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Holaphyllamine, a steroid, is able to induce defense responses in *Arabidopsis thaliana* and increases resistance against bacterial infection

Abderrakib Zahid^{1,6} · Rim Jaber¹ · Ferdousse Laggoun¹ · Arnaud Lehner¹ · Isabelle Remy-Jouet² · Olivier Pamard³ · Sandra Beaupierre³ · Jérôme Leprince⁴ · Marie-Laure Follet-Gueye¹ · Maité Vicré-Gibouin¹ · Xavier Latour⁵ · Vincent Richard² · Catherine Guillou³ · Patrice Lerouge¹ · Azeddine Driouch¹ · Jean-Claude Mollet^{1,7} 

Abstract

Main conclusion A chemical screen of plant-derived compounds identified holaphyllamine, a steroid, able to trigger defense responses in *Arabidopsis thaliana* and improve resistance against the pathogenic bacterium *Pseudomonas syringae* pv tomato DC3000.

A chemical screen of 1600 plant-derived compounds was conducted and allowed the identification of a steroid able to activate defense responses in *A. thaliana* at a concentration of 1 μ M without altering growth. The identified compound is holaphyllamine (HPA) whose chemical structure is similar to steroid pregnanes of mammals. Our data show that HPA, which is not constitutively present in *A. thaliana*, is able to trigger the formation of reactive oxygen species,

deposition of callose and expression of several pathogenesis-related genes of the salicylic and jasmonic acid pathways. In addition, the results show that pre-treatment of *A. thaliana* seedlings with HPA before infection with the pathogenic bacterium *Pseudomonas syringae* pv tomato DC3000 results in a significant reduction of symptoms (i.e., reduction of bacterial colonies). Using *A. thaliana* mutants, we have found that the activation of defense responses by HPA does not depend on BRI1/BAK1 receptor kinases. Finally, a structure/function study reveals that the minimal structure required for activity is a 5-pregnen-20-one steroid with an equatorial nucleophilic group in C-3. Together, these findings demonstrate that HPA can activate defense responses that lead to improved resistance against bacterial infection in *A. thaliana*.

Keywords Callose · Chemical screen · Elicitor · Holaphyllamine · *PATHOGENESIS-RELATED* · Plant defense · *Pseudomonas syringae* pv tomato DC3000 · ROS · Steroid

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Abbreviations

BRI1/BAK1	BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR-LIKE KINASE1
dpi	Days post-inoculation
flg22	Flagellin22
HPA	Holaphyllamine
PR	Pathogenesis related
<i>Pst</i>	<i>Pseudomonas syringae</i> pv tomato DC3000

Introduction

Plant protection against pathogens is one of the major challenges for agriculture to ensure good crop yields, food security and sustainable farming (Walters et al. 2013). Plants are subjected to attack by a wide range of pathogens and, in response, they have developed a number of defense mechanisms (Glazebrook 2005). Plants respond to pathogen attacks using constitutive chemical or mechanical barriers and inducible defense systems (Klarzynski et al. 2003; Thatcher et al. 2005). Plant defenses are triggered by a variety of elicitors that originate either from microorganisms (microbe-associated molecular patterns, MAMPs) or from the plants themselves (damage-associated molecular patterns, DAMPs) and are recognized by a variety of receptors (Ma et al. 2012). Within minutes of the pathogen recognition, a number of early events occur including ion fluxes across the plasma membrane leading to an increase of cytosolic Ca^{2+} , production of reactive oxygen species (ROS) and phosphorylation and dephosphorylation cascades (Garcia-Brugger et al. 2006). These events lead to the expression of pathogenesis-related (PR) genes coding defensins, chitinases, glucanases and others, accumulation of secondary metabolites such as phytoalexins, tannins and phenolic compounds and reinforcement of the cell wall by callose and lignin deposition (Van Loon et al. 2006). The formation of a callose–cellulosic network upon perception of a fungal pathogen was recently highlighted and prevented fungal invasion (Eggert et al. 2014). The induction of these defense responses may ultimately lead to an enhanced resistance against pathogens.

Elicitors were used primarily for their ability to induce the production of phytoalexins, but they are now commonly used for stimulating any type of plant defense mechanism (Walters et al. 2013). Many natural-occurring elicitors have been isolated from bacteria, oomycetes, algae, fungi, whereas relatively few of them have been isolated from plants. Elicitors belong to different classes of compounds including proteins (harpin), peptides (flagellin22, flg22), glycoproteins (peptidoglycan), sterol derivatives (ergosterol) and oligosaccharides (laminarin, chitin, oligogalacturonide) (Nürnberg 1999). One of the best-characterized

MAMPs so far is the bacterial peptide flg22 composed of 22 amino acids and its pattern recognition receptor (PRR), a leucine-rich repeat receptor kinase (LRR-RK), FLAGELLIN SENSITIVE2 (FLS2). It is the recognition of flg22 by FLS2 in association with BRI1-ASSOCIATED RECEPTOR-LIKE KINASE1 (BAK1), another LRR-RK acting as a co-receptor for the brassinolide receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1) (Boller and Felix 2009; Nicaise et al. 2009). BRI1 is implicated in the expression of numerous defense-related genes and triggers resistance to pathogenic bacteria in wild-type plants (Gómez-Gómez and Boller 2000). Laminarin, an oligomeric β -(1,3)-glucan naturally abundant in marine brown alga, has been shown to elicit a variety of defense responses in tobacco via the ethylene (ET)-dependent signaling pathways (Klarzynski et al. 2000; Ménard et al. 2004). The sulfated β -(1,3)-glucan induces the production of acidic isoforms of PR proteins via the salicylic acid (SA) and ET-dependent signaling pathways in tobacco, *A. thaliana* and grapevine (Ménard et al. 2004; Gauthier et al. 2014). Two of the key enzymes in plant defense are the phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX). PAL is one of the first enzymes of the phenylpropanoid pathway that leads to the synthesis of SA in tobacco, lignin and phytoalexin precursors (Dixon et al. 2002). LOX is involved in the conversion of lipid compounds to JA in the oxylipin pathway (Shah 2005). Brassinosteroids (BR) are other hormone actors involved in modulating plant immunity and improving plant resistance against biotic and abiotic stresses (Krishna 2003; Kanwar et al. 2012; Albrecht et al. 2012; Wang 2012). They are a unique class of plant hormones that are related to animal steroids (Clouse 2002). It has been shown that BR perception in rice increases resistance to biotic and abiotic stresses (Nakashita et al. 2003; Divi et al. 2010). Exogenous application of 24-epibrassinolide was shown to prevent the development of disease symptoms on tomato plants inoculated with *Verticillium dahliae* (Krishna 2003). Potato plants treated with BR showed an improved resistance to the infection by *Phytophthora infestans* which was associated with an increase in ABA and ET contents (Krishna 2003). BR can interact with the ABA, ET and SA pathways and mediate stress tolerance in *A. thaliana* (Divi et al. 2010).

Moderate- to high-throughput screening of small compounds (<500 Da) was used to identify new “primers” or elicitors of defense mechanisms. Small compound screens carried out on model biological system can be applied to other species including economically important crops (Robert et al. 2009; Bektas and Eulgem 2015). By screening the Library of Active Compounds in *A. thaliana* (LATCA library), Schreiber et al. (2008) identified a class of sulfanilamides that induced immunity and decreased pathogen numbers in the leaf tissues of soil-grown plants. Knoth et al. (2009) performed a high-throughput screen of 42,000

diverse compounds. One of the effective compounds, the 3,5-dichloroanthranilic acid, improved resistance of *A. thaliana* to the bacterium *Pseudomonas syringae* pv tomato DC3000 (*Pst*) and was significantly more potent than SA. Another screen with 80 compounds from the TimTec natural product collection identified two compounds: sulfamethoxazole and the indole alkaloid gramine called donaxine. Both compounds could reduce the severity of symptoms caused by *Fusarium graminearum* in *A. thaliana* and wheat (Schreiber et al. 2011). Finally, a priming compound was identified by screening the 10,000 DIVERS library and was shown to inhibit the SA glucosyltransferase activity resulting in the accumulation of lower level of the inactive SA-*O*- β -D-glucoside (Noutoshi et al. 2012).

In this study, a chemical library of 1600 small natural compounds isolated and purified from a variety of plant species was screened. The screen allowed the identification of a new compound holaphyllamine (HPA) of the steroid family that was able to induce defense mechanisms in *A. thaliana* and improves its resistance against the bacterium *P. syringae* pv tomato DC3000. In addition, a structure/function study revealed that the minimal structure of the active molecule is a 5-pregnen-20-one steroid with an equatorial nucleophilic group in C-3. Finally, the use of *A. thaliana* mutants showed that the induction of the immune response by HPA does not depend on the BRI1 and BAK1 receptors.

Materials and methods

Plant cell culture

Nicotiana tabacum cv. BY-2 suspension-cultured cells were grown in the dark at 25 °C in the MS medium (Murashige and Skoog 1962) containing 4.33 g L⁻¹ MS (w/v), 3% (w/v) sucrose, 1 mg mL⁻¹ thiamin, 50 mg L⁻¹ myo-inositol, 0.22 mg L⁻¹ 2,4-D and 200 mg mL⁻¹ KH₂PO₄ at pH 5 on a continuous rotary shaker at 120 rpm. Cells were sub-cultured into a fresh medium every 7 days.

Plant growth conditions

Arabidopsis thaliana seeds (ecotype Columbia-0) were sterilized with 75% (v/v) ethanol for 5 min and 10% (v/v) bleach for 5 min. Seeds were washed five times in sterile water and deposited on the surface of a sterile solid ½ MS medium containing 0.8% (w/v) Bacto Agar in 24-well plates (Nunc). Seeds were stratified for 48 h at 4 °C in the dark. For all experiments, *A. thaliana* seedlings were grown for 10 days at 22 °C with 16-h light and 8-h dark cycles. To determine the effect of the selected small compounds on plant growth, seeds were germinated on solid medium containing

0.1–10 μ M of the molecule and the root length was measured with ImageJ after 10 days of culture.

The ICSN chemical library

Part of the ICSN (Institut de Chimie des Substances Naturelles, CNRS, Gif-sur-Yvette France) chemical library (1600 compounds), which contains 5400 structurally diverse pure molecules that are either natural products (30%) (terpenes, steroids, flavonoids, sugars, nucleosides, alkaloids, etc.), derived from natural substances (30%) or synthetic compounds (40%) was screened. For the primary screen on the tobacco BY-2 suspension-cultured cells, the plant-derived compounds were provided in 96-well plates at 10 mg mL⁻¹ in 100% DMSO and diluted with the ½ MS medium. In the subsequent screens, the selected compounds were prepared at 10 mM in 100% DMSO and diluted with ½ MS medium.

Detection of the oxidative burst in tobacco BY-2 suspension-cultured cells

The primary screen was carried out with the tobacco BY-2 suspension-cultured cells. A microplate fluorescence detection of ROS was developed based on Gerber and Dubery (2003) using the fluorescent probe 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl-ester (CM-H₂DCF-DA) (Invitrogen) that reacts with a wide range of ROS including H₂O₂ and hydroxyl radicals (OH^{*}) (Kristiansen et al. 2009). Commercially available elicitors: laminarin from *Laminaria digitata* (Sigma-Aldrich) and peptidoglycan from *Bacillus subtilis* (Sigma-Aldrich) were tested at 50, 100 and 200 μ g mL⁻¹. Prior to performing the primary chemical screen, the reproducibility of the biological assay was estimated by calculating the Z'-factor (Zhang et al. 1999) with 96 assays using laminarin as a positive control and 0.25% (v/v) DMSO as a negative control. Four-day-old cells were diluted in a pre-incubation medium composed of 0.25 \times MS medium and 3% (w/v) of sucrose to reach a final concentration of 0.1 g (FW) mL⁻¹ and were incubated for 3 h at room temperature on a shaker at 120 rpm. The cells were transferred into 96-well plates (Costar) and were treated with DMSO, laminarin, peptidoglycan or the ICSN compounds to reach the final concentrations of 0.25%, 100 and 25 μ g mL⁻¹ (~50–60 μ M), respectively. CM-H₂DCF-DA stock solution was added to obtain a final concentration of 20 μ M. The emitted fluorescence resulting from the oxidative burst was followed every 5 min for 2 h with the FlexStation3 microplate reader (Molecular devices) at 490 nm (excitation) and 527 nm (emission). The chemical screen was carried out in triplicate. Inhibition of the oxidative burst was analyzed by adding the

diphenylene iodonium (DPI) 1 h before elicitation at 5, 10 and 20 μM (final concentrations) to the cell suspension.

Reactive oxygen species detection and quantification

Two staining methods were used to study the induction of ROS accumulation in roots and leaves in the presence of the selected small molecules. In situ detection of total ROS was performed by incubating the roots with CM-H₂DCF-DA as previously described (Plancot et al. 2013). Roots were mounted on a microscope slide with phosphate buffer 20 mM pH 6.1, then incubated with 30 μL of 20 μM CM-H₂DCF-DA. Roots were treated with 50 μL of 0.01% (v/v) DMSO, 1 μM flg22 (synthesized by Dr. J. Leprince, PRIMACEN platform, Normandie Univ) or 1 μM of the selected compounds. The induction of ROS was followed by confocal laser scanning microscopy (CLSM) (Leica TCS SP2 AOBS; excitation filter, 488 nm; barrier filter, 510 nm, 550 mV).

A second method was used on leaves (Tyburski et al. 2009). Leaves of 10-day-old seedlings were treated for 1 h with 0.01% (v/v) DMSO, 1 μM flg22 or 1 μM of the selected compounds. ROS were detected by incubating the seedlings in 0.05% nitroblue tetrazolium chloride (NBT) dissolved in 100 mM phosphate buffer, pH 7.5 for 10 min. The reaction was stopped by replacing the NBT solution with phosphate buffer. The sites of ROS accumulation stained in dark blue were observed with a binocular microscope (Leica M125). Images were acquired with the DFC295 camera (Leica). Each experiment was repeated three times.

Free radicals production was also quantified by electron paramagnetic resonance (EPR) spectroscopy (Bellien et al. 2012). *Arabidopsis thaliana* seedlings were treated with 0.01% DMSO, 1 μM flg22 and different concentrations (0.1, 1 and 10 μM) of HPA to estimate the dose-dependent free radical production. The seedlings were incubated at 22 °C for 60 min in Krebs HEPES buffer containing 5 μM diethyl dithiocarbamate, 25 μM deferoxamine, and the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine hydrochloride (CMH; 500 μM ; Noxygen, Elzach, Germany). Spectra of the oxidized product of CMH (CM.) were recorded from frozen samples with a X-band spectrometer (MS-400; Magnettech, Berlin, Germany) with the following acquisition parameters: microwave power, 20 mW, modulation amplitude, 5G; sweep time, 60 s; and 1 scan. After correction of the baseline, the total amplitude of the signal was measured for 60 min in seedlings and the incubation medium. Measurements are the mean from nine biological replicates.

Callose staining

In situ callose detection was performed using decolorized aniline blue staining adapted from previously described

methods (Millet et al. 2010). Briefly, 10-day-old wild-type and mutants *bak1* (*bak1/serk3*) (SALK_034523C), *fls2* (SALK_062054C) and *bri1-9* (gift of Pr. Jianming Li, University of Michigan) seedlings grown in 24-well plates were treated for 48 h with 1 μM of the selected compound, 1 μM flg22 or 0.01% (v/v) DMSO. Seedlings were fixed in acetic acid/ethanol (1:3, v/v) for 2 h followed by successive ethanol treatments with 75, 50, 25% (v/v) for 2 h and finally with H₂O overnight. Seedlings were incubated with 10% (w/v) NaOH at 37 °C for 1 h and then overnight in 0.01% (w/v) aniline blue dissolved in 150 mM K₂HPO₄ at pH 10. Seedlings were mounted on slides and callose deposition was observed by CLSM (Leica TCS SP2 AOBS; excitation filter, 359 nm; barrier filter, 461 nm). The callose deposition pattern obtained with the selected compounds was compared with the ones obtained with plants treated for 48 h with 1 μM flg22 and commercially available compounds such as the steroid (pregnenolone sulfate) and one relative of the plant hormone brassinolide (24-epibrassinolide) (Sigma-Aldrich). For the quantification of the aniline blue-emitted fluorescence by the callose deposits, the ImageJ program was used (Abramoff et al. 2004). After defining the surface of the leaf, the area of fluorescence was calculated and expressed in arbitrary unit per mm².

RNA isolation and qRT-PCR

Total RNA for qRT-PCR was isolated from 10-day-old seedlings grown in 24-well plates. After treatments for 1, 3, 6, 12, 24, and 48 h with 0.01% DMSO, 1 μM flg22 or 1 μM of the selected compound, total RNA was extracted using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. Prior to qRT-PCR, RNA was treated with RNase-free DNase I (Qiagen) to eliminate any contaminating genomic DNA. cDNA was synthesized from 1 μg of total RNA using high-capacity cDNA reverse transcription kit (Applied biosystems) following the manufacturer's instructions. The PCR mix included 1X SYBR Green Master Mix (Applied Biosystems), cDNA template and each of the forward and reverse primers (Suppl. Table S1). The PCR cycling parameters were 20 s at 95 °C, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s and finally 15 s at 95 °C. After completion of the cycling parameters, dissociation melt curve analyses (60 °C for 1 min, followed by 15 s at 95 °C and 15 s at 60 °C) were conducted to eliminate the effects of primer dimer formation and contamination. The qRT-PCR was performed in triplicate and the negative controls included water and mRNA prior to reverse transcription. The genes investigated were *PATHOGENESIS-RELATED-1* (*PR-1*), *PR-2*, *PR-3*, *PR-4*, *PDF-1.2* (*PLANT DEFENSIN-1.2*), *PHENYLALANINE AMMONIA LYASE1* (*PAL1*), *LIPOXYGENASE3* (*LOX3*), *PHYTOALEXIN*

DEFICIENT3 (PAD3) and the transcription factors *WRKY-29* and *WRKY-40*. The list of primers used is shown in Suppl. Table S1. *Arabidopsis thaliana ACTIN11* and *ELONGATION FACTOR1 α (EF1 α)* genes were used as internal controls for the levels of cDNA. The relative fold changes were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Pathology tests

Pseudomonas syringae pathovar tomato strain DC3000 (*Pst*) was used as a pathogenic model to infect 10-day-old seedlings and 5-week-old plants (Buell et al. 2003). *Pst* was grown at 26 °C in Luria–Bertani (LB) broth medium supplemented with 100 $\mu\text{g mL}^{-1}$ of rifampicin under agitation (180 rpm).

Inoculation of 10-day-old seedlings with *Pst* was performed using the flood-inoculation method developed by Ishiga et al. (2011) with minor modifications. *Pst* suspension cells (after 5 h of growth in a new medium) was centrifuged and diluted in sterile physiological saline solution (0.9% NaCl, pH 7) to reach a final density of 10^5 CFU mL^{-1} . After treatments with 1 μM of HPA, 1 μM flg22 or 0.01% DMSO for 48 h, 2 mL of bacterial suspension was poured into the plate and incubated for 2–4 min at room temperature. The bacterial suspension was then removed and seedlings were incubated in a growth chamber at 22 °C for 4 days. The relative pigment content of the leaves (chl *a*, *b* and carotenoids) was estimated by spectrophotometry at 460, 645 and 663 nm after 80% (v/v) acetone extraction according to Ni et al. (2009).

Inoculation and bacterial count on 5-week-old plants were performed according to Yao et al. (2013) with modifications. Briefly, leaves were sprayed with 2 mL of 0.01% DMSO, 1 μM ergosterol (98%, Acros Organics), 1 μM flg22 or 1 μM HPA containing 0.02% Silwet L-77. After 2 days, 2 mL of bacterial suspension (4×10^8 CFU mL^{-1}) containing 0.02% Silwet L-77 was sprayed on the rosettes. After 17 days, three leaves per plant were harvested, surface-sterilized with EtOH 70% and rinsed with sterile water. Three discs (0.24 cm^2 per disc) per leaf were excised using a cork borer, pooled and ground with a plastic pestle in 300 μL of physiological saline solution (0.9% NaCl, pH 7). The extract was diluted by serial dilution at 1/10, spotted on solid LB medium containing 100 $\mu\text{g mL}^{-1}$ of rifampicin and incubated for 30 h at 28 °C. The number of bacterial colony (CFU) was counted and expressed as the CFU cm^{-2} . The experiments were carried out three times with two plants per condition (i.e., $12 \leq n \leq 18$ leaves/condition and three technical replicates/leaf). For the negative controls (plants treated with DMSO but non-inoculated with *Pst*), no bacterial colony was detected.

Extraction and detection of HPA in *Arabidopsis* seedlings

About 5 g of 10-day-old *A. thaliana* seedlings was extracted by refluxing for 2 h with 70% ethanol (v/v, 2×100 mL). After filtration, the solvent was removed from the combined extract by rotary evaporation at 60 °C. The residue (17 mg) was then dissolved in 25 mL of 70% (v/v) methanol and filtered through a 0.22- μm membrane before analysis. A stock solution of HPA was prepared in 70% (v/v) methanol and diluted to yield a solution at 0.14 mM and 5 $\mu\text{g mL}^{-1}$ for the *A. thaliana* extract.

MS analyses were performed using a UPLC1200 system coupled to a Q-TOF 6520 mass spectrometer equipped with an Electrospray source (Agilent Technologies). Briefly, *A. thaliana* extract and HPA standard were separated on an Acquity HSS T3 (1.8 μm particle size) C18 column (50 mm long \times 2.1 mm inner diameter; Agilent Technologies). A 10 min linear gradient (15–100% acetonitrile in 0.1% formic acid) at a flow rate of 0.5 mL min^{-1} was used, and separated steroids were analyzed with a Q-TOF mass spectrometer. Full autoMS1 scans from 100 to 3000 m/z were recorded.

Statistical analysis

Statistical analyses were performed on GraphPad Prism 5 Program (GraphPad Software, Inc, La Jolla, CA, USA) using the non-parametric Mann–Whitney test ($\alpha = 0.05$) or the parametric one-way ANOVA test ($\alpha = 0.05$). The results were presented by asterisks that indicate significant differences between mean values: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$).

Results

Primary chemical screen revealed compounds able to induce the oxidative burst in tobacco BY-2 suspension-cultured cells

We have first optimized the oxidative burst assay in 96-well plates using tobacco BY-2 suspension-cultured cells and CM-H₂DCF-DA, a probe that reacts with ROS and converts it to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Tobacco BY-2 suspension-cultured cells were used instead of *A. thaliana* seedlings to avoid artifacts originating from chl fluorescence. The results revealed that the highest fluorescence intensity was obtained with a cell concentration of 0.1 g (FW) mL^{-1} and a probe concentration of 20 μM . Similarly, among the two commercially available elicitors tested (laminarin and peptidoglycan), laminarin could induce the highest fluorescence intensity at the lowest concentration (100 $\mu\text{g mL}^{-1}$). With this optimized assay, the

calculated factor $Z' = 0.81$ indicated that the biological assay was suitable for a chemical screen. Only the compounds able to induce an equivalent or higher ROS production than peptidoglycan were selected. Among the 1600 candidates, two quenched completely the ROS production due to their anti-oxidant properties (flavanones) and 16 compounds were able to induce an equivalent or higher ROS production than peptidoglycan (Fig. 1). Measurement of the oxidative burst with CM-H₂DCF-DA in the presence of DPI, an inhibitor of ROS production, showed that the fluorescence intensity decreased with increasing DPI concentrations indicating that the emitted fluorescence was correlated with ROS production of the treated cells and was not due to the direct oxidation of the probe by the compounds (Suppl. Fig. S2). The 16 compounds (Figs. 3, 10 and Suppl. Fig S1) were investigated in subsequent screens on *A. thaliana* seedlings (Fig. 2). Five compounds were able to induce ROS production and callose deposits without affecting growth. The five compounds which passed the secondary screen were tested for their abilities to induce the expression of defense-related genes (Fig. 2). Among them, HPA was the most promising compound and was further investigated for its ability to reduce disease symptoms.

The most effective compound is holaphyllamine

Among the five selected compounds, HPA was found to be the most effective in activating defense responses. HPA belongs to the steroid family. It has a pregnane core structurally related to pregnenolone sulfate and pregnenolone of mammals (Fig. 3). The main difference is the replacement

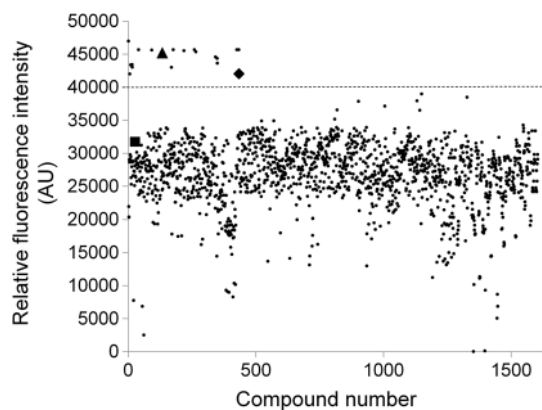


Fig. 1 Scatter plot of the relative fluorescence intensity from reactive oxygen species (ROS) produced by tobacco BY-2 suspension-cultured cells treated for 2 h with DMSO (0.01%) (filled square), laminarin (100 $\mu\text{g mL}^{-1}$) (filled triangle), peptidoglycan (100 $\mu\text{g mL}^{-1}$) (filled diamond) and the 1600 ICSN compounds (filled circle). Fluorescence originates from the reaction between CM-H₂DCF-DA and ROS produced by the viable treated cells. The chemical screen was carried out in triplicate

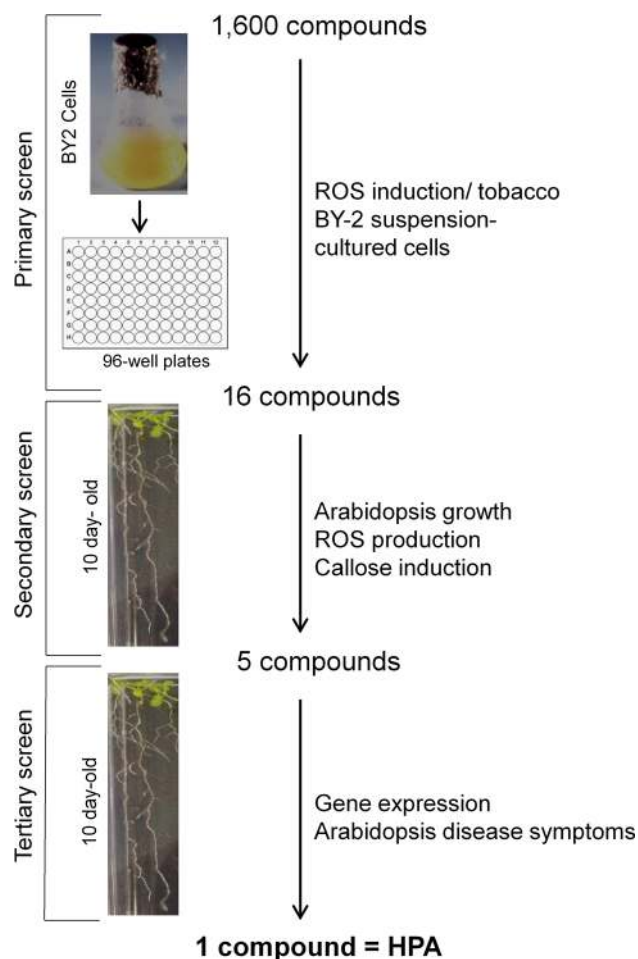


Fig. 2 Flowchart of the different steps used for the chemical screening performed on tobacco BY-2 suspension-cultured cells and then *Arabidopsis* seedlings. Primary screen: 1600 compounds from the ICSN chemical library were added to suspension cells and the production of ROS was assessed over time with a microplate reader. Secondary screen: the 16 selected compounds (Figs. 3, 10, Suppl. Fig. S1) were applied to *Arabidopsis* seedlings to assess plant growth, ROS production and callose deposition. Five compounds at 1 μM were selected to perform the tertiary screen based on the induction of defense-related genes. The most potent compound holaphyllamine (HPA) (see Fig. 3) was selected for investigating its ability to induce defense responses

of the 3-sulfate or 3-hydroxyl groups of the A cycle by an amino group.

Holaphyllamine is not detected in untreated *A. thaliana* seedlings

We checked whether HPA was part of the secondary metabolites produced by *A. thaliana* seedlings using mass spectrometry. Our data showed no ion related to HPA (m/z of HPA is 316.2806) indicating that the compound does not occur constitutively in *A. thaliana* seedlings (Suppl. Fig. S3).

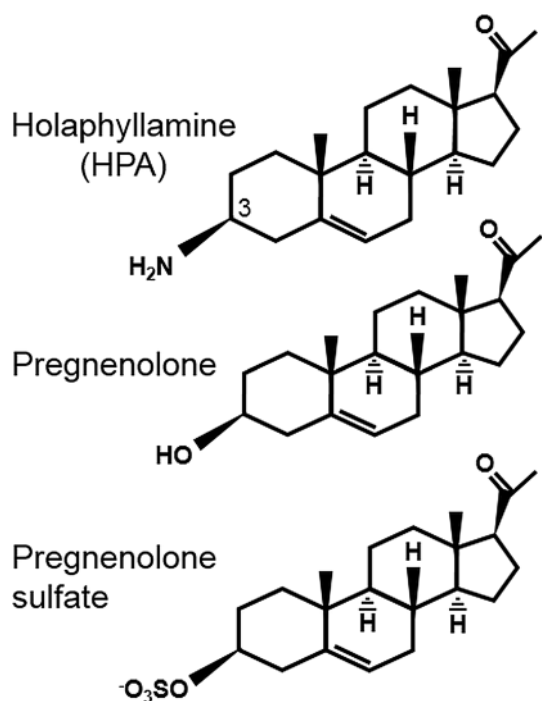


Fig. 3 Chemical structure of HPA selected through the screens. The structure of the compound is closely related to certain bioactive molecules/hormones such as pregnenolone and pregnenolone sulfate of mammals

Holaphyllamine does not affect *A. thaliana* growth at 1 μM

To evaluate the possible inhibitory effect of HPA on the growth of *A. thaliana* seedlings, seeds were germinated in the presence of different concentrations of the compound (0.1, 1.5, 2.5, 5, 7.5 and 10 μM). As illustrated in Fig. 4a, b, HPA did not influence the growth and development up to a concentration of 2.5 μM (also supported by the fact that no visual phenotypic alterations were observed in the cotyledons compared to the control). However, a retardation of the primary root growth was observed at 5 μM and higher concentrations (Fig. 4b, c). Subsequently, 1 μM HPA was used for further experiments and compared to 1 μM flg22, a well-characterized PAMP in plant immunity studies (Zipfel and Robatzek 2010).

Holaphyllamine induces ROS production in *A. thaliana* roots and leaves

Histochemical detection of ROS was visualized using the NBT reagent. The purple-blue formazan precipitate indicative of ROS occurrence was observed in leaves treated for 1 h either with 1 μM flg22 or HPA (Fig. 5a). In contrast, no precipitate was detected in leaves treated with 0.01% DMSO.

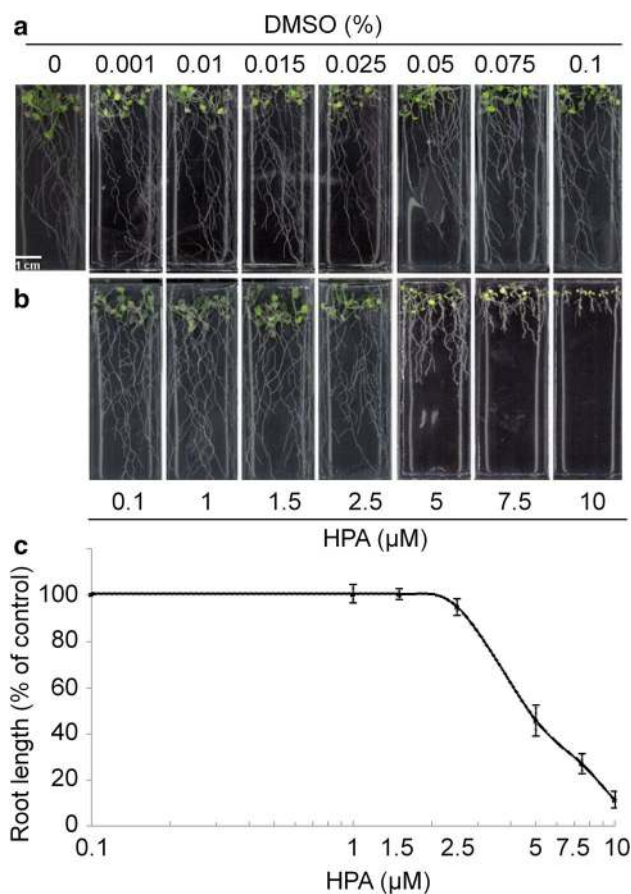


Fig. 4 Effect of HPA on *Arabidopsis* germination and seedling growth. **a, b** Images of untreated and treated *Arabidopsis* seedlings with different concentrations of DMSO or HPA. The seeds were germinated in the presence of DMSO or HPA and images were acquired 10 days later. Scale bar 1 cm. **c** Effect of different concentrations of HPA on the root length. Root length was measured with the ImageJ software. Fifteen roots were measured per condition with four biological replicates

In roots treated with flg22 or HPA, the DCF fluorescence emitted from the oxidation of the non-fluorescent H_2DCF by ROS increased rapidly overtime starting from the root tip and propagating in the area localized between the quiescent center and the elongation zone (Fig. 5b). In the negative control, only a slight fluorescence was detected in roots likely to originate from the constitutive metabolic ROS production by the cells.

The production of free radicals was quantified in seedlings and the incubation medium by EPR spectroscopy (Fig. 5c). When compared with untreated or DMSO-treated seedlings, treatments with HPA and flg22 caused a significant increase of free radical production in both the seedlings and incubation media, the latter showing always the highest accumulation. An increase of HPA concentrations induced a higher production of free radicals in the incubation medium and in the seedlings.

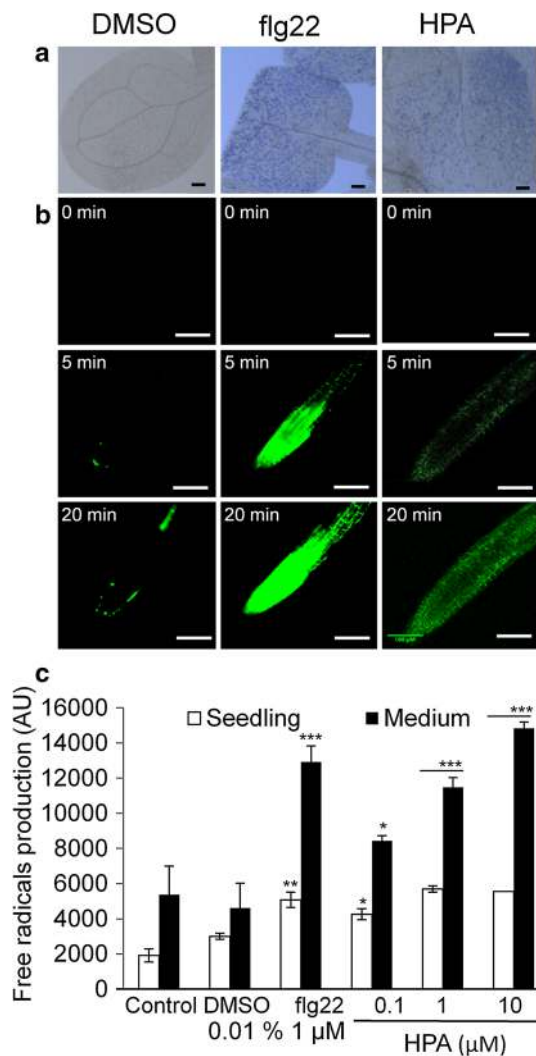


Fig. 5 Induction of ROS in 10-day-old *Arabidopsis* seedlings treated with 0.01% DMSO, 1 μM flg22 or 1 μM HPA. **a, b** Detection of ROS with NBT in treated leaves and time course of ROS induction in roots using the fluorescent probe CM-H₂DCF-DA. Images shown are representative of 12 leaves and 12 roots from three independent experiments. Scale bars 100 μm. **c** Quantification of free radicals by electron paramagnetic resonance (EPR) spectroscopy, in 10-day-old *Arabidopsis* seedlings untreated and treated with 0.01% DMSO, 1 μM flg22, and 0.1, 1 and 10 μM HPA. The free radical production was measured in *Arabidopsis* seedlings and the incubation medium. Values are the means of 16 *Arabidopsis* seedlings. Data are a comparison of the treatments (HPA or flg22) with the DMSO-treated plants. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Holophyllamine induces callose deposition in *A. thaliana* leaves

Unlike ROS, callose induction is a more stable marker of plant defense (Millet et al. 2010; Luna et al. 2011). After 48 h of treatment with 1 μM HPA, a significant accumulation of callose in leaves was detectable (Fig. 6a). The pattern of callose deposition was very similar when plants were treated

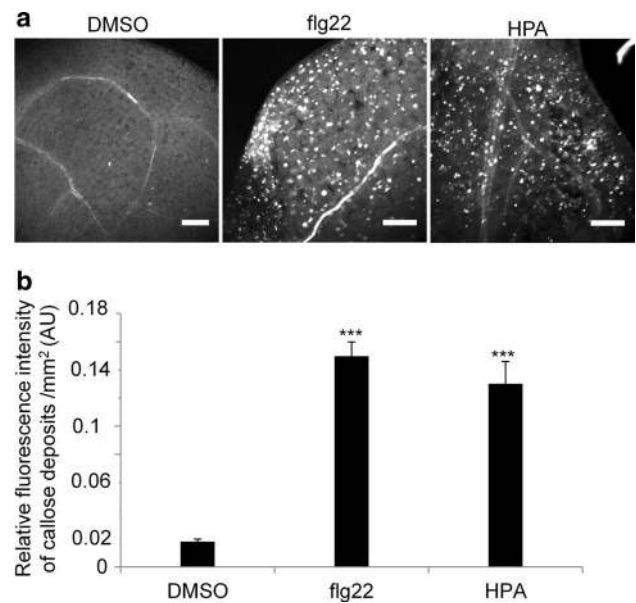


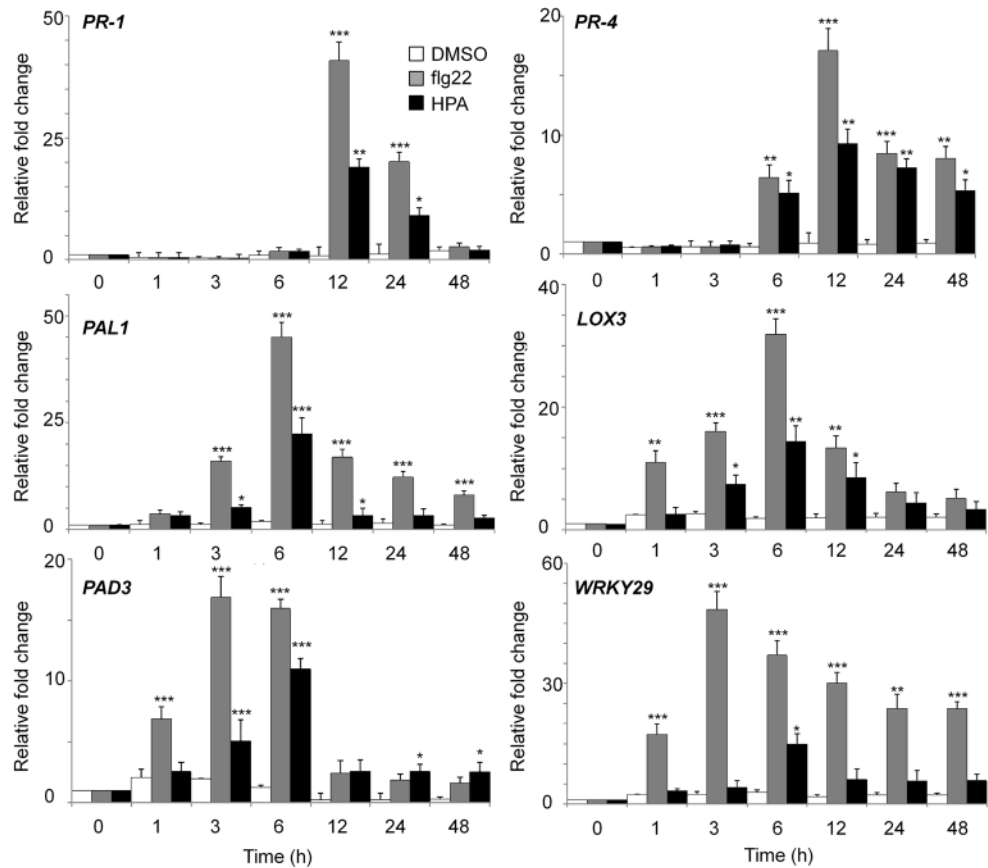
Fig. 6 Induction of callose deposition in leaves of 10-day-old *Arabidopsis* seedlings treated for 48 h with 0.01% DMSO, 1 μM flg22 or 1 μM HPA. **a** Visualization of callose deposits in leaves after aniline blue staining. Scale bars 100 μm. **b** Callose deposit quantification using the ImageJ program. Data are the mean of 12 leaves per condition and three independent experiments. Data compare the treatments (HPA or flg22) with the DMSO-treated plants. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

with 1 μM flg22 and is in agreement with previous reports (Denoux et al. 2008; Millet et al. 2010; Plancot et al. 2013). In contrast, treatment with 0.01% DMSO induced little or no callose accumulation (see Fig. 6a). Quantification of the fluorescence intensity in the decolorized leaves revealed that flg22 and HPA induced significantly much more callose deposits per surface (mm²) compared to DMSO-treated seedlings (Fig. 6b).

Holophyllamine induces the expression of defense-related genes

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to measure the transcript levels of a number of defense-related genes between 0 and 48 h of treatment with 0.01% DMSO, 1 μM flg22 or 1 μM HPA. Our result showed that the expression level of *PR-1* in *A. thaliana* seedlings treated with HPA or flg22 increased at 12 h and then returned to the basal level after 48 h (Fig. 7). The *PR-1* expression was about twofold higher in plants treated with flg22 compared to HPA. The expression levels of *PR-2*, *PR-3* and *PDF1.2* did not change dramatically in plants treated with HPA except for *PDF1.2*, a slight 1.5-fold increase was observed after 24 h. In contrast, seven- and tenfold increases in the expression of *PR-2* and *PR-3* were detected with plants treated with flg22 after 48 h

Fig. 7 Time course of *PR1*, *PR4*, *PAL1*, *LOX3*, *PAD3* and *WRKY29* gene expressions using qRT-PCR in *Arabidopsis* wild-type seedlings after 0, 1, 3, 6, 12, 24 and 48 h of treatment with 0.01% DMSO (white bars), 1 μ M flg22 (gray bars) or 1 μ M HPA (black bars). The values represent the mean fold-changes from three biological replicates. Data compare the treatments (flg22 or HPA) with DMSO at the same induction time. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$



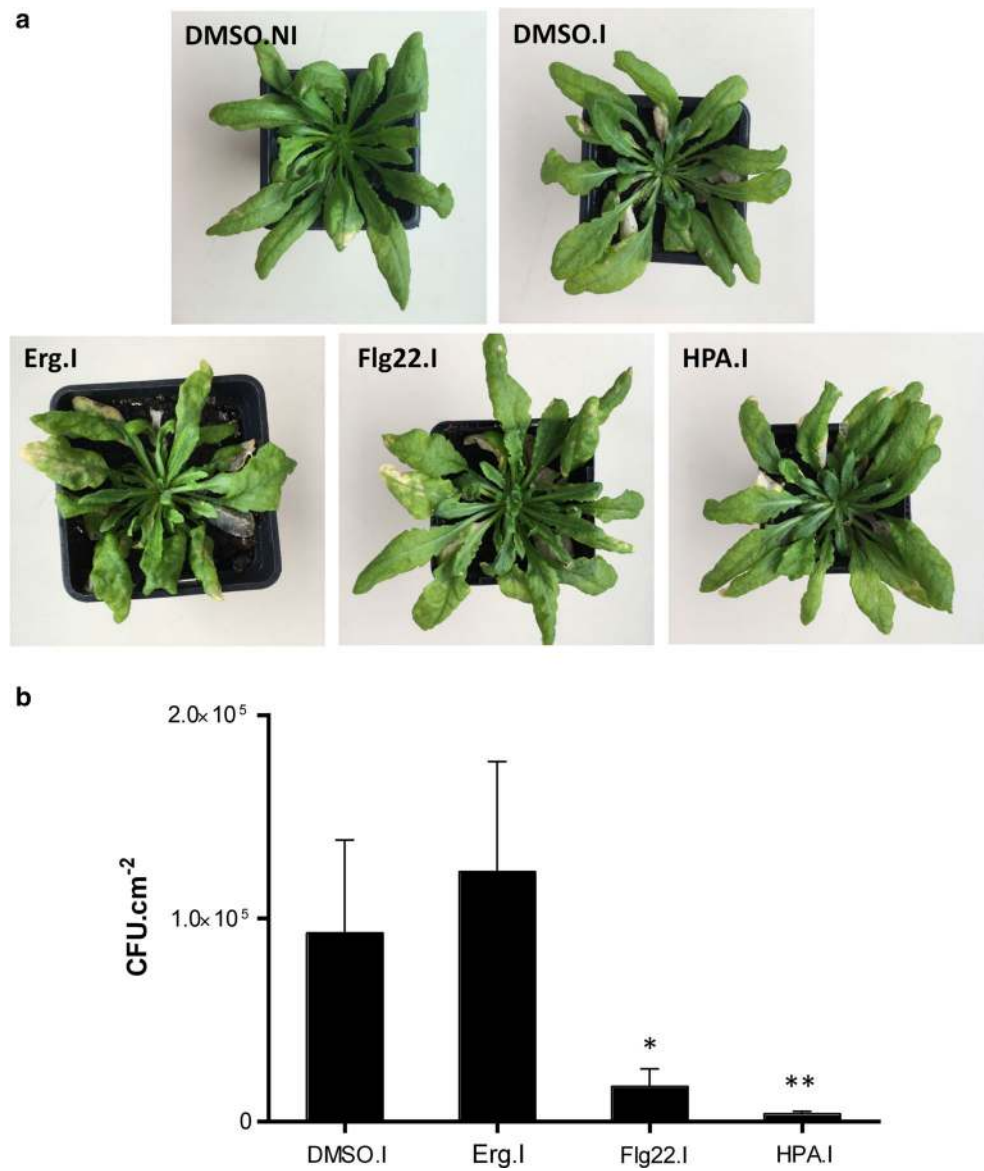
(Suppl. Fig. S4). The expression profile of *PR-4* was quite similar to that observed for *PR-1*, with an increase of *PR-4* expression starting at 6 h and reaching a maximum at 12 h before declining afterwards but without reaching basal levels at 48 h. The expression of *PR-4* was about twofold lower than that of *PR-1*. The expression of *PAL-1*, *LOX-3* and *PAD-3* was induced more rapidly (between 1 and 3 h post-treatment) in plants treated with flg22 or HPA. The expression levels reached their maxima 6 h post-treatment and then declined (Fig. 7). In plants treated with HPA, the relative expression changes were the highest (~20-fold increase) for *PAL-1* and *PR-1*, and were comprised between 10- and 15-fold for *PR-4*, *LOX-3* and *PAD-3*. *WRKY40* was strongly induced by flg22 with its expression reaching a peak after 1 h (50-fold change) and was still strong after 3 h (Suppl. Fig. S3). On the other hand, with the HPA treatment, the expression level of *WRKY40* did not exceed a fivefold change (Suppl. Fig. S4). In contrast, *WRKY29* appeared more expressed (15-fold) (Fig. 7) than *WRKY40* in plants treated with HPA with a peak at 6 h post-treatment (Fig. 7). In all the conditions, treatments of plants with 0.01% DMSO did not induce the expression of any of the investigated genes (Fig. 7).

Holophyllamine-treated plants have reduced symptoms when infected with the pathogenic bacterium *P. syringae* pv tomato DC 3000

Pseudomonas syringae pv tomato DC 3000 is a widespread plant pathogenic bacterium. It causes leaf spots and other foliar necrosis in host plants and elicits the hypersensitive response (HR) in non-hosts (Mansfield et al. 2012). It is a well-studied pathogen on tomato and *A. thaliana* (bacterial speck) known to produce more than 30 effector proteins (Buell et al. 2003; Guo et al. 2009). Here, we have used this strain to infect *A. thaliana* seedlings and generate disease symptoms. The severity of the symptoms was then assessed on plants pre-treated or not with flg22, HPA and the fungal ergosterol prior to infection.

First, we examined the effect of HPA on the bacterial growth. At 5 μ M, HPA did not show any effect on the growth of the bacteria compared to the controls (mock or DMSO) (Suppl. Fig. S5). Control plants treated with DMSO 0.01% and inoculated with bacteria showed chlorotic leaves (Fig. 8a) compared to the untreated and uninfected plants. In contrast, in plants pre-treated for 48 h with HPA or flg22 before inoculation with the bacteria, the chlorosis was less

Fig. 8 Disease symptoms in mature *Arabidopsis* leaves caused by *Pst* infection. **a** Disease symptoms on plants sprayed with 0.01% DMSO, 1 μ M ergosterol (Erg), 1 μ M flg22 or 1 μ M HPA, incubated for 48 h and sprayed with 4×10^8 CFU mL⁻¹ of *Pst*. Pictures were acquired at 17 dpi. Note that, unlike on Erg-treated leaves, less symptoms were observed on those treated with flg22 or HPA. No bacterial colony was detected on the negative controls (plants treated with DMSO but non-inoculated with *Pst*). **b** Number of counted bacterial colonies (CFU cm⁻²) isolated from treated and inoculated leaves. Data compare the treatments (Erg, Flg22 or HPA) with the DMSO-treated plants and each bar represents the mean of $12 \leq n \leq 18$ leaves \pm SE. Statistical analyses were performed using the Mann-Whitney test ($\alpha = 0.05$). Asterisks significant differences between the mean values: * $P < 0.05$, ** $P < 0.01$. I inoculated



pronounced (Suppl. Fig. S6a–e). To confirm this observation, the pigment content of the leaves was quantified (Suppl. Fig. S6f). Pre-treatment of plants with flg22 or HPA before bacterial inoculation showed a slight decrease of the chl *a* and *b* content although without significant differences ($P < 0.05$) compared to the pigment content of plants pre-treated with mock or DMSO without any bacterial inoculation (Suppl. Fig. S6f). In contrast, a significant ($P < 0.05$) decrease of the pigment content (~45%) was observed in plants treated with mock or DMSO and inoculated with bacteria. Both chl *a* and *b* decreased proportionally. Finally, the carotenoid content did not change dramatically between the different conditions.

Five-week-old *A. thaliana* plants were first sprayed with 1 μ M flg22, HPA, ergosterol (used based on the closely related structure with HPA) or 0.01% DMSO, then with the

bacteria 48 h later and symptoms and colony numbers were determined. The data showed that symptoms were much more pronounced on leaves treated with DMSO or ergosterol and inoculated (Fig. 8a) than on plants pre-treated with flg22 or HPA. Consistently, the bacterial CFU cm⁻² was significantly reduced on flg22 or HPA-treated plants as compared to the ones treated with DMSO and ergosterol (Fig. 8b).

Holophyllamine perception seems to be independent of BRI1/BAK1 receptors

To understand the molecular mechanisms of HPA perception, mutants impaired in their abilities to perceive brassinolide or flg22 were used. As shown in Fig. 9, HPA clearly induced significant deposition of callose in leaves of the three mutants *bri1*, *fls2* and *bak1*. As expected, flg22 did

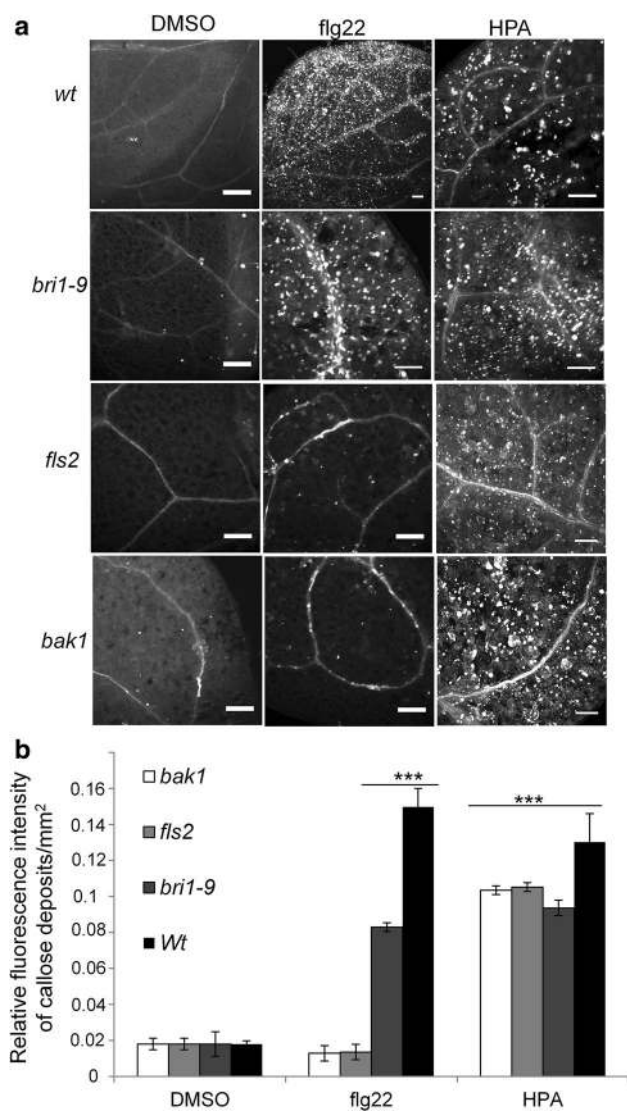


Fig. 9 Induction of callose deposition in leaves of 10-day-old wild-type and mutant (*bak1*, *fls2* and *bri1-9*) plants treated for 48 h with 0.01% DMSO, 1 μ M flg22 or 1 μ M HPA. **a** Visualization of callose deposition by aniline blue staining. Scale bars 100 μ m. **b** Callose deposit quantification. Data are the mean of 12 leaves per condition and three independent experiments. Data compare the treatments (HPA or flg22) with the DMSO-treated plants. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

not induce callose deposition in the *fls2* and *bak1* mutants unlike the *bri1* mutant (Fig. 9a, b). Treatments of wild-type, *bri1*, *bak1* and *fls2* plants with the mammal pregnenolone sulfate also induced callose deposition in leaves (Suppl. Fig. S7). Moreover, treatments of plants with the 24-epibrassinolide, a representative of the plant hormone brassinosteroid, also caused callose deposition in leaves of wild-type, *bak1* and *fls2* plants and a slight reduction in the *bri1* mutant (Suppl. Fig. S7). Based on these results,

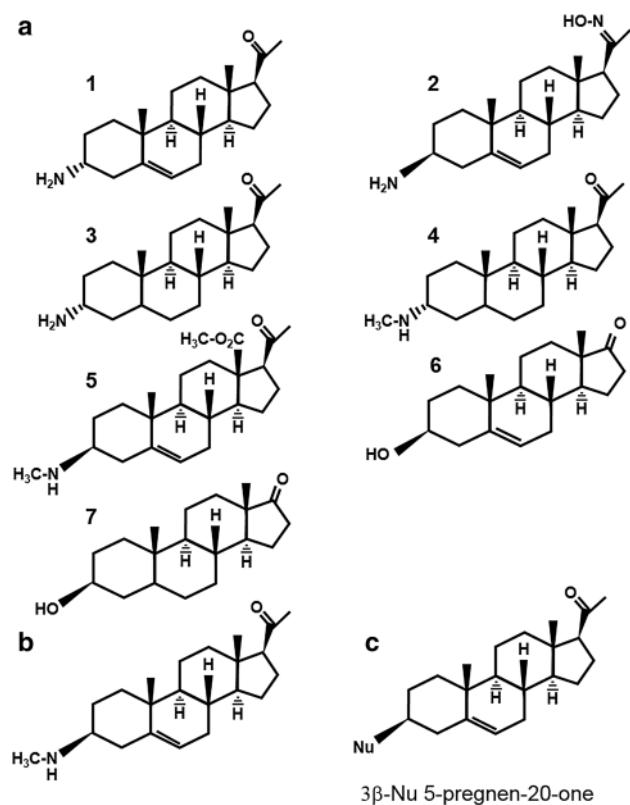


Fig. 10 Chemical structures of steroids closely related to HPA. **a** Structure of the compounds tested that were unable to induce callose deposition in *Arabidopsis* leaves. Compounds (1–5) were part of the ICSN library. Compounds 6 and 7 trans-dehydroandrosterone and trans-androsterone, respectively, are commercially available. **b** Structure of one compound (HP holaphylline) from the ICSN library that could induce ROS production and callose deposition in addition to the three others presented in Fig. 3. **c** Common 3 β -nucleophile 5-pregnen-20-one structure required for the elicitation of plant defense (i.e., callose deposition)

it appears that HPA acts independently of the BRI/BAK1 receptors.

Structural requirements for activation of plant defense responses

The screening of the chemical library resulted in the removal of non-active compounds that exhibit chemical structures closely related to HPA including their C3-epimers (Fig. 10a, compounds 1–5). With the objective to determine the minimal structural feature required for the elicitation of plant defenses, commercially available animal steroids were also assessed for their ability to induce callose deposition in *A. thaliana*. Strong activity was observed for pregnenolone and its sulfate derivative (Suppl. Fig. S7) whereas androstane steroids (Fig. 10a compounds 6 and 7) did not induce any callose deposition (Suppl. Fig. S8). In contrast, one compound present in the library with a methylamine group on

the C-3 of the A cycle (Fig. 10b) instead of the amino group found in HPA (Fig. 3) was able to induce callose deposition. Based on these observations, we postulate that plant defense can be elicited by 5-pregnen-20-one steroid having an equatorial nucleophilic group in C-3 (Fig. 10c).

Discussion

The primary screen performed on suspension-cultured cells has allowed a faster and easier selection of candidate elicitors than screening on *A. thaliana* plants. We screened 1600 small natural compounds isolated from various plant species on tobacco BY-2 suspension-cultured cells and identified a steroid able to induce ROS production and callose deposition. This was identified as HPA, a steroid isolated from the leaves of the plant species *Holarrhena floribunda* of the Apocynaceae family (Janot et al. 1960). Interestingly, the chemical structure of HPA is closely related to pregnanes that serve as precursors of steroid hormone biosynthesis in mammalian cells.

Plants produce a large variety of small compounds called secondary metabolites (D'auria and Gershenzon 2005; Iriti and Faoro 2009) and several studies have reported that plants can synthesize molecules chemically related to mammalian steroids such as progesterone, 3-*O*-sulfate pregnenolone, 3-epi-gitingensine (Thummel and Chory 2002; Simersky et al. 2009) and others that belong to the pregnane alkaloids or glycosides (C18–C21) (De Leo et al. 2005; Devkota et al. 2008). Supporting this, Rhanier et al. (2006) have characterized an *A. thaliana* bi-functional enzyme, the 3 β -hydroxysteroid dehydrogenase/C-4 decarboxylase (At3- β HSD/D) involved in sterol synthesis. The 3- β HSD/D enzyme is known to convert pregnenolone into progesterone in mammalian cells (Do Rego et al. 2009). This enzyme was the first HSD to be characterized in plants (Rhanier et al. 2006). Moreover, other studies have shown that progesterone does occur in a variety of monocot and eudicot species including *A. thaliana* plants and is able to impact the growth at low doses (Iino et al. 2007). These reports indicate that plants and animals may share evolutionary common steroid-synthesizing pathways, whose products act as inter-kingdoms signaling molecules. Despite the fact that HPA was not detected in untreated *A. thaliana* seedlings and that HPA was first isolated and characterized from plants growing in Central Africa (Janot et al. 1960), this compound may be present in other plants, yet undetermined, growing in the distribution area of *A. thaliana* and/or closely related compounds to HPA are present in *A. thaliana* such as the 5- β -pregnane-3,20-dione (<http://pmn.plantcyc.org/cpd-search.shtml>) produced from progesterone that may have the same activity. Alternatively, HPA may also mimic very closely related steroids originating from fungi, oomycetes and/or herbivores. For example, ergosterol,

one of the main components of fungal membranes, was able to enhance protection of grapes against *Botrytis cinerea* at 200 μ M (Laquitaine et al. 2006). However, it seems unlikely as in our conditions, ergosterol did not improve the protection of *Arabidopsis* against *Pst*. The chemical library screened in this study contained five other compounds structurally close to HPA (compounds 1–5, Fig. 10a). It is noteworthy that among the 16 compounds selected after the primary screen, compound 1 (Fig. 10a) (a stereoisomer of HPA) was selected but was not as effective as HPA in the following screens. In our assay conditions, the other molecules were found to be inactive, showing that the test could discriminate between closely related structures in series. Such specificity was also reported by Granado et al. (1995) using structural analogues of ergosterol. Ergosterol was able to induce rapid extracellular alkalinisation at subnanomolar concentrations on tomato cultured-cells. Other structurally related sterols such as stigmasterol, vitamin D₂, 7-dehydrocholesterol and others were not as efficient at this concentration indicating a specific response. From the comparison of the compounds (Figs. 3, 10), it appears that the stereochemistry of the amino bond in position 3 and the presence of a double bond in position 5–6 are important structural features for the biological activity of HPA. Based on this, the 5-pregnen-20-one steroid with an equatorial nucleophilic group in C-3 appeared to be the minimal structure required for elicitor activity. Further work on the structure–activity relationship should help identifying the active group/moiety of the molecule.

Previous studies have shown that innate immune responses induction and growth inhibition can be linked (Gómez-Gómez and Boller 2000; Boller and Felix 2009). Therefore, we investigated the effects of HPA on seedling growth. Under our conditions, treatments of the seedlings with HPA at 5 μ M induced a retardation of primary root growth. It is noteworthy that *flg22* causes also a retardation of root growth but at much lower concentrations (1 and 10 nM) (Lee et al. 2011). Interestingly, similar effects could be observed on plants inoculated with plant growth-promoting rhizobacteria (Shi et al. 2010; Vacheron et al. 2013). *PR-1* and *PAL-1* are generally considered as molecular markers of the salicylic acid (SA)-dependent pathway, whereas *PR-4*, *LOX* and *PAD-3* are often used as markers of the jasmonic acid (JA)-dependent signaling pathways (Kunkel and Brooks 2002). Our data show that transcriptional responses to HPA and to *flg22* are highly correlated in the induction kinetics for the *PR* genes but the expression fold changes were generally higher with *flg22* than HPA. Like *flg22*, HPA was able to induce the expression of marker genes of both signaling pathways (*PR-1*, *PAL-1*, *PR-4*, *LOX-3*, *PAD-3*). However, the markers of the SA pathway (*PR-1* and *PAL-1*) were consistently much more expressed as compared to JA-induced genes. This suggests that the induction of defense mechanisms by HPA might preferentially occur via the SA-dependent pathway. In

support of this is the observation that HPA does not induce the expression of *WRKY-40*, a modulator of the SA and JA pathways that functions as an activator of the JA-dependent defense pathway and a repressor of the SA signaling pathway (Xu et al. 2006; Zheng et al. 2006).

The hemibiotroph *P. syringae* is a plant pathogen able to suppress the plant innate immune system triggered by MAMPs, such as flg22, by translocating effector proteins through a type III secretion system (Zipfel et al. 2004). The bacterium is known to induce necrotic leaf symptoms in *A. thaliana* and tomato. Under our conditions, the number of colonies was significantly reduced when *A. thaliana* seedlings were treated with HPA prior to inoculation with the bacterium suggesting that the resistance was improved. At the tested concentrations (5 μ M), HPA did not show any antibacterial effect indicating that the induced resistance was essentially due to the activation of *A. thaliana* defense mechanisms. This contrasts with the observed effect of rhamnolipids isolated from *Pseudomonas aeruginosa* that were shown to improve resistance against the necrotrophic fungus *Botrytis cinerea* and the oomycete *Hyaloperonospora arabidopsidis* (Sanchez et al. 2012). Rhamnolipids were shown to have an inhibitory effect on the fungus contributing directly but not exclusively to the protection of the plants.

Both HPA and flg22 triggered callose deposition in leaves of wild-type and *bri1-9* mutant seedlings. Unlike flg22 (Belkhadir et al. 2012), HPA was also able to induce callose deposition in *bak1* and *fls2* seedlings. The *bri1-9* mutation confers only weak insensitivity to BRs (Belkhadir et al. 2010) because of partially functional receptor (Jin et al. 2007). Therefore, we cannot rule out that HPA could be perceived by BRI1. BAK1 is a co-receptor with BRI1 (Wang et al. 2008) and other LRR-RK receptors such as FLS2 (Belkhadir et al. 2012). HPA was able to induce callose deposition in *bak1* seedlings. These data suggest that HPA triggered immune signaling response independently of the BRI1 and BAK1 receptors. However, several reports have shown that in addition to BAK1/SERK3, SERK1 and BAK1-Like1/SERK4 (BKK1/SERK4) can also interact with BRI1 (Karlova et al. 2006; Albrecht et al. 2008; Kemmerling and Nürnberger 2008) and are often functionally redundant (Albrecht et al. 2008). Furthermore, transcripts of SERK1, SERK2, and BKK1/SERK4 are up-regulated in response to PAMP or pathogen inoculation (Postel et al. 2010) supporting a potential role of these SERKs in the innate immunity. Thus, SERK receptors are possible candidates for HPA perception. Another possibility due to the nature of the compound is that HPA may be up taken at the plasma membrane and formed and/or perturbed lipid raft or microdomain structures such as detergent-resistant membrane where the ROS producing NADPH oxidases and receptor-like kinases have been found (Rossard et al. 2010; Takahashi et al. 2013; Klemptner et al. 2014). However, further studies, using, for example, chemical

modification of the compound that may potentially allow target identification, are needed to verify this hypothesis as we cannot rule out that HPA could be perceived by internal receptors in the cytosol or the nucleus as commonly observed for steroid perception in animal cells (Carson-Jurica et al. 1990).

Our findings highlight the ability of plant secondary metabolites, here compounds with a minimal structure of 5-pregnen-20-one steroid closely related to mammalian steroids, to be perceived by plant cells and stimulate defense responses that lead to improved resistance against bacterial infection. Further studies should help elucidate the mechanism of action of the steroid investigated here, HPA, and its ability to stimulate defense responses in other plant species and improve their resistances against other pathogens including necrotrophic or biotrophic bacteria, fungi and oomycetes.

Author contribution statement AZ developed and carried out the chemical screen. AZ performed light microscopy and scanning laser confocal imaging with the help of MVG. AZ performed quantitative PCR analyses. RJ, FL, M-LFG and XL were involved in the phytopathology tests. JL synthesized flg22. OP, SB and CG were implicated in the purification of the natural compounds, supplied the chemical library and provided the compounds. AZ, IR-J and VR were implicated in the quantification of ROS by EPR. AZ, AL and PL were involved in the structure–function studies. PL, AD and J-CM designed the study. AZ, PL, AD and J-C M analyzed the data and AZ, AD and J-CM wrote the article.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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