Supplementary Information

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

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Figure S1 Graphical methods summary

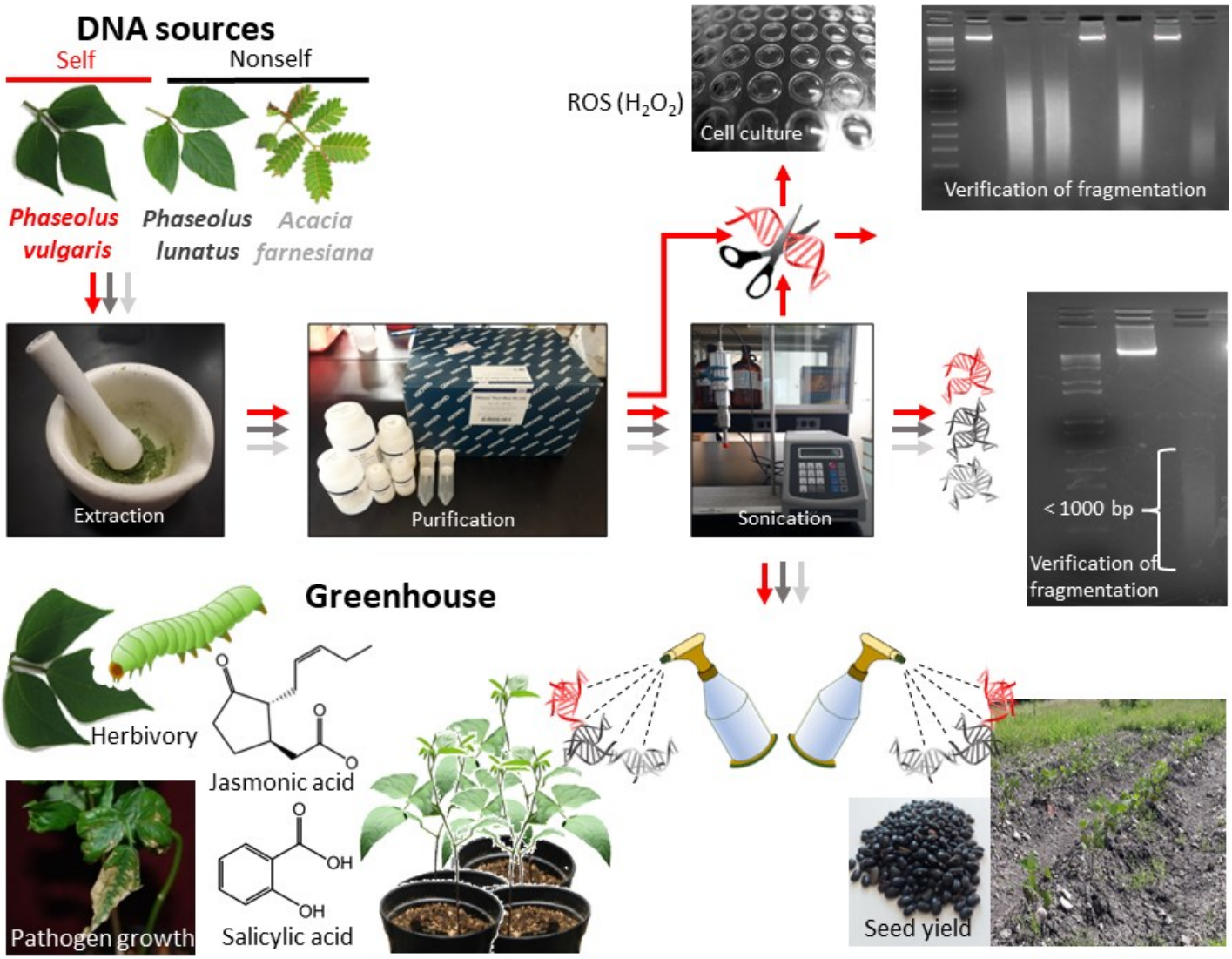


Figure S2 Direct effects of plant DNA on fungal and bacterial pathogens

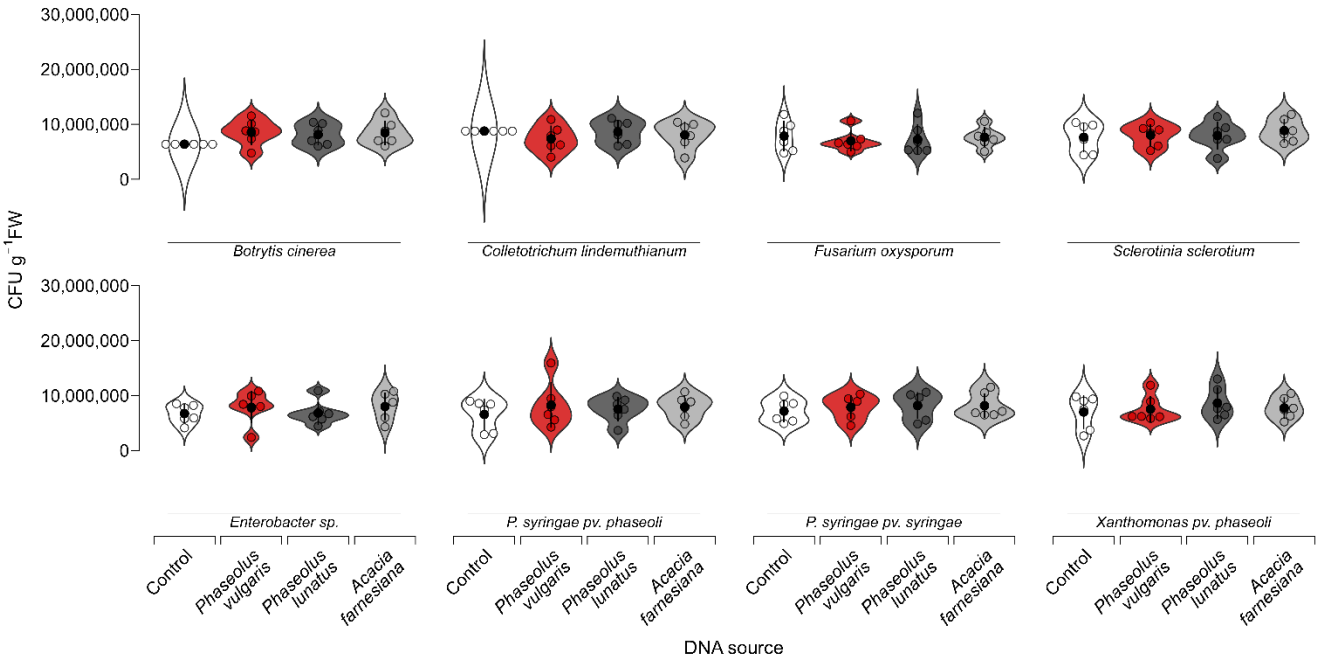


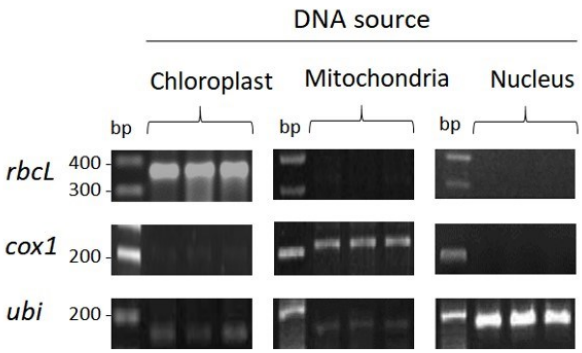
Figure S2 Direct effects of plant DNA on fungal and bacterial pathogens.

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* quantified 2 days and 4 days for bacteria and fungi, respectively, after inoculating Petri dishes of solid medium that had been pre-treated with 100 µl of 50 µg ml^{-1} fragmented plant DNA from *P. vulgaris* (red) *P. lunatus* (dark grey) or *A. farnesiana* (light gray). Insets within violins indicate means (black dots) ± 1 SD, shared letters above bars indicate absence of significant differences among treatments (univariate ANOVA and post-hoc Tukey test: $P > 0.05$, $n = 6$).

Figure S3 Localization and spatial design of the field experiment

Aerial view, terrestrial view and distribution of plants for the four different treatments and their replicates in the field. Plants were grown at distances of 35 cm in a 9 x 3 block arrangement. All 27 plants in each block received the same treatment, but only the inner seven plants (inside of the dotted line) were used to quantify seed production. Each block was separated 1 m from the other, and the entire experiment comprised three spatially independent replicates, with different spatial arrangements of the blocks.

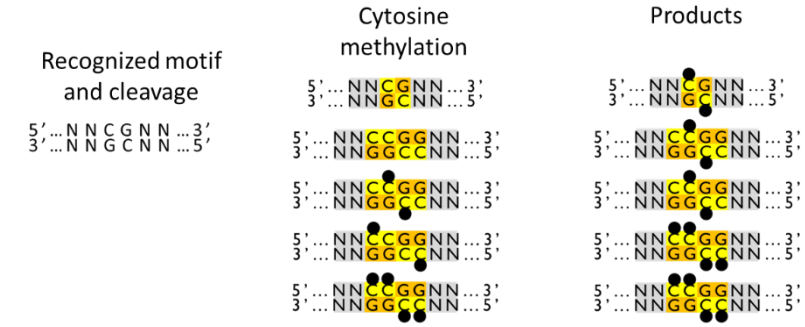
Figure S4 PCR of marker genes confirms purity of DNA from subcellular compartments



Semi-quantitative PCR of marker genes shows that the nuclear DNA (nDNA) was practically free of chloroplast and mitochondrial DNA (cpDNA and mtDNA, respectively), while a low cross-contamination with nDNA can be detected in the cpDNA and mtDNA fractions. Each PCR amplification was performed in triplicate and the products were separated on a 1.2 % agarose gel.

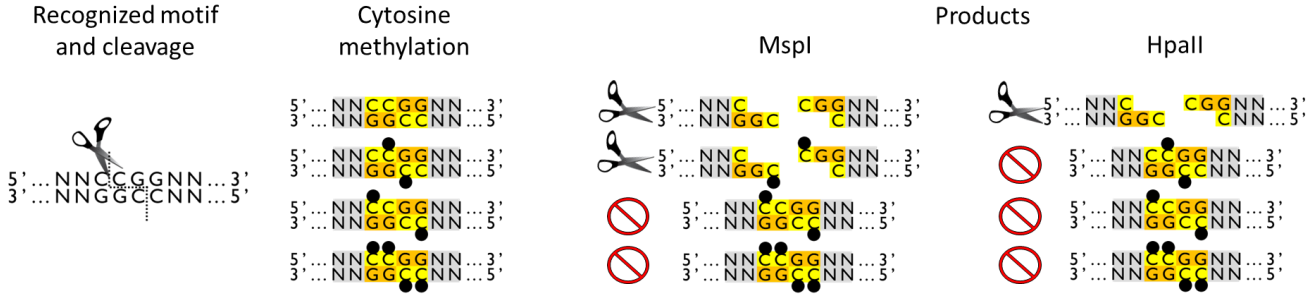
Figure S5 Methylation-dependent cleavage of M.SssI-methylated self-DNA by MspI and HpaII confirms complete methylation of 5’-CCGG-3’ motifs

(a) Methyltransferase M.SssI



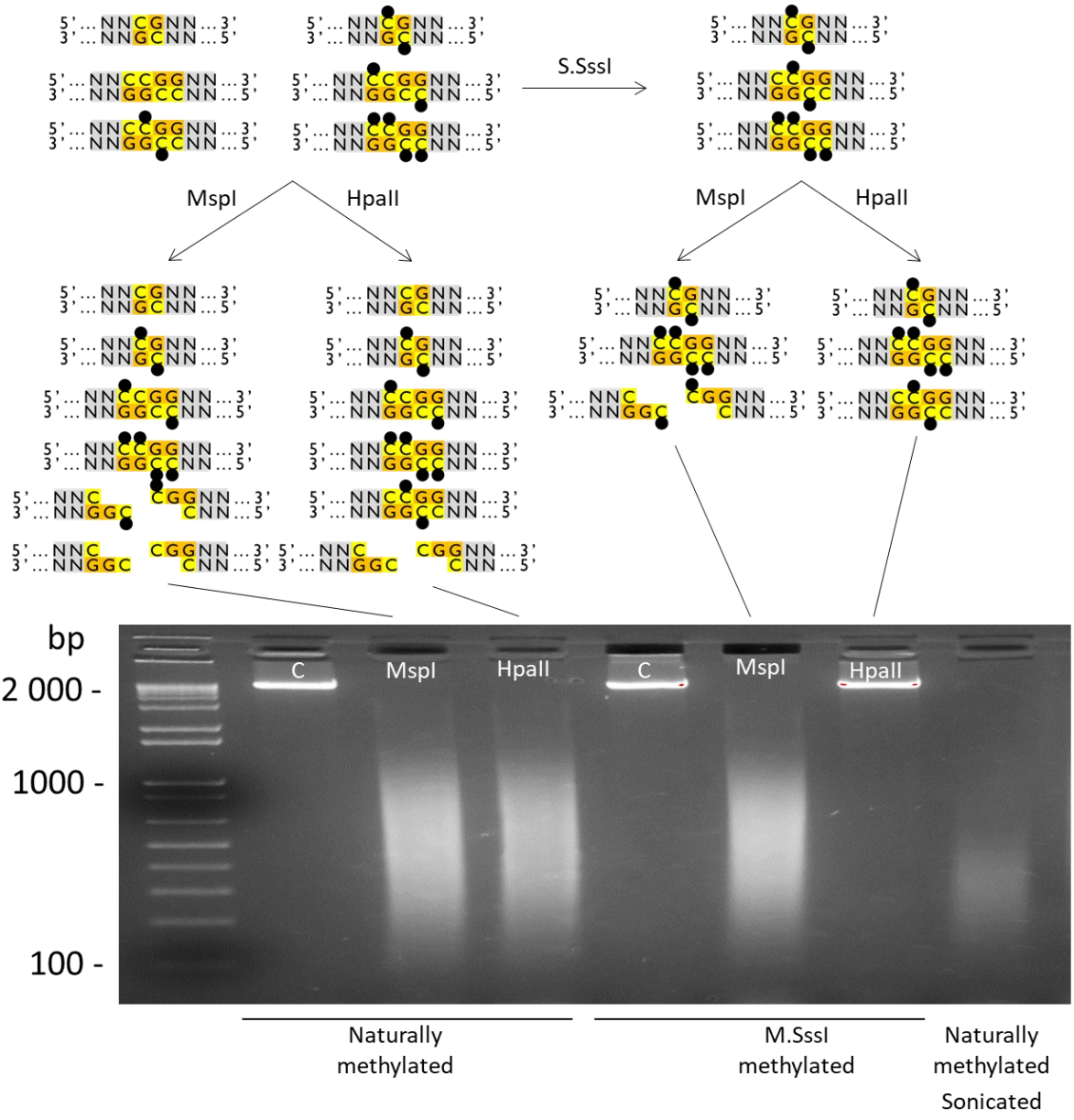
The DNA methyltransferase M.SssI methylates all cytosines in 5’-CG-3’ motifs. In plants, both the external and the internal cytosine in 5’-CCGG-3’ motifs can be methylated (5’-^{m}C^{m}CGG-3’). Here, we assume that M.SssI can methylate the internal cytosine in a 5’-CCGG-3’ motif even when the external cytosine residue was already methylated, i.e., generate 5’-^{m}C^{m}CGG-3’ from 5’-^{m}CCGG-3’. Cytosines are marked in yellow, guanines are marked in brown, methylation is indicated as black dots.

(b) Restriction enzymes MspI and HpaII



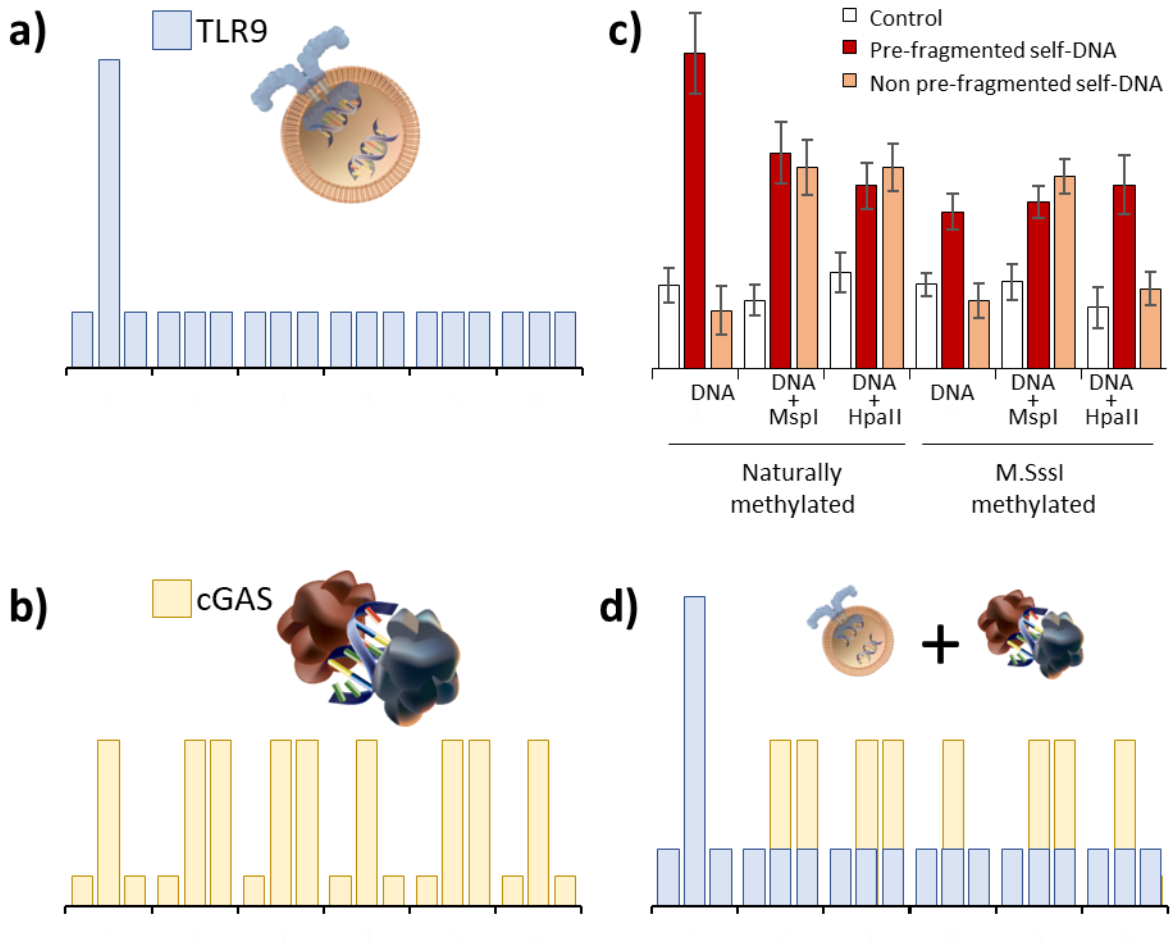
The restriction enzymes MspI and HpaII recognize 5’-CCGG-3’ motifs and cleave between the two cytosines. Both enzymes can cleave when both cytosines are unmethylated (5’-CCGG-3’) and none of them can cleave when the external cytosine is methylated (5’-^{m}CCGG-3’). However, MspI can cleave the sequence when the internal cytosine is methylated (5’-C^{m}CGG-3’), whereas HpaII cannot. Cytosines are marked in yellow, guanines are marked in brown, methylation is indicated as black dots.

Figure S6 Cleavage of M.SssI-methylated self-DNA by MspI and HpaII confirms complete methylation of internal cytosines in 5’-CCGG-3’ motifs



Both restriction enzymes could cleave the natural self-DNA, confirming the presence of unmethylated 5’-CCGG-3’ sequences. Since HpaII could not cleave the M.SssI-treated DNA, we conclude that M.SSI-treatment resulted in a complete methylation the internal cytosines in all 5’-CCGG-3’ motifs, that is, conversion of 5’-CCGG-3’ to 5’-C^{m}CGG-3’ and of 5’-^{m}CCGG-3’ to 5’-^{m}C^{m}CGG-3’.

Figure S7 Effects of differentially methylated bean self-DNA on ROS production in bean suspension cells as compared to predicted activation of Toll-like 9 (TLR9) and the dsDNA sensor cyclic AMP-GMP synthase (cGAS)



Comparison of **(a)** the predicted activation of TLR9 and of **(b)** the predicted activation of cGAS with **(c)** the observed induction of H_2O_2 in suspension cells by the differently treated self-DNA (taken from Figure 6b) and **(d)** a hypothetical additive effect of a TLR9-like response and a cGAS-like response. Drawings of TLR9 and cGAS by Enrique Hurtado Bautista.

Methods S1 Supplementary Methods

DNA extraction

Leaves of *P. vulgaris*, *P. lunatus* or *A. farnesiana* were ground with liquid nitrogen. 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 per 5 g of ground tissue in 50 ml tubes, shaken on a vortex and heated to 65 °C for 10 min in a water bath. Then, 6.6 ml of 5 M potassium acetate were added followed by incubation on ice for 30 min. The tubes were centrifuged at 12000 *g* for 20 min at 4 °C: the supernatant was transferred to a new 50 ml tube. Next, 20 ml of precooled isopropanol were added to the supernatant, which was kept at –20 °C. After 1 h, the tubes were centrifuged at 12000 x *g* for 20 min at 4°C, the supernatant was discarded and the pellet was dried for 5 min at room temperature before washing by adding 5 ml of 70 % ethanol and shaking. After further centrifugation at 12000 x *g* for 10 min a 4 °C, the supernatant was discarded and the pellet was dried for 5 min and suspended in 1 ml of sterile distilled water and purified using a Maxi DNA purification Kit (Qiagen).

Jasmonic acid (JA) extraction

In brief, 0.5 ml of ethyl acetate and 20 µl of 0.1 mg ml^{-1} (9,10- H2)-dihydrojasmonic acid (Internal standard; Sigma-Aldrich) were added to 250 mg of tissue, shaken and kept at 4 °C overnight. After centrifugation at 14000 x *g* for 15 min at 4 °C, the supernatant was collected 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 and 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 of HPLC grade methanol. This suspension was used to JA quantification using gas chromatography.

Salicylic acid (SA) extraction

We added 750 µl of 90 % methanol and 250 ng ml^{-1} of ortho-anisic acid (Internal standard) to 250 mg of ground tissue and kept at 4 °C overnight. After centrifugation at 13000 x *g* for 15 min at 4 °C, the supernatant was collected, the pellet was re-extracted with 750 µl of 90 % methanol, and both supernatants were combined and dried in a Concentrator plus (Eppendorf) for 4 h. The residue was re-suspended with 500 µl of TCA 5 % and centrifuged at 4000 x *g* for 10 min at 4 °C. The supernatant was mixed with two volumes of ethyl acetate-hexane (1: 1 v v^{-1}) and incubated at room temperature for 10 min. The organic phase (upper phase) was recovered and dried with gaseous nitrogen. The residue was derivatized by mixing with 20 µl of pyridine and 80 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma-Aldrich) and incubated at 80 °C for 1 h, using an extraction hood. The resulted mix was used for SA quantification using gas chromatography.

Cell culture

In order to reduce the quantity of purified DNA required for some experiments, we produced cells of *P. vulgaris* in liquid culture. Common bean seeds were surface-sterilized with 70 % ethanol for 1 min and with 20 % sodium hypochlorite for 10 min. The seeds were rinsed with sterile water and placed individually in a 500 ml transparent-glass flask containing 2 cm Murashige and Skoog (MS) solid medium at pH 5.8 and 3 % sucrose (Murashige and Skoog, 1962). The flasks were maintained under sterile conditions in a growth room at 25 °C and a light : dark regime of 16 h : 8 h. After seven days, we cut 3 mm from the tip of the apical meristem and transferred this tissue to solid MS medium enriched with 0.5 mg l^{-1} of indol-3-acetic acid (IAA) and 5 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) (both from Sigma-Aldrich). After incubation for four weeks at 25 °C, the callus formed was gently disintegrated mechanically and then 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 was maintained on a shaker (160 rpm) in the growth room to obtain the cell culture. The cells were subcultured every two weeks transferring 2 ml of culture to a new flask with MS liquid medium and used for experiments seven days after subculturing.

DNA extraction from chloroplasts, mitochondria and nuclei

To extract DNA from chloroplasts 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. One 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 tissue. The suspension was vortexed and filtered through a nylon net (0.25 mm mesh size) into a clean 2 mL test tube. The nylon net and its contents were discarded. To discard the cell debris and nuclei that remained in the filtered preparations, the tubes were centrifuged at 500 x *g* for 5 min at 4 °C. The supernatant was transferred to a fresh 2 mL tube, and then used for chloroplast enrichment. All samples and reagents were kept cold on an ice bath. The supernatant from was centrifuged at 2000 x *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 x *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 in 50 mM Tris-HCl (pH 8.0) and 25 mM EDTA (pH 8.0). Aliquots of 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 were delicately placed on its surface. Then, 300 µL of the chloroplast suspension were gently and slowly placed on top of the sucrose gradient, one drop at a time to avoid mixing. The tube was centrifuged at 7000 x *g* for 1 h 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 mesh size) into a fresh 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 x *g* for 5 min at 4 °C. The supernatant was carefully transferred to a new 2 mL tube and centrifuged at 2000 x *g* for 10 min at 4 °C to pellet chloroplasts, which were removed. This step was repeated

twice. After centrifugation, the supernatant was transferred to a fresh tube, and centrifuged at 16000 x *g* for 10 min at 4 °C to pellet the 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 16000 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. Aliquots of 300 µL of mitochondrial suspension were added, one drop at a time to avoid mixing. This tube was centrifuged at 22000 x *g* for 1 h at 4 °C. The mitochondria accumulated 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 16000 x *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 cooled on an ice bath. Aliquots of 20 mL of nucleus 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 remaining on cheesecloth were resuspended in another 20 mL of NIB with 1 % PVP and filtered 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 provoke the lysis of the contaminating organelles. The tubes were centrifuged at 1800 x *g* for 10 min and the pellet was resuspend in 10 mL of NIB. Density gradient was employed to selectively separate nuclei. Then, 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 x *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 x *g* for 10 min. The pellet was resuspended on 3 mL of NIB and centrifuged at 1200 x *g* for 10 min twice.

The pellet obtained from the chloroplasts, mitochondria or nuclei enrichment steps 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 14000 x *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 14000 x *g* for 10 min at 4 °C. The supernatant was discarded, and the remaining ethanol was evaporated at room temperature for 1 h. 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.

References

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