA (*P.v.*) or non-self eDNA (*Phaseolus lunatus, P.l.;* or *Acacia farnesiana*, *A.f.*). The experiments were repeated three times with similar results.

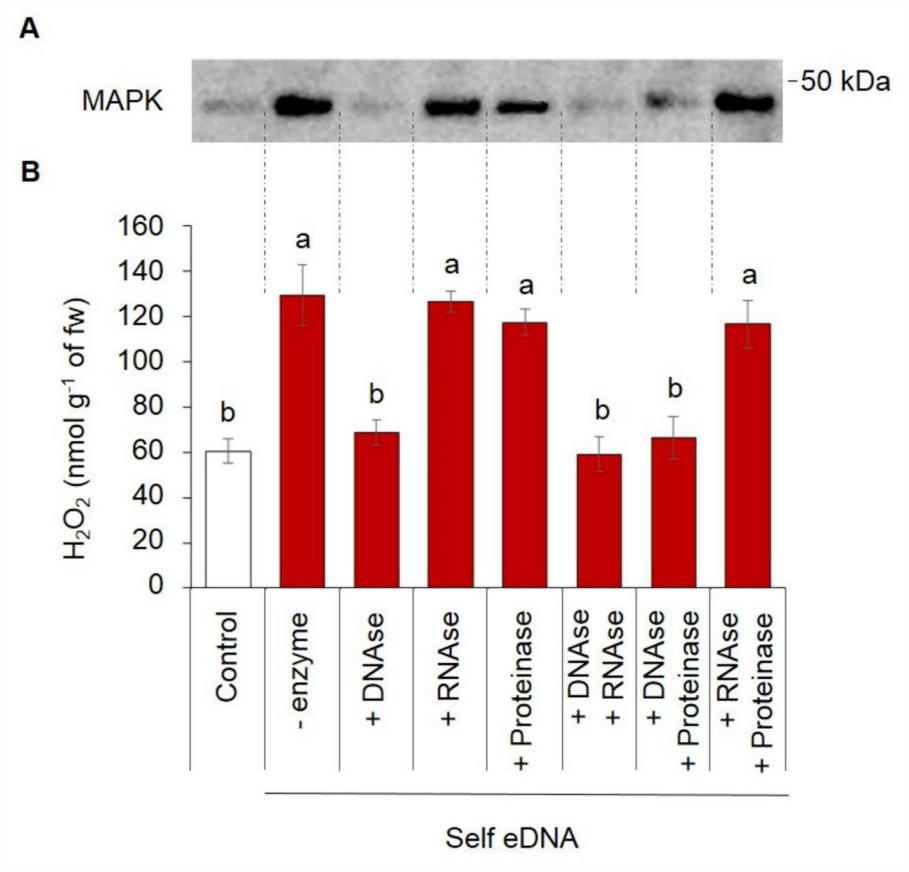

Fig. 4. Digestion of self eDNA eliminates its resistance-inducing effects. Extracellular DNA from common bean (*Phaseolus vulgaris*) was treated with DNAse, RNAse, proteinase, or combinations, and applied to common bean leaves. (A) The activation of MAPKs was tested 30 min after applying 200 μg mL^{-1} of eDNA fragments. The experiment was repeated three times with similar results. (B) The concentration of H_2O_2 in nanomole per gram of fresh mass 2 h after applying 200 μg mL^{-1} of DNA fragments is depicted as mean ± SE. The control treatment (C) consisted of the application of a solution of 0.05% (v/v) Tween 20. Different letters above bars indicate significant differences among treatments (univariate ANOVA and *post hoc* Tukey test: $p < .05$, $n = 9$).

questions that might serve as guidelines for future research. First, what is the ecological or evolutionary relevance of a species-specific recognition of eDNA? Second, are there alternative explanations that remain to be tested? Third, how is eDNA recognized in plants and how similar are the respective mechanisms among plants and mammals?

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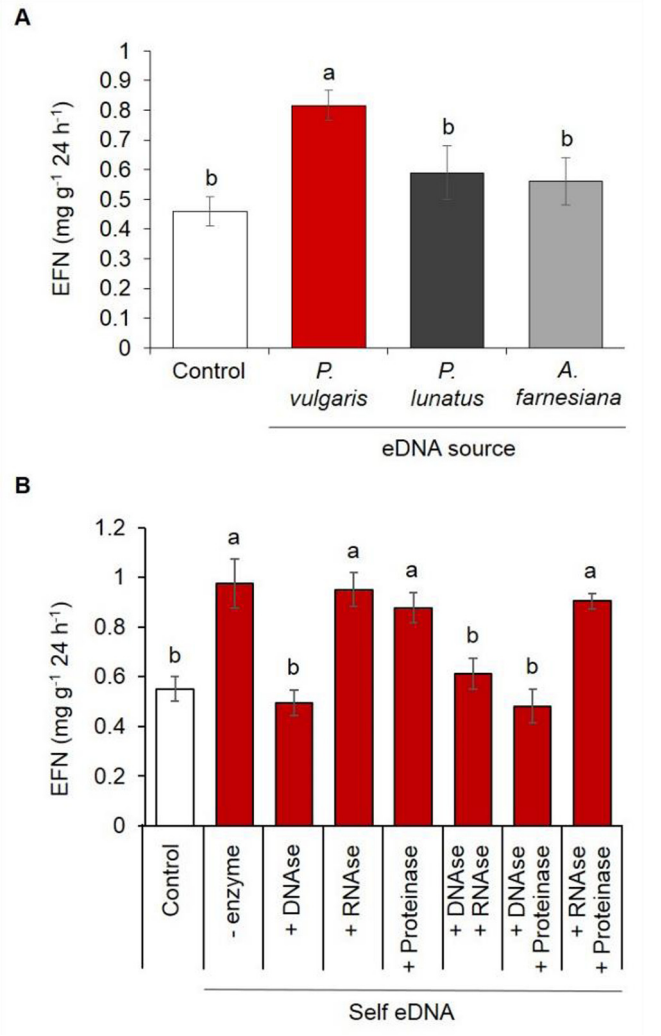

Fig. 5. Extracellular self-DNA induces EFN secretion (A) The EFN secretion by common bean (*Phaseolus vulgaris*) plants is depicted as mean ± SE of mg of soluble solids per gram of dry leaf mass quantified 24 h after treatment with 50 μg mL^{-1} of self eDNA (*P. vulgaris*) or non-self eDNA (*Phaseolus lunatus* or *Acacia farnesiana*). (B) The EFN secretion by common bean after treatment with 50 μg mL^{-1} of self eDNA to which DNAse, RNAse, proteinase or combination of these, had been added. Controls were treated with a solution of 0.05% (v/v) Tween 20. Different letters above bars indicate significant differences among treatments (univariate ANOVA and *post hoc* Tukey test: $p < .05$, $n = 6$).

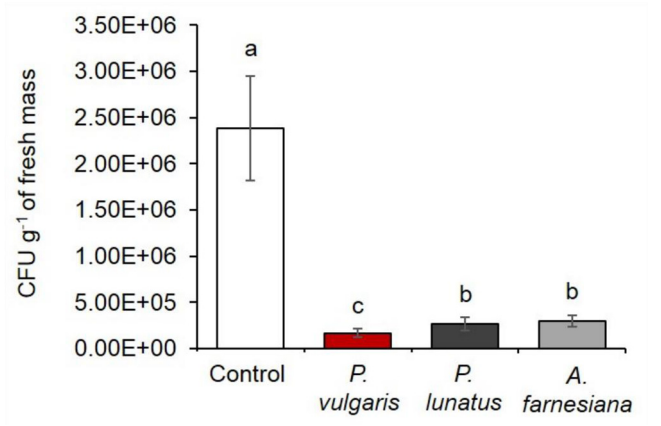

Fig. 6. Extracellular DNA reduces the infection by the bacterium, *P. syringae*. Numbers of colony forming units (CFU) per g of *Phaseolus vulgaris* leaf fresh mass are depicted as mean ± SE. Plants had been treated with self (red bar) or non-self eDNA (grey bars, from *Phaseolus lunatus* or *Acacia farnesiana*), controls were treated with a solution of 0.05% (v/v) Tween 20. Different letters above bars indicate significant differences among treatments (univariate ANOVA and *post hoc* Tukey test: $p < .05$, $n = 7$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

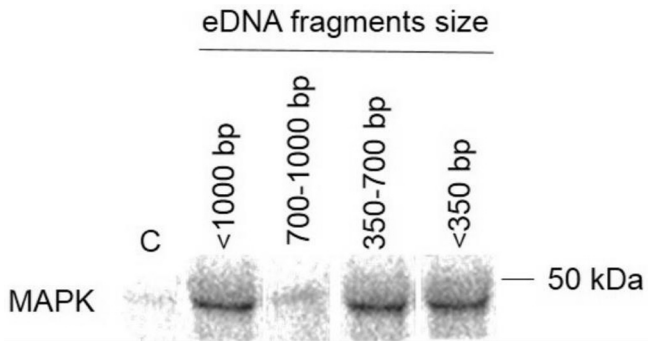

Fig. 7. Small extracellular DNA fragments activate MAPKs in common bean. Sonicated self eDNA was separated on 3% agarose gels and fragments were re-extracted from regions corresponding to different size ranges (<1000 bp, 700–1000 bp, 350–700 bp or <350 bp) and applied at 200 μg mL^{-1} of eDNA to suspension culture cells of *Phaseolus vulgaris*. The activation of MAPKs was tested after 30 min. Water was used as the control treatment (C). The experiment was repeated three times with similar results.

4.2.1. Relevance in natural settings

Are our observations likely to reveal a process of relevance for plant immunity in nature? The experimental conditions undoubtedly were highly artificial, and we are not aware of reports on an active export of DNA from infected or dying plant cells, as it is known from mammals (Takahashi et al., 2017; Toussaint et al., 2017, and references cited therein). However, tissue disruption inevitably releases DNA into the extracellular space (Duran-Flores and Heil 2016). Chewing herbivores in particular continuously disrupt plant cells during feeding, and they regurgitate a part of their gut content into the feeding site (Duran-Flores & Heil 2016). Necrotrophic pathogens secrete a plethora of lytic enzymes to kill plant cells (Mengiste, 2012), and the plant hypersensitive response to biotrophic pathogens (Stotz et al., 2014) represents an immunity-related programmed cell death, equivalent to apoptosis, necroptosis or NETosis: important sources of eDNA in mammals (Hanson, 2016; Kaczmarek et al., 2013;Toussaint et al., 2017). Moreover, eDNA is a common component of biofilms that are formed by pathogenic bacteria (Möllerherm et al., 2016), including plant pathogens (Tran et al., 2016). Thus, the presence of eDNA in plant tissues occurs in multiple natural situations in which plants require an adequate immunity response.

Nevertheless, plants usually don't predate on each other, a fact that causes doubts concerning the selective advantages of a specific recognition mechanism. One possibility is that, due to the abundance of eDNA in soil and litter, discriminating exogenous eDNA from wound-derived self eDNA would allow to restrict the immune responses to the perception of the latter (M. Schuman, personal communication). Furthermore, eDNA induced phenotypic resistance traits that depend on two independent signalling pathways: the salicylic acid pathway controls resistance to biotrophic pathogens like *P. syringae*, whereas the jasmonate signalling cascade that controls plant defence against chewing herbivores, including extrafloral nectar secretion. Since these two pathways usually inhibit each other, our findings indicate the possibility that eDNA triggers resistance via an additional, as yet unknown mechanism. Therefore, the plant response to eDNA should be further studied,

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e.g. by analysing the transcriptomic changes that are triggered by eDNA.

4.2.2. Alternative explanations

Which alternative explanations for our results remain to be excluded? Protocols for the extraction and purification of DNA are not optimised for the complete removal of other molecules, and leaf homogenates contain a complex mixture of DAMPs, including cell wall fragments, eATP, fructans, peptides, or RNA (Duran-Flores and Heil 2015; Heil 2009; Versluys et al., 2017). In fact, eRNA from both the self and the non-self triggers plant immunity responses (Barbero et al., 2016; Lee et al., 2016; Paungfoo-Lonhienne et al., 2010; Wen et al., 2009; Yakushiji et al., 2009). We tried to control for possible effects of RNA or peptides by treating our eDNA preparation with RNase, DNase and proteases. DNase 1 is secreted from cells in animals and plants to degrade eDNA that leaked from dying cells (Hawes et al., 2015). At the experimental level, DNase is frequently used to support, e.g., the recognition of eDNA by a specific receptor (Barton et al., 2006), the role of eDNA in bacterial biofilm formation in vitro (Okshevsky et al., 2015), or its contribution to allergic and immune responses (Toussaint et al., 2017). In our experiments, DNase treatment completely abolished the inducing properties of our eDNA preparation, whereas RNase and protease had no effect. These observations are fully consistent with eDNA being the active principle.

However, future studies will have to control for a possible role of DNA-binding peptides and proteins, which act as DAMPs in mammals. For example, HMGB 1–3 are chromatin proteins that act as DAMPs when appearing in the extracellular space (Klune et al., 2008), and complexes formed by DNA and HMGB1 have stronger pro-inflammatory and immunomodulating effects than the pure molecules (Jounai et al., 2013; Tang et al., 2012). Similarly, host defence peptides – short, cationic amphipathic peptides with direct antimicrobial activity – can bind to eDNA and facilitate its uptake into host cells (Hancock et al., 2016; McGlasson, 2017McGlasson, 2017; and references therein). Thereby, these peptides can enhance the pro-inflammatory effects of eDNA (Hancock et al., 2016), e.g. via a stimulation of CD4+ T cells (Toussaint et al., 2017), and contribute to the differential responses to bacterial (non-self) and mammalian (self) eDNA (Takaoka et al., 2007). Unfortunately, as long as we do not know how plants sense eDNA (Bhat and Ryu 2016), it is difficult to optimise the protocols for the preparation of eDNA for the plant sciences.

4.2.3. What can we learn concerning eDNA recognition in plants?

Research over the last years revealed that plants and mammals share several DAMPs and downstream signalling cascades, but it remains an open question to which degree these similarities represent homologies or analogies (Heil et al., 2016). How similar are our observations to the reported effects of eDNA in mammals, and what can we learn concerning a putative recognition mechanism in plants? In contrast to mammalian cells, plant cells are surrounded by a cell wall, although the hydrophilic nature of this compartment and the network-like structures formed by the major structural macromolecules (lignin and cellulose) make it unlikely that the cell wall represents an obstacle to eDNA mobility. By contrast, larger fragments of DNA are less likely to pass membranes and at least in mammals, the re-uptake of eDNA into living cells is critical for its recognition, because mammalian DNA receptors are located within the cell (Desmet and Ishii, 2012; Gallucci and Maffei, 2017; Hornung et al., 2009; Schlee and Hartmann 2016; Takaoka et al., 2007). Accordingly, 25-bp fragments of a nuclease-resistant analogue of DNA were taken up by Arabidopsis root cells (Paungfoo-Lonhienne et al., 2010), endocytosis inhibitors significantly reduced the immunity-inducing activity of bacterial eDNA in Arabidopsis (Yakushiji et al., 2009), and only fragments <700 bp in length caused significant effects on various immunity-related responses (this study, and Barbero et al., 2016). All these observations make it tempting to speculate that the effects of eDNA on the plant immune system also require its uptake into living cells.

Toll-like receptors are central players in the recognition of eDNA in mammals and sequence-dependent as well as sequence-independent mechanisms contribute to the specificity in the recognition process. Recent studies identified an unmethylated cyto sine–phosphate–guanine (CpG) dideoxynucleotide motif as crucial for the recognition of viral or bacterial DNA by TLR9 (Ohto et al., 2015). Interestingly, the recognition of bacterial eDNA in Arabidopsis required the same motif (Yakushiji et al., 2009). However, TLRs have not been described for plants (Couto and Zipfel, 2016). In plants, PAMPs and DAMPs are mainly recognised via receptor-like kinases. Leucine-rich repeat (LRR)-containing pattern recognition receptors (PRRs) preferentially bind proteins or peptides such as bacterial flagellin (a PAMP) or endogenous AtPep peptides (DAMPs). However, the nucleotide-binding leucine-rich repeat protein (Rx NLR) of potato also binds nucleic acids, with similar preferences for single-stranded and double-stranded DNA (Fenyk et al., 2016). This low degree of specificity makes it unlikely that this receptor allows for a species-specific recognition of eDNA. Further receptors of DAMPs in plants comprise lectin-type PRRs, which bind extracellular ATP, and PRRs with epidermal growth factor (EGF)-like ectodomains, which recognize plant cell-wall fragments (Couto and Zipfel, 2016).

Besides epigenetic or sequence-dependent motifs, DNA recognition in mammals can depend on fragment length, and self versus non-self discrimination is partly achieved via the localization of the respective receptors at the subcellular level (Schlee and Hartmann, 2016). As an alternative, receptor-independent explanation for the specificity of the effects of eDNA on plants, Mazzoleni et al. (2014) Duran-Flores and Heil (2015) speculated that fragments of eDNA, after their uptake into intact cells, could bind to mRNA or to proteins and thereby interfere with essential biological processes, such as transcriptional or enzymatic activities. In short, we are not aware of any report on a plant receptor that recognizes DNA with a level of sequence-specificity that could explain our observations, and the mechanisms that underlie the species-specific responses of plant cells to eDNA remain matter of speculation.

5. Conclusions

Fragments of self eDNA triggered various immunity-related responses in bean plants and the effects of self versus non-self eDNA were species-specific. Non-self eDNA triggered significantly lower responses, or no responses at all, even when obtained from a congeneric plant. To the best of our knowledge, this level of taxonomic specificity in the effects of eDNA has not been reported so far. We suggest that eDNA plays a role as a DAMP in plants and that the plant and the mammalian immune system might share more common elements than it is currently appreciated. However, future work will be required to understanding the selective benefits of a species-specific discrimination of self eDNA from non-self eDNA and to identify the molecular mechanisms that allow for this degree of specificity.

Acknowledgments

We thank Meredith Schuman, Luis Herrera-Estrella, Alfredo Herrera-Etrella, Polly Matzinger, Nico Geldner and many other colleagues for insightful comments on our observations, Johannes Stratmann, Rosa-María Adame-Alvarez and José-Luis Hernández-

Flores for technical help and Caroline Woods for correcting an earlier version of this manuscript. We thank CONACYT de México for financial support (Grant 258119 to MH and Grant 394371 to DDF).

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Please cite this article in press as: Duran-Flores, D., Heil, M. Extracellular self-DNA as a damage-associated molecular pattern (DAMP) that triggers self-specific immunity induction in plants. Brain Behav. Immun. (2017), https://doi.org/10.1016/j.bbi.2017.10.010

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