CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

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El papel del ADN como patrón molecular asociado a daño (DAMP) en la especificidad del auto-reconocimiento de daño en frijol (*Phaseolus vulgaris*)

Tesis que presenta

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Role of DNA as damage-associated molecular pattern (DAMP) in the specificity of damaged-self recognition in bean (*Phaseolus vulgaris*)

Thesis presented by

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
AOS	Allene oxide synthase
bp	Base pairs
BSA	Bovine serum albumin
CAT	Catalase
CFUs	Colony forming units
cGAS	Cyclic-GMP-AMP (cGAMP) synthase
CpG	Cytosine–phosphate–guanine
DAI	DNA-dependent activator of IFN-regulatory factors
DAMPs	Damage-associated molecular patterns
DAPI	4’,6-Diamidino-2-phenylindole dihydrochloride
eATP	Extracellular ATP
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eRNA	Extracellular RNA
H_2O_2	Hydrogen peroxide
HAMPs	Herbivore-associated molecular patterns
IAA	Indol-3-acetic acid
IC	Isochorismate
ICS	Isochorismate synthase
IFNs	Interferons
IRF	Interferon regulatory factor
JA	Jasmonic acid
JA-Ile	Jasmonic acid-isoleucine conjugate
LA	Linolenic acid
LOX	Lipoxygenase

LRR	Leucine-rich repeat
MAPKs	Mitogen-activated protein kinases
MeSA	Methyl salicylate
MS	Murashige and Skoog
NAA	α-naphtaleneacetic acid
NIB	Nuclei isolation buffer
OPDA	12-oxo-phytodienoic acid
OPR	Reductase
PAL	Phenylalanine ammonia-lyase
PAMPs	Pathogen-associated molecular patterns
Phe	Phenylalanine
PI	Proteinase inhibitors
POX	Peroxidases
PR	Pathogenesis-related
PRRs	Pattern recognition receptors
PRRs	Recognition receptors
PYD	Pyrin domains
RLKs	Receptor-like kinases
RLPs	Receptor-like proteins
ROS	Reactive oxygen species
SA	Salicylic acid
***t*-CA**	*trans*-cinnamic acid
TF	Transcription factors
TLRs	Toll-like receptors
VOCs	Volatile organic compounds
WIPK	Wound-induced protein kinase

ABSTRACT

In nature, plants are threatened by bacteria, fungi and herbivores. All of these enemies have different lifestyles and infection strategies and thus, they can cause different types of damage to the plant. Plants can detect the kind of enemy through the perception of pathogen- or herbivore- associated molecular patterns (PAMPs or HAMPs), whereas cell disruption is detected through the perception of damage-associated molecular patterns (DAMPs): endogenous indicators of injury that trigger resistance responses, from the activation of early signaling pathways through phenotypic resistance traits. Interestingly, the application of leaf homogenates –which arguably contain a blend of DAMPs– can cause species-specific responses in plants. However, the active principle that causes this specificity remained unknown. Based on the fact that extracellular DNA (eDNA) causes a species-specific growth inhibition in plants and other organisms and that mammals sense self and non-self eDNA as DAMP or PAMP, respectively, I hypothesized that eDNA acts as a DAMP in plants and that it contributes to self- versus non-self discrimination. I exposed plants and suspension-cultured cells of common bean (*Phaseolus vulgaris*) to fragmented eDNA of common bean (self eDNA), lima bean and acacia (*Phaseolus lunatus* and *Acacia farnesiana*; non-self eDNA) and quantified the responses of several resistance-related traits. Self eDNA induced early (H_2O_2 generation and MAPK signaling) and late (jasmonic and salicylic acid) signaling responses and, consecutively, the phenotypic defense against several types of enemies. Plants treated with self eDNA exhibited an enhanced indirect defense against herbivores (extrafloral nectar secretion) and an enhanced direct defense against the larva *Spodoptera frugiperda*, and they exhibited decreased levels of infection by pathogenic bacteria (*Enterobacter* sp*, Pseudomonas syringae* pv*. phaseoli and* pv*. syringae* and *Xanthomonas phaseoli)* and fungi (*Sclerotinia sclerotiorum*, *Fusarium oxysporum* and *Botrytis cinerea*). All these enhanced traits resulted in an increase of yield on field conditions. By contrast, non-self DNA had significantly lower or no detectable effects on the same traits. Only fragments below 700 bp were active and treating the eDNA preparation with DNAse abolished its inducing activity, whereas treatment with RNAse or proteinase had no effect; indicating that small RNAs, single nucleotides or proteins in the applied eDNA preparation did not account for the observed effects. However, treatments with self eDNA previously digested with CpG methylation-sensitive and non-sensitive indicated an influence of non-methylated CpG sites in the observed effects. I conclude that self eDNA functions a DAMP in plants and that plants can discriminate self from non-self eDNA, even among eDNA preparations that stem from species in the same genus. Further studies will be required to understand the species-specific action of eDNA in plants and its specific role in plant damaged-self recognition.

RESUMEN

En la naturaleza, las plantas se enfrentan a diversos enemigos, entre ellos, bacterias, hongos y herbívoros, con diferentes estilos de vida y estrategias de infección y, por lo tanto, pueden causarle diferentes tipos de daño. Las plantas detectan el tipo de enemigo por medio de la percepción de patrones moleculares asociados a patógenos o herbívoros (PAMPs o HAMPs, por sus siglas en inglés), mientras que la ruptura celular se detecta a través de la percepción de patrones moleculares asociados a daños (DAMPs): indicadores endógenos de lesión que provocan respuestas, desde la activación de vías de señalización tempranas hasta rasgos de resistencia fenotípicos. La aplicación de homogeneizados de hojas de una especie, que posiblemente contienen una mezcla de DAMPs, causa respuestas relacionadas con la resistencia en plantas de la misma especie. Aún se desconoce el principio activo que causa esta especificidad. Sin embargo, el ADN extracelular (ADNe) propio de la especie causa una inhibición de crecimiento en plantas y otros organismos y los mamíferos perciben el ADNe propio y no propio como DAMP o PAMP, respectivamente, y responden activando su sistema inmune. Se hipotetizó que el ADNe actúa como DAMP y contribuye al auto-reconocimiento en plantas. Se expusieron plantas y células cultivadas en suspensión de frijol común (*Phaseolus vulgaris*) al ADNe fragmentado del frijol común (ADNe propio), frijol lima y acacia (*Phaseolus lunatus* y *Acacia farnesiana*; ADNe no propio) y se cuantificaron varias respuestas relacionadas con la resistencia. El eDNA propio indujo respuestas de señalización temprana (generación de H_2O_2 y activación de MAPK) y de señalización tardía (Ácido jasmónico y ácido salicílico), aumentó la resistencia indirecta contra herbívoros (secreción de néctar extrafloral) y la defensa directa contra larvas de *Spodoptera frugiperda,* y disminuyó la infección por patógenos bacterianos (*Enterobacter sp., Pseudomonas syringae pv. Phaseoli y pv. Syringae y Xanthomonas phaseoli*) y fúngicos (*Sclerotinia sclerotiorum, Fusarium oxysporum y Botrytis cinerea).* Todo esto dio como resultado un aumento en el rendimiento de semilla en condiciones de campo. Por el contrario, el ADN no propio tuvo efectos significativamente menores o no detectables en los mismos rasgos. Solo los fragmentos menores a 700 pb fueron activos y el tratamiento del ADN con ADNasa eliminó su actividad inductora, mientras que el tratamiento con ARNasa o proteinasa no tuvo efecto; lo que indica que el ARN, los nucleótidos individuales o las proteínas en la solución de ADN aplicada no influyen en los efectos observados. Sin embargo, los tratamientos con ADNe propio previamente digerido con enzimas sensibles y no sensibles a sitios CpG metilados indicaron una influencia de los sitios CpG no metilados en los efectos observados. Se concluyó que el ADN funciona como DAMP en plantas y que éstas pueden discriminar entre ADN propio y no propio. Se requirieren más estudios para comprender cómo funciona esta discriminación y su papel específico en el auto-reconocimiento de daño en plantas.

1 INTRODUCTION

1.1 Plant resistance-related traits

In plants, mechanical damage or wounding can occur as a result of abiotic factors such as rain or wind, but much more commonly, plants are damaged by their natural enemies: herbivores and pathogens. The different groups of plant enemies have different lifestyles and infection strategies and, thus, can cause different types of damage to the plant. Therefore, plants have evolved to detect damage (Green & Ryan, 1972) and to defend themselves via the expression of resistance-related traits that reduce herbivore or pathogen survival, reproduction, or preference for a plant, and thereby enhance the plant´s fitness in the presence of natural enemies (Karban & Baldwin, 1997). However, due to the costs of defense (Herms & Mattson, 1992), plants cannot continuously maintain defenses against all types of enemies but express defense upon attack. Therefore, the complete spectrum of 'resistance-related traits' involve early and late signaling pathways as well as phenotypic traits that directly interact with the enemy or that act indirectly, e.g. via an attraction of the natural enemies of the plant enemy (Heil, 2008; Heil & Ton, 2008). Moreover, induced defense responses can occur locally, i.e. only in the damage tissue, or as a systemic response, i.e. in undamaged organs that are at long distance from the initial damage (Schilmiller & Howe, 2005).

1.1.1 Early signaling

The first, 'early signaling' steps in plants can be observed within a time range from a few seconds to a few hours, after attack by enemies or mechanical damage (Bricchi *et al.*, 2010). Among the earliest responses to a wide type of damage are the depolarization of membranes (Thain *et al.*, 1995; Maffei *et al.*, 2004), followed by the elevation of intracellular levels of calcium as a result of changes in calcium influxes and mitogen-activated protein kinases (MAPKs). Calcium it is a second messenger that is involved in

numerous signaling actions in all eukaryotes (Dodd *et al*., 2010; Steinhorst and Kudla, 2014). In plants, the discrimination among various stimuli can result from the generation of 'calcium signatures', which shape downstream local and systemic resistance responses (Sanders *et al.*, 2002; Yuan *et al.*, 2017). Among others, changes in calcium influxes are a critical detonator of the oxidative burst, which occurs in form of the accumulation of so-called reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide ion O_2^{2-} in mitochondria, chloroplasts and peroxisomes; and even on the external surfaces of plasma membranes (Kimura *et al.*, 2012). Central enzymes involved in the formation of ROS are NADPH oxidases, peroxidases (POX) and catalase (CAT) (Minibayeva *et al.*, 2015). In plants, ROS formation has been recognized in plant-pathogen interactions (Lamb & Dixon, 1997; O'Brien *et al.*, 2012), in herbivory-induced responses (Bi & Felton, 1995) and after mechanical wounding (Orozco-Cardenas & Ryan, 1999). Specifically, H_2O_2 is associated with a wound healing effect and necrosis and direct resistance against pathogens (Bajji *et al.*, 2007).

As mentioned for ROS, MAPKs signaling is a well-conserved pathway in all eukaryotes that regulates various cellular processes through their activation by phosphorylation (Ausubel, 2005) and that occurs early after attack by plant enemies (Hettenhausen *et al.*, 2015) In plants, many studies have demonstrated the critical roles of MAPKs and their homologues (wound-induced protein kinase; WIPK) in plant resistance against pathogens and herbivores, but also in response to mechanical damage (Nakagami *et al.*, 2005; Hettenhausen *et al.*, 2015).

1.1.2 Late signaling

'Late signaling' in plants occurs from a few minutes to a few hours (or until days) after attack by enemies or mechanical damage (Bricchi *et al.*, 2010). The mayor players of this response are the phytohormones jasmonic acid (JA) and salicylic acid (SA) (Choi *et al*., 2016a). Upon damage, JA is synthesized in chloroplasts and peroxisomes via the octadecanoid pathway,

which starts with the hydrolysis of chloroplast membrane lipids by phospholipase A to release free linolenic acid (LA). Via a series of reactions catalyzed by enzymes localized in chloroplasts (lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), LA is further converted to 12-oxo-phytodienoic acid (OPDA). After being transported to peroxisomes, OPDA is catalyzed by an OPDA reductase (OPR) and after three steps of β-oxidation, JA is formed (Wasternack, 2007). Ultimately, the JA-isoleucine conjugate (JA-Ile) is formed from JA and Ile: JA-Ille has been identified as the molecule that activates the majority of JA-induced molecular responses (Staswick & Tiryaki, 2004), such as genes encoding proteinase inhibitors (PI) or the toxic protein thionin, among many others (Creelman & Mullet, 1997; Heil, 2012). JA induce direct resistance traits such as trichomes and phenolic compounds in leaves and roots (Feng *et al.*, 2012), but also it is involved on the induction of indirect resistance responses (that attract beneficial insects to the plant) such the emission volatile organic compounds (VOCs) (Thaler, 1999; Kessler *et al.*, 2004) or the extrafloral nectar (EFN) secretion (Heil *et al.*, 2001; Radhika *et al.*, 2010). Endogenous level of JA depends on the damage level and responds locally and systemically (Peña-Cortés *et al.*, 1995; Heil *et al.*, 2012). Ultimately, the JA-triggered responses enhance the resistance against chewing herbivores (Thaler, 1999) and against those pathogens which derive nutrients from dead or dying cells, called necrotrophic pathogens (Kniskern *et al.*, 2007; Aleandri *et al.*, 2010; Pieterse *et al.*, 2012).

The SA pathway is primarily activated in response to biotrophic pathogens or insects causing little damage, such as phloem-feeding aphids and spider mites (Pieterse *et al.*, 2012). Plants utilize the isochorismate (IC) and the phenylalanine ammonia-lyase (PAL) pathways to synthesize SA, although neither route for SA biosynthesis is completely understood (Dempsey & Klessig, 2017). It is known that PAL converts phenylalanine (Phe) to *trans*-cinnamic acid (*t*-CA). Depending on the plant species, *t*-CA is converted to SA via the intermediates *ortho*-coumaric acid or benzoic acid (BA). The conversion of BA to SA probably occurs via BA 2-hydroxylase. The synthesis of SA via the IC pathway involves isochorismate synthase (ICS) and isochorismate pyruvate lyase, which converts

isochorismate to SA and pyruvate. However, no plant gene corresponding to isochorismate pyruvate lyase has been identified in plants. Following its synthesis, ICS is imported to the chloroplast stroma, where SA synthesis occurs (Strawn *et al.*, 2007; Dempsey & Klessig, 2017). Then, SA is transported to the cytosol and consecutively activates resistance responses, e.g. via the activation of genes encoding pathogenesis-related (PR) proteins, some of which have antimicrobial activity (van Loon *et al.*, 2006).. Once in the cytoplasm, SA can be methylated generating methyl SA (MeSA). MeSA is a phloem-mobile signal that travels from the infected leaf to the systemic tissues, where it activates resistance following its conversion back to SA (Park *et al.*, 2007).

Several reports suggest overall negative interactions between JA and SA in defense signaling. Exogenous application of SA suppresses the expression of JA biosynthesis genes, suggesting that SA may target the JA biosynthesis pathway to suppress downstream JA signaling, and vice versa, JA suppresses SA synthesis (Pena-Cortés *et al.*, 1993; Doares *et al.*, 1995a; Spoel & Dong, 2008). However, synergistic interactions between JA and SA pathways have been also observed in plant resistance against herbivores and have been suggested to strongly depend on concentration and timing (Mur *et al.*, 2006).

1.1.3 Phenotypic responses

At the phenotypic level, damage induces diverse resistance-related responses. Among these, the formation of physical barriers such as callose, lignin and suberin prevents the potential pathogenic microbe penetration and even the spread of viruses (Vance *et al.*, 1980; Hawkins & Boudet, 1996; Chen & Kim, 2009; Benitez-Alfonso *et al.*, 2011; Bellincampi *et al.*, 2014; Vishwanath *et al.*, 2015). Further direct defense responses comprise secondary metabolites such as toxins, phytoalexins and volatile organic compounds (VOCs) and proteins such as inhibitors of insect digestive enzymes, all of which directly affect the physiology and/or behavior of pathogens or herbivores (Rempt & Pohnert, 2010; Scala *et al.*, 2013; Maag *et al.*, 2015; Quintana-Rodriguez *et al.*, 2015; Kumar-Meena *et al.*, 2017).

However, VOCs released from damaged tissue can also attract beneficial organisms which parasite herbivores and then act as indirect defense trait, (Turlings *et al.*, 1990; Whitman & Eller, 1990; De Moraes *et al.*, 1998) similar to EFN (Koptur, 1992; Heil, 2011; Heil, 2015).

1.1.3.1 Extrafloral nectar (EFN)

Extrafloral nectar is an aqueous solution of sugars, amino acids, lipids and other organic compounds (Koptur, 1992; Heil *et al.*, 2000; González-Teuber & Heil, 2009) that is secreted by extrafloral nectaries (i.e., nectar secreting glands that are mostly located outside the flowers) and is not involved in pollination (Figure 1) (Bentley, 1977). Extrafloral nectaries have been reported from plants in at least 300 genera (Heil, 2009; Weber et al., 2015 http://www.extrafloralnectaries.org). Many studies have shown that EFN plays an important role in plant defense against herbivores (Koptur, 1992; Heil, 2011; Heil, 2015). Ants prefer to feed on plants with extrafloral nectaries (Barton, 1986; Oliveira *et al.*, 1999), and most ant and wasp species act as predators or defend the EFN-bearing parts of the plant against other insects, thereby reducing the number of herbivores and hence the damage caused by herbivores (O'Dowd, 1979; Oliveira *et al.*, 1987; Torres-Hernández *et al.*, 2000). The secretion of EFN is regulated via the octadecanoid pathway and thus, it can be induced by natural or mechanical damage and exogenous JA (Heil *et al.*, 2001; Heil, 2011).

Figure 1. Extrafloral nectar (EFN) of common bean. EFN is secreted by nectaries located on the stipules.

1.2 Perception of dangerous signals in plants through damaged-self and non-self recognition

The necessity to activate wound sealing, resistance, and tissue repair, can be perceived via two major pathways. 'Damaged-self recognition' is mediated via the perception of damage-associated molecular patterns (DAMPs) (Heil, 2009), whereas 'non-self recognition' is mediated via the perception of herbivore-associated molecular patterns (HAMPs) in the case of herbivory, and by microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) in the case of infection by pathogens (Janeway *et al.*, 2001). HAMPs and PAMPs are molecules derived from the herbivore or pathogens and thus, in principle, allow for a species-specific recognition of the attacking enemy. Examples of HAMPs group comprise caeliferins, which are composed of saturated and monounsaturated, sulfated α-hydroxy fatty acids (Alborn *et al.*, 2007); bruchins, a group of egg-derived compounds (Doss *et al.*, 2000); insect-derived effectors, which are secreted by specialized herbivores to overcome the host resistance (Hogenhout & Bos, 2011); and insect pheromones (Fatouros *et al.*, 2008; Helms *et al.*, 2013; Fatouros *et al.*, 2015).

Several studies have demonstrated that HAMPs such as caeliferins, bruchins, benzyl cyanide and male sex pheromone trigger plant resistance against herbivores when they have been exogenously applied in maize, tomato, cabbage or tall goldenrod (Doss *et al.*, 2000; Alborn *et al.*, 2007; Fatouros *et al.*, 2008; Helms *et al.*, 2013; Duran-Flores & Heil, 2016). PAMPs group comprises a wide kind of compounds and are sensed by membrane-associated pattern recognition receptors (PRRs), which include receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Saijo *et al.*, 2018). The most common PAMPs are the protein flagellin and the polysaccharide chitin, these have been successfully applied to enhance resistance against various pathogens in plants such as cucumber, been, tobacco, wheat, sunflower, beet, passion fruit, rice and tomato (Ben-Shalom *et al.*, 2002; Hofgaard *et al.*, 2005; Falcón-Rodríguez *et al.*, 2007; Nandeeshkumar *et al.*, 2008; Boller & Felix, 2009; Mazaro *et al.*, 2009; Hao *et al.*, 2014; Xing *et al.*, 2015).

Research into damaged-self recognition mediated by DAMPs has more than 20 years of history in the medical sciences (Land *et al.*, 1994; Matzinger, 1994). For plants, the concept was formalized only few years ago (Boller & Felix, 2009; Heil, 2009), likely because research into induced resistance to natural enemies focused on non-self recognition.

1.2.1 Damage-associated molecular patterns (DAMPs)

Herbivory and the infection by pathogens inevitably cause injury and, thus, the release of cellular content from disrupted cells and the fragmentation of macromolecules by lytic enzymes. All these endogenous compounds indicate tissue damage when they appear in the extracellular space and, thus, can act as DAMPs or 'danger signals': early - and likely conserved - triggers of resistance in plants (Boller & Felix, 2009; Heil, 2009; Heil & Land, 2014; Acevedo *et al.*, 2015; Quintana-Rodriguez *et al.*, 2018). In fact, Green & Ryan demonstrated in 1972 that mechanical wounding *per se* can increase the resistance in tomato (*Solanum lycopersicum*). This was the first study that showed that plants could perceive damage based on the recognition of endogenous molecules.

The DAMPs have been conceptualized only recently as endogenous molecules that stem from the damaged plant tissue itself and only few DAMPs have been identified in plants (Table 1), for example: nucleotides such as extracellular ATP (eATP) (Tanaka *et al.*, 2014) and extracellular DNA (eDNA); saccharides such extracellular sucrose, cell wall fragments, proteins, and peptides (Doares *et al.*, 1995b; Pearce & Ryan, 2003; Thibaud *et al.*, 2004; Huffaker *et al.*, 2006; Aziz *et al.*, 2007; Yamaguchi *et al.*, 2011; Choi *et al.*, 2016b; Souza *et al.*, 2017). The responses that are induced by DAMPs range from early signaling cascades (e.g., Ca^{2+} influxes, H_2O_2 and MAPK) to phenotypic resistance traits. Extracellular ATP elicited Ca^{2+} influxes (Roux & Steinebrunner, 2007), caused depolarization in Arabidopsis root hairs (Lew & Dearnaley, 2000), and induced the formation of ROS in *Medicago truncatula* (Kim *et al.*, 2006) and the alga, *Dasycladus vermicularis* (Torres *et al.*, 2008). At

the phenotypic level, eATP induced the resistance to viral and bacterial pathogens in tobacco (Chivasa *et al.*, 2009) and the secretion of EFN in lima bean, *Phaseolus lunatus* (Heil *et al.*, 2012). Extracellular ATP represents the only DAMPs for which a receptor has been identified in plants: DORN1, a lectin receptor kinase with an extracellular nucleotide-binding domain with preferred affinity for ATP that is required for ATP-induced Ca^{2+} influxes and MAPK-dependent signaling (Choi *et al.*, 2014). Sucrose induced the expression of several PR genes in Arabidopsis suspensions cells through a SA dependent pathway (Thibaud *et al.*, 2004) whereas in lima bean, maize, sesame and Arabidopsis, exogenous application of sucrose enhanced the levels of endogenous JA (Heil *et al.*, 2012). Cell wall components like cutin can elicit H_2O_2 formation, as shown in cucumber plants (Fauth *et al.*, 1998; Kauss *et al.*, 1999). Peptides like systemin, which in response to attack are liberated from larger endogenous precursor proteins, elicit high levels of PI, JA and ABA, and the release of VOCs in tomato, maize, potato, soybean and Arabidopsis (Pearce *et al.*, 1991; Peña-Cortés *et al.*, 1995; Albert, 2013; Huffaker *et al.*, 2013; Tavormina *et al.*, 2015). Peptides are perceived via leucine-rich repeat (LRR) protein kinases, at least in the case of systemin (receptor: SR160). Ultimately, the fatty-amino acid conjugates like volicitin were originally considered to be HAMPs, although their – at least partial - origin as a plant molecule clearly allows to classify these compounds as DAMPs (Duran-Flores & Heil, 2016). These fatty-amino acid conjugates elicited JA, ET and VOCs in maize, soybean, eggplant and tobacco (Alborn *et al.*, 1997; Alborn *et al.*, 2003; Huffaker *et al.*, 2013; Yoshinaga *et al.*, 2014).

Table 1. Resistance-related traits induced by DAMPs in plants (Quintana-Rodriguez *et al.*, 2018).

	DAMP	Plant in which DAMP was identified	Induced resistance-related trait	References
Nucleotides	Extracellular ATP	Arabidopsis (*Arabidopsis thaliana*), Barrel medic *(Medicago truncatula),* Tobacco (*Nicotiana tabaccum*) and Lima bean (*Phaseolus lunatus*)	Membrane depolarization, Ca^{2+} influxes, ROS, SA, EFN and resistance to viral and bacterial pathogens.	(Lew & Dearnaley, 2000; Kim *et al.*, 2006; Roux & Steinebrunner, 2007; Chivasa *et al.*, 2009; Heil *et al.*, 2012; Tanaka *et al.*, 2014)
	Extracellular NAD(P)	Arabidopsis (*Arabidopsis thaliana*)	PR genes, SA and disease resistance to a bacterial pathogen	(Zhang & Mou, 2009)
	Extracellular DNA	Common bean (*Phaseolus vulgaris*), Lima bean (*Phaseolus lunatus*) and Maize (*Zea mays*)	Ca^{2+} influxes, membrane depolarization, H_2O_2, MAPK, EFN and resistance against bacterial pathogen.	(Barbero *et al.*, 2016; Duran-Flores & Heil, 2018)
Proteins and peptides	Systemin	Tomato *(Solanum lycopersicum)*	PI (1 and 2), JA	(Green & Ryan, 1972; Doares *et al.*, 1995a; Ferrari *et al.*, 2013)
	Inceptin (ATPase fragment)	Caupi (*Vigna unguiculata*) *and* common bean (*Phaseolus vulgaris*)	JA, SA, ET, VOCs and cystatin PI gene	(Schmelz *et al.*, 2006; Schmelz *et al.*, 2007)
	HMGB3	Arabidopsis (*Arabidopsis thaliana*)	Defense-related gene expression, MAPK, callose deposition, and enhanced resistance to fungal disease caused by *Botrytis cinerea*.	(Choi *et al.*, 2016)
	HypSys	Solanaceus: black nightshade (*Solanum nigrum*), in tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), petunia (*Petunia hybrida*), potato (*Solanum tuberosum*) and sweet potato (*Ipomoea batatas*).	PI (2)	(Pearce & Ryan, 2003; Pearce *et al.*, 2009)
	AtPeps	Arabidopsis (*A. thaliana*)	Defensin gene expresion, H_2O_2 and resistance to both *Pythium irregulare* and *Pseudomonas syringae*.	(Huffaker *et al.*, 2006)

	GmSubPep	Soybean (*Glycine max*)	Expression of defense-related genes encoding proteins, such as *cytochrome*, chitinase, salicylic acid–inducible ATP-binding cassette transporte (*PDR12)*, and chalcone synthase.	(Pearce *et al.*, 2010)
	ZmPeps	Maize (*Zea mays*)	ET, JA and resistance related genes, disease reduction of southern leaf blight and anthracnose stalk rot caused by *Cochliobolis heterostrophus* and *Colletotrichum graminicola*	(Huffaker *et al.*, 2011)
	GmPep914	Soybean (*Glycine max*)	Expression of genes involved in phytoalexins synthesis	(Yamaguchi *et al.*, 2011)
	GmPep690	Soybean (*Glycine max*)	Expression of genes involved in phytoalexins synthesis	(Yamaguchi *et al.*, 2011)
	PIPs	Arabidopsis (*A. thaliana*	Resistance against bacteria *P. syringae* and the fungus *Fusarium oxysporum* and amplification of immune response.	(Hou *et al.*, 2014)
Saccharides	Oligogalacturonides (derivatives of cell wall)	Tomato (*S. lycopersicum*) Arabidopsis (*A. thaliana*), Soybean (*Glycine max*), grapevine and tobacco.	Defensive genes against pathogens, PR proteins, phytoalexins and disease reduction against pathogens.	(Doares *et al.*, 1995b; Ferrari *et al.*, 2013)
	Sucrose	Arabidopsis (*A. thaliana*) and lima bean (*P. vulgaris*).	PR genes and corresponding proteins PR-2 and PR-5, JA VOCs and EFN.	(Thibaud *et al.*, 2004; Heil *et al.*, 2012).
	Cellodextrins (derivatives of cell wall)	Grapevine (*Vitis vinifera*)	Ca^{2+} influxes, H_2O_2, PR genes, chitinase and β-1,3-glucanase.	(Aziz *et al.*, 2007).
	Cellobiose (derivatives of cell wall)	Arabidopsis (*A. thaliana*)	Ca^{2+} influxes, MAPK, PR genes, genes that encode to suberin biosynthesis.	(Souza *et al.*, 2017)
Other derivatives of cell wall	Cutin	Cucumber	H_2O_2 and enhance the activity of a fungal elicitor of H_2O_2.	(Fauth *et al.*, 1998; Kauss *et al.*, 1999)

		(*Cucumis sativus*) and tomato (*S. lycopersicum*)		
	Methanol (derived from cell wall pectins)	Tobacco (Specie not specified)	Enhances the resistance of the non-wounded, neighboring "receiver" plants to bacterial pathogens.	(Komarova *et al.*, 2014).
Fatty-aminoacid conjugates (Plant + insect origin)	Volicitin (N-(17-Hydroxy linolenoyl)-l-Gln)	Maize *(Zea mays*)*, Soja* (*Glycine max*) and *eggplant* (*Solanum melongena*)	VOCs, JA and ET	(Alborn *et al.*, 1997)
	18:3-Glu (*N*-linolenoyl-l-Glu)	Tobacco (*Nicotiana attenuate*)	JA, VOCs and defense-related mRNAs	(Alborn *et al.*, 1997)
	18:3-Gln (N-linolenoyl-l-Gln)	Maize (*Z. mays*)	JA, ET, VOCs. expression of allene oxide synthase and allene oxide cyclase	(Alborn *et al.*, 1997; Huffaker *et al.*, 2013)
	18 OH-volicitin or 18-hydroxy-18:3-Gln (N-(18- Hydroxy linolenoyl)-l-Gln)	*Maize* (*Z. mays*)*,* *Tobacco* (*N. tabaccum*) *and eggplant* (*S. melongena*)	VOCs	(Yoshinaga *et al.*, 2014)

ET = Ethylene; HMGB3 = high mobility group box 3; HypSys = hydroxyproline-rich glycoprotein systemins; JA = jasmonic acid; NAD(P) = Pyridine nucleotide; PI = Proteinase inhibitor; PR = pathogenesis-related (PR) genes; SA = salicylic acid; ROS = reactive oxygen species; VOCs = Volatile organic compounds.

1.2.2 Specificity in the resistance-related responses to leaf homogenates

Numerous studies reported that the application of leaf extracts or leaf homogenate elicits resistance-related responses against herbivores, pathogens or both (Heil, 2009; Quintana-Rodriguez *et al.*, 2018). An extract is obtained by the separation of the substances of interest from their original source, generally with a solvent in which the substance(s) of interest dissolve well, while a homogenate consists of a blend of well mixed substances (obtained by extraction or not) (Tejeda *et al.*, 1995). Hereinafter, the term 'extract' is used collectively for all preparations that contain the products of mechanically damaged plant tissues in a solvent. Such extracts evidently contain DAMPs, and their application to plant results in the presence in the extracellular space of molecules such as sucrose, ATP and nucleic acids, which in the intact tissue would be localized within the cell (Heil, 2009; Duran-Flores & Heil, 2014). Among the reported effects, leaf extract of devil´s trumpet elicited resistance in pearl millet to downy mildew caused by an oomycete (Devaiah *et al.*, 2009), extract of rhubarb roots and alder buckthorn bark protected grapevine leaves from infection with the oomycete, *Plasmospora viticola*, (Godard *et al.*, 2009), leaf extract of Zimmu enhanced resistance of banana fruits to fungal diseases caused by *Lasiodiplodia theobromae* and *Colletotrichum musae* (Sangeetha *et al.*, 2013) and leaf extract of common bean, tecomari bean, lima bean, acacia and maize elicited resistance to pathogenic *Pseudomonas syringae* in common bean (Duran-Flores & Heil, 2014) and leaf extracts of billygoat-weed, garlic, neem, devil´s trumpet, false daisy, big-sage, basil and air plant elicited rice resistance against bacterial leaf blight (Khoa *et al.*, 2017).

Whereas in the abovementioned studies the species used to prepare the extract ('source') was different to the treated target plant ('receiver'), other studies applied conspecific plant extracts and observed the emission of VOCs from cabbage or maize plants (Turlings *et al.*, 1993; Mattiacci *et al.*, 1995), the secretion of EFN in lima bean (Heil *et al.*, 2012) and common bean (Duran-Flores & Heil, 2014), and enhanced endogenous levels of JA, a central

hormone in plant resistance to herbivores, in lima bean, Arabidopsis, sesame and tomato (Heil *et al.*, 2012). In fact, conspecific extract caused an overall transcriptomic response in lima bean that was very similar to the response to exogenous JA application (Heil *et al.*, 2012). Hence, it appears that plant extracts obtained from conspecific plants usually induce the resistance to herbivores, whereas extracts from heterospecific plants often induce the resistance to pathogens (Quintana-Rodriguez *et al.*, 2018). Unfortunately, very few studies tried to understand the mechanistic basis of these effects, although the taxonomic diversity of extract sources and observed resistance effects makes it tempting to speculate that the observed effects are based on a general principle: the enhancement of plant resistance in response to 'damaged-self recognition' (Heil, 2009; Quintana-Rodriguez *et al.*, 2018).

Recent evidence indicates a certain level of species-specificity in the plant responses to DAMPs of different taxonomic origin. For example, treating intact leaves of common bean (*Phaseolus vulgaris*) with leaf extracts induced the generation of ROS as an early general response to stress and the secretion of EFN as a late, phenotypic resistance, but only when using extracts prepared from conspecific leaves (Duran-Flores & Heil, 2014). Even the application of leaf extracts of the closely related lima bean (*Phaseolus lunatus*) led to a significantly reduced response or no response (Duran-Flores & Heil, 2014). Which molecules among all the molecules that are released from damaged leaf tissue account for this specificity in the immune response remains an open question.

1.3 DNA in damaged-self recognition

Common knowledge holds that DNA is organized in chromosomes or plasmids and serves as the carrier of an organism's genetic information, but research over the last decades demonstrated several additional functions of DNA, particularly of fragmented eDNA. eDNA represents the vector for horizontal gene transfer (Thomas & Nielsen, 2005) and it contributes to the structuring of microbial biofilms (Whitchurch *et al.*, 2002) or of neutrophil extracellular traps (structural components of the mammalian immune system that disarm

and kill bacteria; see (Brinkmann *et al.*, 2004)). In plants, eDNA can be required for the resistance in root tips to pathogen infection (Wen, F *et al.*, 2009) or can even serve as a source of nutrients, particularly as a source of phosphorus (Paungfoo-Lonhienne *et al.*, 2010b). Moreover, DNA is a common DAMP in mammals (Jounai *et al.*, 2013; Dempsey & Bowie, 2015).

1.3.1 The extracellular DNA (eDNA) as DAMP in mammals

In mammals, well-studied DAMPs include high-mobility group box proteins (HMGBs), eATP, or eDNA. Whereas eDNA molecules of nuclear and mitochondrial origin are considered DAMPs, bacterial and viral DNA molecules are considered MAMPs or PAMPs (Tang *et al.*, 2012; Jounai *et al.*, 2013; Kaczmarek *et al.*, 2013) although it remains matter of discussion whether mitochondrial DNA is perceived as DAMP or rather, due to its biochemical similarities to bacterial DNA, as 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; specifically the intracellular TLR9 that exhibits a preference for unmethylated cytosine–phosphate–guanine (CpG) dideoxynucleotide motif-rich DNA) (Hemmi *et al.*, 2000), DNA-dependent activator of IFN-regulatory factors (DAI), interferon regulatory factor (IRF), or the NACHT, LRR and pyrin domains (PYD)-containing protein 3 (NLPR3) inflammasome (Figure 2) (Takaoka *et al.*, 2007; Patel *et al.*, 2011; Magna & Pisetsky, 2016; Schlee & Hartmann, 2016; Takahashi *et al.*, 2017). The activation of these sensors triggers immunity-related responses like MAPKs signaling, the formation of ROS, the synthesis of interferons (IFNs) and multiple other signaling processes that lead to inflammation, the maturation of dendritic cells to antigen-presenting cells and, ultimately, to an adaptive immune response (Land, 2015). Research into the mechanisms that enable the mammalian immune system to detect

nucleic acids as danger signals has mainly focused on the differentiation of host (self) versus microbial (non-self) eDNA (Schlee & Hartmann, 2016).

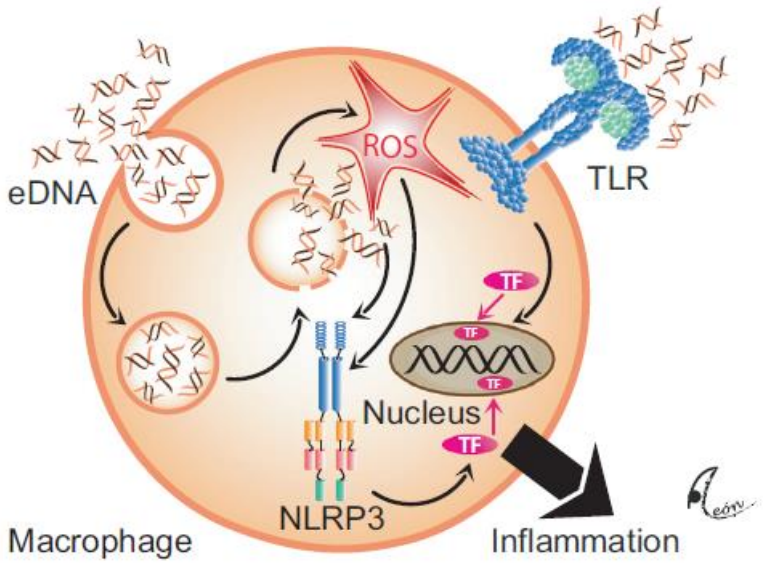

Figure 2. Extracellular and intracellular perception of extracellular DNA (eDNA). Mammalian macrophages perceive eDNA both within and outside the cell. Toll-like receptors (TLRs) sense eDNA at their extracellular domains and release transcription factors (TF) that induce gene-expression leading to pro-inflammatory responses. Alternatively, fragmented eDNA can be taken up via phagocytosis, re-released into the cell plasma and then directly or indirectly (via the formation of reactive oxygen species, ROS) activate the NOD-like receptor family protein 3 (NLRP3) inflammasome to trigger proinflammatory responses. Figure published in (Duran-Flores & Heil, 2015) with elements taken and re-arranged from Fig. 4 in Heil & Land (2014).

1.3.2 eDNA as a possible source of specificity in plants

Recently, eDNA has been suggested to act in plant resistance (Hawes *et al.*, 2011; Duran-Flores & Heil, 2015), mainly because non-self eDNA was reported as an indicator of bacterial infection in *Arabidopsis thaliana* (Yakushiji *et al.*, 2009) and as an inducer of resistance to fungal infections in pea roots (*Pisum sativum*) (Wen, F *et al.*, 2009). Moreover, alterations in the conformation of nuclear DNA by ultraviolet irradiation and DNA dyes or other DNA-intercalating compounds induced resistance responses in pea (Hadwiger & Schwochau,

1970; Hadwiger & Schwochau, 1971a; Hadwiger & Schwochau, 1971b). Most recently, self-eDNA was reported as a trigger of Ca^{2+} signalling and membrane depolarization in lima bean and maize (Barbero *et al.*, 2016). 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 (*Phaseolus lunatus*) or an insect did not result in membrane depolarization in maize (*Zea Mays*) (Barbero *et al.*, 2016), and also the inhibitory effect of eDNA on the growth of organisms in different phyla (Mazzoleni *et al.*, 2014; Mazzoleni *et al.*, 2015a; Mazzoleni *et al.*, 2015b) showed taxonomic specificity: eDNA of garden cress (*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 these observations, we speculated that eDNA functions as a DAMP that contributes to damaged-self recognition (Duran-Flores & Heil, 2015) because the allocation of resources to resistance expression comes at a cost and usually causes a transient decrease in primary metabolism and, thus, growth (Herms & Mattson, 1992; Cipollini & Heil, 2010; Accamando & Cronin, 2012). Therefore, resistance induction by eDNA could lead at the phenotypic level to the growth arrestment phenomenon that was observed by Mazzoleni *et al.* (2015a,b).

1.4 The bean plant (*Phaseolus* sp.)

In the recent years, bean has been widely used as a model to study different aspects of plant defense. For example, lima bean (*Phaseolus lunatus*) is a common model plant used in genetic, biochemical or ecological studies due to their characteristic emission of VOCs or EFN secretion that attracts beneficial insects (Dicke *et al.*, 1990; Dicke *et al.*, 1993; Dicke, 1994; Kost & Heil, 2005).

In Mexico, common bean (*Phaseolus vulgaris*) is the most important legume for human consumption and the second most important product in the agri-food sector, not only as a source of income for thousands of agrarians, but also due to its important role in the diet

of the population (SIACON-SAGARPA, 2016). Like the majority of crops, beans are susceptible of contracting diseases or being infested by pests (Campos-Ávila, 2015). For example, bean blight and bacterial brown spot caused by *Xanthomonas phaseoli* and *Pseudomonas syringae* pv. *syringae* and pv. Phaseoli, respectively, are among the most common foliar bacterial diseases on bean. Whereas the anthracnosis caused by Colletotrichum lindemuthianum, white mold caused by Sclerotinia sclerotiorum and wilt caused by Fusarium oxysporum and are the most common fungal diseases on bean. These pathogenic disease currently are controlled with pesticides which pollute the environment (Schwartz, 2014; Campos-Ávila, 2015). Therefore, the common bean is also turning into a model for the study of inducible resistance-related traits (Córdova-Campos *et al.*, 2012; Duran-Flores & Heil, 2014; Quintana-Rodriguez *et al.*, 2015). In a previous study it was reported that common bean plants increased the formation of H_2O_2 and the secretion of EFN in response to application of leaf homogenates obtained from leaves of the same species, whereas, the application of homogenate from the closely related lima bean led to a significantly reduced response demonstrating a specificity in damaged-self recognition (Duran-Flores & Heil, 2014).

2 HYPOTHESIS

Extracellular DNA can act as damage-associated molecular pattern (DAMP) that activates resistance-related traits in plants and allows to distinguish between 'self' and 'non-self' in plants.

3 OBJETIVES

3.1 Main objective

To determine whether DNA is involved in the activation of plant resistance mechanisms contributing to damaged-self recognition in common bean (*Phaseolus vulgaris*) in a species-specific way.

3.2 Particular objectives

- To determine whether extracellular self-DNA (eDNA) inhibits primary root growth of common bean seedlings.
- To determine whether self eDNA induces resistance-related responses at the level of early (ROS, MAPKs) and late (JA and SA) signaling, and at the phenotypic level (EFN and resistance against herbivory and pathogens).
- To compare the effects of self and non-self eDNA on primary root growth and resistance-related responses (ROS, MAPKs, JA, SA, EFN) in common bean.
- To determine the phenotypic resistance in common bean plants against herbivores and pathogens as a result of the application of self and non-self eDNA.
- To compare yield of common bean plants treated with self and non-self eDNA and homogenates under field conditions.
- To provide first insights into possible mechanisms of eDNA perception in plants, e.g. by characterizing the size of fragments that are perceived, determining a potential role of DNA methylation, and studying if eDNA enters the cells and which organelle is the principal source of active eDNA to induce resistance-related responses.

4 MATERIALS AND METHODS

4.1 Biological material and growing conditions

4.1.1 Plants

For all experiments using living plants, four-week-old common bean plants (*Phaseolus vulgaris*, Negro San Luis variety; seeds were obtained from the national germplasm collection at INIFAP, Celaya, GTO, México) were used as receivers 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 distilled water. Wild acacia (*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).

4.1.2 Suspension cells

In order to reduce the quantity of purified DNA required for some experiments, we produced cells of common bean in suspension culture. We tested four different combinations of phytohormones which have been used to induce a viable callus for suspension cell culture of common bean (Mahamune *et al.*, 2011; Thao *et al.*, 2013) or other bean species such as lima bean (Kanchiswamy & Maffei, 2008) to select the best combination to induce callus of common bean, Negro San Luis variety. Surface-sterilized common bean seeds were germinated under sterile conditions in solid Murashige and

Skoog (MS) medium (Murashige & 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 either of four phytohormones combinations: 1) 0.05 mg L^{-1} of indol-3-acetic acid (IAA) and 0.5 mg L^{-1} of thidiazuron (TDZ), 2) 0.25 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.75 mg L^{-1} of α-naphtaleneacetic acid (NAA), 3) 0.5 mg L^{-1} of IAA and 5 mg L^{-1} of 2,4-D or 4) 0.5 mg L^{-1} of IAA and 0.5 mg L^{-1} of kinetin (All from Sigma-Aldrich). The tips were 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. Based on these experiments, we selected 0.5 mg L^{-1} of IAA and 5 mg L^{-1} of 2,4-D as the best combination. After the four weeks, 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 growth conditions of the callus. 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.

4.1.3 Herbivores

Larvae of the generalist herbivore *Spodoptera frugiperda* were collected from maize crop fields in Irapuato, Guanajuato, México. The larvae were brought to the laboratory, identified according to the illustrated guide by Caballero *et al.* (2012) and placed to their development on 1-2 cm^2 of artificial diet (Hernández *et al.* (1989) (Table 2) in individual 100 ml plastic cups with perforated lid, allowing ventilation. The adult insects were provided with a 10 % v v^{-1} of honey solution in a wet cotton ball.

Table 2. Ingredients of artificial diet for *S. frugiperda* larvae.

Ingredient	Quantity
Water	759.5 ml
Agar	18.3 g
Corn semoline	128.4 g
Wheat germ	32.1 g
Brewer yeast	34.3 g
Ascorbic acid	4.5 g
Benzoic acid	1.3 g
Nipagin	1.1 g

We wait until the third generation to obtain parasite free larvae. For the herbivory experiment, larvae in the fifth instar (14 days after the oviposition), and with 12 h of starvation, were used.

4.1.4 Phytopathogens

In order to determine levels of phenotypic resistance to pathogens, we used some bacterial and fungal strains that cause the most important diseases of common bean in Mexico (Campos-Ávila, 2015). The bacterium *Xanthomonas phaseoli* was provided by Dr. Gabriel Gallegos-Morales (UAAAN, Saltillo, Coahuila, Mexico) and cultivated at 28 °C on agar plates with yeast dextrose carbonate (YDC) medium (20 g l−1 CaCO3, 10 g l−1 yeast extract and 20 g l−1 dextrose) (Cruz-Izquierdo *et al.*, 2001). *Pseudomonas syringae* pv. *phaseoli* strain NPS3121 was provided by Dr José-Luis Hernández-Flores (Cinvestav, Irapuato, Guanajuato, Mexico) and cultivated at 28 °C on agar plates with KB medium (20 g l^{-1} bactopeptone, 1.5 g l^{-1} K_2HPO_4 and 15 ml l^{-1} glycerol) King *et al.* (1954). The rifampicin-resistant *P. syringae* pv. *syringae* strain 61 was provided by Dr. Choong-Min Ryu (KRIBB, Daejeon, South Korea) and cultivated at 28 °C on KB medium with 50 mg l^{-1} rifampicine, and *Enterobacter sp*. strain

FCB1 was previously isolated and identified in the laboratory (Córdova-Campos *et al.*, 2012) and cultivated at 28 °C on YDC medium.

Among the fungal pathogens, *Colletotrichum lindemuthianum* strain 1088 was donated by Dr. June Simpson, (CINVESTAV, Irapuato, Guanajuato, Mexico), *Sclerotinia sclerotiorum* was donated by Dr. Victor Olalde-Portugal (CINVESTAV, Irapuato, Guanajuato, Mexico), and *Fusarium oxysporum* and *Botrytis cinerea* were provided by Dr. Alfredo Herrera-Estrella (UGA, Irapuato, Guanajuato, Mexico). All fungi were cultivated on plates with potato dextrose agar (PDA) medium at 28 °C except for *S. sclerotiorum* that was cultivated at 20 °C.

4.2 Extraction 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). The DNA

from common bean was used 'self eDNA' and the DNA from lima bean or acacia was used as 'non-self eDNA'.

4.3 Fragmentation of DNA

The purified DNA was fragmented by sonication using an ultrasonic processor (Misonix XL2020). We tested different times of sonication to obtain DNA fragments shorter than 1000 bp because the conditions reported by Mazzoleni *et al.* (2015a) (12 min at 90% power level, 0.9 s pulse 'On' and a 0.9 s pulse 'Off') resulted in completely degraded DNA using our ultrasonic processor (see section 5.1). A solution of 500 µg mL^{-1} of DNA was prepared with sterile distilled water and sonicated for 1, 2, 3, 4 and 6 min at 55 % power level, 1 s pulse 'On' and a 1 s pulse 'Off'. The successful fragmentation of DNA was verified comparing with non-sonicated DNA on a 3% agarose gel using ethidium bromide. We selected the time of 6 min to fragment the DNA for the subsequent experiments.

4.4 Effect of self and non-self eDNA on the 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 15-cm Petri dishes on sterile filter paper imbibed with 15 mL of different concentrations (0, 2, 20, 50, 100, 150 or 200 µg $mL^{-1;}$) of self eDNA fragments suspended in sterile water. Sterile distilled water was 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 15 mL of 200 µg mL^{-1} of self eDNA or non-self eDNA (n = 9 seeds per treatment). The Petri dishes were placed in the growth room and the primary root length was measured after four days.

4.5 Effect of self and non-self eDNA on early signaling molecules related to resistance

4.5.1 Quantification of ROS (H_2O_2) formation in leaves and suspension cells

To determine whether self eDNA activates H_2O_2 formation we first tested different concentrations of fragmented self eDNA and quantified the response at different time points after treatment. 0, 2, 20, 50, 100, 150 or 200 µg mL^{-1} of the eDNA in 0.05 % v v^{-1} Tween 20 were applied with micropipette on both sides of the three youngest leaves per plant until the surface was completely wet. Eleven groups of six plants were treated with either of the different concentrations tested. Each group was used to take samples at 0, 1, 3, 5, 10, 15 or 30 min and 1, 2, 4, 8 h) after treatment. At each sampling time, 10 discs of 1-cm diameter were punched out of each treated 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}.

To determine whether H_2O_2 formation in suspension cells responds to self eDNA like in leaves, we used 200 µg mL^{-1} of self eDNA to treat the cells and quantify H_2O_2 at different times after treatment. 1 mL of suspension culture containing 1×10^8 cells was centrifuged at 6 000 *g* for 10 min, the supernatant was discarded and 1 mL of fresh MS medium was added to the cells. These steps were repeated three times to wash the cells and remove any extracellular H_2O_2. To assess the effect of self eDNA in each time period on the suspension

cell cultures, 1 mL of washed cell culture (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 100 µL of sterile water were used as controls. After 0, 1, 3, 5, 10, 15 or 30 min and 1, 2, 4 or 8 h min of treatment, the cell suspension was centrifuged at 6 000 *g* for 10 min. Next, 10 µL of the supernatant were transferred to a 96 multiwell plate and mixed with 90 µL of the substrate solution from Hydrogen Peroxide Assay Kit to quantify H_2O_2 as we mentioned above.

To determine whether self eDNA and non-self eDNA differentially induce H_2O_2 formation, 200 µg mL^{-1} of self eDNA or non-self eDNA fragments in 0.05% (v v^{-1}) Tween 20 were applied to common bean plants as we mentioned above in this section. Groups of nine plants were used for each treatment. Plants treated with 0.05% Tween 20 were used as controls (Duran-Flores & Heil, 2014). H_2O_2 was quantified 2 hours after the treatment.

4.5.2 Determination of the activation of MAPKs in leaves and suspension cells

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 leaves. Three plants were used per time point and the three youngest leaves per plant were treated by applying 200 µg mL^{-1} of self eDNA fragments suspended in 0.05% v v^{-1} Tween 20 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 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) (Duran-Flores & Heil, 2014). After the beforementioned time spans, the 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). In short, 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* for 15 min. 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. Next, 2 mL of the resulted 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* for 15 min. 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.

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).

4.6 Effect of self and non-self eDNA on late signaling molecules related to resistance

4.6.1 Quantification of jasmonic acid (JA)

In order to determine whether endogenous JA responds to self eDNA and to define the time of maximum effect, the JA was quantified at different time points (1, 3, 5, 10, 15, 30, 60 and 120 min) after applying different concentration of fragmented self eDNA suspended in Tween 20 0.05% v v^{-1} (0, 2, 20, 50, 100, 150 or 200 µg mL^{-1}) as mentioned in section 4.5.1. Tween 20 0.05% v v^{-1} solution were used as negative controls. One independent group of six plants was used per time point and each self eDNA concentration.

Jasmonic acid was quantified in common bean leaves by gas chromatography coupled to mass spectrometry (GC-MS). The three youngest treated leaves were cut off at the base at 0, 2, 5, 10, 15, 30 or 45 min and 1, 2, 4, 8, 12, 24 or 48 h after treatment and kept it in liquid nitrogen. One independent group of six plants was used per time point and each self eDNA concentration. The frozen leaves were ground in a mortar with liquid nitrogen; 250 mg of the resultant powdery material were transferred to a 2 mL Eppendorf tube. Jasmonic acid was extracted with ethyl acetate following a method based on Pluskota *et al.* (2007). 0.5 mL of ethyl acetate and 20 µL of 0.1 mg mL^{-1} (9,10- H2)-dihydrojasmonic acid (internal standard) were added to each tube and shaken on vortex shaker. The tubes were kept at 4 °C overnight. Then, the tubes were centrifuged at 14 000 *g* for 15 min at 4 °C. The supernatant was collocated in a new Eppendorf tube of 1.5 mL and the pellet was re-extracted with 0.5 mL of ethyl acetate and centrifuged. The supernatants were combined and completely evaporated with gaseous nitrogen. The residue was derivatized (Mueller & Brodschelm, 1994) adding 100 µL of N´N´-disopropylethylamine, 100 µL of chloroform and 10 µL of pentafluorobenzyl bromide (all from Sigma-Aldrich) and keeping the mixture at 60

°C. After 30 min, the resultant liquid was cooled on ice and completely evaporated with gaseous nitrogen. The residue was re-suspended with 100 µL on HPLC grade methanol.

One mL of each sample was injected in the splitless mode and analyzed by gas chromatography-single ion-mass spectrometry in an Agilent Technologies Gas Chromatograph 7890A using a DB-1MS column (60 m x 0.5 µm Agilent Technologies) coupled to a MSD 5973 detector in SIM mode for 141, 181, 390 and 392 m z^{-1}. The GC-MS operating conditions were described by (Ramírez-Chávez *et al.*, 2004). These were: an injector temperature of 200°C, and the oven temperature was programmed with an initial temperature of 150°C for 3 min, increasing at the rate of 4°C min^{-1} to a final temperature of 300°C, which was maintained for 20 min. Helium was used as carrier gas with a constant flow of 1 mL min^{-1}. For quantification, a standard curve of pure JA (Sigma-Aldrich) was run and peak areas were evaluated with reference to the internal standard.

In order to compare the effect of self to non-self eDNA in plants, we used 50 µg mL^{-1} of self or non-self eDNA to treat groups of six plants. JA was quantified in leaves 30 min after treatments.

4.6.2 Quantification of salicylic acid (SA)

In order to determine whether endogenous SA responds to self eDNA and to define the time of maximum effect, we used the same samples used for JA quantification. The extraction of SA was performed according to previous studies (Malamy *et al.*, 1992; Meuwly & Metraux, 1993) with some modifications. Samples were collected at 0, 2, 5, 10, 15, 30 or 45 min and 1, 2, 4, 8, 12, 24 or 48 h after treatment and 250 mg of ground tissue was mixed with 750 µL of methanol 90% and 250 ng mL^{-1} of ortho-anisic acid as an internal standard, and incubated at 4°C all night. After incubation, the samples were centrifuged at 13 000 *g* for 15 min, the supernatant was recovered and stored in a new 2 mL tube. The pellet was resuspended on 750 µL of 100% methanol and centrifuged again at 13 000 *g* for 15 min.

Both supernatants were combined and dried in a Concentrator plus (Eppendorf) for approximately 4 h. The pellet was re-suspended with 500 µL of TCA 5% and centrifuged at 4 000 *g* for 10 min. The resulting supernatant was mixed with two volumes of ethyl acetate-hexane (1: 1 v/ v) in an extraction hood and incubated at room temperature for 10 min. The organic phase (upper phase) was recovered and dried with gaseous nitrogen in 2 mL tubes. The resulting pellet was derivatized mixing with 20 µL of pyridine and 80 µL of BSTFA (Sigma-Aldrich) and incubated at 80°C for 1h, using an extraction hood. One microliter of each sample was injected in the splitless mode and analyzed by gas chromatography-single ion-mass spectrometry in an Agilent Technologies Gas Chromatograph 7890A using a DB-1MS column (60 m length, 0.25 mm diameter, 0.25 µm film; Agilent Technologies) coupled to a MSD 5973 detector in SIM mode for 73,135, 267, and 282 m z^{-1}. The GC-MS oven program used was: 150°C for 3 min; then, 4°C/ min to 260°C; then hold at 260°C for 25 min. Helium was used as carrier gas with a constant flow of 1 mL min^{-1}. For quantification, a standard curve of pure SA (Baker) was run and peak areas were evaluated with reference to the internal standard.

In order to compare the effect of self to non-self eDNA in plants, we used 50 µg mL^{-1} of self or non-self eDNA to treat groups of six plants. SA was quantified in leaves 24 h after treatments.

4.7 Effect of self and non-self eDNA on phenotypic resistance responses

4.7.1 Quantification of extrafloral nectar secretion (EFN)

In order to determine whether self eDNA activates the EFN secretion in common bean, we quantified the EFN secreted by plants treated with different concentrations of self eDNA. At 9:00 am the extrafloral nectaries of 42 plants were washed with distilled water until there was no trace of EFN. After 1 h, groups of six plants were treated with 0, 2, 20, 50, 100, 150 or 200 µg mL^{-1} of fragmented self eDNA in Tween 20 0.05% v v^{-1}. The self eDNA suspension

was applied with micropipette on both sides of the four youngest leaves until the surface was 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; Heil *et al.*, 2001)

In order to compare the effect of self to non-self eDNA, we used 50 µg mL^{-1} of self or non-self eDNA to treat groups of six plants. Plants treated with 0.05% Tween 20 were used as controls. The EFN was quantified on extrafloral nectaries 24 h after treatments.

4.7.2 Resistance against herbivory

In order to determine the effects eDNA on herbivory, we offered leaves of common bean plants treated with self and non-self eDNA to larvae of the generalist herbivore *Spodoptera frugiperda*. We used the most active concentrations of self eDNA on JA and SA induction to treat the plants. 50 or 200 µg mL^{-1} of self or non-self eDNA were applied to each one leaf of seven plants, Tween20 0.05% v v^{-1} was used as control. After 30 min, when the leaf surface was dry, one larva of *S. frugiperda* in the fifth instar with a previous period of 12 h in starvation, was placed on the leaf. The leaf was covered with a mesh cloth bag (Figure 3). After 24, the feeding damage produced by the larvae was quantified with the ImageJ program (https://imagej.nih.gov).

Figure 3. Larva of *Spodoptera frugiperda* on common bean leaf covered with a mesh cloth bag during the herbivory experiment.

4.7.3 Resistance against bacterial and fungal phytopathogens

In order to test for induced resistance to bacterial and fungal phytopathogens, suspensions of 50 µg mL^{-1} of self or non-self eDNA fragments in 0.05% v v^{-1} Tween 20 were applied with a micropipette to both sides of the leaves of common bean plants until the surface was completely wet (ten plants per treatment). Five minutes or 24 hours after the treatment, the plants were inoculated by spraying 10 mL per plant of a suspension of the pathogens (bacteria at 1 x 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; fungi at 1 x 10^7 cells mL^{-1}, counted in a Neubauer Chamber). Infection levels were quantified eight or fifteen days after challenging with bacteria or fungi, respectively, in a pool of three randomly selected leaves per plant. Leaf material was weighed and ground in a mortar with 1 mL of sterile distilled water. The resulting liquid was decanted and completed to 2 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 the corresponding solid medium for each pathogen, previously indicated above, in section 4.1.4. After two days, bacterial colonies

were counted to express infection rates as the colony forming units (CFUs) of pathogen per g of fresh leaf mass. The fungal colonies were counted four days after plating.

Putative direct effects of the eDNA solutions on the pathogens were tested by plating 100 µL of each of the eDNA or of the control treatment (Tween20 at 0.05 %, n = 6 repetitions) on Petri dishes the respective medium. After 5 min, 20 µL of a 1:10 1:100, 1:1 000 or 1: 10,000 v v^{-1} dilution of 1 x 10^7 cells mL^{-1} bacteria suspension were spread on the same plates. Six 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. This test was also performed with fungal pathogens after 5 min or 24 h after plating each eDNA type because plants showed resistance to fungi when they were inoculated 5 min or 24 h after eDNA treatments (see results section 5.4).

4.8 Effects of self and non-self treatments in Common bean growing on the field

In order to determine the effect of eDNA treatments on development and yield of common bean plants, four-week-old common bean were used as receivers and compared with plants treated with homogenates or mechanical damage. The plants were cultivated in blocks of 3x9 plants with 35 cm between each of them under field conditions (Campus of CINVESTAV Irapuato, central highlands of Mexico, state of Guanajuato, 2,000 m above sea level; 20 °43'13" N; 101° 19'43"W). Each block was separated from the others by 1 m of distance. This experiment was carried out in April to July 2018, during the early rainy season, which represents the dominant cultivation period for common bean in the region (Acosta-Díaz *et al.*, 2009). 50 µg mL^{-1} of self or non-self eDNA fragments in 0.05% v v^{-1} Tween 20 were applied to three random blocks of 27 plants (Figure 4). Plants treated with self and non-self homogenates or mechanical damaged were used as positive controls, whereas 0.05% Tween 20 were used as negative control (Duran-Flores & Heil, 2014). We counted the number of leaves weekly for 5 weeks after treatments of the seven plants in the center of

each block in order to avoid the border effects. The time of flowering, pod filling and plant dead were registered. The pods were collected and the seeds per plant were weighed.

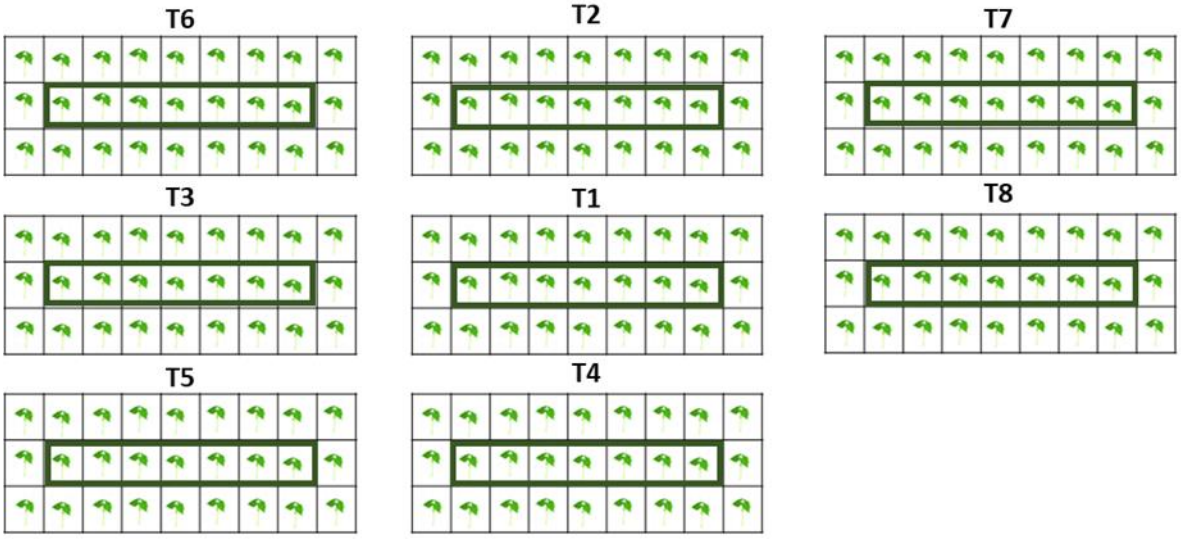

Figure 4. Experimental design on the field. Example of one of three replicates of the distribution of the plant's blocks. The treatments (T) were: 1) 0.05% Tween 20, 2) mechanical damage, 3) self homogenate (common bean), 4) non-self homogenate obtained from lima bean, 5) non-self homogenate obtained from acacia, 6) self eDNA, 7) non-self eDNA obtained from lima bean and 8) non-self eDNA obtained from acacia. The plants in green rectangle were used to register the time of flowering, pod filling, and plant dead and to count the number of leaves and quantify the yield.

4.9 Confirming eDNA as the active principle

4.9.1 Using enzymes and individual nucleotides

In order to confirm that the effects observed in early signaling and phenotypic response were due to eDNA and not caused by impurities such as small RNAs or proteins, fragments of common bean DNA shorter than 1000 bp were treated with either of both nucleases, DNase 1 (Invitrogen) or RNase A (Invitrogen), and with proteinase K (Thermo Fisher Scientific) or combination of all these enzymes, according to product manuals. 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 nuclease- or proteinase-treated self eDNA 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 deactivated enzyme solutions to leaves. Two hours after the treatment with 200 µg mL^{-1} of enzyme-treated self eDNA, 10 discs of 1 cm in diameter were punched out of each leaf and H_2O_2 was quantified as indicated above in section 4.5.1 (Quantification of ROS (H2O2) formation in leaves and suspension cells). Further plants were treated in the same manner and after 30 min of treatment with 200 µg mL^{-1} of enzyme-treated self eDNA, the leaves were excised and frozen in liquid nitrogen and used for the MAPK activation test as indicated in section 4.5.2 (Determination of the activation of MAPKs in leaves and suspension cells). And the four youngest leaves of each of nine plants were treated with 50 µg mL^{-1} of enzyme-treated self eDNA and used to quantify the EFN secretion 24 h after treatment as indicated in section 4.7.1 (Quantification of extrafloral nectar secretion (EFN)).

Finally, in order to test possible effects of individual nucleotides released by DNAse, we used deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), each of them individually, or in equal proportions, at 50, 100, 150 or 200 µg mL^{-1} to treat $1x10^8$ suspension cells (*n* = 6 per treatment). H_2O_2 formations was quantified 2 h after treatment as indicated in section 4.5.1 (Quantification of ROS (H2O2) formation in leaves and suspension cells).

4.9.2 Using random amplification of sequences from genomic DNA

In order to avoid possible effects of DNA methylation marks, proteins or other impurities in eDNA sample, we carry out a random amplification of the whole genome of common bean

to obtain 'synthetic' self-eDNA. The arbitrary oligonucleotide 5’ GTTGCTCC 3´ was used as primer for the amplification. Because the primer concentration affects the size of amplified fragments (Arnheim & Erlich, 1992), we tested different concentrations. A 100 µL PCR reaction was performed with 2, 4, 6 or 8 µM of the primer, 10 ng of common bean DNA, 250 µM of dNTPs and 4 U of DNA polymerase (Invitrogen). The PCR proceeded using the following program: an initial cycle of denaturalization at 94 °C for 4 min followed by 40 cycles as follows: 94 °C for 1 min, alignment at 24 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were separated by gel electrophoresis on 3% agarose gel. We selected the concentration of 8 µM of the primer to synthetize the DNA used to evaluate early signaling responses in suspension cells. 'Syntetic' DNA was purified with QIAquick PCR purification kit (Qiagen) and applied to 1 mL containing $1x10^8$ suspension cells to a final concentration of 200 µg mL^{-1} (*n* = 9 repetitions). The H_2O_2 formation and the activation of MAPK was determined 2 h and 30 min, respectively, after treatment as indicated in sections 4.5.1 and 4.5.2. Cells treated with sterile distilled water were used as negative control and cells treated with 'natural' fragmented DNA (extracted from common bean) were used as positive controls.

4.10 Searching for more details of eDNA perception in plants

4.10.1 Determination of active size of fragments

In order to define the size range of the eDNA fragments that activate early signaling, 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 suspension cell culture containing 1×10^8 cells was treated with 100 µL of 2200 µg mL^{-1} of these eDNA fragments. Cells treated 100 µL of sterile distilled 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 in section 4.5.2 and used for the MAPK assays. This experiment was only performed using suspension cells to determine MAPKs because a larger quantity of DNA would have been needed to perform this experiment using entire plants.

4.10.2 Influence of DNA methylation on the induction of resistance responses

In order to determine if DNA methylation marks are involved in self eDNA activity to induce early responses, we used CpG methylation-sensitive and non-sensitive restriction enzymes to evaluate the effects in early responses. To evaluate the effectiveness of enzymes, 1 µg of non-fragmented DNA from common bean was methylated with CpG methyltransferase M.SssI (Thermo-Scientific) according to product manual. 1 µL of 1 µg μL^{-1} methylated or non-methylated DNA was treated with the CpG methylation non-sensitive restriction enzyme MspI (Thermo-Scientific) or with the CpG methylation sensitive restriction enzyme HapII according to product manual. MspI cut in methylated and no methylated CpG motifs whereas HapII cut in non-methylated CpG motifs. The activity of the restriction enzymes was confirmed on a 2% agarose gel. Complete self DNA or fragments shorter than 1000 bp were methylated with CpG methyltransferase M.SssI and methylated and non-methylated self DNA were treated with MspI or HapII according to product manuals. Each product was applied to 1 ml containing $1x10^8$ suspension cells at final concentration of 200 µg mL^{-1} (n = 5 repetition per treatment), 2 h after treatment, the H_2O_2 formation was quantified as indicated in section 4.5.1. Cells treated with water or enzymes solutions were used as controls.

4.10.3 Visualization and search of eDNA fragments in intracellular space

In order to observe weather DNA fragments enter to plant cells, we performed a DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) staining of common bean leaves treated with fragmented and non-fragmented self eDNA based on a previous method (Otto, 1990). 200 µg mL^{-1} of fragmented or non-fragmented self eDNA were applying on both sides of three

common bean leaves until the surface was completely wet. Leaves treated with 0.05 Tween 20 were used as controls. 30 min after treatment, 1 cm^2 was cut off and individually washed three times in 1 mL of phosphate buffer (pH 7.2) and suspended in 1 mL of 10% sucrose. After 3 h the leaf pieces were transferred to 1 mL of 20% sucrose, after 3 h the leaf pieces were transferred to 1 mL of 30 % sucrose and stored at 4 °C overnight. The sucrose solutions were prepared in phosphate buffer. The leaf piece was fixed with polyethyleneglycol on a glass slide and cut sections of 18 µm with a microtome. 50 µL of 1 µg mL^{-1} of DAPI were applied onto each leaf section and it was kept in the dark for 15 min. After that time, the stain solution was removed, and the sections were washed two times with phosphate buffer. The sections were observed by fluorescence microscopy.

4.10.4 Determination of organelle source of DNA that cause resistance effects

In order to evaluate the activity of self eDNA obtained from a specific organelle to induce early responses, we extracted DNA from either chloroplast, mitochondria and nucleus and applied fragments shorter than 1000 bp to suspension cells (n=9 repetitions) to quantify H_2O_2 and to determine MAPK activation.

To extract DNA from chloroplast or mitochondria, we followed a method based on Morales-Gutiérrez *et al.* (2016). Leaves of common bean plants were ground in a mortar with liquid nitrogen. 1 mL of cold extraction buffer (350 mM sorbitol, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 15 mM β ME and 0.1 % BSA) was added to 0.5 g of ground, vortexed and filtered through a nylon net (0.25 mm hole size) into a clean 2 mL test tube. The nylon net and its contents were discarded. To discard the cell debris and nuclei in the filtered, the tubes were centrifuged at 500 *g* for 5 min at 4 °C. The supernatant was transferred to a new 2 mL tube, and then used for chloroplast enrichment. All samples and reagents were kept cold in an ice bath. The supernatant from was centrifuged at 2000 *g* for 10 min at 4 °C. After centrifugation, the supernatant was removed carefully without touching the chloroplast pellet at the bottom. The pellet was dissolved and washed in 500 µL wash buffer (350 mM

sorbitol, 50 mM Tris-HCl pH 8.0, 25 mM EDTA and 0.1 % BSA). The suspension was washed and centrifuged at 2000 *g* for 10 min at 4 °C twice, removing the supernatant after each centrifugation. The resulting pellet was suspended in 500 µL wash buffer. To enrich the suspension for chloroplasts, we used the density gradient centrifugation method. The gradient was constructed by preparing two molecular grade sucrose solutions at 1.75 M and 1.08 M prepared in 50 mM Tris-HCl (pH 8.0) and 25 mM EDTA (pH 8.0). 700 µL of the 1.75 M sucrose solution were transferred to a 2 mL tube, and then 900 µL of 1.08 M sucrose solution was delicately placed on top of it. Three hundred (300) µL of the chloroplast suspension was gently and slowly placed on top of the sucrose gradient, one drop at a time to avoid mixing. The tube was centrifuged at 7000 *g* for 1 hour at 4 °C. After centrifugation, the green pellet formed was carefully collected with a micropipette and placed in a new tube. The pellet was suspended in three volumes of buffer (175 mM sorbitol, 50 mM Tris-HCl pH 8.0, 25 mM EDTA). Finally, the tube was centrifuged at 2000 x*g* for 10 min at 4 °C.

For mitochondrial DNA isolation, 1 mL of extraction buffer (400 mM sucrose, 50 mM Trizma base, 1 mM EDTA, 10 mM KH_2PO_4, 4 mM cysteine and 1 % BSA; pH 7.6) was added to 0.5 g of ground tissue, vortexed and filtered through nylon net (0.25 mm hole size) into a new 2 mL test tube. The nylon net and its contents were discarded. To discard the cell debris and nuclei the tubes were centrifuged at 500 *g* for 5 min at 4 °C. The supernatant was carefully transferred to a new 2 mL tube and centrifuged at 2000 *g* for 10 min at 4 °C to pellet chloroplasts for removal. This step was repeated twice. After centrifugation, the supernatant was transferred to a new tube, and centrifuged at 16000 x*g* for 10 min at 4 °C to pellet mitochondria. The supernatant was carefully removed. The pellet was then dissolved in 500 µL of wash buffer (400 mM mannitol, 10 mM KH_2PO_4 and 0.5 % BSA, pH 7.2). Then the tubes were centrifuged at 16 000 x*g* for 10 min at 4 °C and the supernatant was removed twice. Density gradient centrifugation was employed to selectively separate mitochondria from other subcellular organelles. Two sucrose solutions at 0.6 and 1.8 M prepared in 50 mM Tris-HCl pH 8.0 and 10 mM KH_2PO_4 pH 8.0. The gradient was constructed in a 2 mL tube by adding 700 µL of 1.8 M sucrose, followed by careful and slow addition of

900 µL of 0.6 M sucrose on top, without mixing. 300 µL of mitochondrial suspension were added, one drop at a time to avoid mixing. This tube was centrifuged at 22 000 *g* for 1 hour at 4 °C. The mitochondria were on the tube sidewall as a green-yellow pellet. The mitochondrial fraction was collected with a micropipette and placed in a new tube. Three volumes of dilution buffer (175 mM mannitol, 10 mM KH_2PO_4, pH 7.2) were added, and the test tube was centrifuged at 16 000 *g* for 10 min at 4 °C.

To extract DNA from nuclei, we followed a method based on Peterson *et al.* (1997). All solutions were cold on ice bath. 20 mL of nuclei isolation buffer (NIB) with 1% PVP were added to 2g of ground tissue in 50 mL tube, vortexed and decant homogenates through two layers of pre-wetted cheesecloth. The debris on cheesecloth were resuspended in another 20 ml of NIB with 1% PVP and pass through the same cheesecloth. Then, Triton X-100 to a final concentration of 0.5% was added and the tubes were shaken slowly for 20 min at +4°C to the lysis of contaminating organelles. The tubes were centrifuged at 1800 *g* for 10 min and the pellet was resuspend in 10 mL of NIB. Density gradient was employed to selectively separate nuclei. 3 ml of 60% Percoll (Sigma-Aldrich) solution were carefully overlayed on 3 mL of 2.5 M sucrose in a chilled 15 mL tube. The crude preparation of nuclei was carefully loaded on the top of the density gradient. The tubes were centrifuged at 1200 *g* for 30 min at 4 °C. The liquid above the gradient was removed and the 60% Percoll layer containing most of the nuclei was carefully collected, diluted with 3 volumes of NIB and centrifuged at 1800 *g* for 10 min. The pellet was resuspended on 3 ml of NIB and centrifuged at 1200 g for 10 min twice.

The pellet obtained from chloroplasts, mitochondria or nuclei enrichment was dissolved in 500 µL of lysis buffer (50 mM Tris- HCl pH 8.0, 20 mM EDTA, 2 % N-lauroylsarcosine sodium salt) and kept at room temperature for 15 min to promote organelle rupture. At the end of the incubation time, 1 mL of phenol:chloroform:isoamyl alcohol (25:24:1) solution was added. The tube was shaken in a vortex and then centrifuged at 14 000 *g* for 10 min at 4 °C. The supernatant was collected and mixed again with phenol:chloroform:isoamyl alcohol

(25:24:1) solution. The DNA was precipitated with 2.5 volumes of cold absolute ethanol and maintained overnight at -20 °C. Then, the tubes were centrifuged at 14 000 *g* for 10 min at 4 °C. The supernatant was discarded, and the remaining ethanol was evaporated at room temperature for 1 hour. The DNA pellet was dissolved in 30 µL of sterile distilled water. The DNA was quantified using a NanoDrop 2000 spectrometer (Thermo Scientific) and the integrity was checked on a 1.2 % agarose gel.

The extracted DNA from chloroplast, mitochondria or nuclei was confirm by the amplification of reference genes. Three genes were selected for their exclusive location to chloroplast (Rubisco large subunit), mitochondria (Cytochrome oxidase) or nucleus (Ubiquitin). Phaseolus vulgaris gene sequences were obtained from http://www.ncbi.nlm.nih.gov/nucleotide/; GenBank numbers for each gene used as template for primer design are: rubisco large subunit, YP_001122790; cytochrome oxidase, XP_007142315 and ubiquitin, AGV54749.1. Primers were designed according to their coding sequence (CDS) using Primer3 v.4 (http://primer3.ut.ee/; Table 3). The amplification of these genes was performed by PCR in the follow reaction mix: 1 U of DNA polymerase (Invitrogen), 40 ng of DNA, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs and 20 pM for each primer. The PCR program was: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, and 72 °C during 10 min. PCR products were observed in 1.2 % agarose gel with ethidium bromide.

Table 3. Primers for reference genes from chloroplast, mitochondria and nucleus.

Location	**Gene**	**Sequence of primers**	
Chloroplast	Rubisco large subunit (*rbcL*)	Forward	5´GGACAACTGTGTGGACCGAT3´
		Reverse	5´AAACGGTCTCTCCAACGCAT3´
Mitochondria	Cytochrome oxidase (*cox1*)	Forward	5´CAGCGGTTTCCTGTCTCCAA3´
		Reverse	5´TTTCCGCTTTATGCGTTGCC3´
Nucleus	Ubiquitin (*ubi*)	Forward	5´TTGGGACGGAGGGAGTATGG3´
		Reverse	5´GTGGGATCCCTTCCTTGTCC3´

4.11 Confirming the role of DNA in leaf homogenate as an active factor to induce resistance responses.

In order to know the quantity of released DNA during the leaf homogenate preparation (Duran-Flores & Heil, 2014), 30 mL of leaf homogenate (1 g of ground leaf in 0.05% Tween 20) were centrifuged at 12 000 *g* for 20 min. The supernatant was separated, 20 mL of precooled isopropanol were added and 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 0.1 mL of sterile distilled water. The DNA was quantified using a NanoDrop 2000 spectrometer (Thermo Scientific) and visualized in 3 % agarose gel.

In order to evaluate the activity of DNA contained in leaf homogenate, self homogenate was treated with either of both nucleases, DNase 1 (Invitrogen) or RNase A (Invitrogen), and with proteinase K (Thermo Fisher Scientific) or combination of all these enzymes, according to product manuals. The enzymes in the eDNA solution were deactivated before the use of it according to product manual. Leaf homogenate treated with a solution of nuclease or proteinase was used to treat common bean plants (n = 7 per treatment) as we previously reported (Duran-Flores & Heil, 2014). The solution was applied with a soft brush on both sides of the four youngest 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 homogenate without nuclease were used as positive controls. After 24 h, the EFN secretion was quantified as indicated in section 4.7.1 (Quantification of extrafloral nectar secretion (EFN)).

5 RESULTS

5.1 INA and 2,4-D in combination induced appropriate callus formation to obtain suspension cell culture

In order to avoid a larger quantity of purified DNA for some experiments, we produced suspension cell culture of common bean. We tested four different combinations of phytohormones which has been used to induce a viable callus for suspension cell culture of common bean or other bean species such as lima bean. Three of four of these combinations induced the formation of root-like structures in common bean explants (Figure 5). Only 0.5 mg L^{-1} of IAA and 5 mg L^{-1} of 2,4-D combination was effective to induce the formation of a viable callus for suspension cell culture (Figure 5) four weeks after the explants were transferred to solid MS medium. This combination of phytohormones was selected to produce the suspension cells.

5.1 Fragments of DNA shorter than 1000 bp were obtained by sonication

The sonication conditions reported by Mazzoleni et al 2015a (12 min at 90% power level, 0.9 s pulse 'On' and a 0.9 s pulse 'Off') resulted in completely degraded DNA (Figure 6a) using our ultrasonic processor. After trying various power levels and times of sonication, we obtained fragmented DNA (Figure 6 b,c) at 55 % power level, 1 s pulse 'On' and a 1 s pulse 'Off'. We obtained DNA fragments shorter than 1000 bp with 6 min of sonication (Figure 6c). This time were used to fragment the DNA for the subsequent experiments.

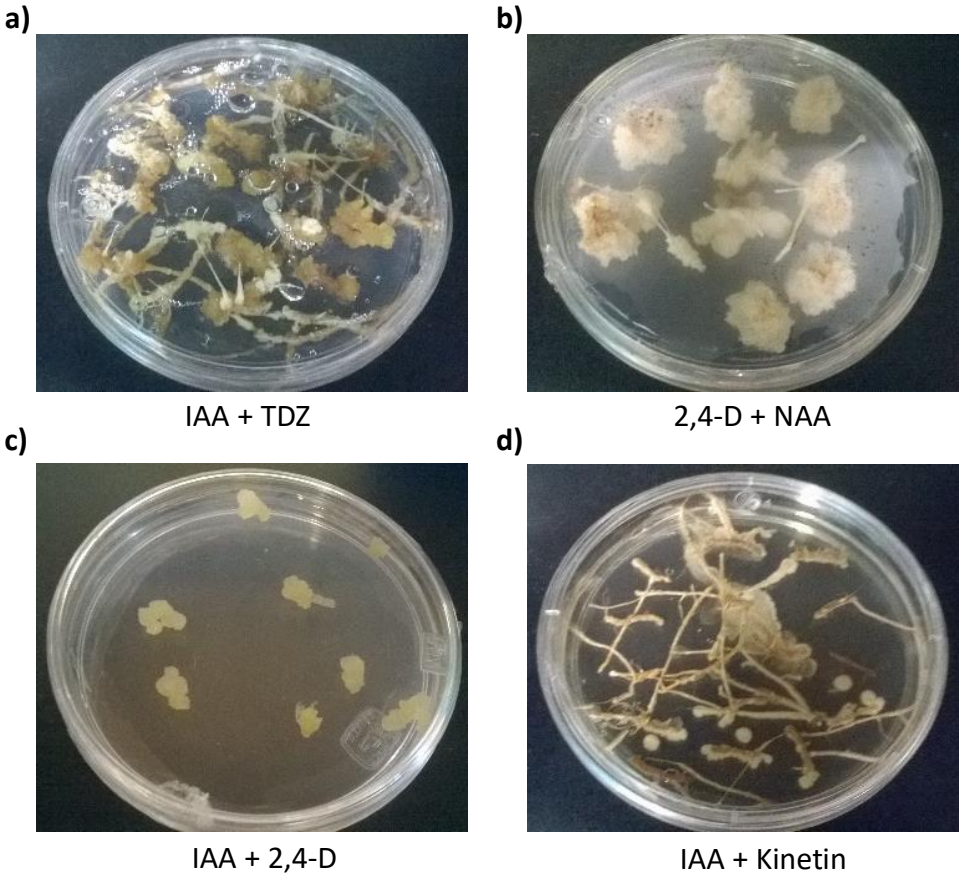

Figure 5. Effect of different combinations of phytohormones in common bean explants. Apical meristems of common bean were transferred to Murashige and Skoog solid medium supplemented with different combinations of phytohormones: **a)** 0.05 mg L^{-1} of indol-3-acetic acid (IAA) and 0.5 mg L^{-1} of thidiazuron (TDZ), **b)** 0.25 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.75 mg L^{-1} of α-naphtaleneacetic acid (NAA), **c)** 0.5 mg L^{-1} of IAA and 5 mg L^{-1} of 2,4-D and **d)** 0.5 mg L^{-1} of IAA and 0.5 mg L^{-1} of kinetin. Pictures were taken after four weeks.

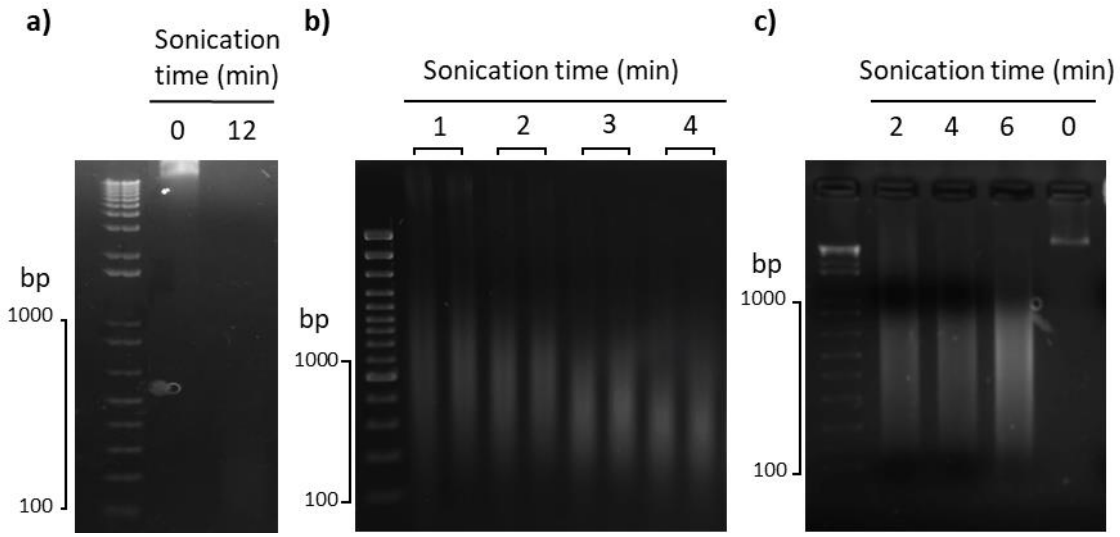

Figure 6. Fragments of DNA obtained by sonication. **a)** DNA sonicated under conditions reported by Mazzoleni et al 2015a (12 min at 90% power level, 0.9 s pulse 'On' and a 0.9 s pulse 'Off') was completely degraded. Different times of sonication were tested at 55 % power level, 1 s pulse 'On' and a 1 s pulse 'Off'; 1, 2, 3 and 4 min are shown in **b)** and 2, 4, 6 and 0 min are shown in **c)**. Fragments shorter than 1000 bp were obtained with 6 min of sonication.

5.2 Self eDNA inhibited primary root growth.

Self eDNA inhibited the growth of the primary root (

Figure 7a) of common bean seedlings in a dosage-dependent manner. A significant inhibition was observed at a concentration of 50 µL mL^{-1} of fragmented self eDNA, but higher concentrations had a stronger effect (

Figure 7b). Based on these results, we selected the concentration of 200 µg mL^{-1} for use in the subsequent experiments. The observed effect showed 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 (Figure 8a,b). Non-fragmented DNA did not cause any detectable inhibition of root growth.

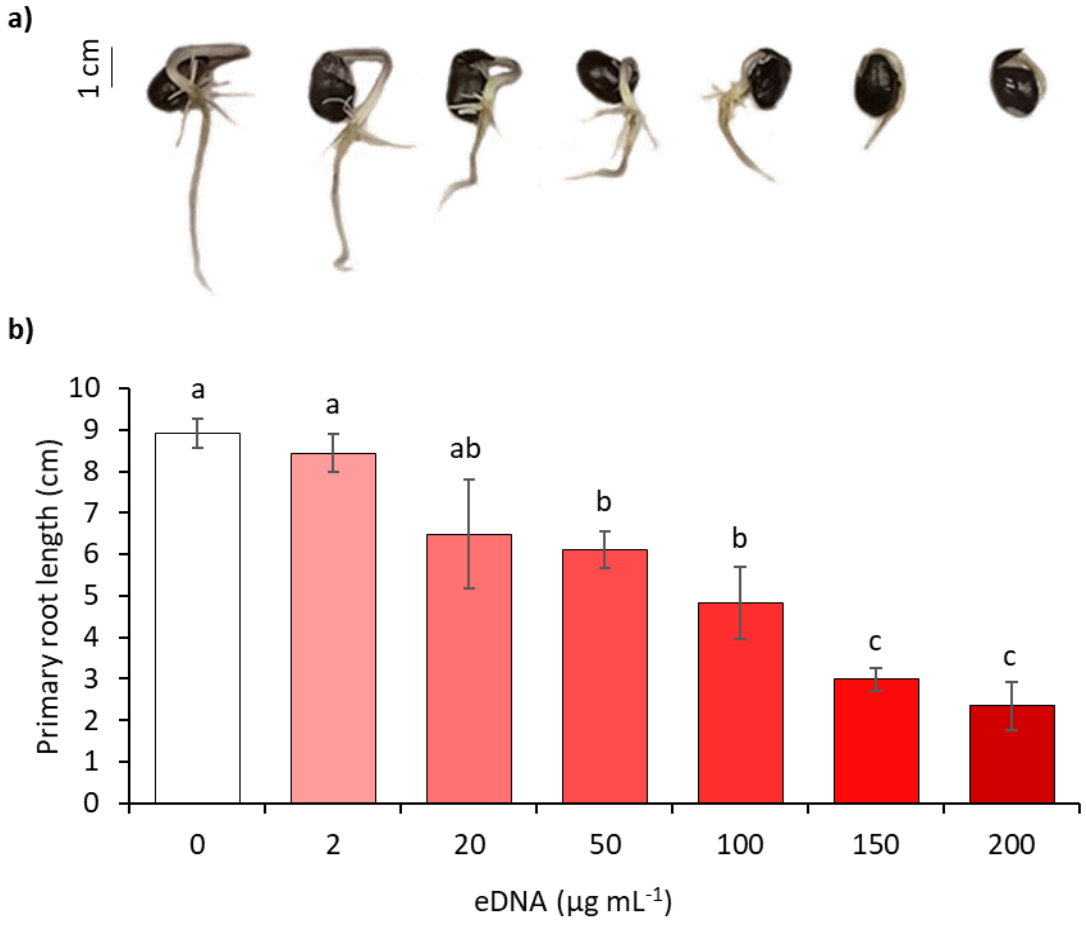

Figure 7. Extracellular self-DNA (eDNA) inhibits primary root growth in a concentration-dependent manner. **a)** The length of the primary root of common bean seedlings after four days in germination medium containing different concentrations of self eDNA (Common bean) is depicted in **b)** as the mean ± the SE. As the concentration of eDNA increases, the bars are depicted in a more intense red color; 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: $p < 0.05$, $n = 9$).

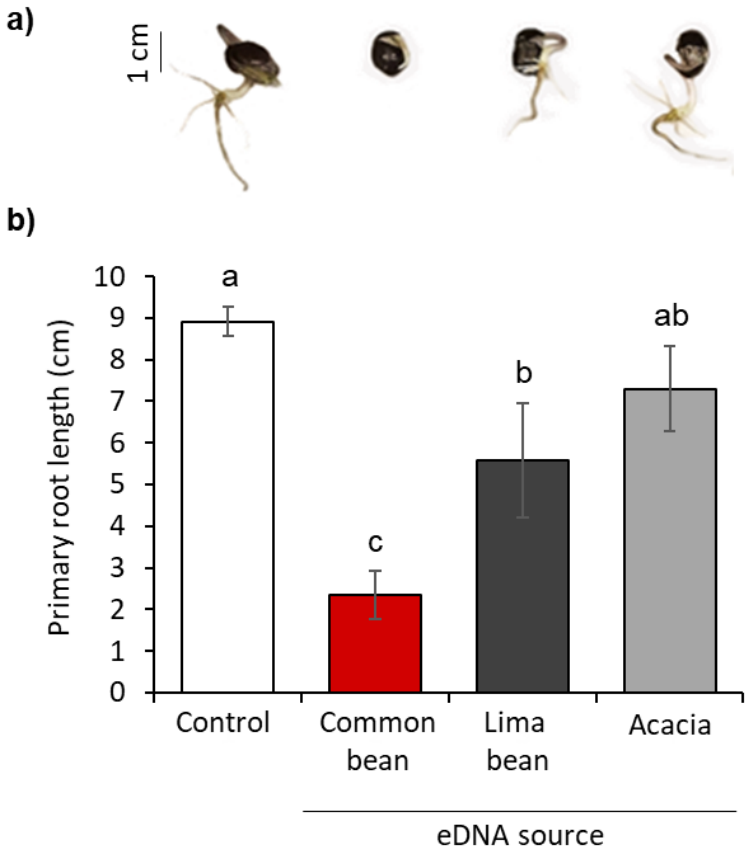

Figure 8. Primary root growth is differently affected by extracellular self and non-self DNA (eDNA). **a)** The length of the primary root of common bean seedlings after four days in germination medium containing 200 µg mL^{-1} of eDNA is depicted in **b)** as the mean ± the SE. White bar represents the control (0 µg mL^{-1} of eDNA), red bar represents self eDNA (Common bean), grey bars represent non-self eDNA (Lima bean or Acacia). Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 9$).

5.3 Self eDNA induced both, early and late signaling molecules (ROS, MAPKs, JA and SA)

The effect of different concentration of self eDNA on the formation of H_2O_2 in the leaves of common bean was evaluated at different times in order to select the optimal time for subsequent experiments. The results showed an increase on the formation of H_2O_2 3 minutes after treatments with 50 to 200 µg mL^{-1} of self eDNA, however, maximum level was reached 2 hours after treatment (

Figure ***9***a). We used 200 µg mL^{-1} of eDNA to test the effect of self eDNA on the formation of H_2O_2 in suspension cells at different times. In this case, we observed higher levels of H_2O_2 at 1 and 2 hours after treatment (
Figure ***9***b). We selected 2 hours to test the formation of H_2O_2 in subsequent experiments in which suspension cells were involved.

The effect of eDNA on the formation of H_2O_2 in the leaves showed taxonomic specificity. Self eDNA caused a significant (approximately three-fold) increase in H_2O_2. The effect of non-self eDNA was significantly lower, yet still significant with respect to the control (
Figure ***10***). 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
Figure ***11***a). 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 (
Figure ***11***a). Again, MAPKs responded to eDNA in a species-specific way. Self eDNA caused the 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 (
Figure ***11***b). Self eDNA also affected the endogenous levels of the phytohormones JA and SA. JA was induced by self eDNA in a concentration dependent manner, reaching the highest level 30 min after treatment (Figure 12a), and self eDNA induced significantly higher JA levels than non-self eDNA (Figure 12b). SA reached the highest level 24 h after applying eDNA, showing a significant response already to 20 µg mL^{-1} and a significantly stronger response to concentrations to all tested eDNA concentrations > 50 µg mL^{-1} (Figure 13a). In contrast to JA, no significantly different responses were induced by self or non-self eDNA 24 h after applying 50 µg mL^{-1} of eDNA (Figure 13a).

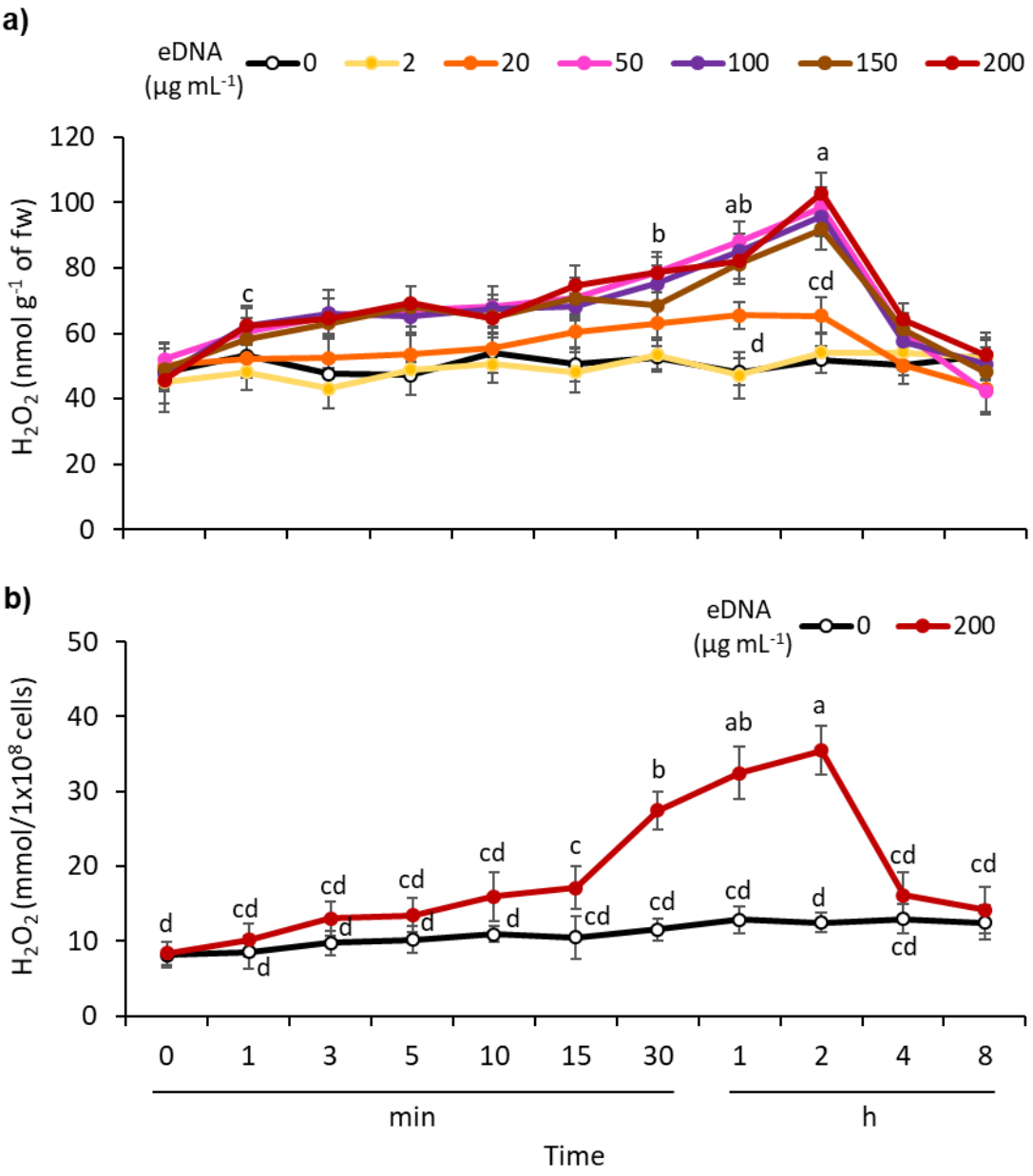

Figure 9. Extracellular self-DNA (eDNA) induces H_2O_2 formation in leaves and suspension cells. The concentration of H_2O_2 at different times after treatment with self eDNA is depicted as the mean ± the SE in **a)** nanomole per gram fresh mass after applying 0, 2, 20, 50, 100, 150 or 200 µg mL^{-1} of eDNA on leaves and in **b)** millimole per $1x10^8$ suspension cells after applying 0 or 200 µg mL^{-1} of self eDNA. Different letters indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 6$ in **a** and $n = 9$ in **b**).

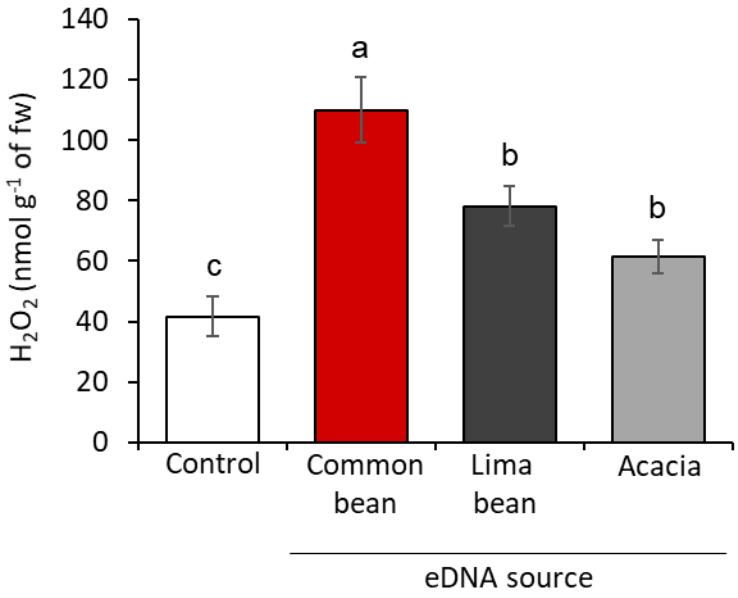

Figure 10. Response of H_2O_2 levels to extracellular self and non-self DNA (eDNA). The concentration of H_2O_2 in nanomol per gram fresh mass 2 h after applying 200 µg mL^{-1} of eDNA is depicted as the mean ± the SE. The white bar represents the control (0 µg mL^{-1} of eDNA), the red bar represents self eDNA (Common bean) and grey bars represent non-self eDNA (Lima bean or Acacia). Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 9$).

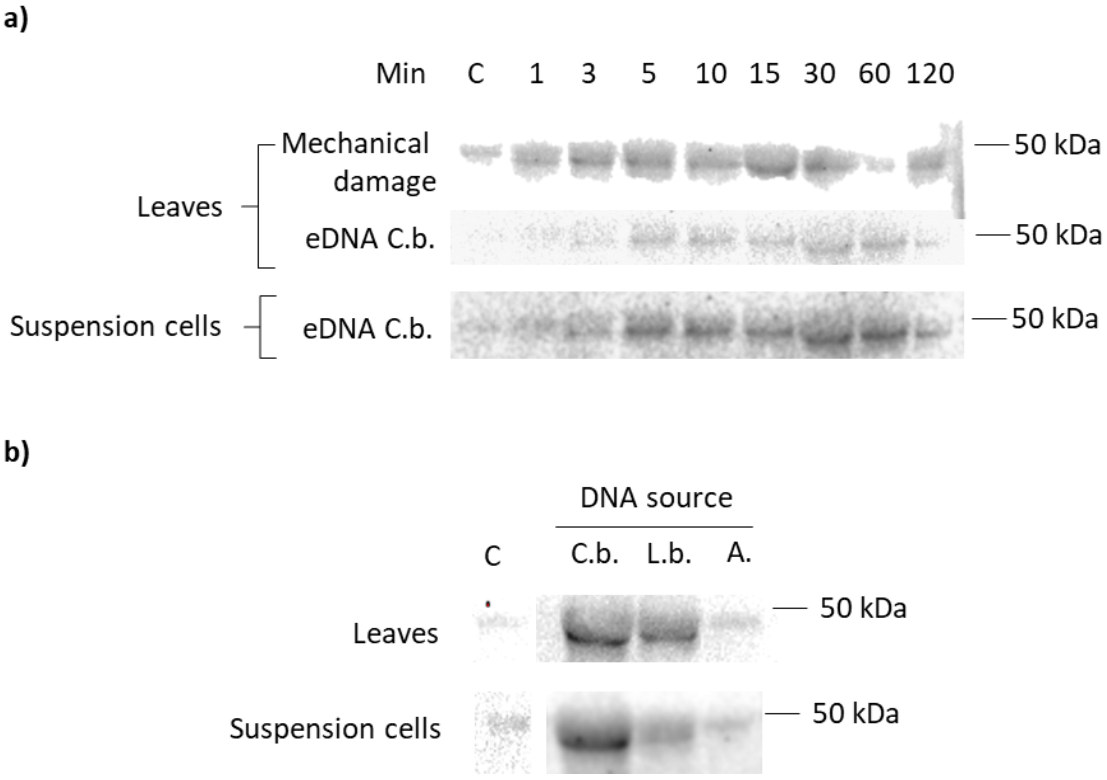

Figure 11. Extracellular DNA (eDNA) activates mitogen-activated protein kinases (MAPKs). **a)** The activation of MAPKs in leaves or suspension cells of common bean was tested at different times after treatment with 200 µg mL^{-1} of self-eDNA or mechanical damage (Positive control, only leaves). Intact leaves and suspension cells treated with 0 µg mL^{-1} of eDNA served as negative 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 (Common bean, C.b.) or non-self eDNA (Lima bean, L.b.; or Acacia, A.).

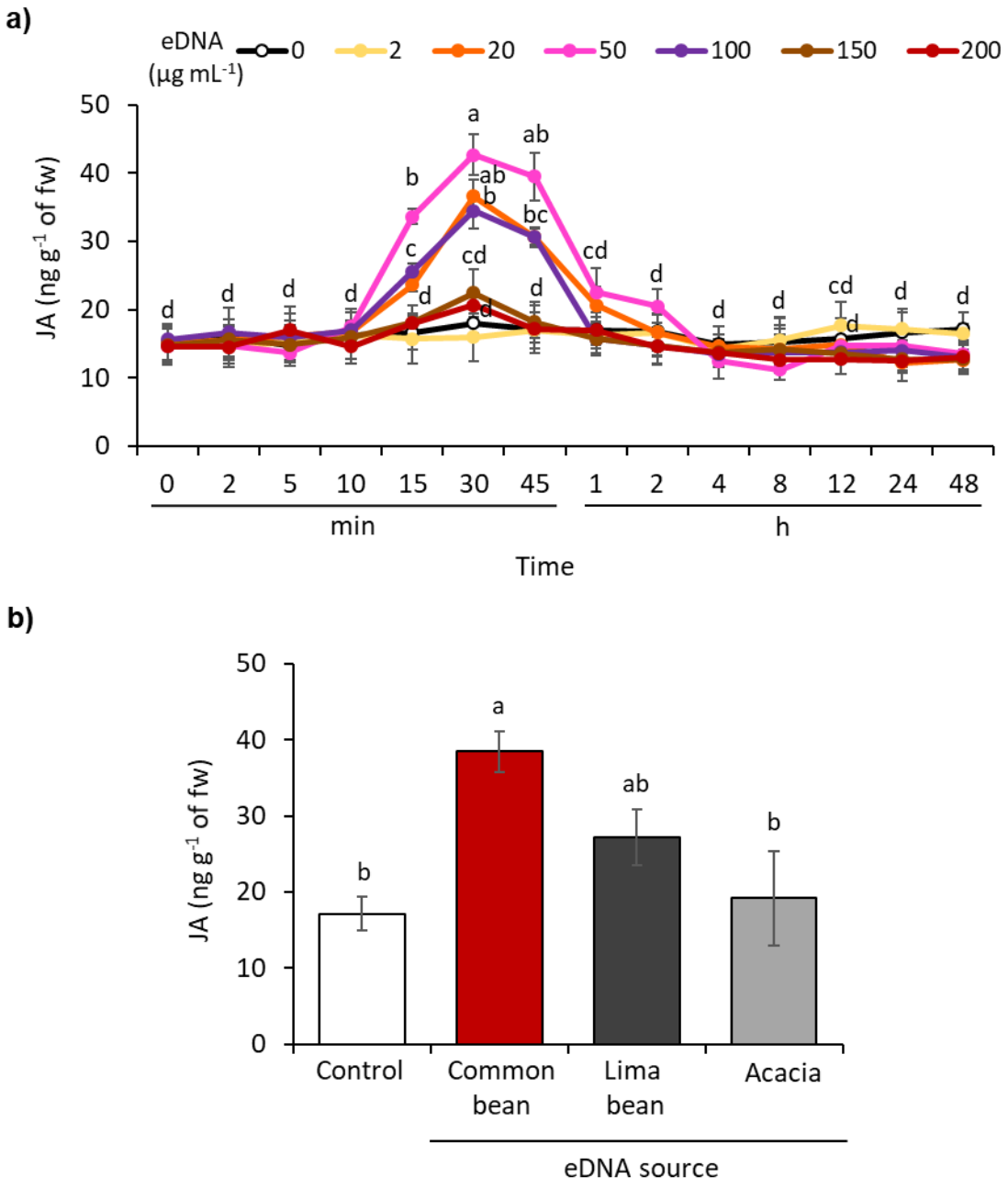

Figure 12. Extracellular DNA (eDNA) affects endogenous levels of jasmonic acid (JA). The concentration of JA in nanograms per gram of fresh weight **a)** at different times after applying 0, 2, 20, 50, 100, 150 or 200 µg mL^{-1} of self eDNA on leaves and **b)** 30 min after applying 50 µg mL^{-1} of eDNA from self (Common bean; red bar) or non-self (Lima bean and Acacia; grey bars) sources is depicted as the mean ± the SE. White bar represents the control (0 µg mL^{-1} of eDNA). Different letters indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 6$).

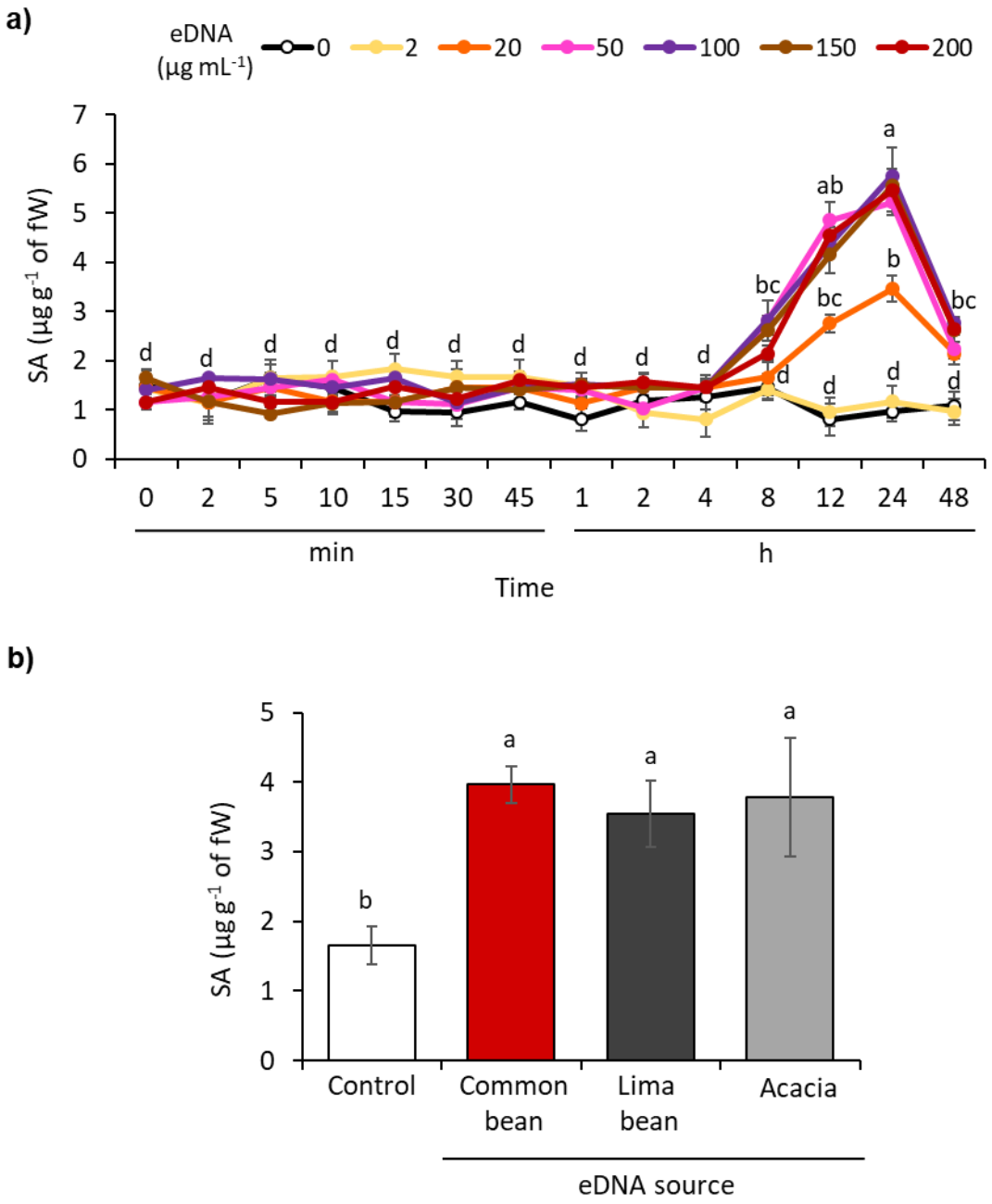

Figure 13. Extracellular DNA (eDNA) affects endogenous levels of salicylic acid (SA). The concentration of SA in micrograms per gram of fresh weight **a)** at different times after applying 0, 2, 20, 50, 100, 150 or 200 µg mL^{-1} of self eDNA on leaves and **b)** 24 h after applying 50 µg mL^{-1} of eDNA from self (Common bean; red bar) or non-self (Lima bean and Acacia; grey bars) sources is depicted as the mean ± the SE. White bar represents the control (0 µg mL^{-1} of eDNA). Different letters indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 7$).

5.4 Self eDNA induced phenotypic resistance (EFN and resistance against herbivory and pathogens)

The secretion of EFN was significantly induced 24 h after applying 50 or 100 µg mL^{-1} of self eDNA (Figure 14a), whereas higher concentrations (150 and 200 µg mL^{-1}) of self eDNA inhibited the EFN secretion (Figure 14a). The induction of EFN depended on the source of eDNA as it responded significantly only to 50 µg mL^{-1} of self eDNA, but not non-self eDNA (Figure 14b). The effects of eDNA on the phenotypic resistance to *Spodoptera frugiperda* also was species-specific: self-eDNA applied at 50 or 200 µg mL^{-1} of self eDNA decreased the percentage of lost leaf area by ca. 95%, whereas nonself eDNA from lima bean at 200 µg mL^{-1} decreased leaf area loss by ca. 90% , while eDNA from Lima bean at 50 µg mL^{-1} or eDNA from Acacia at both concentrations did not cause a significant effect (Figure 15).

Plants that were treated with either self or non-self eDNA exhibited significantly lower infection rates by the bacterial phytopathogens tested (*Enterobacter* sp*, Pseudomonas syringae* pv*. phaseoli and* pv*. syringae* and *Xanthomonas phaseoli*) when they were inoculated 5 min after eDNA treatments (Figure 16a). By contrast, eDNA treatment did not significantly affect infection levels when bacteria were inoculated 24 h after treatments (Figure 16b). Self and no-self eDNA also caused a lower infection by fungal pathogens (Figure ***17***). Nevertheless, infection rates by the necrotrophic fungi *B. cinerea* and *S. sclerotiorum* were significantly lower when they were inoculated 5 min after eDNA treatments (
Figure ***17***a) whereas the hemibiotrophic fungi *F. oxysporum* and *C. lindemuthianum* and the necrotrophic *S. sclerotiorum* showed lower infection when they were inoculated 24 h after treatments, although only *F. oxysporum* infection was significantly lower (
Figure ***17***b). When we tested for putative direct effects of the eDNA solutions against bacterial and fungal pathogens in Petri dish*,* no effect was observed (Figure 18 and Figure 19).

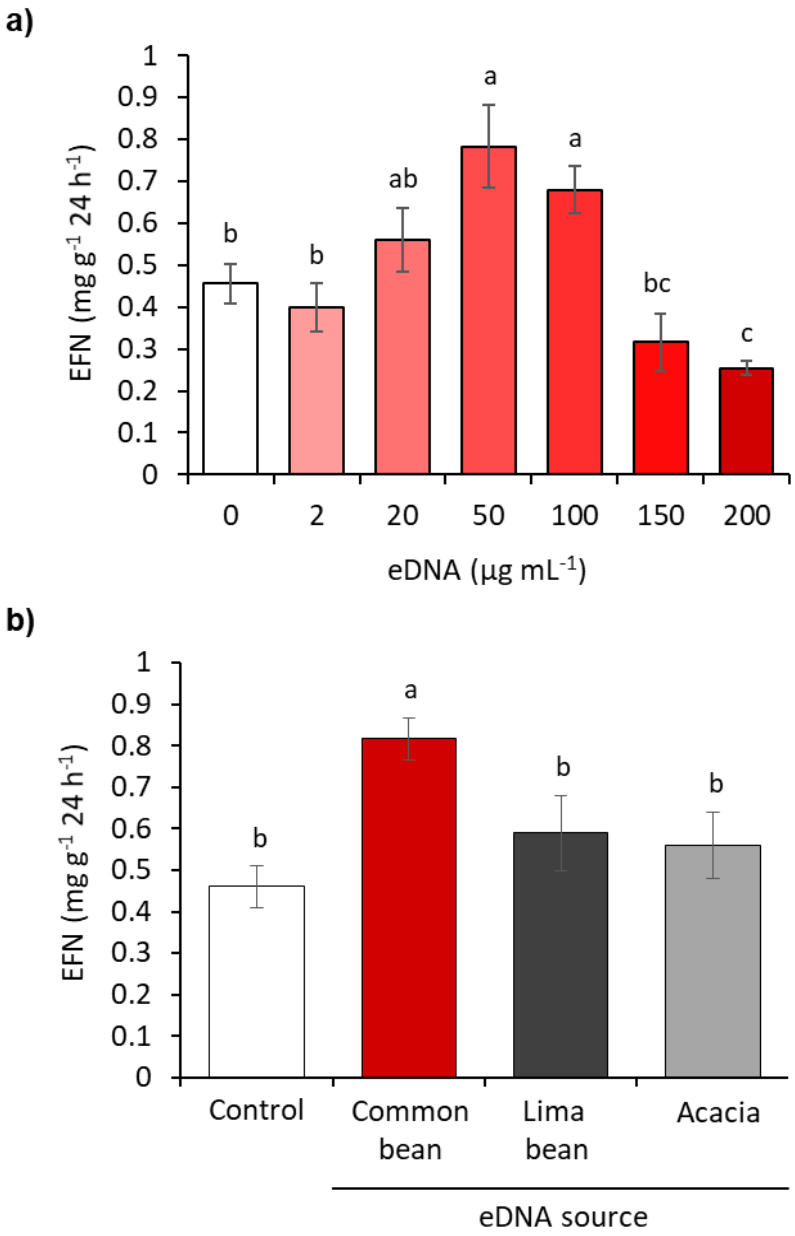

Figure 14. Extracellular DNA (eDNA) induces extrafloral nectar (EFN) secretion. The EFN secretion by common bean plants is depicted as the mean ± the SE of mg of soluble solids per gram of dry leaf mass quantified 24 h after applying **a)** 0, 2, 20, 50, 100, 150 or 200 µg mL^{-1} of self eDNA or **b)** 50 µg mL^{-1} of self eDNA (Common bean; red bar) or non-self eDNA (Lima bean or acacia; grey bars). As the concentration of eDNA increases in **a)**, the bars are depicted in a more intense red color. White bar represents the control (0 µg mL^{-1} of eDNA). Different letters indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 6$).

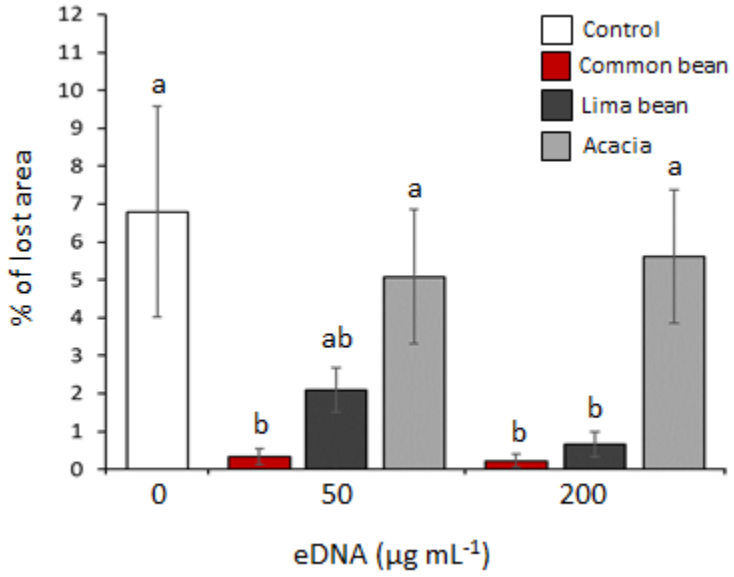

Figure 15. Extracellular DNA (eDNA) induces resistance against herbivory. The percentage of lost leaf area by herbivory 24 h after the exposure to *Spodoptera frugiperda* of common bean plants treated with 50 and 200 µg mL^{-1} of self (Common bean; red bars) or non-self eDNA (Lima bean or acacia; grey bars). The white bars represent the controls (0 µg mL^{-1} of eDNA). Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 7$).

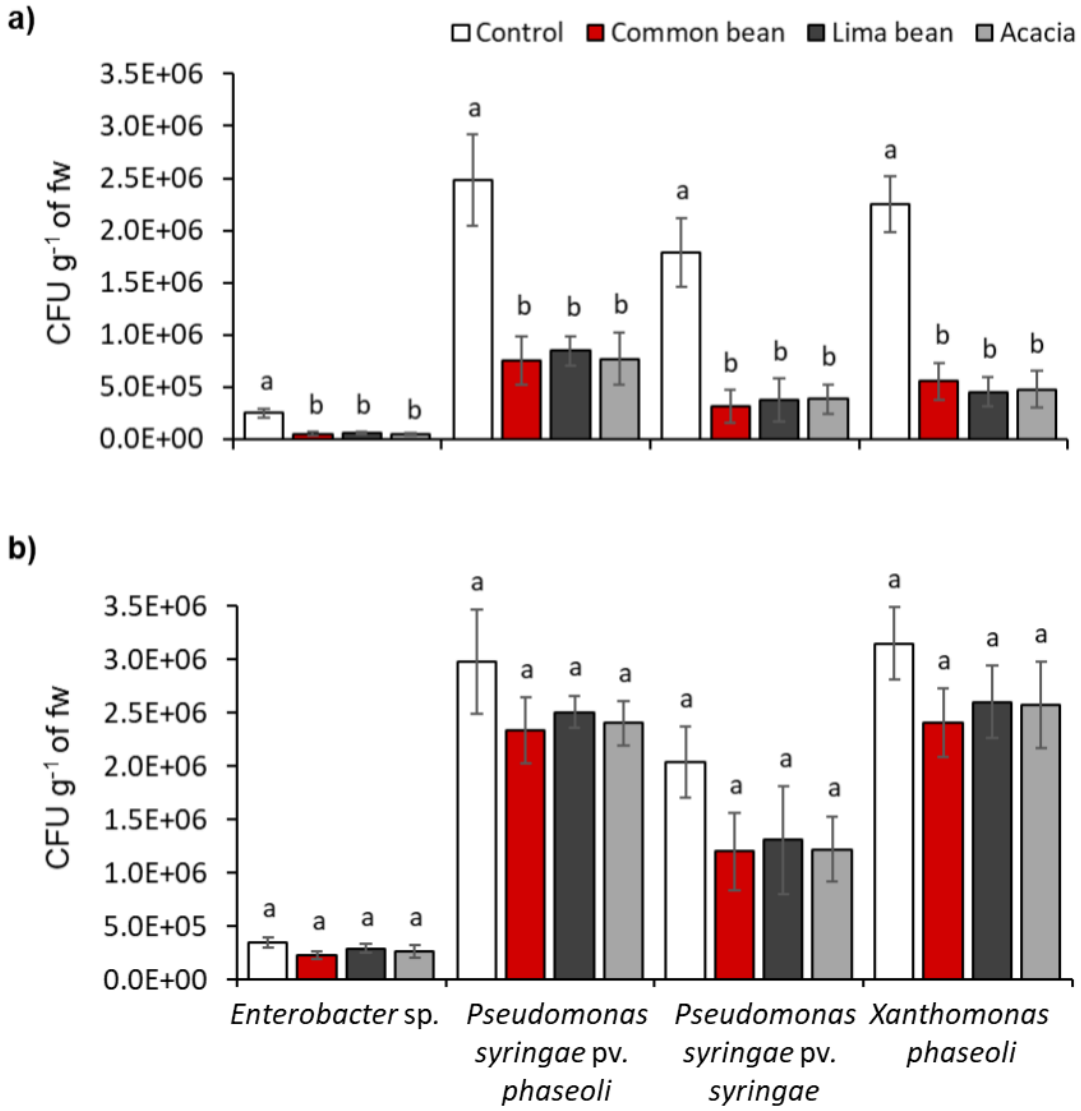

Figure 16. Extracellular DNA (eDNA) induces the resistance against bacterial phytopathogens. Colony-forming units (CFU) per gram of leaf fresh weight are depicted for *Enterobacter* sp, *Pseudomonas syringae* pv. *phaseoli* and pv. *syringae* and *Xanthomonas phaseoli* on common bean plants treated with 50 µg mL^{-1} of self (Common bean) or non-self eDNA (Lima bean and acacia). **a)** CFU in plants inoculated with bacterial pathogens 5 min after treatments with eDNA. **b)** CFU in plants inoculated 24 h after treatments. All samples were taken 8 days after inoculation. Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 10$).

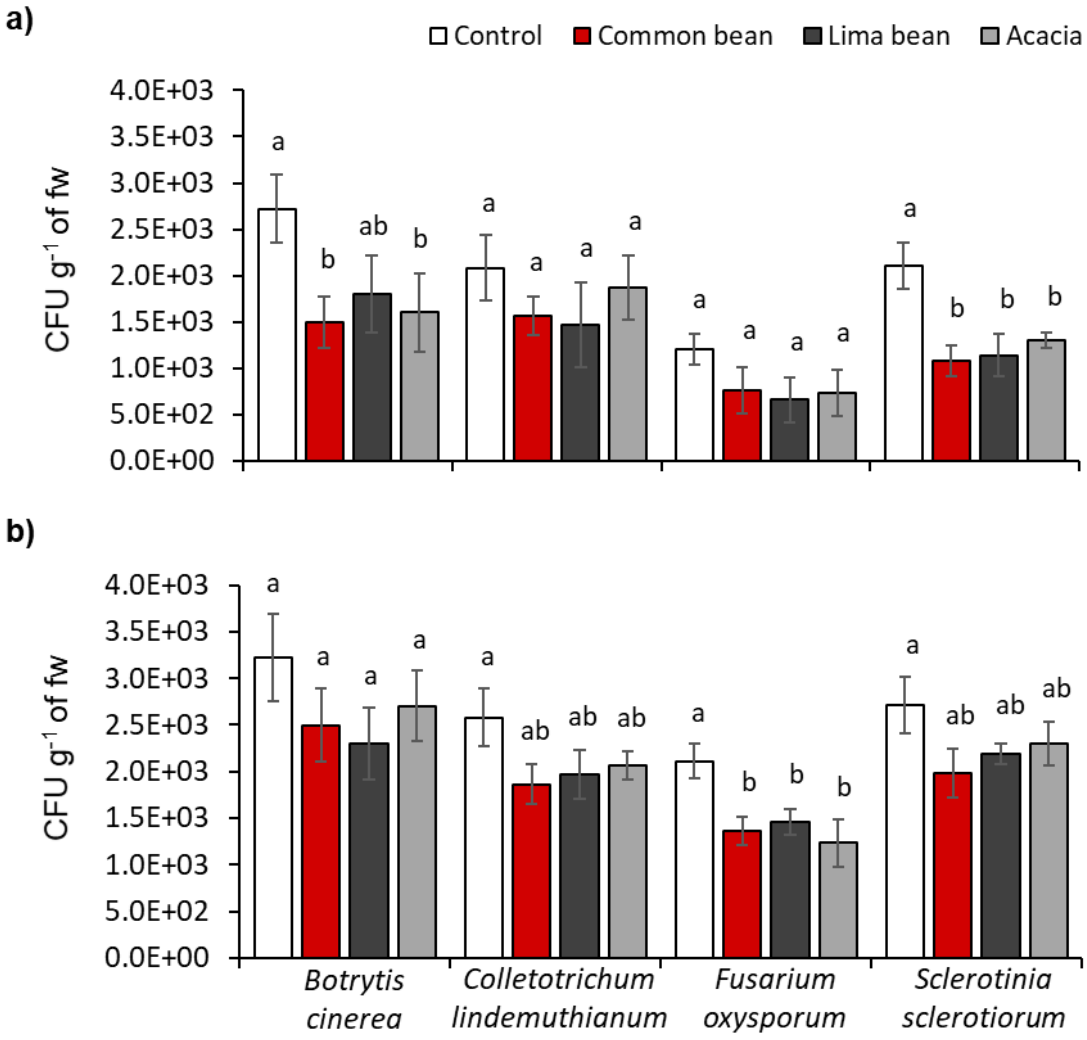

Figure 17. Extracellular DNA (eDNA) reduces the infection by fungal phytopathogens. Colony-forming units (CFU) per gram of fresh weight of leaf are depicted for the fungi *Botrytis cinerea*, *Colletotrichum lindemuthianum*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* on Common bean plants treated with 50 µg mL^{-1} of self (Common bean) or non-self eDNA (Lima bean and Acacia). **a)** CFU on plants inoculated with fungal pathogens 5 min after treatments with eDNA. **b)** CFU on plants inoculated 24 h after treatments. All samples were taken 15 days after inoculation. Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 10$).

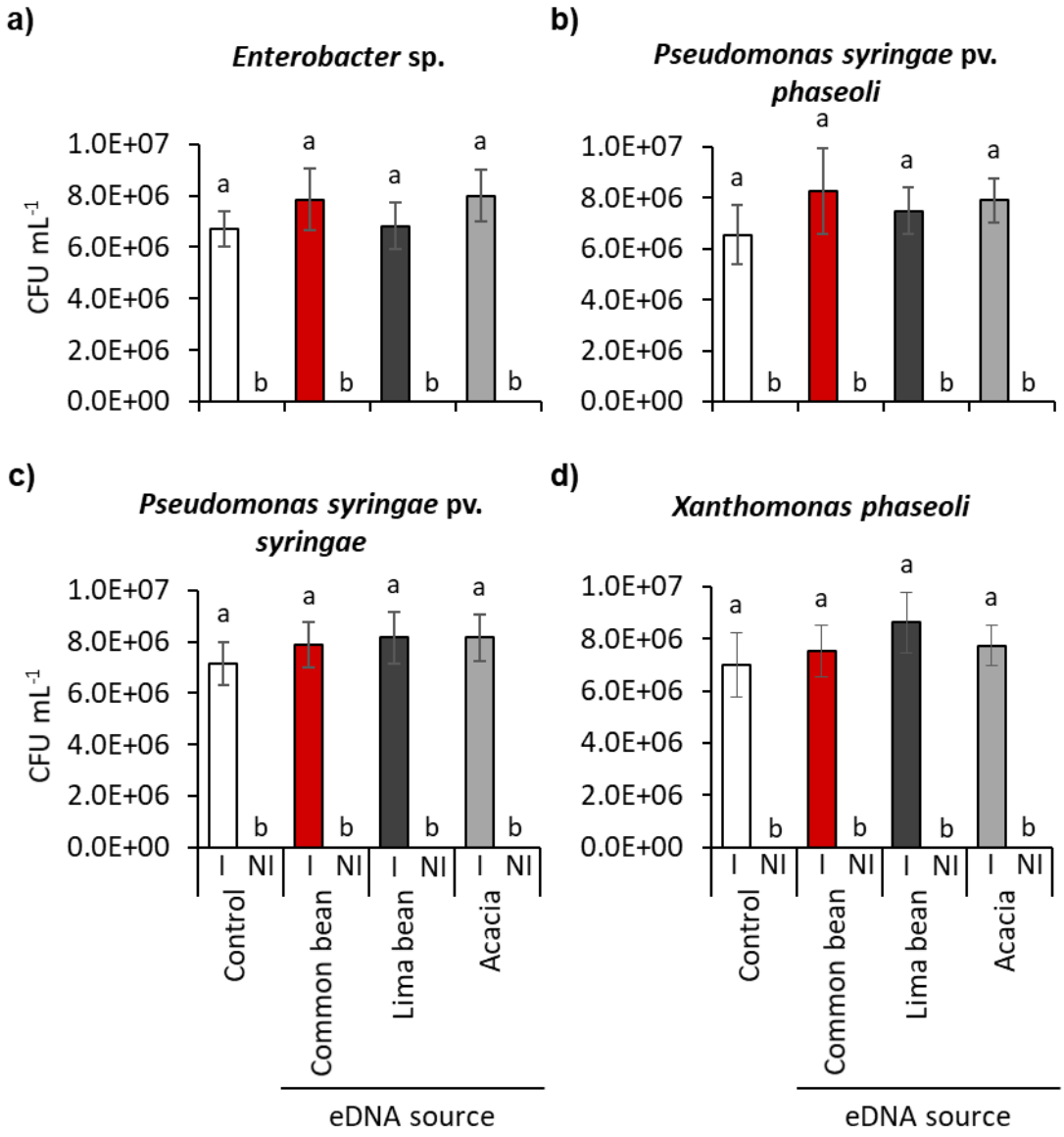

Figure 18. Extracellular DNA (eDNA) does not directly inhibit bacterial phytopathogens. The bars indicate the number of colony-forming units (CFU) observed two days after inoculating (I) the bacterium **a)** *Enterobacter* sp*,* **b)** *Pseudomonas syringae* pv *phaseoli,* **c)** *P. Syringae* pv*. Syringae and* **d)** *Xanthomonas phaseoli* on Petri dishes that 5 min before had been spread with eDNA from different plant sources. Negative controls were not inoculated (NI) with bacterial phytopathogens to ensure that the eDNA samples themselves did not carry bacteria. Bars indicate means ± SE of n = 6 repetitions and different letters indicate significant difference among treatments (Tukey test: $p<0.05$).

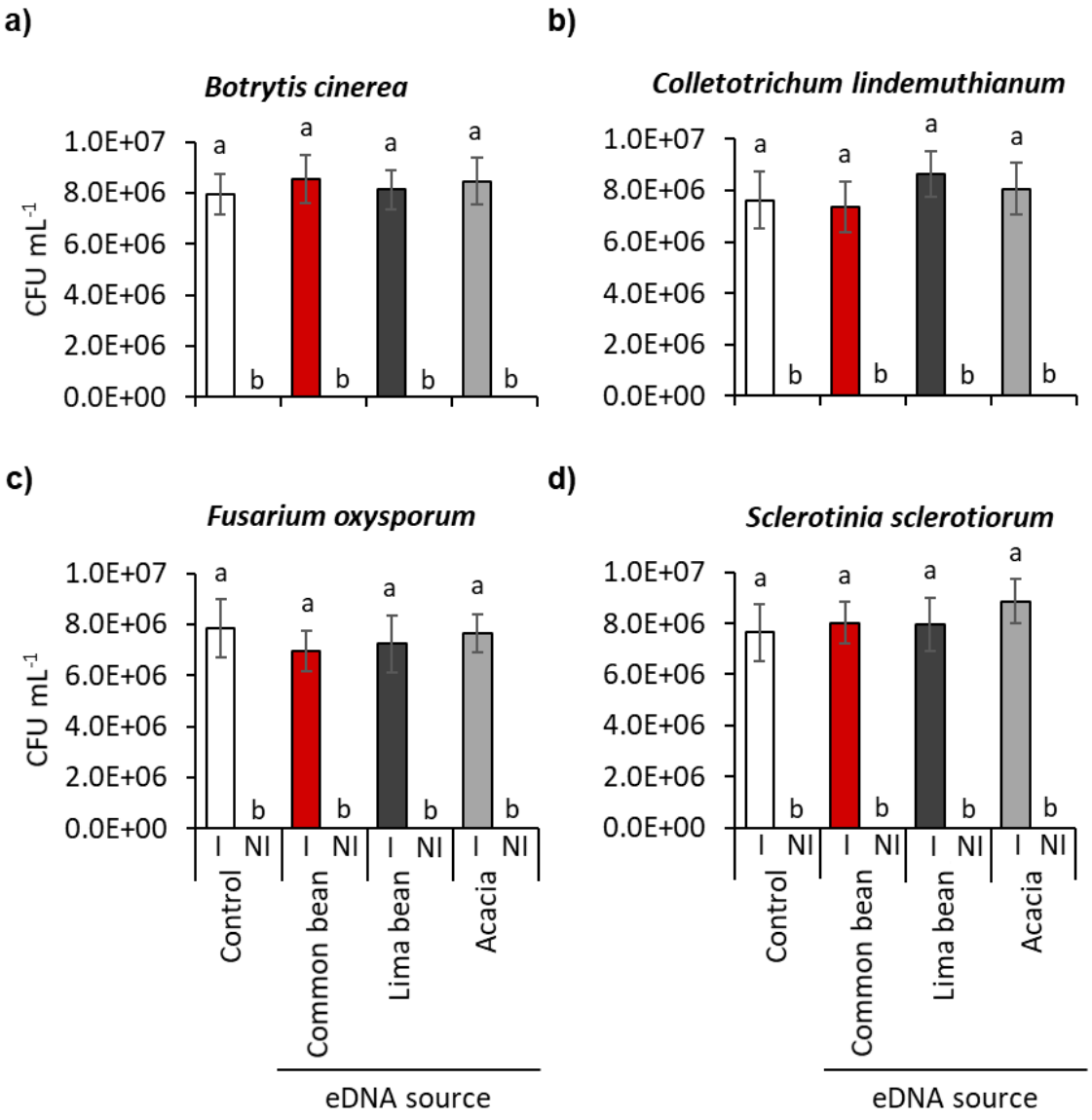

Figure 19. Extracellular DNA (eDNA) do not directly inhibit fungal phytopathogens. The bars indicate the number of colony-forming units (CFU) observed 4 days after inoculating (I) the fungus **a)** *Botrytis cinerea,* **b)** *Colletotrichum lindemuthianum*, **c)** *Fusarium oxysporum* or **d)** *Sclerotinia sclerotiorum* on Petri dishes that 5 min (a and d) or 24 h (b and c) before had been spread with eDNA from different plant sources. Negative controls were not inoculated (NI) with fungal phytopathogens to ensure that the eDNA samples themselves did not carry fungi. Bars indicate means ± SE of n = 6 repetitions and different letters indicate significant difference among treatments (Tukey test: $p<0.05$).

5.5 Self eDNA treatment modified phenological stages and enhanced yield

Under field conditions, treatment with eDNA, leaf homogenates or mechanical damage affected the phenological stages (Figure 20), the number of leaves (Figure 21) and the yield (Figure 22) of common bean plants. We observed a delayed flowering after treatment with self eDNA whereas mechanically damaged plants flowering earlier than plants exposed to other treatments. The pod filling occurred first in mechanically damaged plants, then, one week later, in plants treated with self eDNA or self homogenate, and another week later, in all other plants. However, apparently the pod filling was accelerated just after the flowering with self eDNA treatment occurring faster than in plants with either other treatment, just 3 weeks after flowering, whereas in plants treated with mechanical damage or self homogenate the pod filling occurred 4 weeks after flowering and after 5 weeks in plants treated with non-self eDNA or homogenates. Dead of plants also occurred first with mechanically damaged plants, one week later, dead occurred in self eDNA treated plants and another week later in the other plants (Figure 20).

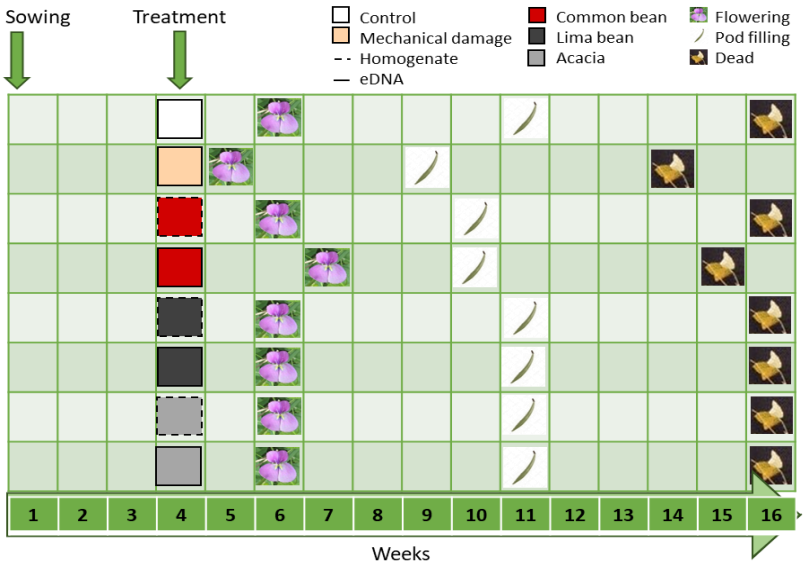

Figure 20. Time of flowering, pod filling and dead of common bean are affected by extracellular self eDNA (eDNA). Time of flowering, pod filling and dead of common bean are affected by extracellular self eDNA (eDNA). Figure shows the observations about the time of flowering, pod filling and dead along the weeks of common bean plants development after applying self and non-self eDNA or homogenates. Mechanical damaged plants were used as positive DAMPs control. Plants without treatment were used as negative control.

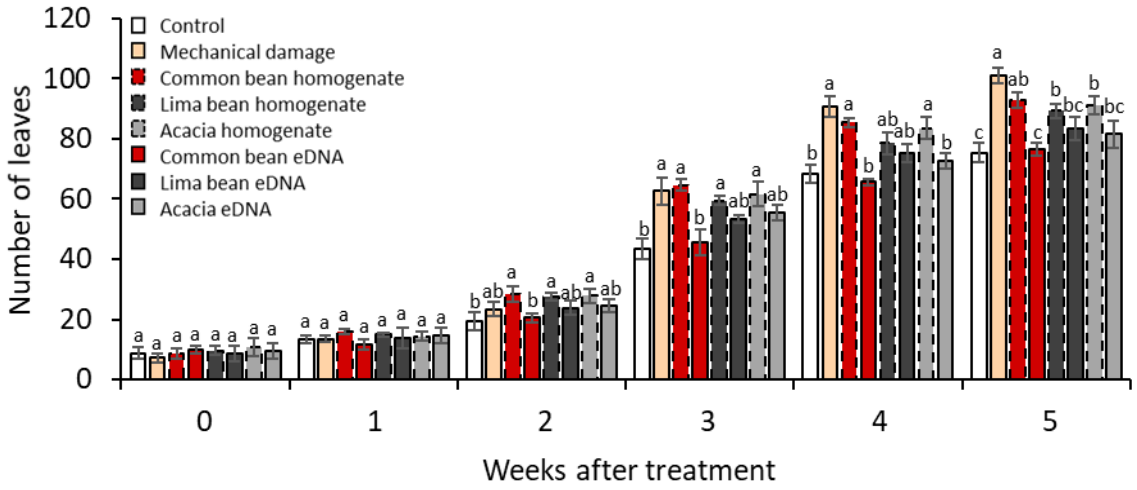

Figure 21. The number of leaves is not affected by extracellular DNA (eDNA). The means ± SE of number of leaves in common bean plants along the 5 weeks after applying self and non-self eDNA or homogenates. Mechanical damaged plants were used as positive DAMPs control. Plants without treatment were used as negative control. Different letters indicate significant differences among treatments per week (ANOVA and post hoc Fisher's LSD test: $p < 0.05$, n = 3 blocks of 7 individuals).

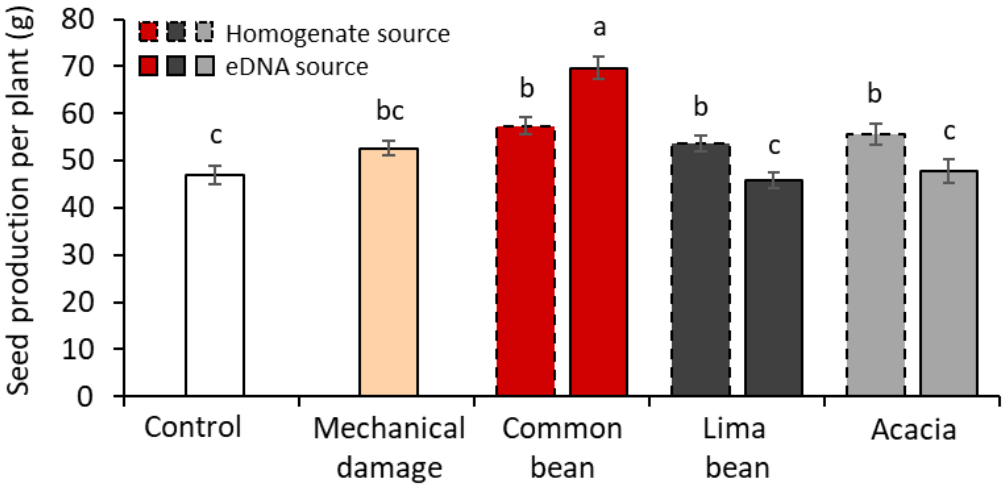

Figure 22. Treatment with extracellular self-DNA (eDNA) enhances yield. Seed production by common bean plants after treatment with self and non-self eDNA or homogenates are depicted as the mean of grams produced per plant ± SE. Mechanical damaged plants were used as positive DAMPs control. Plants without treatment were used as negative control. Different letters indicate significant differences among treatments (ANOVA and post hoc Fisher's LSD test: $p < 0.05$, n = 3 blocks of 7 individuals).

5.6 Fragments of DNA were confirmed as active principle.

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 (Figure 23a), the formation of ROS (Figure 23b) and the EFN secretion in common bean plants. Whereas the treatment with RNAse or proteinase did not detectably affect the activation of MAPKs, the formation of H_2O_2 or EFN secretion by self eDNA, no effects could be detected when the self eDNA had been treated with DNAse before its application (Figure 23**¡Error! No se encuentra el origen de la referencia.**a,b,c). When we used deactivated enzymes (Figure 24) or individual nucleotides (Figure 25), no changes to the inducing effects were observed. Furthermore, a random amplification of common bean DNA using a concentration of 8 µM of primer resulted in the amplification of fragments shorter than 1000 bp (Figure 26a), however, this 'synthetic' self-eDNA did not cause any detectable effect on the activation of MAPKs (Figure 26a) or on the formation of H_2O_2 (Figure 26c).

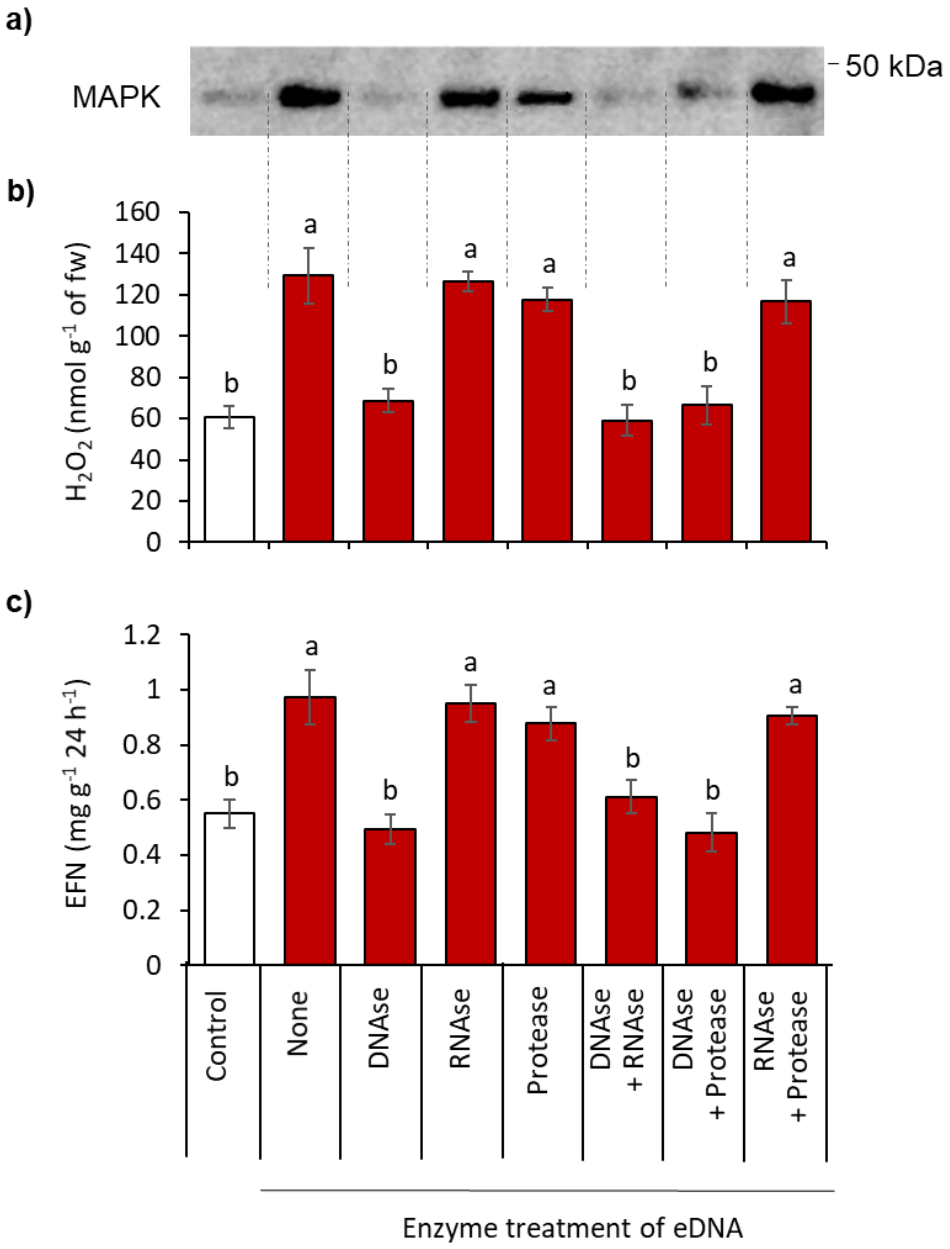

Figure 23. Digestion of self eDNA eliminates its resistance-inducing effects**.** Extracellular DNA from common bean was treated with enzyme (DNAse, RNAse, proteinase or combination of these) and applied to common bean leaves. **a)** The activation of MAPKs was tested 30 min after applying 200 µg mL^{-1} of eDNA fragments. The experiment was repeated three times with similar results. **b)** The concentration of H_2O_2 in nanomol per gram of fresh mass 2 h after applying 200 µg mL^{-1} of DNA fragments and **c)** the secretion of extrafloral nectar (EFN) in mg per gram of dry mass 24 h after applying 50 µg mL^{-1} of eDNA fragments is depicted as the mean ± the SE. The control treatment (C) consisted of the application of a solution of 0.05 % v v^{-1} Tween 20. Different letters above bars indicate significant differences among treatments (univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 9$).

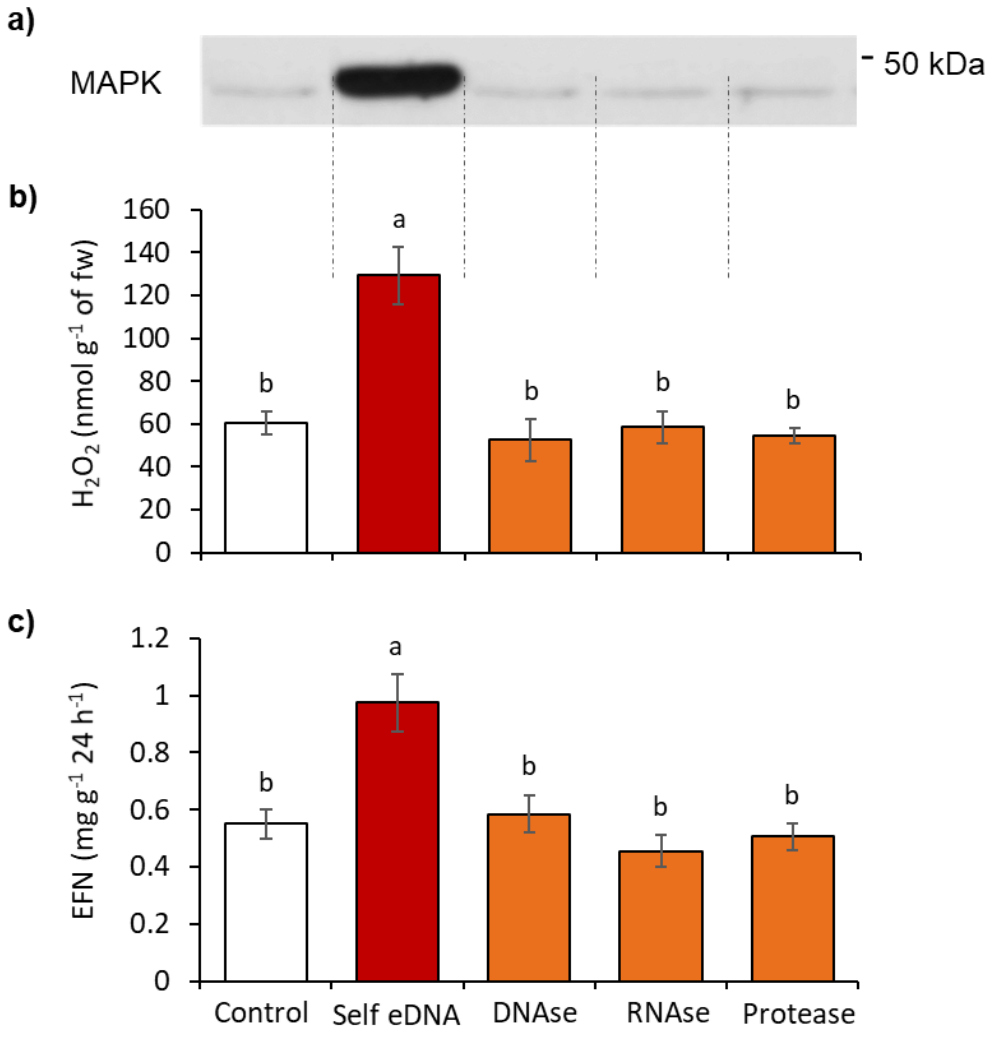

Figure 24. Deactivated DNAse, RNAse and protease do not reduce the resistance-inducing effect. Common bean leaves were treated with enzymes that were used to confirm eDNA as active principle. **a)** The activation of MAPKs was tested 30 min after treatment, **b)** the concentration of H_2O_2 in nanomol per gram of fresh mass 2 h after treatment and **c)** the secretion of extrafloral nectar (EFN) in mg per gram of dry mass 24 h after treatment are depicted as the mean ± the SE. The control treatment (white bar) consisted of the application of a solution of 0.05 % v v^{-1} Tween 20 and treatment with self eDNA (red bar) was used as positive control. Different letters above bars indicate significant differences among treatments (univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 9$).

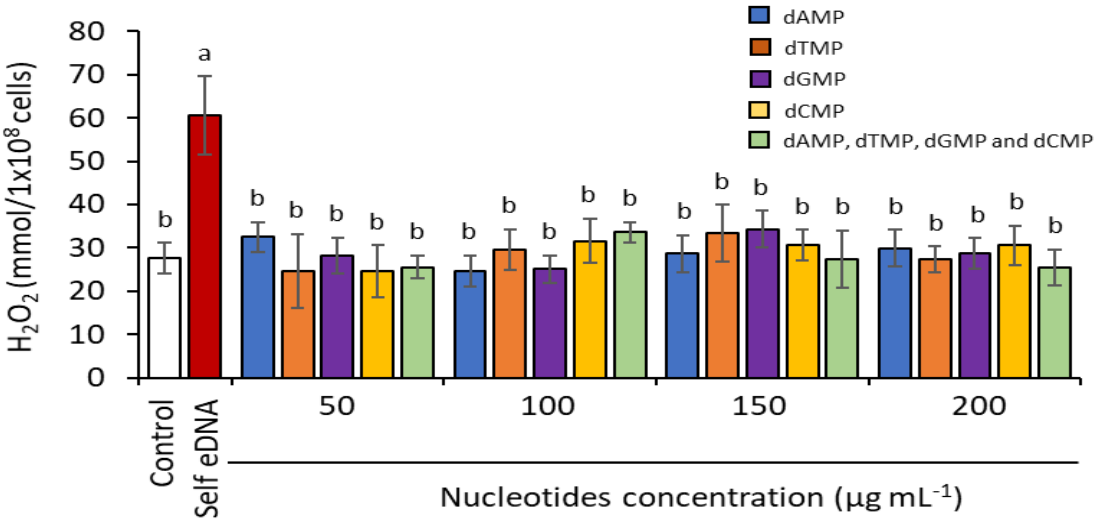

Figure 25. Individual nucleotides do not induce H_2O_2 generation. Millimoles of hydrogen peroxide generated by 1x10^8 of common bean suspension cells 2 h after applying individual nucleotides at different concentrations (50, 100, 150 and 200 µg mL^{-1}). Nucleotides were: deoxyadenosine monophosphate (dAMP), deoxythymidine monophosphate (dTMP), deoxyguanosine monophosphate (dGMP) and deoxycytidine monophosphate (dCMP). Control was treated with water (white bar) and we use self eDNA treatment as positive control (red bar). Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 3$).

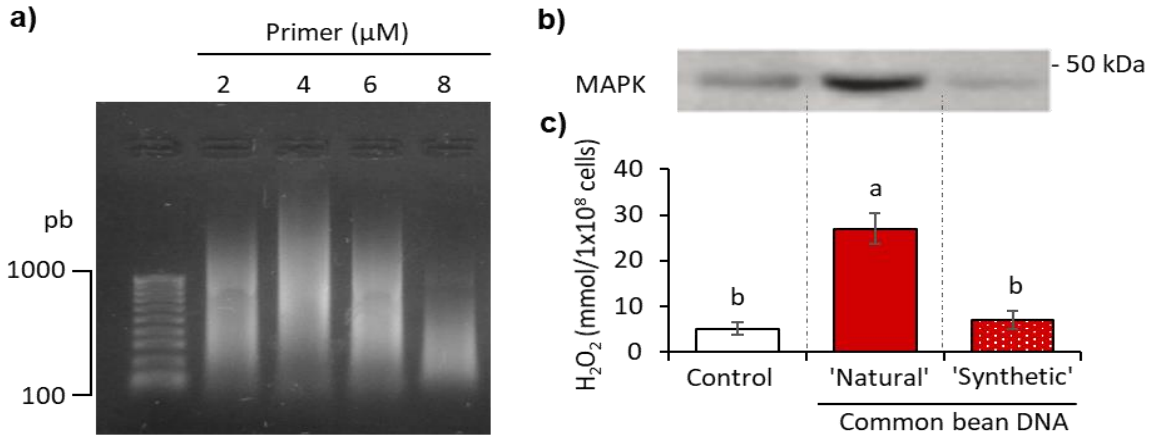

Figure 26. Random amplified fragments of self DNA do not affect early signaling. **a)** Random amplification of common bean DNA by PCR at different concentrations (2, 4, 6 and 8 µM) of the primer 5' GTTGCTCC 3´ used to obtain 'synthetic' self-DNA. a) The activation of MAPKs was tested 30 min after applying 200 µg mL^{-1} of 'natural' or 'synthetic' self-DNA in suspension cells. **b)** Millimoles of H_2O_2 per 1x10^8 suspension cells 2 h after applying 200 µg mL^{-1} of 'natural' or 'synthetic' self-DNA. Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 9$).

5.7 More details about eDNA perception in plants

5.7.1 The size of active eDNA fragments was <750 pb

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 (Figure 27).

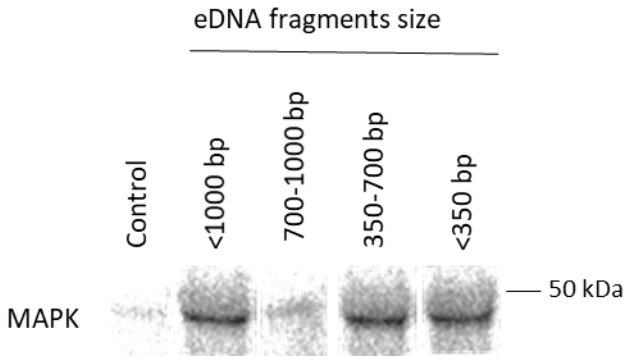

Figure 27. Small extracellular DNA fragments activate MAPKs in common bean. Sonicated self eDNA was separated on 3% agarose gels and fragments were re-extracted from regions corresponding to different size ranges (<1000 bp, 700−1000 bp, 350−700 bp or <350 bp) and applied at 200 µg mL^{-1} of eDNA to suspension culture cells. The activation of MAPKs was tested after 30 min. Water was used as the control treatment.

5.7.2 CpG methylation in self eDNA influences its effect on resistance responses.

In order to determine if DNA methylation marks are involved in the effects of self eDNA, we used CpG methylation-sensitive (HapII) and non-sensitive restriction (MspI) enzymes to evaluate the effects on H_2O_2 formation. The effectiveness of the enzymes was confirmed by the restriction of common bean eDNA which previously was methylated with the methyltranseferase M.SssI (Figure 28a). These digestions showed that common bean DNA naturally contains methylated and non-methylated CpG sites. An increase in H_2O_2 formation

was observed as long as the eDNA was either fragmented by sonication or digested by the enzymes MspI or HapII. However, the effect always was lower when the eDNA had been digested in non-methylated CpG sites or they were completely methylated (Figure 28b).

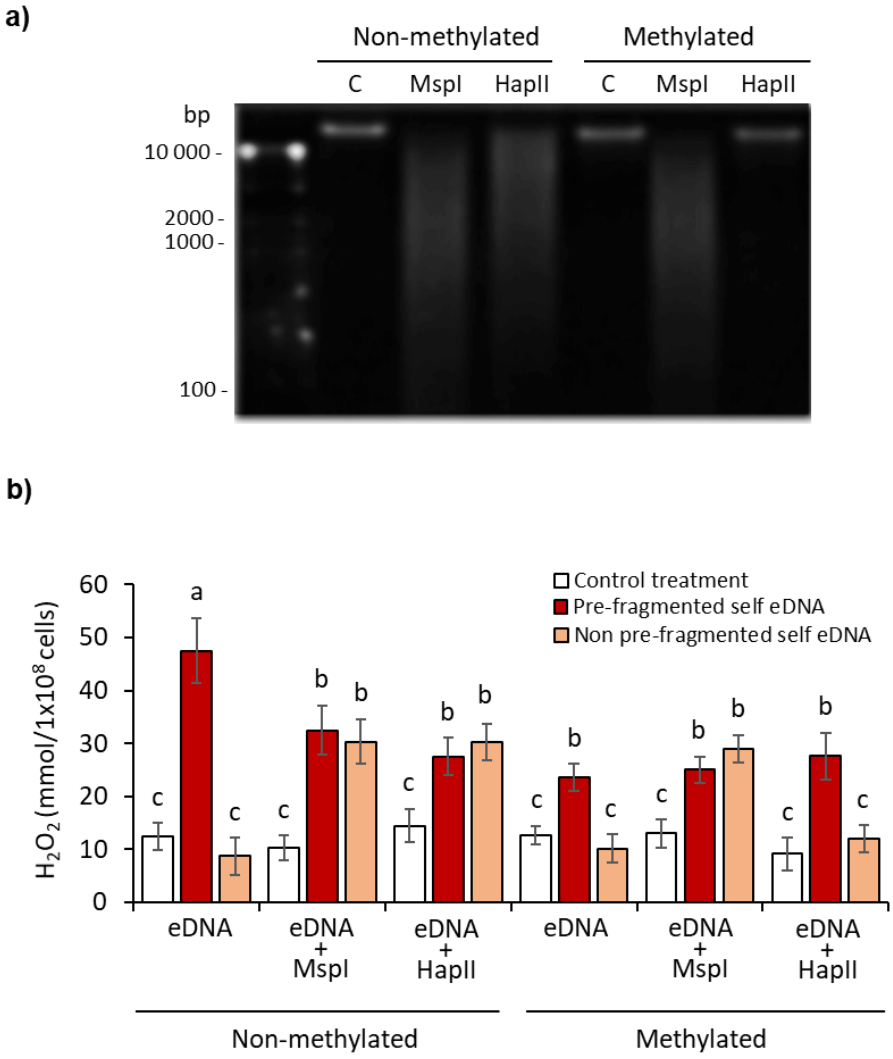

Figure 28. Non-methylated CpG sites in extracellular self eDNA (eDNA) influence the effects on H_2O_2 formation. **a)** The activity of CpG methylation-insensitive MspI and CpG methylation-sensitive HapII enzymes was confirmed by the digestion of non-methylated and methylated DNA from common bean. The CpG methyltransferase (M.SssI) was used to methylate the DNA. b) H_2O_2 in millimoles per $1x10^8$ suspension cells after applying self eDNA that previously was fragmented or non-fragmented by sonication, methylated or non-methylated with M.SssI and digested with MspI or HapII. Enzyme solutions were used as control treatments. Different letters indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 5$).

5.7.3 Fragments of self eDNA can be detected on cells from self eDNA treated plants.

In order to observe weather DNA fragments enter into plant cells, we performed a DAPI staining of common bean leaves treated with fragmented or non-fragmented DNA self eDNA. DNA could be observed by fluorescence microscopy. Treatments with fragmented or non-fragmented DNA self eDNA resulted in more fluorescence zones around the nuclei than control treatment (Figure 29). However, we could not distinguish whether these fluorescence zones represent extracellular or intracellular DNA.

5.7.1 Nucleus was the principal source of eDNA that induce early signaling (ROS and MAPK)

In order to evaluate the activity of self eDNA obtained from a specific organelle to induce early responses, we extracted DNA from either chloroplast, mitochondria and nucleus enriched solution and applied to suspension cells, although so far there are no techniques to guarantee the obtention of a 100% pure suspension of each mentioned organelle. The purity test of each type of DNA showed a cross contamination of chloroplast DNA with DNA from either mitochondria (*cox1*) and nucleus (*ubi*). Mitochondria DNA also showed a cross contamination with DNA from chloroplast (*rbcL*) and nucleus. Whereas DNA from nucleus did not showed a detectable cross contamination with any of the other two types of DNA (Figure 30). Suspension cells treated with self eDNA from the nucleus exhibited stronger levels of MAPK activation and H_2O_2 formation than those treated with self eDNA from chloroplast (Figure 31 a,b). H_2O_2 formation with self eDNA from nucleus was similar to treatment with total DNA. Whereas suspension cells treated with self eDNA from mitochondria did not exhibit a significative difference of H_2O_2 level respect to both control and self eDNA from nucleus treatments (Figure 31b).

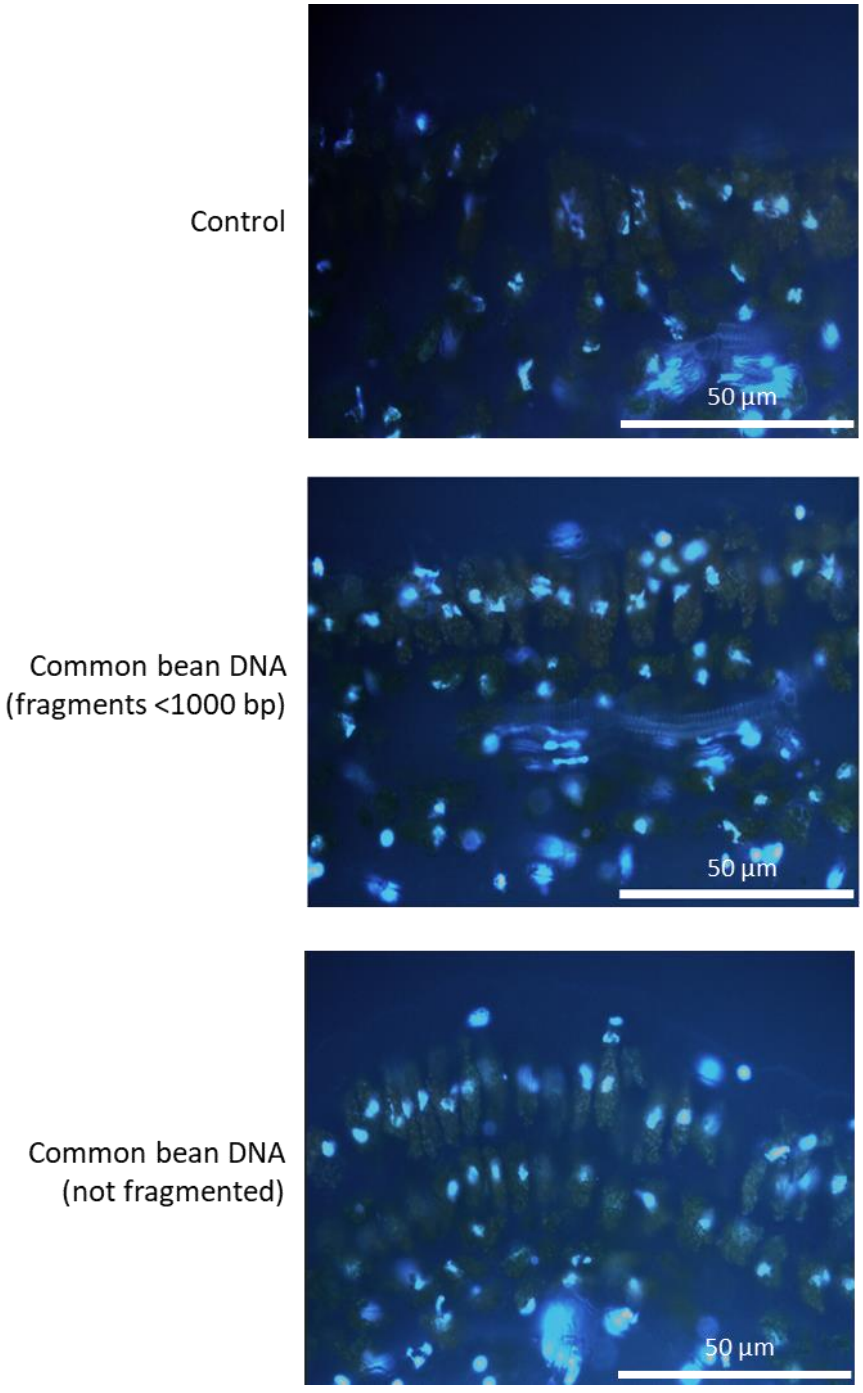

Figure 29. Self eDNA in common bean leaves. Observation by fluorescence microscopy of common bean leaves 30 min after applying 200 µg mL^{-1} of self eDNA. Pictures represent portions of leaf blade where the blue fluorescence indicates the presence of DNA. Scale bar (50 µm) is indicated on the figures.

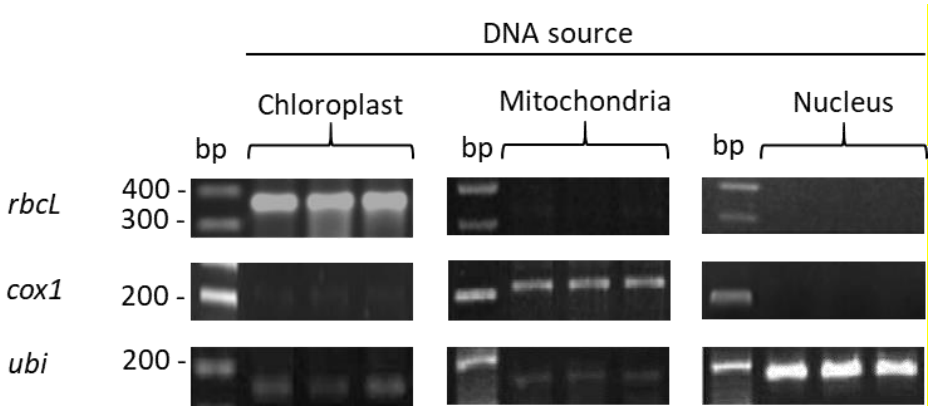

Figure 30. Purity check for DNA extracted from chloroplast, mitochondria and nucleus. PCR amplification of reference genes present only in chloroplast (Rubisco large subunit, *rbcL*), mitochondria (cytochrome oxidase, *cox1*) and nucleus (ubiquitin, *ubi*). Each PCR amplification was performed by triplicate and showed on agarose gel.

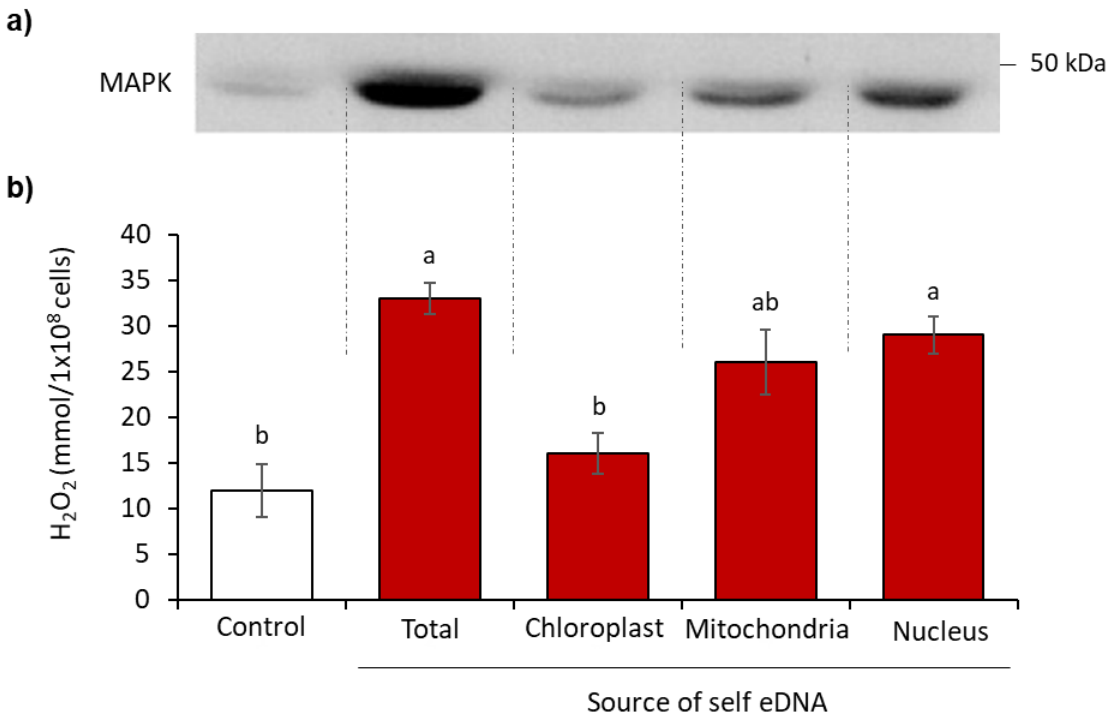

Figure 31. Nuclear extracellular self-DNA (eDNA) activates MAPKs and induce H_2O_2 formation. Self eDNA from chloroplasts, mitochondria or nuclei was tested to induce MAPKs activation or H_2O_2 formation in suspension cells. **a)** The activation of MAPKs after applying 200 µg mL^{-1} of eDNA. **b)** Millimoles of H_2O_2 per $1x10^8$ cells 2 h after applying 200 µg mL^{-1} of eDNA (red bars). Water was used as control treatment (white bar). Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 9$).

5.8 DNA is an active component in the effects of leaf homogenates on the resistance responses in plants.

The DNA in leaf homogenate was purified and quantified with NanoDrop spectrometer. The results showed the leaf homogenate contains on average 234±23 SE μg mL^{-1} of eDNA and it is partially fragmented because a smear resulted in the agarose gel electrophoresis (Figure 32a). When leaf homogenate was treated with enzymes to degrade DNA, RNA or proteins, the treatment with RNAse did not significantly affect the EFN-inducing effect, whereas most other enzymatic treatments tended to reduce the EFN-inducing effect of the leaf homogenates. However, only the treatment with DNAse + protease caused a statistically significant effect (Figure 32b).

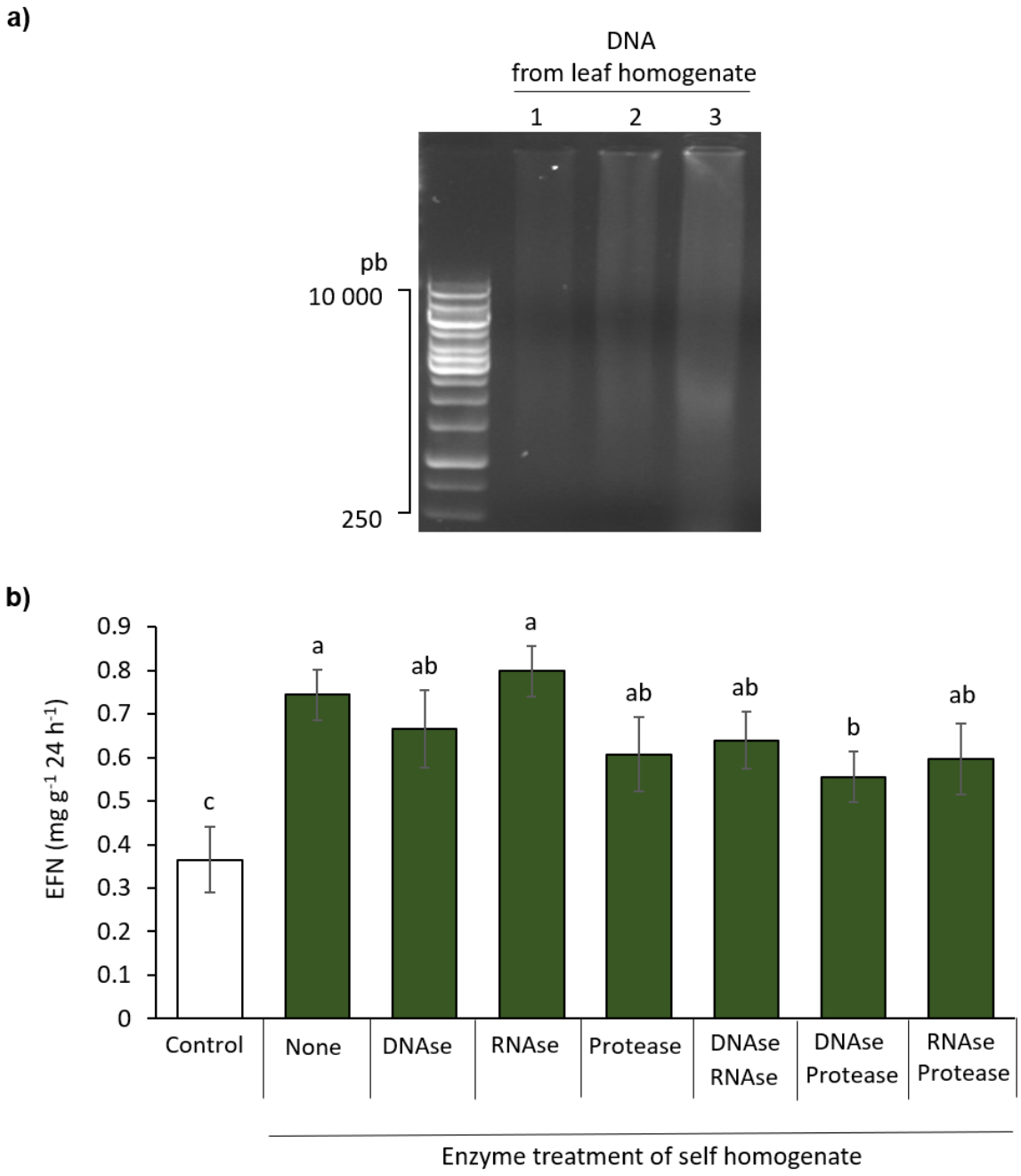

Figure 32. The DNA in leaf homogenates contributes to induce the phenotypic response extrafloral nectar (EFN). **a)** DNA purified from three replicates (1-3) of leaf homogenate of common bean. **b)** Self leaf-homogenate was treated with enzyme (DNAse, RNAse, proteinase or combination of these) and applied to common bean leaves. The secretion of EFN in mg per gram of dry mass 24 h after applying the treated homogenate (green bars) is depicted as the mean ± the SE. The control treatment (white bar) consisted of the application of a solution of 0.05 % v v^{-1} Tween 20. Different letters above bars indicate significant differences among treatments (Univariate ANOVA and post hoc Tukey test: $p < 0.05$, $n = 7$).

6 DISCUSSION

6.1 Confirming eDNA as a DAMP in plants that contributes to damaged-self recognition

In this study, we asked whether eDNA can act as a DAMP in plants and, thereby, contributes to self versus non-self discrimination during plant damaged-self recognition. Fragmented eDNA from three different plant species induced resistance-related traits in common bean, in general patterns that were similar to the effects of leaf homogenates obtained from the same three species (Duran-Flores & Heil, 2014). Self- eDNA triggered early and late signaling cascades and induced phenotypic resistance to an herbivore and several bacterial and fungal pathogens, although the resistance to bacterial pathogens was only observed when the plants were challenged almost immediately after eDNA application whereas in the case of the fungi, only two out of four strains tested were significantly affected. With the exception of the SA response and the resistance to microbial pathogens, all effects showed species-specificity with respect of the eDNA source: 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, and no type of non-self eDNA had a detectable effect on endogenous levels of JA and on the secretion of EFN. At the phenotypic level, self eDNA caused stronger resistance against herbivory than non-self eDNA from lima bean, although the latter also caused resistance at the higher concentration tested. Similarly, the growth-inhibition effects of eDNA 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 resistance (Duran-Flores & Heil, 2015). However, strong negative correlations among DNA-induced growth inhibition and resistance expression in plants have also been reported by others (Yakushiji *et al.*, 2009) and in general terms, a transient reduction in growth during the activation of a resistance response is a commonly observed outcome of the costs of resistance expression (Herms & Mattson, 1992; Cipollini & Heil,

2010; Accamando & Cronin, 2012). 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 they 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 on resistance-related traits or growth are frequently reported, but as to the best of our knowledge, the comparisons are usually made at higher taxonomic levels. 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; Mazzoleni *et al.*, 2015b), from species of the same plant class but different family (Vega-Muñoz *et al.*, 2018) or, ultimately, the effects of bacterial or viral non-self eDNA versus mammalian self eDNA (Meller *et al.*, 2015; McGlasson *et al.*, 2017). However, we are not aware of a study that compared the resistance responses to eDNA from two species in the same genus. In a preceding study we compared the resistance responses to leaf homogenates from two species in the same genus and found similar differences (Duran-Flores & Heil, 2014). In the present study we show that leaf homogenate contains DNA fragments and that the resistance-related responses to leaf homogenate are significantly reduced (although still present) when both DNA and proteins in homogenate are digested by enzymes. Thus, our study supports the hypothesis that self eDNA is one – but not the only - active principle in the resistance-inducing effects of leaf homogenates.

6.1.1 Relevance of self and non-self eDNA discrimination in natural settings

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 fact, the overwhelming majority of studies conducted in the immunological sciences report stronger immunogenic

properties of nonself- as compared to self-DNA (reviewed in Heil and Vega-Muñoz, in press). Thus, in our study, we found the opposite effects. Are our observations likely to reveal a process of relevance for plant immunity in nature? The experimental conditions undoubtedly were highly artificial and in fact, it appears likely that the receiver plants had to 'solve a problem' that they would never face in a natural setting. Plants suffer infection by microorganisms and attack by herbivores, but plants usually don't predate on each other. We are also 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). However, tissue disruption definitely can release DNA into the extracellular space (Duran-Flores and Heil 2016), eDNA is an abundant environmental molecule (Mazzoleni *et al.*, 2015a), and 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 self- and non-self eDNA in plant tissues occurs in multiple natural situations.

Concerning the putative selective advantages of a specific recognition mechanism, the presence of eDNA in the formation of biofilms and the multitude of plant-pathogenic microorganisms clearly create scenarios in which discriminating self (plant) eDNA from non-self (microbial) eDNA can have a selective benefit. However, this argument only applies at the highest taxonomic level. In the specific case of plants, possible selective benefits of discriminating non-self (but nevertheless, plant-derived) eDNA from self eDNA might apply in the below ground compartment. eDNA is abundant in soil and litter (Nielsen *et al.*, 2007; Nagler *et al*., 2018), and discriminating exogenous eDNA from wound-derived self eDNA would allow to restrict the resistance responses to the perception of the latter. Further work will be required to understand the why of taxonomic specificity in the effects of eDNA on plant resistance.

6.2 Caveats and alternative explanations

Protocols for the extraction and purification of DNA are not optimized 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 (Heil, 2009; Duran-Flores & Heil, 2015; Versluys *et al.*, 2017). In fact, extracellular RNA (eRNA) from both the self and the non-self can trigger plant immunity responses (Wen, A *et al.*, 2009; Yakushiji *et al.*, 2009; Paungfoo-Lonhienne *et al.*, 2010a; Barbero *et al.*, 2016; Lee *et al.*, 2016). 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.*, 2011). 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, when we used 'synthetic' eDNA ('free' of RNA, proteins and methylation marks) obtained by random PCR from the whole genome of self DNA, we did not observe any effect on resistance-related traits. Hence, future studies will have to control for a possible role of DNA methylation patterns or DNA-binding peptides and proteins. In mammals, DNA-associated proteins and peptides are frequently described to act as DAMPs or to enhance the immunogenic properties of eDNA. 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 (Tang *et al.*, 2012; Jounai *et al.*, 2013; Andersson *et al*., 2018). Similarly, host resistance 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 *et al.*, 2017). 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 & Ryu, 2016), it is difficult to optimize the protocols for the preparation of eDNA for the plant sciences.

6.3 eDNA sensing in plants

Research over the last years revealed that plants and mammals share several DAMPs and downstream signaling 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 mammals, the re-uptake of eDNA into living cells is critical for its recognition, because the mammalian DNA sensor cGAS (O'Neill, 2013) and most other receptors for nucleic acids (see Introduction, chapter 1.3.1.) are located within the cell (Takaoka *et al.*, 2007; Hornung *et al.*, 2009; Desmet & Ishii, 2012; Schlee & Hartmann, 2016). In plants, 25-bp fragments of a nuclease-resistant analogue of DNA were taken up by Arabidopsis root cells (Paungfoo-Lonhienne *et al.*, 2010a), endocytosis inhibitors significantly reduced the immunity-inducing activity of bacterial eDNA in Arabidopsis (Yakushiji *et al.*, 2009), fragments < 1000 bp caused significant effects resistance-related responses (Barbero *et al.*, 2016). Similarly, we observed that only fragments < 700 bp in length caused significant effects resistance-related responses. Taken together, 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. We tried to observe by DAPI staining and fluorescence microscopy whether fragmented self DNA is taken up into cells, however, because the limitation of the technic, we could not differentiate or confirm whether the fragments really were inside of the cell since non-fragmented DNA was visualized similarly to fragmented DNA.

The most classical case of a mechanism for self/nonself discrimination in nucleic acid sensing is the sensing of unmethylated CpG motifs by TLR9 (Hemmi *et al*., 2000). Unmethylated CpG motifs are much more frequent in viral or bacterial DNA as compared to eukaryotic DNA and thus, were suggested to allow for a self/nonself discrimination by TLR9 (Ohto *et al.*, 2015). Interestingly, the recognition of bacterial eDNA in Arabidopsis required the same motif (Yakushiji *et al.*, 2009) and vice-versa, a recent report shows that self eDNA can change the CpG methylation levels in genomic DNA of the receiver plant (Vega-Muñoz *et al.*, 2018). In the present study, we show that non-methylated CpG motifs are involved in self eDNA sensing, although our results indicate that additional factors also are important: we could still observe a weaker effect on resistance-related responses when CpG motifs were completely methylated or digested by the non-sensitive enzyme. Moreover, TLRs have not been described for plants (Couto & Zipfel, 2016). In plants, PAMPs and DAMPs are mainly recognized via receptor-like kinases. Leucine-rich repeat (LRR)-containing PRRs preferentially bind proteins or peptides such as bacterial flagellin (a PAMP) or endogenous AtPep peptides (DAMPs) (Saijo *et al.*, 2018). 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 & 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 (O'Neill, 2013; Schlee & Hartmann, 2016). However, in the present study, self- and nonself eDNA was applied in the same way, which makes it unlikely that localization can explain the differential responses observed. As an alternative, receptor-independent explanation for the specificity of the

effects of eDNA on plants, we 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 (Duran-Flores & Heil, 2015). In short, the mechanisms that underlie the species-specific responses of plant cells to eDNA remain matter of speculation.

6.4 Self eDNA as a tool in the biological control of herbivory and plant disease

As to the best of our knowledge, the present study represents the first attempt to use the resistance induction by self eDNA as a tool in biological control and to test its effect on yield under field conditions. Indeed, bean plants there were subjected to a single treatment with self-eDNA produced ca 30% more seeds than control plants, whereas nonself- eDNA did not cause any significant effect on yield. Interestingly, these results were consistent with the patterns that we observed in endogenous levels of JA and the EFN secretion, confirming the importance of JA-dependent signaling for the resistance in plants to chewing herbivores (Thaler, 1999). However, the closely related eDNA (congeneric eDNA) reduced the herbivory at high concentration (200 µg ml^{-1}) at which JA acid nor EFN were not induced. This suggest that an unknown factor is involved in sensing self eDNA and another factor in JA-independent non-self eDNA sensing.

No specificity effects were observed in the induced resistance to biotrophic and necrotrophic pathogens. Self and non-self eDNA reduced the infection rate by the biotrophic bacteria *P. syringae* pv*. phaseoli and* pv*. syringae* and *X. phaseoli* and the necrotrophic bacteria *Enterobacter* sp and the necrotrophic fungal pathogens *B. cinerea* and *S. sclerotiorum* when these pathogens were inoculated 5 min after the eDNA treatment, whereas no significant effect was found when the infection occurred 24 h after DNA application. Interestingly, a reduction of the infection rate by the hemibiotrophic fungal pathogen *F. oxysporum* was observed only when eDNA had been applied 24 h before

inoculation, whereas no effect was observed on the resistance against the hemibiotrophic fungus *C. lindemuthianum*. In general terms, the activation of the JA pathway is related to the resistance against to necrotrophic pathogens whereas the SA pathway acts against biotrophics (Pieterse & Dicke, 2007; Pieterse *et al.*, 2009). In most plant species, these two pathways are subject to a negative crosstalk (Thaler *et al.*, 2012) whereas we observed a strong induction of both pathways by self eDNA. However, the response of JA to self eDNA started at 10 minutes, peaking at 30 minutes and ceased 1h after treatment, whereas SA levels started to increase at 8 h, peaking at 24 h and decreased 48 h after treatment with eDNA. Both hormones showed a concentration-dependent effect but only the induction of JA was species-specific: SA was induced by both, self and non-self eDNA. The time differences observed between the JA peak and the SA peak might explain why we detected no trade-off in our study. More importantly, the differential patterns with respect to the eDNA source that we observed in the induction of the hormones nicely resembled the phenotypic resistance effects: JA was induced in a species-specific manner and so was EFN secretion and the resistance to Spodoptera. By contrast, SA was induced by self- and nonself eDNA and similarly, eDNA induced the resistance to four bacteria independently of its origin, at least pathogen challenging occurred 5 min after the eDNA treatment.

Further studies will be required to explain the strong temporal component in the effects of eDNA application on pathogen infection Additional to the use of JA or SA pathways, the short-term enhancement of pathogen resistance might be explained by a synergic effect of eDNA and PAMPs. In humans, the immunity in intestinal epithelial cells is activated by the PAMP flagellin only in the presence of the nucleotide eATP (Ivison *et al.*, 2011). Furthermore, to successfully face an injury requires multiple actions that are independent of the detailed nature of the injury-causing agent (in this case, eDNA functions as the injury signal). Therefore, plants have to recognize if they have an injury and increase their resistance to avoid opportunistic infections (Komarova *et al.*, 2014). Thus, as soon as a plant is damaged, it must seal the wound and prepare locally for infection.

We compared the effects of eDNA with the effects caused by leaf homogenates on field conditions. Self and non-self leaf homogenates showed no specificity effects on number of leaves, both types homogenates increased the number of leaves produced by plants whereas eDNA did not cause a detectable effect. Furthermore, eDNA increased the yield in a species-specific way, yield increased after self eDNA treatment but not after non-self eDNA treatment. Because leaf homogenates contain a wide variety of compounds, these may play a role as foliar fertilizer and can be absorbed by the leaves (Franke, 1986; Fageria *et al.*, 2009) and this property in not shared with eDNA. However, self eDNA caused increase of yield probably as a result of the effects of all resistance-related responses on the natural biotic factors. Hence, there are no cost of induced resistance in terms of seed production (Herms & Mattson, 1992; Cipollini & Heil, 2010; Accamando & Cronin, 2012) although the flowering stage of plants occurred later (may be by the resistance costs) but pod filling occurred earlier.

7 CONCLUSIONS

Fragments of self eDNA trigger early and late resistance-related signaling cascades and phenotypic resistance in bean plants and, hence, act as a DAMP in plants.

The effects of eDNA were species-specific with respect to their source, showing that self eDNA can contribute to the specificity in damaged-self recognition in plants.

Unmethylated CpG motifs contribute to the effects self eDNA on plant resistance but are not sufficient to explain the observed specificity.

The plant and the mammalian immune system might share more common elements than it is currently appreciated. However, the stronger effects of self-eDNA as compared to nonself-eDNA and to nuclear self-DNA as compared to plastic and mitochondrial DNA indicate the presence of a fundamentally new mechanism in nucleic acid sensing.

The general patterns in the responses of plant hormones and phenotypic resistance indicate that eDNA affects plant resistance via JA- and SA-dependent signaling cascades.

DNA and proteins released in the preparation of leaf homogenates together contribute to induce resistance-related traits in plants.

Self-eDNA bears an important potential as a tool in the biological control of pests and disease

8 OUTLOOK

Future research efforts will be required to understand the mechanisms that allow for the reported species-specific effects of eDNA on the plant immune system and to finally support the proposed role of eDNA as a DAMP that contributes to damaged-self recognition in plants.

Considering that self eDNA caused a wide kind of resistance-related responses, including the phenotypic resistance against herbivory and disease, and that eDNA treatment even increased the yield of bean plants in the field, future studies should test the effect of self eDNA on different crop species and focus on the optimization of DNA extraction and application.

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10 ANNEXES. Published articles related to this work.

10.1 Duran-Flores D, Heil M. 2018. Extracellular self-DNA as a damage-associated molecular pattern (DAMP) that triggers self-specific immunity induction in plants. *Brain, behavior, immunity* 72: 78-88.

10.2 Quintana-Rodriguez E, Duran-Flores D, Heil M, Camacho-Coronel X. 2018. Damage-associated molecular patterns (DAMPs) as future plant vaccines that protect crops from pests. *Scientia Horticulturae* 237: 207-220.

10.3 Duran-Flores D, Heil M. 2016. Sources of specificity in plant damaged-self recognition. *Current Opinion in Plant Biology* 32: 77-87.

10.4 Duran-Flores D, Heil M. 2015. Growth inhibition by self-DNA: a phenomenon and its multiple explanations. *New Phytologist* 207: 482-485

10.5 Duran-Flores D, Heil M. 2014. Damaged-self recognition in common bean (*Phaseolus vulgaris*) shows taxonomic specificity and triggers signaling via reactive oxygen species (ROS). *Frontiers in Plant Science* 5: Art585.

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

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abstract

Mammals sense self or non-self extracellular or extranuclear DNA fragments (hereinafter collectively ter-med 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 vul-garis) 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 (extraßoral nectar secretion). By contrast, non-self DNA (obtained from lima bean, Phaseolus lunatus, and Acacia farnesiana) had signiÞcantly 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 Þndings 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-speciÞc 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 beneÞts of an immunity response that is triggered by eDNA in a species-speciÞc manner.

1. Introduction

Multicellular organisms suffer different types of cellular dam-age that may, or may not, include infectious processes. JanewayÕs classical model states that the immune system evolved to distin-guish 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 ÕdangerÕ or ÕdamageÕ-associated molecular patterns (DAMPs): endogenous indicators of injury (Land et al., 1994; Matzinger 2002, 1994). Dur-ing 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 Õdamaged-self recognitionÕ: an induction of immunity in damaged organisms that is independent of exogenous molecules such as microbe- or pathogen-associated molecular pat-terns (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; VŽnŽreau et al., 2015). For the sake of simplicity, hereinafter we employ the term ÔeDNAÕ collectively for extracellular and extranuclear (i.e., cytoso-lic) 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 discus-sion whether mitochondrial DNA is perceived as DAMP or rather as

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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), inter-feron regulatory factor (IRF), or the NACHT, LRR and PYD domains-containing protein 3 (NLPR3) inßammasome (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 inde-pendently 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 acti-vation 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 inßammation, 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 ÔÔself from non-selfÓ 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 speciÞcity at which DAMPs of different taxo-nomic 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 var-ious immunity-related responses, but only when using homoge-nate prepared from conspeciÞc leaves (Duran-Flores and Heil, 2014). Even the application of homogenate from the closely related lima bean (Phaseolus lunatus) led to a signiÞcantly 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 speciÞcity 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 can-didate of a DAMP that could contribute to the species-speciÞcity in plant damaged-self recognition; mainly for the following reasons. First, delocalized self nucleic acids Ñ such as extranuclear DNA or extracellular RNA Ñ are well-known DAMPs in mammals, ÔÔbe-cause they are reliable indicators of cellular damageÓ (Desmet and Ishii, 2012). Upon its recognition, eDNA triggers the generation of ROS, downstream MAPK signalling cascades, the release of cytokines, inßammation 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 speciÞcity: eDNA of Lepidium sativum inhibited the root growth of Arabidopsis in a dosage-dependent manner, but Ôself eDNAÕ prepared from Ara-bidopsis 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 speciÞcity 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-speciÞc 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 culti-var 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 quantiÞed the generation of ROS and the activation of MAPKs as two early, general responses to stress and the secretion of extraßoral nectar (EFN) and the infection by a bacterial phy-topathogen 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 Õnatural biological controlÕ (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 speci-Þcity 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 collec-tion at INIFAP, Celaya, GTO, MŽxico). The plants were grown under greenhouse conditions and natural light (average day-time tem-perature, 28 LC; night-time temperature, 20 LC), watered on Mon-days, Wednesdays and Fridays, and fertilized weekly with a commercial fertilizer (Ferviafol 20-30-10 , Agroqu'micos Rivas S. A. de C.V., Celaya, GTO, MŽxico). 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 (15L55^{0} N and 097L09^{0} W), and cultivated under greenhouse conditions. Before cultivation, the seeds were surface-sterilized with 70% etha-nol for 1 min and with a 20% hypochlorite solution for 10 min and then washed Þve times with sterile water. Wild Acacia farnesiana was collected from the area around CINVESTAV - Irapuato, in the state of Guanajuato in Central Mexico (20L72^{0} N and 101L33^{0}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

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 LC and a light:dark regime of 16 h: 8 h to enable the undiffer-entiated cells (callus) to proliferate. After that time, the callus was transferred to a 250 mL ßask 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 LC. The cells were continuously subcultured every 2 weeks, transferring 2 mL of cul-ture to a new ßask 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 aca-cia 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 b-mercaptoethanol) were added to each tube and then shaken for a few seconds on a vortex shaker. Next, the tubes were heated to 65 LC for 10 min in a water bath before add-ing 6.6 mL of 5 M potassium acetate and placing the tubes on ice. After 30 min on ice, the tubes were centrifuged at 12000g 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 pre-cooled isopropanol were added to the supernatant, which was then kept at 20 LC for 1 h. The tubes were then centrifuged at 12000g 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 shak-ing. The tubes were centrifuged at 12000g for 10 min, the super-natant was discarded again and the pellet was dried for 5 min and then suspended in 1 mL of sterile distilled water and puriÞed using a Maxi DNA puriÞcation Kit (Qiagen). The DNA was quanti-Þed using a NanoDrop 2000 spectrometer (Thermo ScientiÞc) and then fragmented by sonication to obtain fragments of less than 1000 bp using an ultrasonic processor (Misonix XL2020). A solu-tion of 500 lg 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 ÔOnÕ and a 1 s pulse ÔOffÕ. The successful fragmentation of DNA was veriÞed on a 3% agarose gel using ethidium bromide. The DNA from common bean was used Ôself eDNAÕ; the DNA from lima bean or acacia was used as Ônon-self eDNAÕ.

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

In order to conÞrm 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 germi-nated in 9-cm Petri dishes on sterile Þlter paper imbibed with 5 mL of different concentrations (0, 2, 20, 50, 100, 150, 200 or 250 lg mL ^{1}) of self eDNA fragments in sterile water. Sterile distilled water with 0 lg mL ^{1} of eDNA were used as the control treatment. Petri dishes were placed in a growth room at 25 LC with 16 h of light and 8 h of darkness. The primary root length was measured with a ßexible 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 Þlter paper imbibed with 5 mL of 200 lg 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 lg 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 000g for 15 min. Next, 10 lL of the supernatant were mixed with 90 lL of the substrate solution containing ferrous iron and xylenol orange (Hydrogen Peroxide Assay Kit, National Diagnostics, Atlanta, GA, USA). Blanks were pre-pared 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 deÞne 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 mg 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 con-trols (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 b-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 12000g. 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 equili-bration, 100 mL of 2200 mg mL ^{1} of self eDNA were added to a Þnal concentration of 200 mg 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 000g. 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 mg mL ^{1} and the activation of MAPK was tested 30 min after treatments.

In order to deÞne the size range of the eDNA fragments that activate the MAPKs, eDNA fragments of common bean were sepa-rated 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 puriÞed using a DNA puriÞcation kit (Qiagen). Next, 1 mL of each suspension cell culture (1 10^{8} cells mL^{-1}) was treated with 0.1 mL of 2200 mg 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 per-form 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 stan-dard 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 lm PVDF membrane (Trans-Blot Turbo Mini PVDF transfer pack: Bio-Rad) in a Trans-Blot Turbo Transfer System (Bio-Rad). After transfer, the mem-brane was blocked in 5% BSA TBS-Tween 20 (0.1%) overnight at 4 LC 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 Þve times with 0.1% TBS-Tween 20 [1 M Tris-HCl (pH 7.5), 150 mM NaCl, 1% v v^{-1} Tween 20] and incu-bated with a secondary antibody (anti-rabbit IgG coupled to alka-line phosphatase, Sigma-Aldrich) at 1:20,000 for 1 h at room temperature. The membrane was washed Þve 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 detec-tion of phosphorylated MAPKs in an imaging system (Bio-Rad).

2.7. Confirming eDNA as the active principle

In order to conÞrm that the effects observed were due to eDNA and not caused by impurities such as small RNAs or proteins, frag-ments of common bean DNA of less than 1000 bp were treated with DNase 1 (Invitrogen), RNase A (Invitrogen) or proteinase K (Thermo Fisher ScientiÞc) or combination of these, according to product manual. The enzymes in the eDNA solution were deacti-vated before the use of it according to product manual. The activity of the nucleases was conÞrmed on a 3% agarose gel. Plants were treated with a solution of 200 mg 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 sur-face 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 diam-eter were punched out of each leaf and H_2O_2 was quantiÞed as indicated above (ÔÔEffect of eDNA on H_2O_2 accumulationÓ). Further plants were treated in the same manner and after 30 min of treat-ment, the leaves were excised and frozen in liquid nitrogen and used for the MAPK activation test as indicated above (ÔÔEffect of eDNA on the activation of MAPKs in plants and suspension cells cultureÓ).

2.8. Effect of eDNA on EFN secretion

In order to determine whether eDNA activates a late immunity-response in common bean, we quantiÞed the EFN secreted by plants treated with eDNA. At 9:00 am the plant organs called stip-ules or extraßoral 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 mg 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 trea-ted with 0.05% Tween 20 were used as controls. After 24 h, the EFN was quantiÞed on extraßoral nectaries of each of the four youngest leaves. To quantify EFN, 10 mL of distilled water were applied to each of the four leaf nectaries using a micropipette by expelling and sucking up the water Þve 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 LC 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 conÞrm 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 solu-tions to leaves. Plants treated with 0.05% Tween were used as con-trols. After 24 h, the EFN present in each of the four youngest leaves was quantiÞed.

2.9. Effect of eDNA on immunity against phytopathogen

In order to test for induced immunity to a pathogenic bac-terium, solutions of 200 mg mL^{-1} of self or non-self eDNA frag-ments 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 ScientiÞc Inc, NY, NY, USA). Seven plants were used per treatment, all the infection levels were quantiÞed eight days after inoculation in one randomly selected leaf per plant. Leaf material was weighed and ground in a mortar with approximately 500 lL 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 lL of each dilution were plated on KB medium (B medium as described by (King et al., 1954) with rifampicin (100 lg 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 mL of each of the eDNA or of the control treatment (Tween20 at 0.05%, n = 5 repe-titions) on Petri dishes with KB medium with rifampicin. After 5 min, 20 mL 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 signiÞ-cant inhibition was observed at a concentration of 50 lL mL^{-1} of self eDNA, but higher concentrations had a stronger effect (Fig. 1B). Based on these results, we selected the concentration of

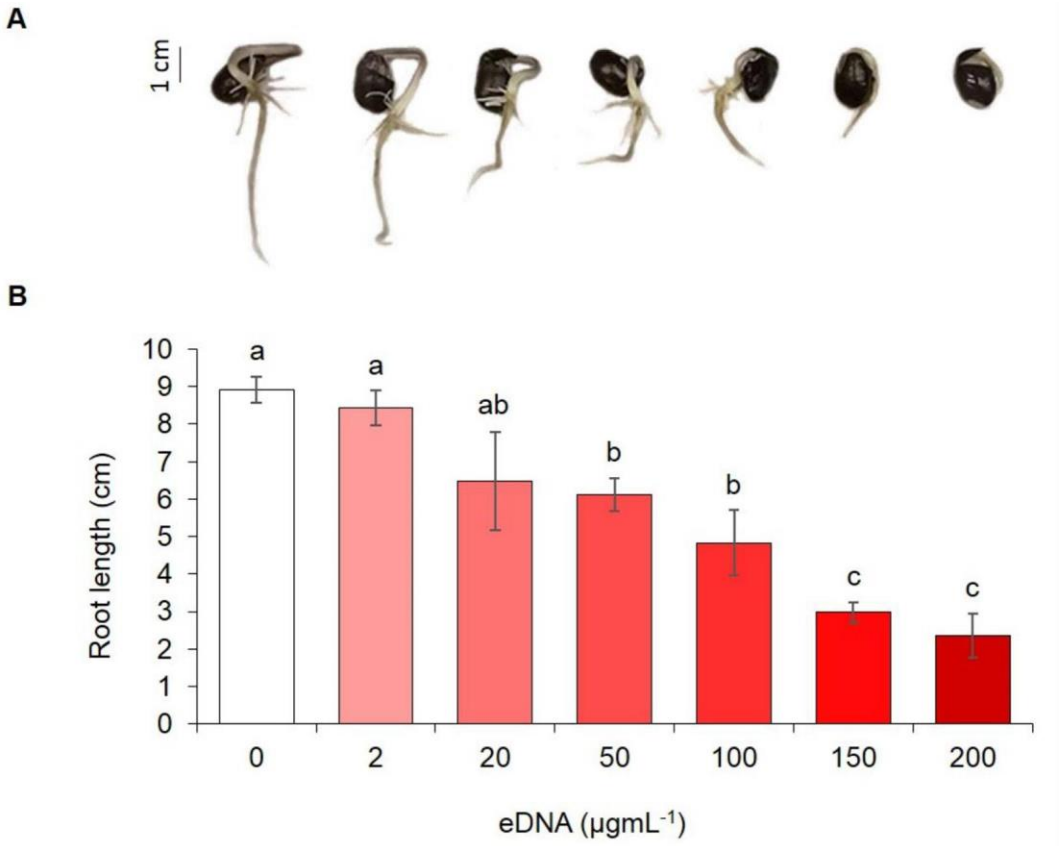

Fig. 1. Extracellular self-DNA (eDNA) inhibits root growth in a concentration-dependent manner. (A) The length of the primary root of common bean (Phaseolus vulgaris) seedlings after four days in germination medium containing different concentrations of self eDNA is depicted in (B) as mean ± SE. As the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

200 lg mL ^{1} for use in the subsequent experiments. The observed effect shows taxonomic speciÞcity: self eDNA inhibited root growth most strongly, non-self eDNA from lima bean caused a weaker, but still signiÞcant effect, whereas non-self eDNA from acacia did not signiÞcantly 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 speciÞcity. Self eDNA caused a signiÞcant (ca. three-fold) increase in H_2O_2, whereas non-self eDNA caused no statistically signiÞcant 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-speciÞc way. Self eDNA caused strongest activation of MAPKs (quantiÞed 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 prepa-ration and followed the activation of MAPKs (Fig.4A) and the for-mation 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 deacti-vated enzymes, no changes to the inducing effects were observed (data not shown).

We observed a signiÞcant 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 secre-tion was not signiÞcantly induced by self eDNA that had been trea-ted with DNAse (Fig. 5B).

Plants that were treated with either self or non-self eDNA exhibited signiÞcantly lower infection rates by P. syringae phy-topathogen (Fig. 6). Nevertheless, infection rates in leaves treated with self eDNA were signiÞcantly 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).

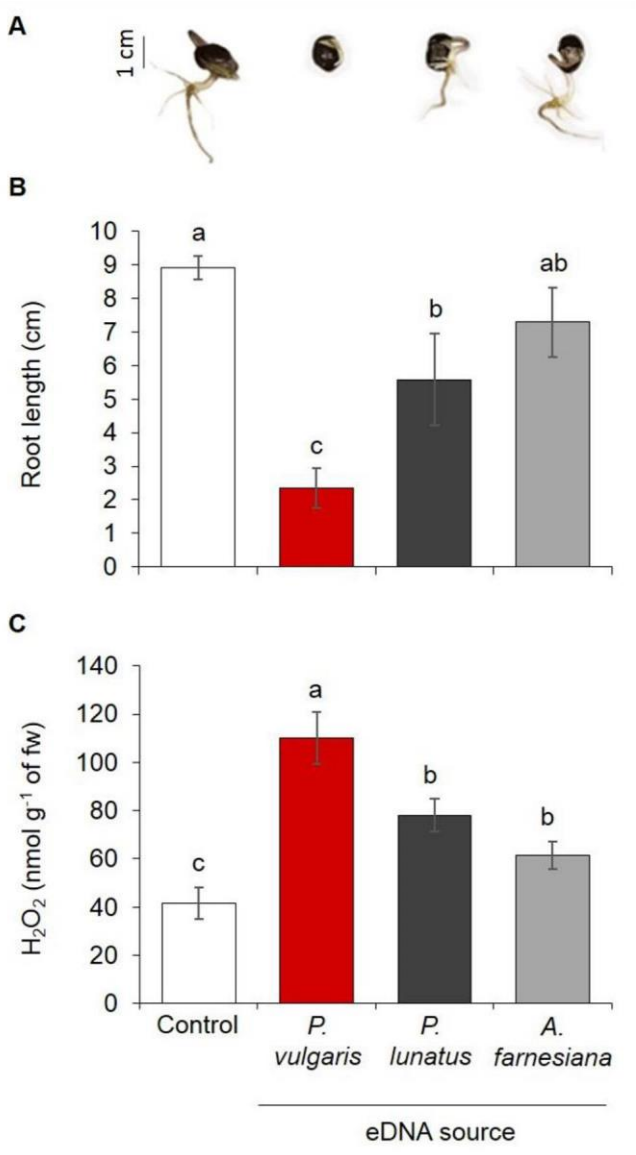

Fig. 2. Root growth and H_2O_2 generation are differently affected by self and non-self eDNA. (A) The length of the primary root of common bean (Phaseolus vulgaris, P. vulgaris) seedlings after four days in germination medium containing 200 mg mL ^{1} of eDNA is depicted in (B) as mean ± SE. (C) The concentration of H_2O_2 in nanomole per gram fresh mass 2 h after applying 200 mg mL ^{1} of eDNA is depicted as mean ± SE. White bars bar represent the control (0 lg 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 signiÞcant 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. Frag-mented self eDNA induced four immunity-related traits in com-mon 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 signiÞcantly 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 reduc-tion in many behavioural activities that is frequently shown by infected or heavily injured mammals, including humans. Further-more, our Þndings complement a recent report on the depolariza-tion of membranes and the inßux 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 con-sistent with the hypothesis that eDNA can contribute to the species-speciÞc discrimination of self versus non-self.

Differential effects of self eDNA versus non-self eDNA are fre-quently reported. For example, others compared eDNA from plants versus animals, bacteria or fungi (Mazzoleni et al., 2015b), eDNA from a bacterium versus a Þsh (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 speciÞcity in a response to eDNA than previously reported. It remains an open question whether this lack of reports indicates that mammalian cells respond less specif-ically to eDNA than plant cells or rather, that this possibility has never been considered. Testing the effects of non-human, mam-malian 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 Þndings might have relevance for the research into the human immune sys-tem. 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-speciÞc response to eDNA, DNA fragments of non-viral origin would be insufÞcient 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 gen-eral 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 bac-terial pathogen as well as of extraßoral nectar, although these responses depend on two different signalling pathways that usu-ally inhibit each other. In the following, we discuss three major

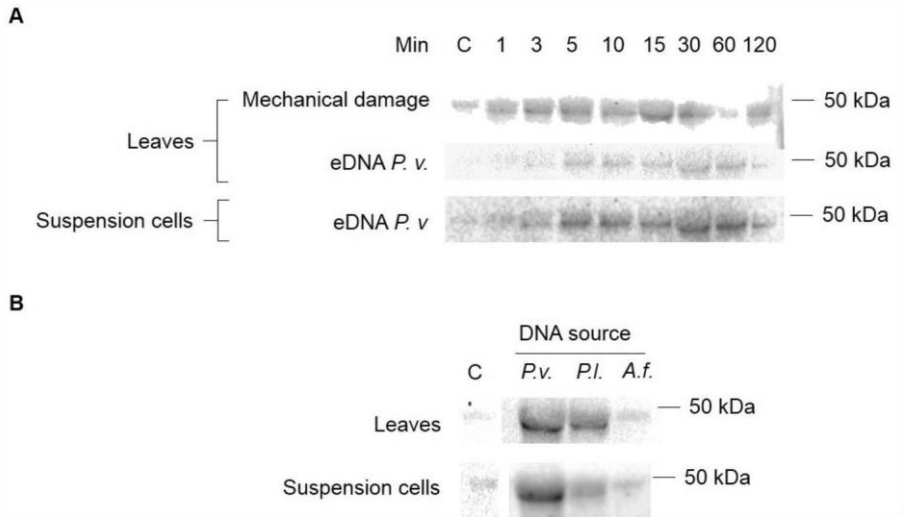

Fig. 3. Extracellular self-DNA (eDNA) activates mitogen-activated protein kinases (MAPKs). (A) The activation of MAPKs in leaves or suspension cells of common bean (Phaseolus vulgaris, P. v.) was tested at different times after treatment with 200 mg mL ^{1} of self-eDNA or mechanical damage (only leaves). 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 mg 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.

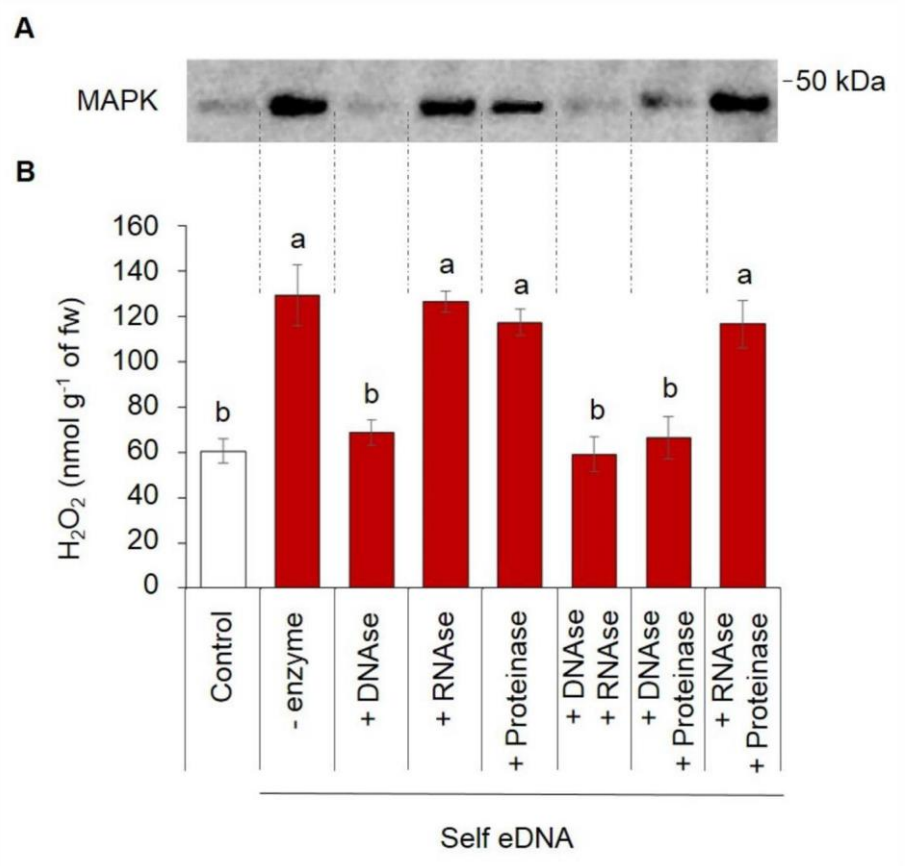

Fig. 4. Digestion of self eDNA eliminates its resistance-inducing effects. Extracellular DNA from common bean (Phaseolus vulgaris) was treated with DNAse, RNAse, proteinase, or combinations, and applied to common bean leaves. (A) The activation of MAPKs was tested 30 min after applying 200 mg mL ^{1} 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 mg 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 signiÞcant 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-speciÞc 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?

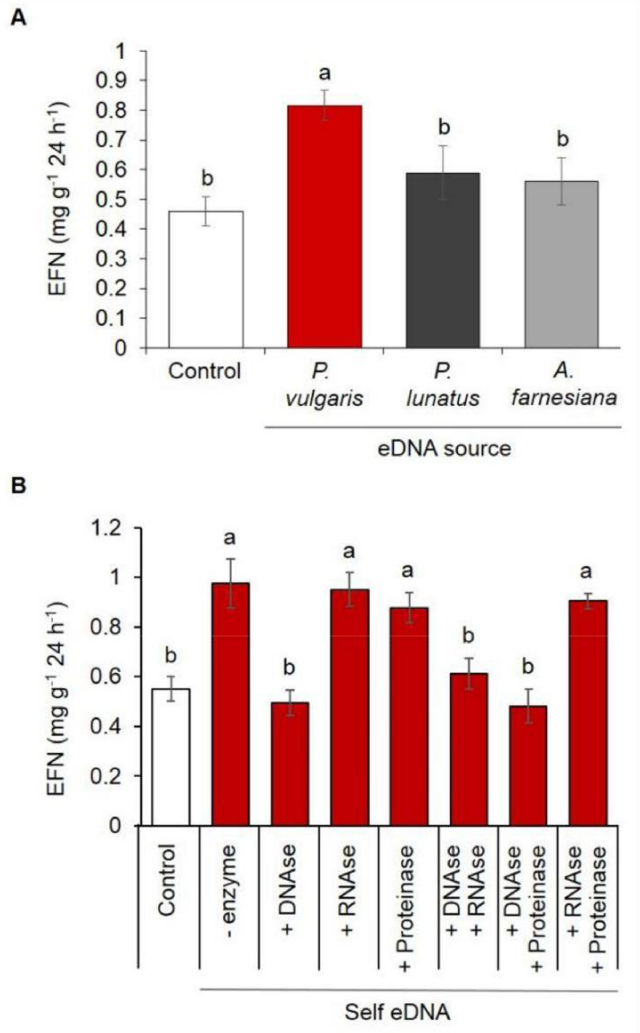

Fig. 5. Extracellular self-DNA induces EFN secretion (A) The EFN secretion by common bean (Phaseolus vulgaris) plants is depicted as mean ± SE of mg of soluble solids per gram of dry leaf mass quantiÞed 24 h after treatment with 50 mg mL ^{1} of self eDNA (P. vulgaris) or non-self eDNA (Phaseolus lunatus or Acacia farnesiana). (B) The EFN secretion by common bean after treatment with 50 mg 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 signiÞcant differences among treatments (univariate ANOVA and post hoc Tukey test: $p < .05$, $n = 6$).

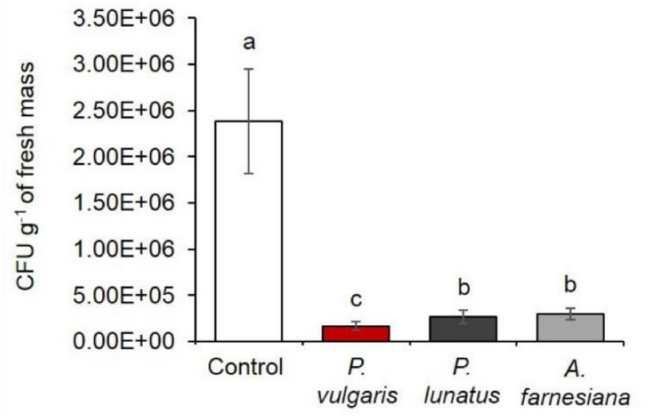

Fig. 6. Extracellular DNA reduces the infection by the bacterium, P. syringae. Numbers of colony forming units (CFU) per g of Phaseolus vulgaris leaf fresh mass are depicted as mean ± SE. Plants had been treated with self (red bar) or non-self eDNA (grey bars, from Phaseolus lunatus or Acacia farnesiana),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

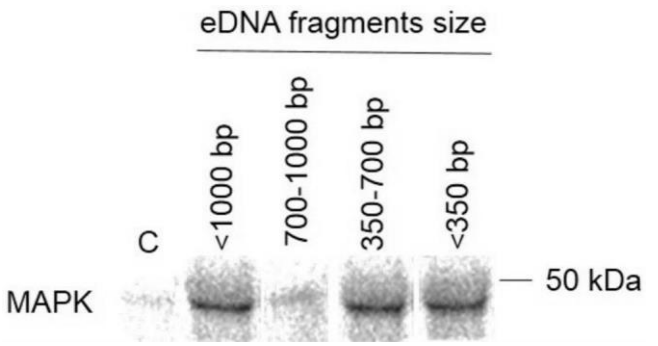

Fig. 7. Small extracellular DNA fragments activate MAPKs in common bean. Sonicated self eDNA was separated on 3% agarose gels and fragments were re-extracted from regions corresponding to different size ranges (<1000 bp, 700-1000 bp, 350-700 bp or <350 bp) and applied at 200 mg mL ^{1} of eDNA to suspension 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 undoubt-edly were highly artiÞcial, 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 continu-ously 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 apop-tosis, necroptosis or NETosis: important sources of eDNA in mam-mals (Hanson, 2016; Kaczmarek et al., 2013;Toussaint et al., 2017). Moreover, eDNA is a common component of bioÞlms that are formed by pathogenic bacteria (Mšllerherm 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 donÕt predate on each other, a fact that causes doubts concerning the selective advantages of a speci-Þc recognition mechanism. One possibility is that, due to the abun-dance 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 resis-tance traits that depend on two independent signalling pathways: the salicylic acid pathway controls resistance to biotrophic patho-gens like P. syringae, whereas the jasmonate signalling cascade that controls plant defence against chewing herbivores, including extraßoral nectar secretion. Since these two pathways usually inhi-bit each other, our Þndings indicate the possibility that eDNA trig-gers resistance via an additional, as yet unknown mechanism. Therefore, the plant response to eDNA should be further studied,

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 puriÞcation 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 immu-nity 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 treat-ing 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 speciÞc receptor (Barton et al., 2006), the role of eDNA in bacterial bioÞlm 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 abol-ished the inducing properties of our eDNA preparation, whereas RNase and protease had no effect. These observations are fully con-sistent 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-inßammatory 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-inßammatory 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 difÞcult 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 repre-sent 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 mecha-nism in plants? In contrast to mammalian cells, plant cells are sur-rounded 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 con-trast, 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 signiÞcantly reduced the immunity-inducing activity of bacterial eDNA in Arabidopsis (Yakushiji et al., 2009), and only fragments <700 bp in length caused signiÞcant 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 liv-ing 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 speciÞcity in the recog-nition process. Recent studies identiÞed 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 Arabidop-sis 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 recogni-tion receptors (PRRs) preferentially bind proteins or peptides such as bacterial ßagellin (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 speciÞcity makes it unlikely that this receptor allows for a species-speciÞc recognition of eDNA. Fur-ther receptors of DAMPs in plants comprise lectin-type PRRs, which bind extracellular ATP, and PRRs with epidermal growth fac-tor (EGF)-like ectodomains, which recognize plant cell-wall frag-ments (Couto and Zipfel, 2016).

Besides epigenetic or sequence-dependent motifs, DNA recogni-tion 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 expla-nation for the speciÞcity 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 activi-ties. In short, we are not aware of any report on a plant receptor that recognizes DNA with a level of sequence-speciÞcity that could explain our observations, and the mechanisms that underlie the species-speciÞc 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-speciÞc. Non-self eDNA triggered signiÞcantly lower responses, or no responses at all, even when obtained from a congeneric plant. To the best of our knowledge, this level of tax-onomic speciÞcity 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 bene-Þts of a species-speciÞc discrimination of self eDNA from non-self eDNA and to identify the molecular mechanisms that allow for this degree of speciÞcity.

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