Single-stranded oligodeoxynucleotides induce plant defence in *Arabidopsis thaliana*

Laila Toum[1], Gabriela Conti[2], Francesca Coppola Guerriero[3,4,5], Valeria P. Conforte[1], Franco A. Garolla[4], Sebastián Asurmendi[2], Adrián A. Vojnov[1] and Gustavo E. Gudesblat[3,4,5]*

[1]Instituto de Ciencia y Tecnología Dr. César Milstein, Fundación Pablo Cassará, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468, C1440FFX C.A.B.A., Argentina; [2]Instituto de Agrobiotecnología y Biología Molecular (IABIMO), CICVyA- Instituto Nacional de Tecnología Agropecuaria (INTA), Consejo Nacional de investigaciones Científicas y Tecnológicas (CONICET), Los Reseros y Nicolas Repeto, Hurlingham, Argentina; [3]Departamento de Fisiología, Biología Molecular y Celular "Profesor Héctor Maldonado"- Instituto de Biociencias, Biotecnología y Biología Translacional (IB3), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Pres. Dr. Raúl Alfonsín S/N, Ciudad Universitaria, 1428EGA, C.A.B.A., Argentina; [4]Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Pres. Dr. Raúl Alfonsín S/N, Ciudad Universitaria, 1428EGA, C.A.B.A., Argentina; [5]Instituto de Biodiversidad y Biología Experimental y Aplicada (IBBEA), CONICET, Argentina

**For correspondence. E-mail: ggudesblat@fbmc.fcen.uba.ar*

- **Background and aims** Single-stranded DNA oligodeoxynucleotides (ssODNs) have been shown to elicit immune responses in mammals. In plants, RNA and genomic DNA can activate immunity, although the exact mechanism through which they are sensed is not clear. The aim of this work was to study the possible effect of ssODNs on plant immunity.
- **Key results** ssODNs IMT504 and 2006 increased protection against the pathogens *Pseudomonas syringae* pv. *tomato* DC3000 and *Botrytis cinerea* but not against Tobacco Mosaic Virus - Cg when infiltrated in *Arabidopsis thaliana*. In addition, ssODNs inhibited root growth and promoted stomatal closure in a dose dependent manner, with half maximal effective concentrations between 0.79 and 2.06 µM. Promotion of stomatal closure by ssODNs was reduced by DNase I treatment. It was also diminished by the NADPH oxidase inhibitor diphenyleneiodonium and by coronatine, a bacterial toxin that inhibits NADPH oxidase-dependent reactive oxygen species (ROS) synthesis in guard cells. In addition it was found that ssODN-mediated stomatal closure was impaired in *bak1-5*, *bak1-5/bkk1*, *mpk3* and *npr1-3* mutants. ssODNs also induced early expression of *MPK3*, *WRKY33*, *PROPEP1* and *FRK1* genes involved in plant defence, an effect that was reduced in *bak1-5* and *bak1-5/bkk1* mutants.
- **Conclusions** ssODNs are capable of inducing protection against pathogens through the activation of defence genes and promotion of stomatal closure through a mechanism similar to that of other elicitors of plant immunity, which involves the BAK1 co-receptor, and ROS synthesis.

Key words: IMT504, 2006, DNA, plant immune system, Arabidopsis thaliana, elicitor, BAK1, stomatal immunity.

INTRODUCTION

Nucleic acids trigger defence against pathogens in all kingdoms of life Given that they are present in both the pathogen and the host during an infective process, these molecules have been proposed to act either as Microbe-Associated Molecular Patterns (MAMPs), or as Damage-Associated Molecular Patterns (DAMPs) (Gallucci and Maffei, 2017, Heil, 2009). In bacteria recognition of foreign DNA by both restriction enzymes and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated endonuclease 9 (CRISPR-Cas) are involved in defence against bacteriophages (Garneau *et al.*, 2010). The mechanisms of DNA recognition have been best characterized in mammals, where their perception promotes immunity against bacteria and viruses. The Toll-like receptor 9 (TLR9) is located in the endoplasmic reticulum and was shown to recognize DNA present in endosomes, which activates the production of pro-inflammatory cytokines (Latz *et al.*, 2004b, Latz *et al.*, 2004a). This receptor preferentially recognizes single stranded unmethylated CpG-rich DNA, although methylated single-stranded CpG DNA and double-stranded DNA can also activate it less effectively (Hartmann and Krieg, 2000, Hartmann, 2017, Krieg, 2002, Ohto *et al.*, 2015, Ohto *et al.*, 2018). The CpG rich single-stranded DNA oligodeoxynucleotide (ssODN) 2006 is a potent activator of TLR9 (Hartmann *et al.*, 1999), while mammalian self-DNA bound to antibodies can be endocytosed and subsequently activates TLR9 (Ohto *et al.*, 2015). It was also demonstrated that RNase 7, a 14.5-kDa antimicrobial ribonuclease constitutively expressed in the human epidermis, promotes self-DNA recognition in keratinocytes and activates antiviral immune responses in the skin (Kopfnagel *et al.*, 2018). In addition, association mapping studies have linked mutations in the TLR9 promoter to inflammatory diseases (Demirci *et al.*, 2007). DNA can also activate inflammatory and local anti-viral responses in the cytoplasm of mammalian cells, where it can be present as a result of viral infection or upon extensive extracellular endocytosis, through the activation of different DNA

receptors such as cyclic GMP-AMP synthase-Stimulator of Interferon Genes (sGAS-STING), Z-DNA-binding protein, the Receptor for Advanced Glycation End-products (RAGE), and several receptors belonging to the PYHIN family (Sun *et al*., 2013, Collins *et al*., 2015, Li and Chen, 2018, Sirois *et al*., 2013). Non-CpG oligonucleotides containing a PyNTTTTGT motif, such as IMT504, have also been shown to stimulate B lymphocytes and plasmacytoid dendritic cells of the human immune system (Elias *et al*., 2003, Rodriguez *et al*., 2006). Both CpG and non-CpG containing oligonucleotides have been shown to improve the activity of vaccines targeting infectious diseases and cancer (Elias *et al*., 2005, Montaner *et al*., 2012, Bode *et al*., 2011, Adamus and Kortylewski, 2018, Jahrsdörfer and Weiner, 2008).

Double-stranded RNA (dsRNA) can also activate antiviral responses in the cytosol upon recognition by RIG1 and MDA5 proteins (Takeuchi and Akira, 2008b, Takeuchi and Akira, 2008a, Ablasser *et al*., 2009). RNA can also be recognized in the endolysosomal compartment by other members of the Toll-like family, TLR3, TLR7 and TLR8 (Gasser *et al*., 2017), and based on genomic structure and sequence similarity, it has been proposed that TLR9 forms a subfamily with TLR7 and TLR8 (Chuang and Ulevitch, 2000, Du *et al*., 2000). It was demonstrated that single stranded RNAs (ssRNA) derived from Human Immunodeficiency Virus-1 (HIV-1) are able to activate TLR7 and TLR8, stimulating dendritic cells and macrophages to secrete interferon α and cytokines as part of the defence response (Heil *et al*., 2004).

Like in animals, plant immunity is also activated by both DNA and RNA. Bacterial DNA was demonstrated to act as an effective elicitor in *Arabidopsis thaliana* plants, triggering reactive oxygen species (ROS) generation and callose deposition, an effect that was reduced by DNA CpG methylation (Yakushiji *et al*., 2009). It was also shown that the extracellular DNA present on pea root cap slime is required for defence against a necrotrophic fungus (Wen *et al*., 2009). In agreement with this observation, the absence of a

single gene encoding a candidate extracellular DNase from the fungal phytopathogen *Cochliobolus heterostrophus* results in reduced virulence (Park *et al*., 2019). Huang *et al*., 2019 have also reported a salivary DNase II from the insect *Laodelphax striatellus* that acts as an effector that suppresses plant defence.

Several works have found evidence that self-DNA, or that of related species, is more active in triggering immunity than foreign DNA. Autotoxicity of aged litter has been linked to its content of fragmented DNA, since inhibition of root growth by self-DNA was stronger than by DNA from unrelated species (Mazzoleni *et al*., 2015a). Inhibition of growth by self but not by foreign DNA was also observed in bacteria, fungi, algae, protozoa and insects (Mazzoleni *et al*., 2015a, Mazzoleni *et al*., 2015b). Similarly, it was found that leaf homogenates from common bean protected this species from the bacterial pathogen *Pseudomonas syringae* and induced H_2O_2 and extrafloral nectar production. Non-self homogenates also elicited common bean defences, but to a lesser extent (Duran-Flores and Heil, 2014). Similar results were obtained when fragmented self-DNA was used instead of leaf homogenates (Duran-Flores and Heil, 2018) or DNA. In lettuce it was also observed that self-DNA induced plant defences, induced changes in CpG methylation and inhibited seed germination and root growth to a higher extent that non-self DNA (Vega-Muñoz *et al*., 2018). By contrast, non-self DNA from plant species phylogenetically distant did not exhibit a protective role on lettuce. It was also shown that self-DNA was more effective than heterologous plant or insect DNA at inducing plasma membrane depolarization and calcium signalling in lima bean and maize (Barbero *et al*., 2016). RNA has also been shown to activate plant defences, since pre-infiltration of total RNAs from *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), but not from *A. thaliana*, activated innate immunity in this plant, reducing susceptibility to both bacterial and fungal pathogens (Lee *et al.*, 2016).

Unlike in mammals, the molecular basis of DNA perception in plants is not clear. It was found that nucleotide-binding leucine-rich repeat (NB-LRR) receptors I2 from tomato (Fenyk *et al*., 2016) and Rx1 from potato (Fenyk *et al*., 2015) can bind both double- and single-stranded DNA as well as single-stranded RNA *in vitro*. Rx1 was shown to regulate the DNA-binding activity of a Golden2-like transcription factor (Townsend *et al*., 2018) and triggered immune responses leading to cell death after activation by a PVX virus capsid (Knip *et al*., 2019). However, neither I2, nor Rx1 have been linked to nucleic acid sensing *in vivo*.

Pattern-triggered immunity (PTI) allow plants to recognize MAMPs or DAMPs through pattern recognition receptors (PRRs), which leads to activation of basal resistance. PRRs are all cell surface-localized receptors, receptor-like kinases (RLKs) or receptor-like proteins (RLPs). RLKs and RLPs differ in that the former possess a cytoplasmic kinase domain, while the latter don´t (Saijo *et al*., 2018). BAK1 (for BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE1) is a RLK belonging to the family of leucine-rich repeat like kinase (LRR-RLKs) receptors and act as co-receptor, or adaptor, of several LRR-RLK receptors involved in MAMP and DAMP perception, such as FLAGELLIN SENSING2 (FLS2) flagellin receptor (Chinchilla *et al*., 2007a, Chinchilla *et al*., 2007b) or PEP RECEPTOR1 (PEPR1), which recognizes endogenous PROPEP peptide-derived DAMPs including PEP1 (Huffaker *et al*., 2006). BAK1 belongs to a subfamily of LRR-RLKs that includes BAK1-LIKE1 (BKK1), which can also heterodimerize with FLS2 or PROPEP1 (Roux *et al*., 2011). BAK1 is shared by several signalling pathways controlling developmental as well as defence responses (reviewed in Chinchilla *et al*., 2009). Consistent with a role in plant immunity, plants depleted for BAK1 showed reduced responses to flg22 and exhibited more symptoms to virulent bacteria (Heese *et al*., 2007, Chinchilla *et al*., 2007b). BAK1 might be involved in sensing of double-stranded RNA, since this nucleic acid

has been shown to activate plant defence against Oilseed Rape Mosaic Virus (ORMV) in a BAK1-dependent manner (Niehl *et al*., 2016).

While the immunostimulatory effect of single-stranded DNA (ssDNA) has been well characterized in mammals, it has not been investigated in plants. In this work we found that ssODNs IMT504 and 2006, previously shown to activate immunity in mammalian cells, can protect *A. thaliana* from *Botrytis cinerea* and from *Pst* DC3000, but not from Crucifer infecting Tobacco Mosaic Virus (TMV-Cg). Both IMT504 and 2006 promoted stomatal closure, activated expression of defence genes and inhibited root growth, in a BAK1-dependent manner.

MATERIALS AND METHODS

Plant material and growth conditions

Plants were grown in petri dishes containing half-strength Murashige and Skoog (MS) medium with 1% sucrose under a 12h:12h light/dark cycle (photon flux density of 90 μE) at 22°C to 23°C. The relative humidity was maintained at 60% to 70%. After a week plants were transferred to a mixture of vermiculite, peat moss and perlite (1:1:1). Experiments were performed using *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) or the following mutants: *npr1*-3 (Cao *et al.*, 1997), *mpk3* (SALK_151594), *bak1-5* (Schwessinger et al., 2011, Nekrasov et al., 2009) and *bak1-5/bkk1* (Schwessinger et al., 2011).

Bacterial strain

Pst DC3000 strain (Ma et al., 1991) was grown overnight at 28°C in Luria-Bertani medium supplemented with kanamycin 50 μg/ml and rifampicin 50 μg/ml.

Bacterial infection assays

Oligonucleotides (4 µM), flg22 (1 µM) or sterile water (mock) were infiltrated into leaves from 4-week-old *A. thaliana* plants using a needleless syringe. 24 h after pre-treatment, *Pst* DC3000 suspensions were sprayed on *A. thaliana* leaves as previously described (Macho *et al*., 2012). Briefly, cells were harvested by centrifugation, and pellets were resuspended in 10 mM $MgCl_2$ at an OD_{600} of 0.1. Immediately prior to spraying, Silwet L-77 0.02% (v/v) was added to the bacterial suspension. Bacteria were sprayed onto leaf surfaces, and plants were not covered. 4 h and 4 days post inoculation, six 0.6-cm^2 leaf discs (3 discs per plant) per treatment were harvested and surface sterilized (30 s in 70% ethanol, followed by 30 s in sterile distilled water). Leaf discs from two different plants were ground in 10 mM $MgCl_2$ in a tube using a plastic pestle. After grinding, samples were serially diluted 1:10 and plated on PYM solid medium (Cadmus *et al*., 1976) supplemented with the appropriate antibiotics. Plates were placed at 28°C for 2 days, after which the colony-forming units were counted.

Botrytis cinerea *infection assay*

Oligonucleotides (4 µM) or sterile water were infiltrated into 4-week-old *A. thaliana* leaves with a needleless syringe. 24 h after pre-treatment, leaves were detached, placed on MS agar plates (without sucrose), and inoculated with conidial suspensions of *B. cinerea* isolate B05.10 (Rossi *et al*., 2011). The inoculum consisted of 5-µl droplets of a conidial suspension (5×10^5 conidia/ml) or medium alone (for Mock treatment) prepared as previously described (Flors *et al*., 2007, Real *et al*., 2004). The adaxial surface of three fully expanded leaves per plant from four plants was inoculated with one droplet on right side of the central vein and incubated in MS agar (without sucrose) petri dishes. Plates were sealed to maintain humidity. Symptoms were recorded and measured 48 h post inoculation and the lesion area was measured using Image J software (Analyze tool).

Virus infection assays

The third oldest expanded leaf of each plant (1.08 stage) was dusted with carborundum. Subsequently, 5 µl of semipurified TMV-Cg virus (Asurmendi *et al*., 2004) diluted in 20 mM phosphate buffer (pH 7) was added and the surface of the leaf and was gently abraded. Mock-inoculated plants were buffer-rubbed. Samples of systemic leaves (half of rosette upper third oldest expanded leaf) were taken at 5 and 7 dpi. Leaves were frozen in individual tubes in liquid nitrogen and stored at −80°C until RNA extraction. The TMV-Cg infection level was determined as TMVCg-CP transcript relative accumulation by RT-qPCR. The following primers were used for qRT-PCR: TMVCg-CP FW´ 5´ TGTCGCAATCGTATCAAAC 3´; TMVCg-CP RV´ 5´ CTGTATCTGGAAACCGCTG 3´. Plants were infiltrated with distilled water or ssODN IMT504 (4 µM) 24 h prior virus infection with TMVCg. Total RNA was isolated from frozen *A. thaliana* leaf tissues using Trizol Reagent (Thermo Fisher, USA) and subsequently treated with DNAse I (Thermo Fisher, USA). For messenger-RNA detection, the first-strand cDNA was synthesized using MMLV reverse transcriptase (Thermo Fisher, USA) according to manufacturer's instructions. All qRT-PCR experiments were carried out using an ABI Prism 7500 Real Time PCR System (Applied Biosystems, USA), following MIQE requirements (Minimun information for publication of quantitative real time PCR experiments requirements). Ubiquitin5 (UBQ5, NM-116090) was used as an internal reference gene (FW 5´ CGGACCAGCAGCGATTGATT 3´, RV 5´ ACGGAGGACGAGATGAAGCG 3').

Chemicals

Oligonucleotides (IMT 504: CATCATTTTGTCATTTTGTCATT and 2006: TCGTCGTTTTGTCGTTTTGT) were synthesized by IDT (Integrated DNA Technologies, USA) in both phosphodiester and phosphorothioate versions. In the latter case, all bonds between nucleotides were phosphorothioate. All ssODNs were solubilized in water. Abscisic

acid (ABA, mixed isomers), coronatine and Diphenyleneiodonium (DPI) were purchased from Sigma (USA), while flg22 was synthesized by GL Biochem (China) and PEP1 by Abbiotec (USA). PEP1 and ABA were dissolved in ethanol, coronatine in methanol and DPI in dimethylsulfoxide (DMSO), taking care to maintain the final concentration of all solvents below 1 % (v/v). For root length and qRT-PCR experiments, phosphorothioate ssODNs were used, while phosphodiester ssODNs were used for the rest of the experiments.

RNA Isolation and qRT-PCR

Gene expression assays were performed on RNA extracted from five seven-day-old seedlings grown in 1X MS liquid medium supplemented with 0.5% sucrose (w/v) (Millet et al., 2010) (four biological replicates per treatment). Seedlings were incubated with H_2O (control), 100 nM flg22 or phosphorothioate versions of the oligonucleotides (4µM) for 4h. Plant material was frozen in liquid nitrogen, and total RNA was extracted using Trizol reagent (Thermo Fisher, USA) according to the manufacturer's instructions. RNA samples were treated with RQ-1 DNA-free DNase I (Promega, USA) and quantified with a Nanodrop spectrophotometer (Biophotometer Plus, Eppendorf). Complementary DNA synthesis was performed using M-MLV reverse transcriptase (Promega, USA) according to the manufacter´s instructions. qRT-PCR experiments were carried out in an StepOne Plus Real Time PCR System (Applied Biosystems, USA) equipment. Elongation factor 1 (EF1) was used as an internal reference gene. Primers used for qRT-PCR are as follows: EF1c (At1g18070) FW 5´ AGCACGCTCTTCTTGCTTTC 3´, EF1c RV 5´ GGGTTGTATCCGACCTTCTTC 3´, MPK3 (At3g45640) FW 5´ TCCCTGGTAAAGACCATGTTCA 3', RV 5´ TCGGTGTGCCAAGCAACTC 3´, WRKY33 (At2g38470) FW 5´ TTCGTATGGCTGCTTCTTTTC 3´, RV 5´ TGAGGTTTAGGATGGTTGTGG 3', PROPEP 1 (At5g64900) FW 5´ TGGCTCTACTCGTCACAACG 3´, PROPEP 1 RV

5´CAATGTAACTTAAAGTGCCTAATTATG 3´ and FRK1 (At2g19190) FW 5´ CAGAAACAGCGCGAAACGA 3´, FRK1 RV 5´ GGTCGGGCGGTCTGAAA 3´. qRT-PCR data analysis and primer efficiencies were obtained using LinReg PCR software (Ramakers *et al*., 2003). EF1c gene was used to standardize the expression of a given target gene; then a ratio between treatments was calculated using the algorithm developed by (Pfaffl, 2001). Relative expression ratios and statistical analysis were performed using fgStatistics software interface (J. A. Di Rienzo, personal communication). The cut-off for statistically significant differences was set at $P < 0.05$.

Stomatal aperture bioassays

Stomatal bioassays were performed as previously described (Gudesblat *et al*., 2009). Epidermal peels from Col-0 or mutant leaves of 4-week-old plants were floated on 10:10 buffer under light (10 mM KCl and 10 mM MES-KOH, pH 6.15) for 2.5 h, then ABA (20 µM), flg22 (5 µM), PEP1 (100nM) or ssODNs IMT504 and 2006 at indicated concentrations were added to the incubation medium and peels were incubated for a further 1.5 h. DPI (20 µM) or coronatine (1.56 µM) were applied 30 minutes prior to addition of flg22, IMT504 or 2006. For the DNase I experiment, IMT504 (1µg) was incubated with or without 1µl (1U) of RQ-DNase I (Promega, USA) in the reaction buffer supplied by the manufacturer for 30 min at 37°C. Aliquots of the reactions were diluted in 10:10 buffer with the epidermal peels so that the final concentration of the initial input of IMT504 was 4 µM. Peels were also incubated with an identical volume of reaction buffer with DNase I as a control. EC_{50} values from dose-response curves were calculated using GraphPad Prism software (v5.01).

Root length measurements

Seedlings were grown on MS agar plates for 2 days and subsequently transferred to 1X MS liquid medium supplemented with 0.5% sucrose (w/v) for a further 5 days in the presence of

phosphorothioate versions of the ssODNs. Root length was measured with Image J software. EC_{50} values from dose-response curves were calculated using GraphPad Prism software (v5.01).

RESULTS

ssODNs elicit plant innate immunity in A. thaliana

To investigate whether ssODNs are able to trigger plant defence, we performed a bacterial infection assay with *Pst* DC3000. Leaves from four-week-old *A. thaliana* were infiltrated with the ssODNs, IMT504 and 2006, the flagellin-derived peptide flg22 (Felix *et al*., 1999), or water as control. Plants were sprayed with *Pst* DC3000 24 h later. We observed that pre-treatment with IMT504 or 2006 reduced pathogen growth to a similar extent as flg22 (Fig. 1A). A similar 24 h pretreatment with IMT504 or 2006 also conferred some protection against the necrotrophic fungus *B. cinerea* (Fig. 1B), thus showing that the assayed ssODNs elicit a defence response against these pathogens. Next, we studied if IMT504 protects against TMV-Cg infection, by monitoring TMV-Cg coat protein (CgCP) mRNA accumulation through qPCR. Pretreatment with the ssODN failed to protect the plant against TMV-CG (Fig. 1C). Thus, the tested ssODNs confer protection against bacterial and fungal infections, but not against TMV-Cg.

SSODNs inhibit root growth and promote stomatal closure in a BAK1-dependent manner

Elicitors capable of triggering pattern-triggered immunity (PTI) such as flg22 (Zipfel *et al*., 2006, Bartels and Boller, 2015) as well as dsRNA (Niehl *et al*., 2016) and exogenous DNA (Vega-Muñoz *et al*., 2018, Mazzoleni *et al*., 2015b, Duran-Flores and Heil, 2018) have been shown to inhibit root growth. Therefore, we tested the effect of IMT504 and 2006 on root growth in *A. thaliana* seedlings. We found no effect on root growth with regular

ssODNs, however when IMT504 and 2006 with phosphorothioate instead of phosphodiester bonds were used we found that both inhibit root growth in a dose-dependent manner with half maximal effective concentrations (EC_{50}s) of 1.037 µM and 2.06 µM respectively (Fig.2A). Phosphorothioate bonds render ssODNs resistant to nucleases (Wickstrom, 1986) Lack of effect of regular ssODNs with phosphodiester bonds might be due nuclease degradation after prolonged incubation in culture medium. Promotion of stomatal closure is another common response triggered by different elicitors of plant defence such as flg22 (Melotto *et al*., 2006, Zhang *et al*., 2008), chitin, chitosan (Gust *et al*., 2007, Lee *et al*., 1999, Amborabe *et al*., 2008) and PEP1(Zheng *et al*., 2018). For this reason, we tested the effect of ssODNs on stomata, and found that IMT504 and 2006 promoted stomatal closure in a dose-dependent manner with EC_{50}s of 1.05 µM and 0.79 µM respectively (Fig. 2B). Promotion of closure by IMT504 was strongly reduced by treatment with DNase I but not by the enzyme buffer alone (Fig. 2C), showing that ssODN integrity is required to elicit stomatal immunity. Next, we tested whether BAK1, a LRR-RLK protein which has been shown to act as co-receptor of multiple proteins involved in MAMP perception (Yasuda *et al*., 2017), is required for the effect of ssODNs. For this purpose we measured inhibition of root growth and promotion of stomatal closure in *bak1-5* and *bak1-5/bkk1*, since BKK1 is a receptor similar to BAK1 and with partially redundant function (Roux *et al*., 2011). We found a partial reduction in inhibition of root growth by IMT504 in both mutants (Fig. 3A), and a strong reduction in promotion of stomatal closure (Fig. 3B). These results indicate that ssODNs activate responses similar to those of other MAMPs and require BAK1 to activate immune responses.

Signalling elements involved in induction of defence by ssODNs

ROS produced by NADPH oxidase RBOHD are required for stomatal closure triggered by MAMPs (Torres and Dangl, 2005, Toum *et al*., 2016, Kadota *et al*., 2014). To find out if NADPH oxidases are also involved in signalling downstream of ssODNs, we

conducted stomatal assays in the presence of the NADPH oxidase inhibitor DPI. As shown in Fig. 4A, we found that DPI significantly reduced closure induced by IMT504 and 2006, consistent with an involvement of ROS in ssODN signalling. This possibility was further strengthened by the observation that coronatine, a bacterial toxin previously shown to inhibit ROS synthesis in guard cells (Toum *et al*., 2016), significantly inhibited ssODNs-induced stomatal closure (Fig.4B). We next investigated sensitivity to ssODNs of *npr1-3* and *mpk3* mutants, previously shown to be affected in promotion of stomatal closure by MAMPs such as flg22 (Macho *et al*., 2012), and found that both mutants were only partially insensitive to ssODNs. By contrast, they were completely insensitive to flg22, as previously reported. Altogether these results indicate that ssODNs share signalling components with other MAMPs such as flg22; however, the fact that *npr1-3* and *mpk3* mutants retain some sensitivity suggest that signalling cascades downstream of ssODNs and flg22 in guard cells are not identical.

Early defence-related genes were upregulated by ssODNs

Since ssODNs protected *A. thaliana* plants from pathogen attack, we investigated the induction of some genes involved in early plant defence. For this purpose, we treated 7-day-old WT, *bak1* and *bak1/bkk1* seedlings with ssODNs and measured expression of the pathogen-inducible genes *MPK3* (Beckers et al., 2009), *PROPEP1* (Huffaker *et al*., 2006), *WRKY33* (Birkenbihl *et al*., 2012) and *FRK1* (Boudsocq *et al*., 2010) by qRT-PCR 4h post incubation. All four genes were induced in response to IMT504 and 2006 in WT, and to a lesser extent in *bak1* and *bak1/bkk1* mutants, albeit not in all cases with statistically significant difference, consistent with a possible role of BAK1 in ssODN signalling (Fig. 5).

DISCUSSION

Previously it was demonstrated that DNA extracted from plants (Wen *et al*., 2009, Mazzoleni *et al*., 2015a, Mazzoleni *et al*., 2015b, Duran-Flores and Heil, 2014, Duran-Flores and Heil, 2018, Vega-Muñoz *et al*., 2018, Barbero *et al*., 2016) or bacteria (Yakushiji *et al*., 2009) can activate plant defence against bacterial or fungal pathogens. In this work we showed that two ssODNs, CpG rich 2006 and CpG poor IMT504, can also activate plant immunity at concentrations in the low micromolar range, similar to those reported for other activators of plant immunity such as several MAMPs and DAMPs. To our knowledge, this is the first report showing that synthetic ssDNA can activate plant immunity against bacterial and fungal pathogens. However, we failed to observe protection by the tested ssODNs against TMV-Cg, a single-stranded RNA virus.

Induction of immunity in *A. thaliana* by IMT504 and 2006 has similarities with that elicited by other MAMPs such as flagellin (Chinchilla *et al*., 2007a, Chinchilla *et al*., 2007b, Melotto *et al*., 2006), since both ssODNs promoted stomatal closure, inhibited root elongation, and induced the transcription of defence genes. The three responses were reduced in *bak1-5* and *bak1-5/bkk1* mutants, affected in co-receptors involved in the perception of

various elicitors of plant defence (Yasuda *et al*., 2017). The effect of these mutants was more marked in promotion of stomatal closure and inhibition of root elongation, and was not apparent in all cases in induction of gene expression. While the mechanism of DNA perception in plants is not clear, our results suggest that ssODNs are perceived by a LRR-RLK receptor that requires association with BAK1 co-receptor in order to activate a downstream signalling cascade. Consistent with this possibility, similar EC_{50}s were observed for inhibition of root elongation and promotion of stomatal closure for both ssODNs, ranging from 0.791 to 2.06 µM. While flg22, a BAK1 dependent elicitor of plant immunity, has been shown to induce extracellular alkalinization with an EC_{50} close to 1 nM (Chinchilla *et al*., 2006), the same ligand only promoted stomatal closure at concentrations of 1 µM and higher (Melotto *et al*., 2006) and inhibited seedling growth with an EC_{50} close to 0.1 µM (Gomez-Gomez *et al*., 1999). As a reference, an EC_{50} close to 1 µM has been observed for NF-κB activation by 2006 in HEK293 cells expressing TLR9 (Latz *et al*., 2004a), although different reports describing activation of different immune responses in mammalian cells by single or double stranded DNA report concentrations from low nM to over 100 µM (reviewed in Heil and Vega-Muñoz, 2019). In plants, the potato NB domain of the NLR R1 receptor was shown to bind to ssDNA, dsDNA and ssRNA *in vitro*, with dissociation constants (Kd) of 3.34, 2.75 and 12.45 µM respectively (Fenyk *et al*., 2015), however this receptor has not been linked to DNA perception *in vivo*. Like ssODNs, dsRNA also stimulates immunity against ORMV, a dsRNA virus, in a BAK1 dependent manner (Niehl *et al*., 2016), indicating that like ssODNs, dsRNAs could also be perceived by a LRR that requires BAK1 as co-receptor. The integrity of ssODNs is required for their effect, since DNase treatment abolished most of its activity.

Previously it has been shown that *A. thaliana* sonicated genomic self-DNA almost completely stopped root growth, while *Lepidium sativum* heterologous DNA only reduced it by around 10% in the same plant (Mazzoleni *et al*., 2015b). In our experiments both ssODNs

reduced growth by around 50% at the highest concentration used, but only when when phosphorothioate versions were used. No inhibition of growth was observed with phosphodiester ssODNs, suggesting that they could be degraded during incubation. It is difficult to make comparisons between both experiments, since the mechanism of perception of genomic DNA and ssODNs could not be identical. Nevertheless, it would be interesting in the future to study the effect of synthetic double and single DNAs of diverse sequence to better understand the molecular mechanism underlying the differential perception of self and heterologous DNA.

It has been proposed that dsDNA acts as a DAMP in plants (Yakushiji *et al*., 2009, Gallucci and Maffei, 2017, Vega-Muñoz *et al*., 2018). ssDNA is not normally found inside plants, however given that TLR9 mammalian receptor can be activated by both single and double-stranded DNA (Gasser *et al*., 2017, Rigby *et al*., 2014), it could be possible that a similar receptor is responsible for the perception of both kind of molecules in plants. Alternatively, ssDNA perception could have evolved as a mechanism of defence against ssDNA viruses such as Geminiviruses, members of *Geminiviridae* family.

Signalling downstream of MAMPs whose perception depends on LRR receptors that associate with BAK1, such as flg22 and elf18 (Chinchilla *et al*., 2007b, Nekrasov *et al*., 2009), is completely dependent on ROS production by RBOHD (Kadota *et al*., 2014). Promotion of stomatal closure by ssODNs was only partially reduced by DPI, a NADPH oxidase inhibitor, or in *mpk3* and *npr1-3* , two mutants completely insensitive to flg22 that presumably act downstream of ROS in the signalling cascade (Toum *et al*., 2016). Further evidence that ROS are involved in ssODN signalling arises from the fact that promotion of closure by these molecules is reduced by coronatine, a bacterial toxin that affects ROS production in guard cells (Toum *et al*., 2016). Therefore, it seems likely that signalling cascades for flg22 and ssODNs share some elements, but do not overlap completely. More

research is needed to find out which are the ROS-independent signalling events triggered by ssODNs.

In this work, we reported that two ssODNs previously shown to stimulate immunity in mammals, CpG rich 2006 and CpG poor (IMT504), could also elicit plant defence. Further research using ssODNs with different sequences should reveal if there is any specificity in sequence recognition, as suggested by previous findings showing that plants and other organisms preferentially activates their defences in response to foreign DNA (Vega-Muñoz *et al*., 2018). The use of synthetic oligonucleotides could shed light on the mechanism of stimulation of immunity by DNA, which might be a promising tool for crop protection and plant disease management.

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LITERATURE CITED

Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V. 2009. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol,* **10**: 1065-72.

Adamus T, Kortylewski M. 2018. The revival of CpG oligonucleotide-based cancer immunotherapies. *Contemp Oncol (Pozn),* **22**: 56-60.

Amborabe BE, Bonmort J, Fleurat-Lessard P, Roblin G. 2008. Early events induced by chitosan on plant cells. *J Exp Bot,* **59**: 2317-24.

Asurmendi S, Berg RH, Koo JC, Beachy RN. 2004. Coat protein regulates formation of replication complexes during tobacco mosaic virus infection. *Proc Natl Acad Sci U S A,* **101**: 1415-20.

Barbero F, Guglielmotto M, Capuzzo A, Maffei ME. 2016. Extracellular Self-DNA (esDNA), but Not Heterologous Plant or Insect DNA (etDNA), Induces Plasma Membrane Depolarization and Calcium Signaling in Lima Bean (*Phaseolus lunatus*) and Maize (*Zea mays*). *Int J Mol Sci,* **17**.

Bartels S, Boller T. 2015. Quo vadis, Pep? Plant elicitor peptides at the crossroads of immunity, stress, and development. *J Exp Bot,* **66**: 5183-93.

Beckers GJ, Jaskiewicz M, Liu Y et al. 2009. Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in Arabidopsis thaliana. *Plant Cell,* **21**: 944-53.

Birkenbihl RP, Diezel C, Somssich IE. 2012. *Arabidopsis* WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiol,* **159**: 266-85.

Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. 2011. CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines,* **10**: 499-511.

Boudsocq M, Willmann MR, McCormack M et al. 2010. Differential innate immune signalling via Ca(2+) sensor protein kinases. *Nature,* **464**: 418-22.

Cadmus MC, Rogovin SP, Burton KA, Pittsley JE, Knutson CA, Jeanes A. 1976. Colonial variation in Xanthomonas campestris NRRL B-1459 and characterization of the polysaccharide from a variant strain. *Can J Microbiol,* **22**: 942-8.

Collins AC, Cai H, Li T et al. 2015. Cyclic GMP-AMP Synthase Is an Innate Immune DNA Sensor for *Mycobacterium* tuberculosis. *Cell Host Microbe,* **17**: 820-8.

Chinchilla D, Bauer Z, Regenass M, Boller T, Felix G. 2006. The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell,* **18**: 465-76.

Chinchilla D, Boller T, Robatzek S. 2007a. Flagellin signalling in plant immunity. *Adv Exp Med Biol,* **598**: 358-71.

Chinchilla D, Shan L, He P, de Vries S, Kemmerling B. 2009. One for all: the receptor-associated kinase BAK1. *Trends Plant Sci,* **14**: 535-41.

Chinchilla D, Zipfel C, Robatzek S et al. 2007b. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature,* **448**: 497-500.

Chuang TH, Ulevitch RJ. 2000. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur Cytokine Netw,* **11**: 372-8.

Demirci FY, Manzi S, Ramsey-Goldman R et al. 2007. Association study of Toll-like receptor 5 (TLR5) and Toll-like receptor 9 (TLR9) polymorphisms in systemic lupus erythematosus. *J Rheumatol,* **34**: 1708-11.

Du X, Poltorak A, Wei Y, Beutler B. 2000. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur Cytokine Netw,* **11**: 362-71.

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). *Front Plant Sci,* **5**: 585.

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 Behav Immun,* **72**: 78-88.

Elias F, Flo J, Lopez RA, Zorzopulos J, Montaner A, Rodriguez JM. 2003. Strong cytosine-guanosine-independent immunostimulation in humans and other primates by synthetic oligodeoxynucleotides with PyNTTTTGT motifs. *J Immunol,* **171**: 3697-704.
Elias F, Flo J, Rodriguez JM, De Nichilo A et al. 2005. PyNTTTTGT prototype oligonucleotide IMT504 is a potent adjuvant for the recombinant hepatitis B vaccine that enhances the Th1 response. *Vaccine,* **23**: 3597-603.
Felix G, Duran JD, Volko S, Boller T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J,* **18**: 265-76.
Fenyk S, Dixon CH, Gittens WH at al. 2016. The Tomato Nucleotide-binding Leucine-rich Repeat Immune Receptor I-2 Couples DNA-binding to Nucleotide-binding Domain Nucleotide Exchange. *J Biol Chem,* **291**: 1137-47.
Fenyk S, Townsend PD, Dixon CH at al. 2015. The Potato Nucleotide-binding Leucine-rich Repeat (NLR) Immune Receptor Rx1 Is a Pathogen-dependent DNA-deforming Protein. *J Biol Chem,* **290**: 24945-60.
Flors V, Leyva Mde L, Vicedo B at al. 2007. Absence of the endo-beta-1,4-glucanases Cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. *Plant J,* **52**: 1027-40.
Gallucci S, Maffei ME. 2017. DNA Sensing across the Tree of Life. *Trends Immunol,* **38**: 719-732.
Garneau JE, Dupuis ME, Villion M et al. 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature,* **468**: 67-71.
Gasser S, Zhang WYL, Tan NYJ et al. 2017. Sensing of dangerous DNA. *Mech Ageing Dev,* **165**: 33-46.
Gomez-Gomez L, Felix G, Boller T. 1999. A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. *Plant J,* **18**: 277-84.
Gudesblat GE, Torres PS, Vojnov AA. 2009. Xanthomonas campestris overcomes Arabidopsis stomatal innate immunity through a DSF cell-to-cell signal-regulated virulence factor. *Plant Physiol,* **149**: 1017-27.
Gust AA, Biswas R, Lenz HD at al. 2007. Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. *J Biol Chem,* **282**: 32338-48.
Hartmann G. 2017. Chapter Four: Nucleid acid immunity. *Advances in Immunology,* **133**: 121-169.
Hartmann G, Krieg AM. 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol,* **164**: 944-53.
Hartmann G, Weiner GJ, Krieg AM. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc Natl Acad Sci U S A,* **96**: 9305-10.
Heese A, Hann DR, Gimenez-Ibanez S et al. 2007. The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc Natl Acad Sci U S A,* **104**: 12217-22.
Heil F, Hemmi H, Hochrein H et al. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science,* **303**: 1526-9.
Heil M. 2009. Damaged-self recognition in plant herbivore defence. *Trends Plant Sci,* **14**: 356-63.
Heil M, Vega-Muñoz I. 2019. Nucleic Acid Sensing in Mammals and Plants: Facts and Caveats. *International Review of Cell and Molecular Biology*: Elsevier.
Huang HJ, Cui JR, Xia X, Chen J, Ye YX, Zhang CX, Hong XY. 2019. Salivary DNase II from *Laodelphax striatellus* acts as an effector that suppresses plant defence. *New Phytol,* **224**: 860-874.
Huffaker A, Pearce G, Ryan CA. 2006. An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proc Natl Acad Sci U S A,* **103**: 10098-103.
Jahrsdörfer B, Weiner GJ. 2008. CpG oligodeoxynucleotides as immunotherapy in cancer. *Update Cancer Therapy,* **3**: 27-32.
Kadota Y, Sklenar J, Derbyshire P et al. 2014. Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. *Mol Cell,* **54**: 43-55.
Knip M, Richard MMS, Oskam L, van Engelen HTD, Aalders T, Takken FLW. 2019. Activation of immune receptor Rx1 triggers distinct immune responses culminating in cell death after 4 hours. *Mol Plant Pathol,* **20**: 575-588.

Kopfnagel V, Wagenknecht S, Harder J et al. 2018. RNase 7 Strongly Promotes TLR9-Mediated DNA Sensing by Human Plasmacytoid Dendritic Cells. *J Invest Dermatol,* **138**: 872-881.
Krieg AM. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol,* **20**: 709-60.
Latz E, Schoenemeyer A, Visintin A et al. 2004a. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol,* **5**: 190-8.
Latz E, Visintin A, Espevik T, Golenbock DT. 2004b. Mechanisms of TLR9 activation. *J Endotoxin Res,* **10**: 406-12.
Lee S, Choi H, Suh S et al. 1999. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol,* **121**: 147-52.
Li T, Chen ZJ. 2018. The cGAS-cGAMP-STING pathway connects DNA damage to inflammation, senescence, and cancer. *J Exp Med,* **215**: 1287-1299.
Ma H, Yanofsky MF, Huang H. 1991. Isolation and sequence analysis of TGA1 cDNAs encoding a tomato G protein alpha subunit. *Gene,* **107**: 189-95.
Macho AP, Boutrot F, Rathjen JP, Zipfel C. 2012. Aspartate oxidase plays an important role in *Arabidopsis stomatal* immunity. *Plant Physiol,* **159**: 1845-56.
Mazzoleni S, Bonanomi G, Incerti G et al. 2015a. Inhibitory and toxic effects of extracellular self-DNA in litter: a mechanism for negative plant-soil feedbacks? *New Phytol,* **205**: 1195-210.
Mazzoleni S, Carteni F, Bonanomi G et al. 2015b. Inhibitory effects of extracellular self-DNA: a general biological process? *New Phytol,* **206**: 127-32.
Melotto M, Underwood W, Koczan J, Nomura K, He SY. 2006. Plant stomata function in innate immunity against bacterial invasion. *Cell,* **126**: 969-80.
Millet YA, Danna CH, Clay NK et al. 2010. Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. *Plant Cell,* **22**: 973-90.
Montaner A, De Nichilo A, Rodrìguez JM et al. 2012. IMT504: A new and potent adjuvant for rabies vaccines permitting significant dose sparing. *World Journal of Vaccines,* **2**: 182-188.
Nekrasov V, Li J, Batoux M et al. 2009. Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO J,* **28**: 3428-38.
Niehl A, Wyrsch I, Boller T, Heinlein M. 2016. Double-stranded RNAs induce a pattern-triggered immune signaling pathway in plants. *New Phytol,* **211**: 1008-19.
Ohto U, Ishida H, Shibata T, Sato R, Miyake K, Shimizu T. 2018. Toll-like Receptor 9 Contains Two DNA Binding Sites that Function Cooperatively to Promote Receptor Dimerization and Activation. *Immunity,* **48**: 649-658 e4.
Ohto U, Shibata T, Tanji H et al. 2015. Structural basis of CpG and inhibitory DNA recognition by Toll-like receptor 9. *Nature,* **520**: 702-5.
Park HJ, Wang W, Curlango-Rivera G et al. 2019. A DNase from a Fungal Phytopathogen Is a Virulence Factor Likely Deployed as Counter Defense against Host-Secreted Extracellular DNA. *MBio,* **10**.
Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* **29**: e45.
Ramakers C, Ruijter JM, Deprez RH, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett,* **339**: 62-6.
Real MD, Company P, Garcia-Agustin P, Bennett AB, Gonzalez-Bosch C. 2004. Characterization of tomato endo-beta-1,4-glucanase Cel1 protein in fruit during ripening and after fungal infection. *Planta,* **220**: 80-6.
Rigby RE, Webb LM, Mackenzie KJ et al. 2014. RNA:DNA hybrids are a novel molecular pattern sensed by TLR9. *EMBO J,* **33**: 542-58.
Rodriguez JM, Elias F, Flo J, Lopez RA, Zorzopulos J, Montaner AD. 2006. Immunostimulatory PyNTTTTGT oligodeoxynucleotides: structural properties and refinement of the active motif. *Oligonucleotides,* **16**: 275-85.

Rossi FR, Garriz A, Marina M et al. 2011. The sesquiterpene botrydial produced by *Botrytis cinerea* induces the hypersensitive response on plant tissues and its action is modulated by salicylic acid and jasmonic acid signaling. *Mol Plant Microbe Interact,* **24**: 888-96.

Roux M, Schwessinger B, Albrecht C et al. 2011. The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell,* **23**: 2440-55.

Saijo Y, Loo EP, Yasuda S. 2018. Pattern recognition receptors and signaling in plant-microbe interactions. *Plant J,* **93**: 592-613.

Schwessinger B, Roux M, Kadota Y et al. 2011. Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet,* **7**: e1002046.

Sirois CM, Jin T, Miller AL et al. 2013. RAGE is a nucleic acid receptor that promotes inflammatory responses to DNA. *J Exp Med,* **210**: 2447-63.

Sun L, Wu J, Du F, Chen X, Chen ZJ. 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science,* **339**: 786-91.

Takeuchi O, Akira S. 2008a. MDA5/RIG-I and virus recognition. *Curr Opin Immunol,* **20**: 17-22.

Takeuchi O, Akira S. 2008b. RIG-I-like antiviral protein in flies. *Nat Immunol,* **9**: 1327-8.

Torres MA, Dangl JL. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr Opin Plant Biol,* **8**: 397-403.

Toum L, Torres PS, Gallego SM, Benavides MP, Vojnov AA, Gudesblat GE. 2016. Coronatine Inhibits Stomatal Closure through Guard Cell-Specific Inhibition of NADPH Oxidase-Dependent ROS Production. *Front Plant Sci,* **7**: 1851.

Townsend PD, Dixon CH, Slootweg EJ et al. 2018. The intracellular immune receptor Rx1 regulates the DNA-binding activity of a Golden2-like transcription factor. *J Biol Chem,* **293**: 3218-3233.

Vega-Muñoz I, Feregrino-Pérez A, Torres-Pacheco I, Guevara-González R. 2018. Exogenous fragmented DNA acts as a damage-associated molecular pattern (DAMP) inducing changes in CpG DNA methylation and defence-related responses in *Lactuca sativa*. *Functional Plant Biology,* **45**: 1065-1072.

Wen F, White GJ, VanEtten HD, Xiong Z, Hawes MC. 2009. Extracellular DNA is required for root tip resistance to fungal infection. *Plant Physiol,* **151**: 820-9.

Wickstrom E. 1986. Oligodeoxynucleotide stability in subcellular extracts and culture media. *J Biochem Biophys Methods,* **13**: 97-102.

Yakushiji S, Ishiga Y, Inagaki Y, Toyoda K, Shiraishi T, Ichinose Y. 2009. Bacterial DNA activates immunity in *Arabidopsis thaliana*. *J Gen Plant Pathol,* **75**: 227-234.

Yasuda S, Okada K, Saijo Y. 2017. A look at plant immunity through the window of the multitasking coreceptor BAK1. *Curr Opin Plant Biol,* **38**: 10-18.

Zhang W, He SY, Assmann SM. 2008. The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. *Plant J,* **56**: 984-96.

Zheng X, Kang S, Jing Y, Ren Z et al. 2018. Danger-Associated Peptides Close Stomata by OST1-Independent Activation of Anion Channels in Guard Cells. *Plant Cell,* **30**: 1132-1146.

Zipfel C, Kunze G, Chinchilla D et al. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell,* **125**: 749-60.

Figure captions

Fig.1 ssODNs induce immunity against *Pst* DC3000 and *B. cinerea* but not against TMV-Cg in *A. thaliana*

(A) ssODNs protect from *Pst* DC3000. Col-0 plants were infiltrated with H_2O (mock), flg22 (1μM), IMT504 (4 μM) or 2006 (4 μM) 24 h prior to infection with *Pst* DC3000. Bacterial growth was measured at 0 and 4 d post inoculation (dpi), cfu, colony-forming units. (B) ssODNs protect from *B. cinerea*. Col-0 plants were infiltrated with H_2O (mock), IMT504 (4 μM) or 2006 (4 μM) 24 h prior to infection with *B. cinerea*, and lesion areas were measured 48 h after inoculation. (C) TMV-Cg accumulation 5- and 7-days post infection (dpi) of Col-0 plants pre-infiltrated 24 h before infection with IMT504 (4 μM) relative to H_20 (mock) control. TMV-Cg infection was determined as TMVCg-CP transcript relative accumulation by RT-qPCR. Different letters indicate statistically significant differences ($p < 0.05$, (A) two-way ANOVA, (B,C) one-way ANOVA). Error bars represent SE from two biological replicates per treatment in three independent experiments (A), from 12 lesion replicates in three independent experiments and 30 replicates in one additional independent experiment (B), and from 8 replicates in two independent experiments (C)

Fig. 2 ssODNs inhibit root growth and promote stomatal closure in a dose-dependent manner

(A) IMT504 and 2006 inhibit Col-0 root growth. Two-day-old seedlings were transferred for five days to liquid MS medium supplemented with IMT504 or 2006 at the indicated concentrations. (B) IMT504 and 2006 promote stomatal closure in Col-0. Stomatal apertures

were measured 1.5 h after incubation with IMT504 or 2006 at the indicated concentrations (C) DNase I treatment reduces promotion of stomatal closure by IMT504. A 30-minute pre-treatment of IMT504 with DNase I, but not with DNase I buffer alone, reduced its capacity to promote stomatal closure. IMT504 was used at 4 µM for the stomatal assay. Different letters indicate significant differences ($p < 0.05$, one-way ANOVA). Error bars represent SE from three independent trials (A, B); n=5 (A), or 40 (B, C) per trial.

Fig. 3 ssODNs inhibit root growth and promote stomatal closure in a BAK1 dependent manner

(A) *bak1-5* and *bak1-5/bkk1* mutants are less sensitive than Col-0 in inhibition of root growth by IMT504 and 2006. Two-day-old seedlings were incubated for five days in liquid MS medium supplemented with phosphorothioate IMT504 (4 µM) or 2006 (4 µM). (B) *bak1-5* and *bak1-5/bkk1* mutants fail to close stomata in response to IMT504 and 2006. Stomatal apertures were measured 1.5 h after incubation with H_2O (mock), ABA (20 µM), flg22 (5 µM), PEP1 (100nM), IMT504 (4 µM) or 2006 (4 µM). Different letters indicate significant differences ($p < 0.05$, two-way ANOVA) (A, B). Error bars represent SE from three independent trials (A, B); n=5 (A) or 40 (B) per trial.

Fig. 4 Promotion of stomatal closure by ssODNs involves ROS production and is reduced in *mpk3* and *npr1-3* mutants

(A) DPI inhibits ssODN-induced stomatal closure. DPI (20 µM) was added to the incubation buffer 10 minutes prior to flg22 (5 µM), IMT504 (4 µM) and 2006 (4 µM). (B) Coronatine (COR, 1.56 µM) partially prevented stomatal closure induced by IMT504 (4 µM) and 2006 (4 µM). (C) *npr1-3* and *mpk3* mutants displayed reduced susceptibility to IMT504 and 2006. Epidermis were incubated with IMT504 (4 µM) and 2006 (4 µM) or flg22 (5 µM). Different letters indicate significant differences ($p < 0.05$ (A), (B) one-way ANOVA, Turkey´s test; (C)

two-way ANOVA). Error bars represent SE from three independent trials, n=40 per trial in all experiments. Stomatal apertures were measured 1.5 h after application of the respective treatments.

Fig. 5 ssODNs induce defence related genes MPK3, PROPEP1 and WRKY33

A. thaliana 7-d-old seedlings were incubated in liquid MS medium with IMT504 (4 µM), 2006 (4 µM), flg22 (1 µM) or H_2O (control, mock). Plants were harvested 4 h post incubation, and expression of MPK3, PROPEP1, WRKY33 and FRK1 was measured by qRT-PCR. Asterisks indicate significant differences relative to mock-treated plants ($p < 0.05$, permutation test, Fg Statistics). Error bars represent SD from three independent trials with 2 biological replicates each.

Figure 1

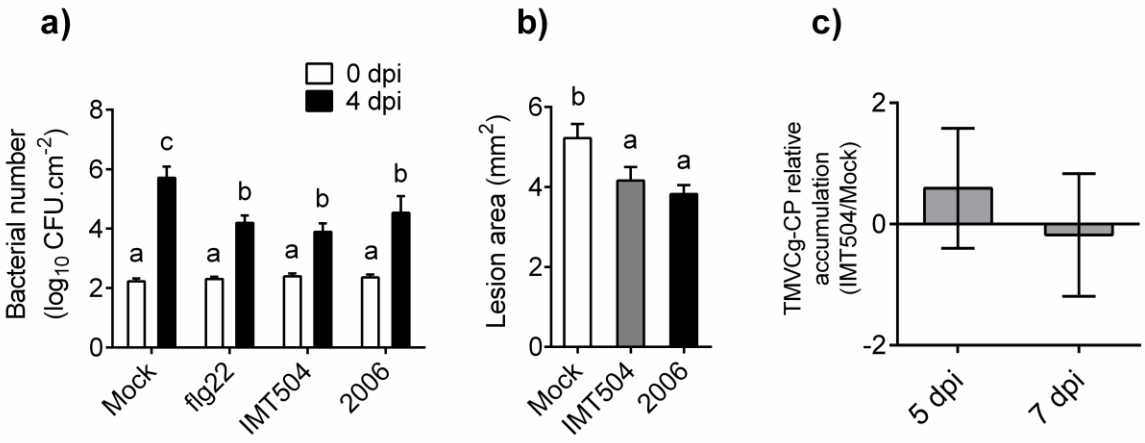

Figure 2

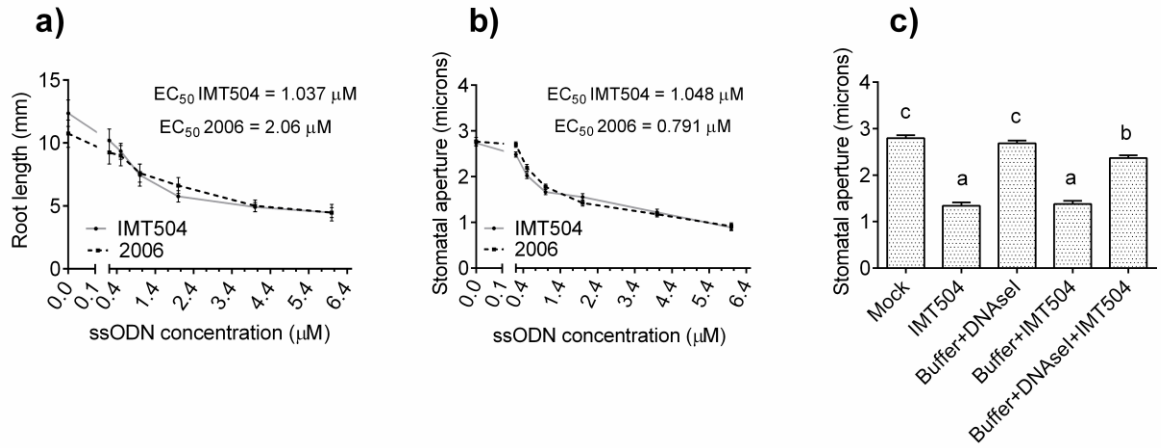

Figure 3

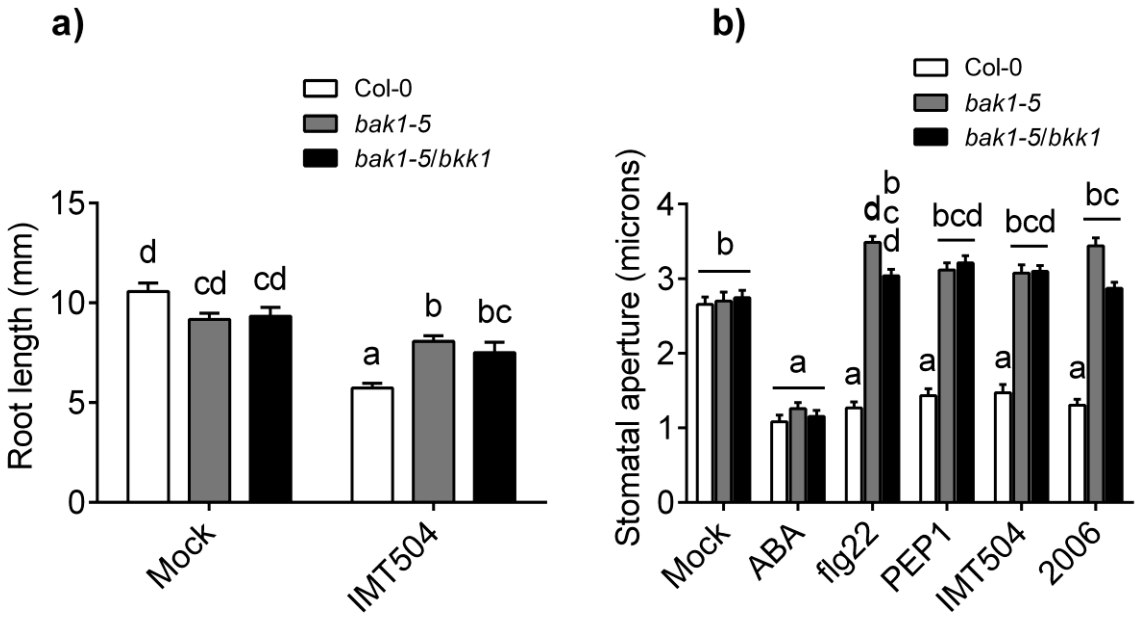

Figure 4

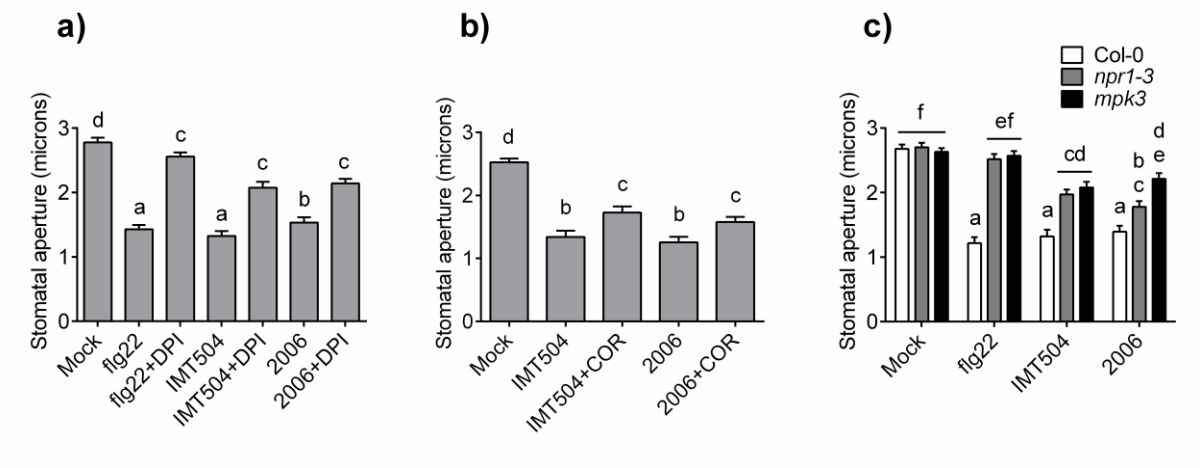

Figure 5