The CpG-dependent plant immune response to self-DNA triggers defence hormone signalling and improves fitness

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Research Article

Keywords: innate immunity, plant vaccine, damage-associated molecular patterns, DAMPs, self-DNA, wound response, immunogenic DNA, disease resistance, biological control, plant–pathogen interaction

DOI: https://doi.org/10.21203/rs.3.rs-2649049/v1

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The CpG-dependent plant immune response to self-DNA
triggers defence hormone signalling and improves fitness

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Abstract

The accumulation of DNA in the cytoplasm or extracellular space is a signal of danger. Plants respond to this signal with a self/non-self-specific activation of early immune signalling events. Here, we asked whether this specificity translates to fitness-relevant resistance to natural enemies. We treated common bean (Phaseolus vulgaris) plants with self-DNA and with non-self-DNA from other plant species. Self-DNA treatment induced jasmonic acid and decreased feeding by a chewing herbivore (Spodoptera frugiperda), while self- and non-self-DNA induced salicylic acid and reduced the population densities of two fungal pathogens (Botrytis cinerea and Sclerotinia sclerotiorum) and of four bacterial pathogens (Enterobacter sp. strain FCB1, Pseudomonas syringae pv. phaseoli and pv. syringae, and Xanthomonas axonopodis pv. phaseoli). Strikingly, a single self-DNA-treatment increased seed production under field conditions in two seasons ca. 1.5-fold and 3.2-fold, while non-self-DNA had lower or no detectable effects. Stronger responses to self- than non-self-DNA seemingly contradict immunological theory. In mammalian immune cells, toll-like receptor 9 (TLR9) is activated by microbial DNA but also by mitochondrial (mt)DNA that is rich in unmethylated CpG motifs. Using bean suspension cells, we observed stronger immunogenic effects of genomic DNA than chloroplast DNA or mtDNA. Moreover, in vitro methylation or cleavage of CpG motifs reduced – without eliminating – the H_{2}O_{2}-inducing properties of DNA. We conclude that the plant immune response to DNA comprises a self/non-self-specific induction of major defence hormones that can be adaptive under natural enemy pressure and that unmethylated CpG motifs contribute to the immunogenic effects of DNA in plants.

The accumulation of DNA in aberrant compartments such the cytoplasm or the extracellular space is a signal of danger. 'Non-self'-nucleic acids would usually stem from infecting microorganisms, i.e., they represent microbe-(or pathogen-) associated molecular patterns (MAMPs/PAMPs). Indeed, treatments with bacterial RNAs, double-
stranded (ds)RNAs and synthetic single-stranded oligodeoxynucleotides (ssODNs) activated early immune signals and induced salicylic acid (SA)-dependent resistance to bacterial or fungal pathogens in diverse plant species, including *Arabidopsis thaliana*, chili pepper (*Capsicum annuum*) and wheat (*Triticum aestivum*)1-7. However, attack by herbivores and pathogens can also provoke the release of the plant’s own DNA. These 'self'-DNA fragments indicate damage and thus, represent damage-associated molecular patterns (DAMPs)8,9: endogenous molecules that – in the words of Emilie Vénérau and colleagues – "have a physiological 'day-time job' inside the cell, and have the additional job of signalling cell damage when they are outside the cell"10.

Self-DNA is increasingly being reported to activate immune responses in plants [reviewed in 11-14]. For example, studies using *A. thaliana*, common bean (*Phaseolus vulgaris*), lima bean (*Phaseolus lunatus*), lettuce (*Lactuca sativa*), maize (*Zea mays*) and tomato (*Solanum lycopersicum*) reported that treatment with fragmented self-DNA triggered Ca\(^{2+}\) fluxes, membrane depolarization, the activation of mitogen-activated protein kinases (MAPKs), the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H\(_2\)O\(_2\)), the expression of diverse resistance-related genes, the secretion of extrafloral nectar [an indirect defence against chewing herbivores that is controlled by the 'wound hormone', jasmonic acid (JA)], and also phenotypic resistance to biological enemies including pathogenic bacteria (*Pseudomonas syringae*) and fungi (*Botrytis cinerea*), the aphid *Myzus persicae* and the oomycete *Hyaloperonospora arabidopsidis*15-19.

Intriguingly, these latter studies reported stronger immune responses to self- as compared to non-self-DNA. Moreover, accumulating evidence characterises the 'Mazzoleni-effect' (a dosage-dependent inhibition of growth by self-DNA, but not non-self-DNA) as a seemingly universal feature of organisms across the tree of life7,15,17,20-23. Immune responses to self-derived molecules seemingly contradict the general immunological paradigm that expects immune responses to non-self because "The immune system evolved to discriminate infectious nonselves from noninfectious self"24, but they support the danger model of Polly Matzinger, who argues that "the immune system is more concerned with entities that do damage than with those that are foreign"25. Evidently, 'immunity' is not restricted to early signalling responses. However, although the potential of self-DNA treatment for biocontrol was seen immediately12,26-28, it remained unknown whether self/non-self-specific effects of DNA on early signalling events modulate the interactions of plants with their biological enemies and how these responses affect plant fitness under enemy pressure. Moreover, self-DNA and non-self-nucleic acids have been reported to induce JA- as well as SA-dependent genes3,18,29, although two recent transcriptomic studies revealed more down-regulated than upregulated genes in self DNA-treated *A. thaliana* or tomato plants,18,29 and in apparent contrast to the stronger responses of early immune signalling events to self-DNA, in *A. thaliana* more genes changed their expression in response to non-self-DNA as compared to self-DNA29.
In short, the available information on plant responses to self-versus non-self-DNA does not provide a satisfying empirical basis to discuss to what degree the plant immune system follows Janeway’s traditional paradigm versus Matzinger’s danger model. Therefore, the principal aim of our present study was to determine the potential of exogenously applied plant DNA to trigger self/non-self-specific induction of two major plant defence hormones (JA and SA) and to test the corresponding effects on the quality of *P. vulgaris* as a host for its biological enemies (see Fig. S1 for a graphical methods summary). Our study shows that self-DNA induced increased JA levels and strongly enhanced resistance to a chewing herbivore, whereas both self- and non-self-DNA induced SA to similar levels and reduced the population densities of various fungal and bacterial pathogens. Moreover, only self-DNA treatment consistently led to enhanced seed production under open field conditions, while non-self-DNA treatment had lower or no detectable effects.

**Results**

**Self-DNA induces the two plant hormones JA and SA.** We aimed to understand the hormonal responses that link early signalling responses stimulated by the application of self or non-self-DNA, to the resistance to biological enemies. Therefore, we first characterised effects of exogenously applied self-DNA on the plant hormones, JA and SA. We extracted, purified and sonicated DNA from *P. vulgaris* leaves following our established protocol and treated *P. vulgaris* plantlets with this self-DNA at different concentrations. Self-DNA induced both hormones, although JA and SA increased at different times after treatment and showed different dose–response relationships (Fig. 1). The concentration of JA depended significantly on both time after treatment and self-DNA concentration (general linear model (GLM) *P* < 0.001, *n* = 6). Endogenous JA levels started to increase at 15 min and peaked at 30 min after the application of self-DNA. However, while concentrations of 20 μg ml⁻¹, 50 μg ml⁻¹ or 100 μg ml⁻¹ of self-DNA had significant effects (Fig. 1a, GLM, *P* < 0.001, Tukey post-hoc test, *P* < 0.05, *n* = 6), we could detect no significant effects of lower (2 μg ml⁻¹) and higher concentrations (150 μg ml⁻¹ and 200 μg ml⁻¹) (GLM, Tukey post-hoc test, *P* < 0.05, *n* = 6). In short, we observed the strongest JA induction (to levels > 40 ng JA g⁻¹ FW) at 30 min after treatment with 50 μg ml⁻¹ self-DNA (Fig. 1a).
Central plant defence hormones are induced by self-DNA at different dose-response relations. Common bean plants were treated with different concentrations of self-DNA and samples were taken at different time points to quantify the concentration of jasmonic acid (JA) in nanogram JA per gram of leaf fresh weight (FW) (panel A) and of salicylic acid (SA) in micrograms per gram of leaf fresh weight (FW) (panel B). Symbols indicate means ± 1 SE; different symbols indicate the different concentrations of self-DNA used and different letters indicate significant differences among treatments at each specific time point (univariate ANOVA and post-hoc Tukey test: $P < 0.05, n = 6$).

Self-DNA also induced SA synthesis in a strongly time- and concentration-dependent manner (Fig. 1b, GLM, $P < 0.05, n = 6$). SA levels started to increase at 8 hours and peaked at 24 hours after the application of self-DNA. Moreover, the minimum concentration of self-DNA that induced a statistically significant increase in endogenous SA levels was 20 μg ml$^{-1}$ (GLM, Tukey post-hoc test, $P < 0.001$), and all higher concentrations tested (50 μg ml$^{-1}$, 100 μg ml$^{-1}$, 150 μg ml$^{-1}$, and 200 μg ml$^{-1}$) induced maximum levels of ca. 5 μg SA g$^{-1}$ FW (Fig. 2a, GLM, $P < 0.001$, Tukey post-hoc test, $P < 0.05, n = 6$). Based on these results, we selected 50 μg ml$^{-1}$ DNA as the optimal concentration for all subsequent experiments.
Self-/non-self-specific effects of DNA on JA and SA. Next, we tested for self/non-self-specific differences in the response of both hormones to exogenous DNA. Aiming to detect a potential phylogenetic signal, we prepared non-self-DNA from *P. lunatus* and *Acacia farnesiana* (two plant species belonging to the same family as *P. vulgaris* that had been used earlier as sources of non-self-DNA. *P. vulgaris* plantlets were treated with self or non-self-DNA at 50 μg ml$^{-1}$ and JA and SA were quantified 30 min and 24 hours later, respectively. Self-DNA induced a statistically significant increase in endogenous JA at 30 min after treatment - confirming the before mentioned results (Fig. 2a, GLM, Tukey post-hoc test: $P < 0.01$, $n = 6$), whereas non-self-DNA from *P. lunatus* had only a slight, non-significant effect (GLM, Tukey post-hoc test, $P > 0.05$, $n = 6$) and non-self-DNA from *A. farnesiana* had no detectable effect (GLM, Tukey post-hoc test, $P > 0.05$, $n = 6$). In contrast to this self/non-self-specific JA response, all three types of DNA caused a significant induction of SA (GLM, $P < 0.001$, $n = 6$), with no statistically significant differences between the types of DNA (GLM, Tukey post-hoc test, $P > 0.05$, $n = 6$ for each DNA type vs control, Fig. 2b).

![Fig. 2. Extracellular plant DNA from different species triggers a self-/nonself-specific induction of JA but not SA. Violin plots depict the frequency distribution of (a) the concentration of jasmonic acid (JA) in nanograms JA per gram of leaf fresh weight (FW) at 30 min after treatment and (b) the concentration of salicylic acid (SA) in micrograms per gram of leaf fresh weight (FW) at 24 h after treating common bean plants with 50 μg ml$^{-1}$ of self-DNA (from *Phaseolus vulgaris*, red) or non-self-DNA (from *Phaseolus lunatus* and *Acacia farnesiana*, dark-grey and light-grey, respectively) or 0 μg ml$^{-1}$ of DNA (control, white violin). Insets within violins indicate means (black dots) ± 1 SD; different letters indicate significant differences among treatments (univariate ANOVA and post-hoc Tukey test: $P < 0.05$, $n = 6$).]
Exogenous DNA triggers self-/non-self-specific resistance to a chewing herbivore. In most plants, JA controls the resistance to chewing herbivores and necrotrophic pathogens whereas SA controls resistance to sucking herbivores and biotrophic pathogens. Nevertheless, exceptions to this general model are common and therefore, we decided to complement the quantification of hormones with bioassays using a diverse spectrum of plant enemies. We selected *Spodoptera frugiperda* as a chewing herbivore, treated bean plantlets with 50 µg ml$^{-1}$ of self- or non-self-DNA and performed no-choice feeding assays to quantify the percentage of leaf area consumed by one caterpillar over the following 24 hours. We observed a significant treatment effect (GLM, $P < 0.05$, $n = 7$) (Fig. 3). However, of the three DNA types tested, only self-DNA reduced leaf area loss significantly, from ca. 7% in controls to ca. 0.3% in treated plants (Tukey post-hoc test $P < 0.05$, $n = 7$). Non-self-DNA from *P. lunatus* reduced leaf area loss to ca. 2%, although this effect was not statistically significant (GLM, Tukey post-hoc test, $P > 0.05$, $n = 7$), and non-self-DNA from *A. farnesiana* had no detectable effect (Fig. 3, GLM Tukey post-hoc test $P > 0.05$, $n = 7$).

**Fig. 3** Self-DNA induces resistance against a chewing herbivore. Violin plots depict the frequency distribution of herbivory by *Spodoptera frugiperda* larvae quantified as the percentage of leaf area lost during 24 h of feeding by one larva per leaf on *Phaseolus vulgaris* leaves after treatment with 50 µg ml$^{-1}$ of self-DNA (*Phaseolus vulgaris*, red) or non-self-DNA (*Phaseolus lunatus* and *Acacia farnesiana*, dark-grey and light-grey, respectively). The white violin represents the control (0 µg ml$^{-1}$ of DNA). Insets within violins indicate means (black dots) ± 1 SD; different letters above bars indicate significant differences among treatments (univariate ANOVA and post-hoc Tukey test: $P < 0.05$, $n = 7$).
Exogenous DNA reduces infection by fungal and bacterial pathogens in a self-/non-self-nonspecific way. To determine the effects of DNA treatment on the quality of the plant as a host for microbial pathogens, we aimed to take the perspective of the microbes and therefore, we decided to quantify population densities reached at a specific time point after challenge as an approximation of microbial reproductive fitness. Among four fungal strains tested, we detected no significant effect of DNA treatment on the density reached by Colletotrichum lindemuthianum or Fusarium oxysporum (GLM, $P > 0.05$, $n = 10$, Fig. 4a), whereas the necrotrophic fungi Botrytis cinerea and Sclerotinia sclerotiorum showed a significant reduction in the densities reached in DNA-treated as compared to control plants (GLM, $P < 0.05$, univariate ANOVA, Tukey post-hoc test, $P < 0.05$, $n = 10$, Fig. 4a).

Fig. 4 Self- and non-self-DNA treatment reduces infection by bacterial and fungal pathogens. Violin plots depict the frequency distribution of colony-forming units (CFU) of (a) the fungal pathogens Botrytis cinerea, Colletotrichum lindemuthianum, Fusarium oxysporum and Sclerotinia sclerotiorum and (b) the bacterial pathogens Enterobacter sp. strain FCB1, Pseudomonas syringae pv. phaseoli, P. syringae pv. syringae and Xanthomonas axonopodis pv. Phaseoli at 4 days or 2 days after inoculation Phaseolus vulgaris plants that had been treated with 50 µg ml$^{-1}$ of self-DNA (Phaseolus vulgaris, red) or non-self-DNA (Phaseolus lunatus and Acacia farnesiana, dark-grey and light-grey respectively). The white violins represent the controls (0 µg ml$^{-1}$ of DNA). Insets within violins indicate means (black dots) ± 1 SD; different letters above bars indicate significant differences among treatments (univariate ANOVA and post-hoc Tukey test: $P < 0.05$, $n = 10$).
Similarly, all four bacterial strains tested (Enterobacter sp. strain FCB1, Pseudomonas syringae pv. phaseoli, Pseudomonas syringae pv. syringae and Xanthomonas phaseoli) showed strongly reduced populations in DNA-treated plants as compared to controls (Fig. 4b, univariate ANOVA, Tukey post-hoc test, $P < 0.05$, $n = 10$). In addition, although 6 of the 8 pathogens tested showed clear differences in colonization levels between DNA-treated and non-DNA-treated plants, post-hoc tests revealed that for each of the 8 individual microbial species, levels of colonization of DNA-treated plants were similar irrespective of the source of DNA used for the treatment (Fig. 4).

We conclude that the effects of exogenous DNA treatment on the quality of P. vulgaris as a host for microbial leaf pathogens are not self/non-self-specific, at least not if we compare DNA from different plant species.

Extracellular DNA can have direct antimicrobial activity, both in experimental setups and in natural settings, for example in plant-DNA-containing extracellular traps that are secreted by plant roots as a means of defence against soil borne pathogens $^{35,36}$. To test for this possibility, we inoculated Petri dishes with solid medium that had been pre-treated with 100 µl of DNA at 50 µg ml$^{-1}$ from each of the three plant species (P. vulgaris, P. lunatus or A. farnesiana) and quantified bacterial colony forming units (CFUs) 2 days after inoculation and fungal CFUs 4 days after inoculation. However, under these experimental conditions, we detected no significant effect of any type of plant DNA on any of the eight microbial strains (Fig. S2).

The plant response to self-DNA enhances seed set in nature. The before mentioned reductions of herbivore-inflicted damage and pathogen populations indicate that the response of P. vulgaris plants to exogenously applied self-DNA is likely to be adaptive for the plant. However, conclusive evidence for the adaptiveness of a trait requires the demonstration of positive effects on fitness, and the ultimate fitness consequences of any plant resistance mechanism are highly dependent on the environmental context $^{37}$. Therefore, we decided to quantify the effects of treatments with self- and non-self-DNA on net seed production of P. vulgaris plants grown under open field conditions, in both the rainy and the dry season (April-July and July-October, respectively). To this end, we established an experimental field plot at CINVESTAV - Irapuato, Guanajuato, Mexico ~20°43′13″ N and 101°19′43″W, 1730 m.a.s.l. (see Fig. S3). Bean seeds were sown directly into the soil, emerging plantlets were treated once with 50 µg ml$^{-1}$ of self- or non-self-DNA and plants were allowed to finish their growth cycle without the application of any pesticide. We observed a
significant effect of the treatment in both seasons (GLM, \( P < 0.05, n = 3 \) blocks of 7 individuals, Fig. 5) although the magnitude and the self/non-self-specificity of the effects strongly differed between the seasons. In the rainy season, treatment with self-DNA increased seed yield ca. 1.5-fold as compared to controls (Tukey post-hoc test, \( P < 0.05 \)), whereas treatment with non-self-DNA had no detectable effect (Fig. 5a). In the dry season, however, all three DNA treatments increased seed yield significantly (Tukey post-hoc test, \( P < 0.05 \)), although at different magnitudes (over 3-fold and ca. 2-fold in response to self- and non-self-DNA, respectively, see Fig. 5b).

**Fig. 5** Treatment with self-DNA enhances seed production under natural enemy pressure. Violin plots depict the frequency distribution of the seed production (in grams seed dry mass per individual) of field-grown *Phaseolus vulgaris* plants in the rainy season (panel a) and the dry season (panel b) that had been treated once (at an age of four weeks) with 50 \( \mu g \) ml\(^{-1} \) of self-DNA (*Phaseolus vulgaris*, red) or non-self-DNA (*Phaseolus lunatus* and *Acacia farnesiana*, dark-grey and light-grey, respectively). The white violins represent the controls (0 \( \mu g \) ml\(^{-1} \) of DNA). Insets within violins indicate means (black dots) ± 1 SD; different letters above bars indicate significant differences among treatments (ANOVA and post-hoc Fisher’s least significant difference test: \( P < 0.05, n = 3 \) blocks of 7 individuals).
Subcellular origin and CpG methylation affect the immunogenic properties of plant DNA. In summary, our results show that the self/non-self-specific response to DNA can shape the interactions with natural enemies and that self-DNA can trigger an adaptive immunity that enhances the fitness of bean plants in nature. However, we lack a mechanistic explanation of these self/non-self-specific responses. The stronger responses to bacterial or viral DNA in mammalian immune cells depend mainly on the preference of toll-like receptor 9 (TLR9) for DNA that is rich in unmethylated 5'−CG−3' motifs, the so-called 'CpG' motif 38: a feature that also explains the inflammatory and immunogenic effects of mitochondrial DNA in mammals. We aimed to explore to what degree the plant immune response to DNA exhibits similarities to the mammalian system and therefore, we decided to compare the effects of self-DNA fractions of different subcellular origin and of self-DNA with experimentally manipulated contents of methylated CpG motifs, using common bean cells in suspension culture as the experimental system.

To this end, we first extracted DNA from suspensions enriched in chloroplasts (cpDNA), mitochondria (mtDNA), and nuclei (nDNA) and used polymerase chain reaction (PCR)-based amplification of three organelle-specific marker genes from the chloroplast (Rubisco large subunit, rbcL), mitochondrion (Cytochrome oxidase, cox1) or nucleus (Ubiquitin, ubi) to test for the purity of these DNA preparations. We detected no cross contamination of nDNA with any of the other two types of DNA (Fig. S4), although cpDNA showed a low degree of cross contamination with mtDNA (cox1) and nDNA (ubi) and mtDNA showed a very low degree of cross contamination with nDNA (Fig. S4). Intriguingly, we observed the strongest activation of MAPKs and the highest concentration of H$_2$O$_2$ in response to the total self-DNA preparation, and stronger effects of nDNA than organelle-derived DNA on MAPK activation, with MAPKs being least activated in response to cpDNA (Fig. 6a). The quantification of H$_2$O$_2$ formation revealed a similar pattern as for MAPK activation and confirmed significantly different responses to self-DNA of different subcellular origin (GLM, P < 0.05, n = 9). The H$_2$O$_2$ formation in response to nDNA was similar to the response
to total self-DNA, while cpDNA did not induce any detectable increase in H$_2$O$_2$ levels as compared to the controls (Fig. 6, Univariate ANOVA, Tukey post-hoc test, $P < 0.05$, $n = 9$).

**Fig. 6** The immunogenic effects of self-DNA fragments on plant cells depend on subcellular origin and the presence of unmethylated 5′-CCGG-3′ motifs. (a) Activation of mitogen-activated protein kinases at 30 min and H$_2$O$_2$ levels at 2 hours after treating suspension cells of *Phaseolus vulgaris* with total self-DNA, chloroplast-derived (cp)DNA, mitochondrial (mt)DNA or genomic (n)DNA at a final concentration of 50 µg ml$^{-1}$ DNA. Photos are representatives of $n = 3$ replicates of an assay for activates mitogen-activated protein kinases (MAPKs), violin plots depict the frequency distribution of H$_2$O$_2$ levels in millimoles per 1 X 10$^8$ cells. Insets within violins indicate means (black dots), different letters indicate significant differences among treatments (univariate ANOVA and post-hoc Tukey test: $P < 0.05$, $n = 9$).

Next, we aimed to generate self-DNA with a different content of methylated CpG motifs. To this end, we pre-treated bean self-DNA with the DNA methyltransferase M.SssI, which methylates all cytosine residues in the 5′-CG-3′ motif, and subsequently subjected fractions of natural and M.SssI-methylated DNA to digestion with each of two restriction enzymes, MspI and HpaII, which recognize 5′-CCGG-3′ motifs and cleave between the two cytosines (see Fig. S5 for a graphical illustration). Both enzymes can cleave unmethylated 5′-CCGG-3′ motifs and neither of them can cleave when the external cytosine residue is methylated (5′-mCCGG-3′), but MspI can cleave when the internal cytosine is methylated (5′-CmCCGG-3′), whereas HpaII cannot. Correspondingly, pre-treatment with M.SssI protected genomic DNA of *Aspergillus fumigatus* from digestion by HpaII, but not MspI, and pre-treatment with each of these enzymes reduced the effects of microbial DNA on cytokine production by mouse bone marrow-derived dendritic cells: both responses considered as canonical TLR9-dependent mammalian immune responses to non-self-DNA. As described for *A. fumigatus* DNA, both restriction enzymes generated fragments from ‘natural’, non-sonicated *P. vulgaris* self-DNA, whereas only MspI generated fragments from the M.SssI-treated self-DNA (Fig. S6). Although enzymatic digestion produced relatively fewer fragments of < 1000 bp than sonication (Fig. S6), these results show that common bean DNA contains unmethylated 5′-CCGG-3′ motifs and that M.SssI methylated the inner cytosine.
nucleotides in these 5’-CCGG-3’ motifs more or less completely. We treated bean cells with the
different DNA fractions and observed an increase in H$_2$O$_2$ accumulation in all cases in which self-
DNA had been fragmented, either by sonication or by digestion with MspI or HpaII (Fig. 6b,
Univariate ANOVA, Tukey post-hoc test, $P < 0.01$, $n = 5$). However, DNA digested with the
restriction enzymes had significantly lower effects on H$_2$O$_2$ production than sonicated DNA.
Similarly, DNA in which all 5’-CG-3’ motifs had been methylated induced H$_2$O$_2$ significantly, but to a
lower degree than naturally methylated DNA (Fig. 6b). These results indicate that the presence of
unmethylated CpG motifs contribute to the immunogenic properties of DNA in plants.

**Fig. 7.** Presence of unmethylated cytosines in 5’-CCGG-3’ motifs affects the H$_2$O$_2$-inducing properties of
self-DNA. Self-DNA was either pre-fragmented with sonication (red bars) or was not pre-fragmented (orange
bars), subsequently methylated with M.SssI ('M.SssI-methylated') or not ('Naturally methylated') and
subsequently digested with one of two restriction enzymes (MspI or HpaII). Enzyme solutions were used as
control treatments (white bars). Violin plots depict the frequency distribution of H$_2$O$_2$ levels in millimoles per
1 X 10$^8$ cells, insets within violins indicate means (black dots), different letters indicate significant differences
among treatments (univariate ANOVA and post-hoc Tukey test: $P < 0.05$, $n = 5$).

**Discussion**

Self-DNA can trigger immune responses in plants. Here, we asked whether this apparent lack of
self-tolerance is beneficial for the resistance and reproduction of plants. Indeed, self-DNA induced
JA and SA production and increased the resistance in common bean to biological enemies.
Strikingly we observed a self-specific induction of JA whereas both self and non-self-DNA induced
Correspondingly, only the self-DNA treatment caused a significant reduction in feeding by a chewing herbivore, while self and non-self-DNA reduced bacterial and fungal infections to similar degrees. Most importantly, a single treatment with self-DNA resulted in significantly higher net seed production by field-grown plants, in two different seasons.

Our study supports the hypothesis that self-DNA acts as a DAMP that triggers an adaptive immune response in plants. Interestingly, two recent studies identified secreted DNAses as effectors that suppress plant resistance in response to feeding by the small brown planthopper (*Laodelphax striatellus*)\(^{44}\) or root infection by the fungus *Cochliobolus heterostrophus*\(^{45}\). Planthoppers in which DNase II had been knocked down were not able to fully eliminate the release of plant self-DNA in the damaged tissue, consequently triggered increased ROS production during feeding and showed reduced performance on rice plants, and similarly, DNase deletion mutants of *Cochliobolus heterostrophus* were less virulent on maize leaves\(^{44,45}\). Taken together, these observations further support a function of self-DNA sensing in the plant immune system.

The induction of JA and SA by plant-derived self-DNA indicates self-DNA as an active principle in the immunogenic effects of conspecific leaf homogenates\(^{16,46-48}\) and might explain, at least in part, the success of multiple plant or algal derived bio-control products\(^{26}\). In more general terms, our findings are consistent with Polly Matzinger’s ‘danger model’\(^{25,49}\). An accumulation of DAMPs, such as cell wall-derived oligogalacturonides or extracellular ATP, indicates massive cellular damage\(^{14,50,51}\). Therefore, DAMPs usually trigger ROS and induce JA thereby inducing resistance to chewing herbivores and necrotrophic pathogens\(^{52,53}\). Correspondingly, wounding and extracellular ATP increased ROS formation and the activity of JA-inducible polyphenol oxidase in *P. vulgaris*\(^{54}\), and a significant percentage of all eATP-responsive genes in *A. thaliana* require an intact JA signalling pathway\(^{55}\). In our study, self-DNA induced JA and reduced feeding by a chewing herbivore and infection by a necrotrophic bacterium (*Enterobacter* sp.) and two necrotrophic fungi (*B. cinerea* and *S. sclerotiorum*). In contrast, both self and non-self-DNA induced SA and reduced the infection levels of three biotrophic bacteria (*P. syringae* pv. *phaseoli* and pv. *syringae*, and *X. axonopodis* pv. *phaseoli*). Whereas the differential effects of self and non-self-DNA on JA versus SA explained most of the phenotypic resistance effects, the specific roles of JA versus SA seem insufficient to explain why self-DNA also induced SA. However, Matzinger argued that damage means danger and, thus, necessitates immunity to (future) infection by any type of pathogen\(^{25}\). Damaged-self recognition represents an important element of mammalian immunity to virus infection\(^{56-58}\) and evidently, damaged plants are more susceptible to subsequent
infections. Therefore, we conclude that the induction of JA and SA by self-DNA and the induction of only SA by non-self-DNA can be adaptive under many ecologically relevant conditions. Furthermore, our results confirm the existence of self/non-self-specific immunogenic effects of DNA at a taxonomic level that has rarely been reported (but see \textsuperscript{15,19}). The possible explanations for this novel discovery range from a true biological difference between plants and mammals to the mere absence of similar studies in mammals. Evidently, studying the effects of, e.g., treating mammalian immune cells with DNA from other primate species is beyond the scope of classical medical research. However, the stronger immunogenic and pro-inflammatory effects of mtDNA as compared to genomic DNA and the role of unmethylated CpG motifs in mammalian TLR9-mediated DNA perception have been confirmed in hundreds of studies (see, e.g., reviews by \textsuperscript{60-62}). In our work, DNA from organelles had lower effects than genomic DNA and self-DNA maintained immunogenic properties after methylation with the CpG methyltransferase M.SssI or cleavage of all unmethylated 5′-CCGG-3′ motifs. Theoretically, these patterns would be consistent with a model that considers DNA fragmentation as a pre-requisite of immunogenic activity and assumes additive effects of a specific response to unmethylated CpG-motifs and of a non-specific response to dsDNA, as exhibited, e.g., by several mammalian DNA sensors, including cyclic GMP-AMP synthase or by absence in melanoma \textsuperscript{61,63} (Fig. S7).

In conclusion, our study supports a role of self-DNA as a DAMP in the plant immune response to wounding and infection, reveals a role of CpG methylation in the immunogenic effects of DNA in plants and demonstrates that testing DNA from different plant species offers a unique opportunity to compare the effects of DAMPs and PAMPs using molecules with the same chemical nature. However, much more work will be required to identify the molecular players that control the self/non-self-specific plant response to DNA and to explore its potential for the development of DNA-based biocontrol strategies.

Methods

**Plants and DNA sources.** We used four-week-old common bean (*Phaseolus vulgaris* L.) plants (variety Negro San Luis) grown in a glasshouse, for the quantification of defence hormones and biological resistance, and *P. vulgaris* plants grown under field conditions (experimental field at CINVESTAV - Irapuato, Guanajuato, Mexico ~20°43′13″ N and 101°19′43″ W, 1730 m a.s.l.) for the quantification of seed production as an approximation of fitness. For the quantification of ROS in
response to DNA with different methylation patterns, we produced cells of *P. vulgaris* in suspension culture as described in 17. *P. vulgaris* seeds were obtained from the national germplasm collection at INIFAP, Celaya, GTO, Mexico. Conditions in the glasshouse were as follows: natural light and photoperiod; average day-time temperature, 28°C; night-time temperature, 20°C. Plants were watered three times a week and fertilized weekly with a commercial fertilizer (Ferviafol 20-30-10®, Agroquímicos Rivas S.A. de C.V., Celaya, GTO, México).

We used leaves from four-week-old common bean plants grown in the glasshouse as sources of self-DNA. We used leaves of lima bean (*Phaseolus lunatus* L.) plants grown in the glasshouse and leaves of wild *Acacia farnesiana* (L.) Willd. plants growing in the area around CINVESTAV – Irapuato as sources of non-self-DNA. Lima bean seeds were collected from a wild population 5-km west of Puerto Escondido, Oaxaca, Mexico (~15°55' N and 097°09' W). All seeds were surface-sterilized with 70% ethanol for 1 min and with 20% hypochlorite for 10 min and then washed five times with sterile distilled water before sowing.

**Herbivore.** Larvae of the generalist herbivore *Spodoptera frugiperda* (J.E. Smith) were collected from maize fields in Irapuato, Guanajuato, Mexico, identified following 64 and reared in individual 100 ml plastic cups with perforated lids, allowing ventilation, on an artificial diet (1–2 cm³) (Methods S1). The colony was maintained until the third generation to increase the probability that the larvae were parasite-free. For the herbivory experiment, we used larvae at the fifth instar (14 days after oviposition) that had been maintained for 12 h without food before starting the experiment.

**Phytopathogens.** The bacterium *Xanthomonas axonopodis pv. phaseoli* was provided by Dr Gabriel Gallegos-Morales (UAAAN, Saltillo, Coahuila, Mexico) and cultivated at 28°C on solid yeast dextrose carbonate (YDC) medium 65. *Pseudomonas syringae pv. phaseoli* strain NPS3121 was provided by Dr José-Luis Hernández-Flores (CINVESTAV, Irapuato, Guanajuato, Mexico) and cultivated at 28°C on solid King’s B (KB) medium 66. The rifampicin-resistant *P. syringae* pv. *syringae* strain 61 was provided by Dr Choong-Min Ryu (KIRIBB, Daejeon, South Korea) and cultivated at 28°C on solid KB medium with 50 mg l⁻¹ rifampicin. The *Enterobacter* sp. strain FCB1 had previously been isolated and identified in our laboratory 67 and was cultivated at 28°C on solid YDC medium. Fungal pathogens: *Colletotrichum lindemuthianum* strain 1088 was donated by Dr June Simpson (CINVESTAV, Irapuato, Guanajuato, Mexico), *Sclerotinia sclerotiorum* was donated by Dr Víctor 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 containing potato dextrose agar (PDA) medium at 28°C, except for *S. sclerotiorum*, which was cultivated at 20°C.

**Extraction and fragmentation of DNA.** The extraction of DNA from leaves of *P. vulgaris*, *P. lunatus* and *A. farnesiana* was based on a method reported by 68 (Methods S1). The DNA was quantified using a NanoDrop 2000 spectrometer (Thermo Scientific) and fragmented by sonication using an ultrasonic processor (Misonix XL2020). To obtain fragments shorter than 1000 bp, a solution of 500 μg ml\(^{-1}\) of DNA in sterile distilled water was sonicated for 3 min at 55% of amplitude and using pulse mode (1 s pulse ‘On’ and a 1 s pulse ‘Off’). The successful fragmentation of DNA was verified by gel electrophoresis on a 3% agarose gel using ethidium bromide staining (see Supporting Information Fig. S1 for a graphical summary).

**JA and SA levels.** In order to quantify the effects of exogenously applied self-DNA on the synthesis of JA and SA, we applied fragmented self-DNA at different concentrations (0, 2, 20, 50, 100, 150 or 200 μg ml\(^{-1}\) in 0.05% v v\(^{-1}\) Tween 20). The solution of self-DNA was applied to both sides of the three youngest leaves of each plant using a micropipette until the leaf surface was completely wet. Independent groups of six plants were used for each time point and for each self-DNA concentration. Treated leaves were cut off at the base at 0, 2, 5, 10, 15, 30 or 45 min or 1, 2, 4, 8, 12, 24 or 48 h after treatment; the three leaves from each plant were pooled and ground with liquid nitrogen. In order to compare the effects of self and non-self-DNA, we used 50 μg ml\(^{-1}\) of DNA to treat groups of six plants and quantified JA 30 min after treatment, whereas SA was quantified 24 h after treatment.

The extraction of JA followed 69 and the extraction of SA followed 70 and 71 (see Methods S1 for details). Both hormones were quantified using gas chromatography coupled to an electronic impact ionization mass spectrometer (GC-EIMS) in an Agilent Technologies Gas Chromatograph 7890A equipped with a DB-1MS UI column (60 m × 0.25 mm × 0.25 μm Agilent Technologies) coupled to a MSD 5973 detector in SIM mode, using ions 141, 181, 390 and 392 m z\(^{-1}\) for JA and 73, 135, 267 and 282 m z\(^{-1}\) for SA. An injection volume of 1 μl of sample was used in the splitless mode. For JA, the operating conditions followed 72, i.e., we used an injector temperature of 200°C and an initial oven temperature of 150°C for 3 min, which was then ramped at 4°C min\(^{-1}\) to 300°C, with the final temperature maintained for 20 min. For SA we used an injector temperature of 200°C and an initial oven temperature of 150°C for 3 min, which was then ramped at 4°C min\(^{-1}\) to
260°C, with the final temperature maintained for 25 min. Helium was used as a carrier gas with a constant flow of 1 ml min\(^{-1}\) and standard curves were prepared using pure compounds (JA, Sigma-Aldrich; SA, Baker) to quantify the respective amounts of JA and SA based on peak areas with reference to the internal standard.

**Herbivore feeding.** Plants of *P. vulgaris* were treated with 50 µg ml\(^{-1}\) of self- or non-self-DNA as described above (\(n = 7\) for each DNA source). After 30 min, one larva of *S. frugiperda* was placed on each leaf, which was then covered with a mesh bag. After 24 h, individual photos of each leaf including a ruler at one side were taken using a J5-Prime Samsung phone camera from 20 cm distance. Feeding damage was quantified as percentage of lost area using ImageJ software (https://imagej.nih.gov/ij/) to measure the lost area by herbivory.

**Bacterial and fungal infection.** Plants of *P. vulgaris* were treated with 50 µg ml\(^{-1}\) of self or non-self-DNA as described above (\(n = 10\) for each DNA source and pathogen). Five minutes after the treatment, plants were sprayed with a 10 ml suspension (1 \(\times\) \(10^7\) cells ml\(^{-1}\)) of one of the seven pathogens. Infection levels of three randomly selected leaves per plant were quantified after eight days (bacteria) or fifteen days (fungi). Leaves were collected, weighed and ground in a mortar with 1 ml of sterile distilled water, diluted 1:100 and 20 µl of the resultant liquid were plated onto the appropriate solid medium as described in the 'Phytopathogens' section to quantify colony forming units (CFUs). The putative direct effects of DNA solutions on pathogens \(^{35}\) were investigated by plating 100 µl of 50 µg ml\(^{-1}\) of DNA of either *P. vulgaris*, *P. lunatus* or *A. farnesiana* DNA (\(n = 6\) for each DNA type and pathogen) on Petri dishes of solid medium. After 5 min, the medium was inoculated with the respective pathogen and CFUs were quantified 2 days after inoculation for bacteria and 4 days after inoculation for fungi.

**Seed production.** Plants of *P. vulgaris* were cultivated in experimental fields at CINVESTAV Irapuato in twelve blocks (three per treatment) of 3 \(\times\) 9 plants, with 35 cm between individuals and 1 m between blocks (Supporting information Fig. S3). This experiment was carried out from April to July 2018, during the early rainy season, which is the main cultivation period for *P. vulgaris* in this region \(^{73}\). The experiment was repeated from July to October 2018. Four-week-old plants were treated by spraying 50 µg ml\(^{-1}\) of self- or non-self-DNA in 0.05% v v\(^{-1}\) Tween 20 on both sides of all leaves until the plants were completely wet. Plants treated with 0.05% Tween 20 were used as controls \(^{48}\). Plants were allowed to finish their growing cycle with no further treatment. Pods
were collected from the seven central plants of each block and the total mass of seeds from each plant was recorded.

**Preparation of DNA from different organelles.** To compare the immunogenic properties of self-DNA from different organelles, we followed to extract DNA from suspensions enriched in chloroplasts or mitochondria, and followed to extract DNA from nuclei (see Methods S1). The extracted DNA was quantified using a NanoDrop 2000 spectrometer (Thermo Scientific) and the integrity was checked on a 1.2% agarose gel. The purity of the different DNA fractions was tested by amplifying reference genes from the chloroplast (Rubisco large subunit, \textit{rbcL}), mitochondrion (Cytochrome oxidase, \textit{cox1}) or nucleus (Ubiquitin, \textit{ubi}) (Supporting Information Fig. S6). \textit{P. vulgaris} gene sequences (\textit{rbcL}, YP_001122790; \textit{cox1}, XP_007142315; and \textit{ubi}, AGV54749.1) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/nucleotide/). Primers were designed according to their coding sequence using the Primer3 v.4 software (http://primer3.ut.ee) and are listed in Table 1. Amplification was performed by PCR using the following 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 of each primer. The following PCR program was used: 95°C for 3 min, 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 40 s, and then 72°C for 10 min. PCR products were checked using 1.2% agarose gel electrophoresis with ethidium bromide staining.

Then, fragmented DNA at a final concentration of 50 µg ml$^{-1}$ was applied to cell cultures [established as described in and Methods S1] to quantify MAPK activation and ROS formation as described below (“\textit{Quantification of MAPK activation and ROS (H$_2$O$_2$) formation in cell culture}”)

**Methylation and CpG-dependent DNA enzymatic digestion of DNA.** Non-fragmented self-DNA or sonicated fragments of < 1000 bp were methylated with the GpG DNA methyltransferase from \textit{Spiroplasma} sp. strain MW1 (M.SssI) according to the product manual. M.SssI methylates “all cytosine nucleotides contained in unmethylated or hemimethylated double stranded DNA in a 5’-CpG-3’ context” (https://www.thermofisher.com/order/catalog/product/EM0821#/EM0821) (see Fig. S5 for predicted products). Subsequently, aliquots of 1 µg µl$^{-1}$ of the M.SssI-treated DNA or of ‘natural’ DNA were digested with a restriction enzyme, either MspI (Thermo-Scientific ER0541) or HpaII (Invitrogen INVN093-6), according to the product manuals. Both enzymes cleave unmethylated 5’-CCGG-3’ motifs between the two cytosines (Fig. S5) and none of them can cleave when the external cytosine residue is methylated (5’-\textit{m}CCGG-3’). However, MspI is an isoshizomer of HpaII that can cleave 5’-CCGG-3’ motifs when the internal cytosine is methylated (5’-\textit{C}\textit{m}CGG-3’),
whereas HpaII cannot \cite{40,41} and

https://www.thermofisher.com/order/catalog/product/ER0541#/ER0541]. The completeness of 5’-CG-3’ methylation and DNA digestion by the restriction enzymes was confirmed on a 2% agarose gel (Fig. S6).

**Quantification of MAPK activation and ROS \((\text{H}_2\text{O}_2)\) formation in cell culture.** To quantify the activation of MAPKs, 1 mL of cell culture suspension \((1 \times 10^{-8} \text{ cells mL}^{-1})\) was transferred to a 24 multiwell plate and shaken at 160 rpm on an orbital shaker at room temperature. After 1 h of equilibration, fragmented DNA from the different organelles was added to a final concentration of 50 µg ml\(^{-1}\), using complete sonicated self-DNA at 50 µg ml\(^{-1}\) as a positive control and 0.1 ml of sterile water as the negative control. After 30 min, the cells were mixed with 1 ml of the extraction buffer and frozen in liquid nitrogen. Next, 2 ml of the suspension culture were transferred to 2 ml tubes, cells were sonicated twice for 20 s (Ultrasonic Processor Misonix XL2020) and centrifuged at 13,000 \(\times\) g. The supernatant was used for the MAPK assays based on established methods \cite{76,77}.

To quantify the production of ROS 1 ml of cell culture [established as described in \cite{17} and Methods S 1] containing \(1 \times 10^{8}\) cells was centrifuged at 6000 \(\times\) g for 10 min at room temperature. The supernatant was discarded, and 1 ml of fresh MS medium was added to the pellet. These steps were repeated three times to wash the cells and to remove any extracellular \(\text{H}_2\text{O}_2\). 1 ml of washed cell culture \((1 \times 10^{8} \text{ cells ml}^{-1})\) was transferred to a 24-well plate and shaken at 160 rpm on an orbital shaker at room temperature. After 1 h of equilibration, DNA from different organelles \((n = 9 \text{ per treatment})\) or DNA with different contents of methylated 5’-CCGG-3’ motifs \((n = 5 \text{ per treatment})\) as added to a final concentration of 50 µg ml\(^{-1}\). After 2 h of treatment, the cell culture was centrifuged at 6000 \(\times\) g for 10 min. Next, 10 µl of the supernatant of the centrifuged cell culture was transferred to a 96-well plate and mixed with 90 µl of the substrate solution from the Hydrogen Peroxide Assay Kit (National Diagnostics). 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.) and compared to a calibration curve obtained using \(\text{H}_2\text{O}_2\) at concentrations of 0–250 nmol ml\(^{-1}\). Samples out of the range of the calibration curve were diluted to quantify \(\text{H}_2\text{O}_2\).
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Acknowledgements

We thank Rosa-María Adame-Álvarez and José-Luis Hernández-Flores for technical support, Isaac Vega-Muñoz for creating the violin plots and Patricia Sanchez-Garcia, Silvia Sanchez-Garcia and Rosa-María Adame-Álvarez for their priceless help with the field work. We are grateful to Stefano Mazzoleni and Marilu’ Chiusano for sharing unpublished data, to Stefano Mazzoleni, Marilu' Chiusano, Jurriaan Ton, June Simpson, Thomas Boller and Simon Stael for valuable comments on an earlier version of this manuscript and Caroline Woods for correcting the English. Financial support from CONACyT de México (Grants 258119 and 278283 to MH and Grant 394371 to DDF) is gratefully acknowledged.

Author Contributions

D.D.F. and M.H. designed the experiments, D.D.F. performed the experiments, evaluated the data and wrote the original draft, M.H. acquired funding and both authors reviewed and edited the final version of the manuscript.

Funding

This work was supported by CONACYT de México, grants #258119 and #278283 to M.H. and grant #394371 to D.D.F.)

Competing Interests

The authors have no competing interests to declare

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/

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**Table 1.** Primers used for the amplification of *Phaseolus vulgaris* chloroplast-, mitochondrion- and nucleus-specific reference genes

<table>
<thead>
<tr>
<th>Location</th>
<th>Gene</th>
<th>Sequence of primers</th>
</tr>
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<tbody>
<tr>
<td>Chloroplast</td>
<td>Rubisco large subunit (rbcL)</td>
<td>Forward: 5'-GGACAACTGTGTGGACCGAT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-AAACGGTCTCTCCAACGCAT-3'</td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>Cytochrome oxidase (cox1)</td>
<td>Forward: 5'-CAGCGGTTTCCTGTCTCCAA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-TTTCCGCTTTATGCCTGGCC-3'</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Ubiquitin (ubi)</td>
<td>Forward: 5'TTGGGACGGAGGGAGTATGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5'-GTGGGATCCCTTCTTGTCC-3'</td>
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