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The nematode signaling molecule ascr#18 induces prepenetration defenses in wheat against a leaf rust fungus

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Abstract

Leaf rust, caused by the pathogenic biotrophic rust fungus *Puccinia triticina* (*Pt*), is one of the most destructive wheat diseases worldwide; its negative impact on crop yields is exacerbated by increasing temperatures due to climate change. Ascarosides are nematode pheromones that induce resistance to microbial pathogens and pests in a wide range of crops, making them valuable components in biocontrol scenarios. We investigated the effect on infection of various wheat (*Triticum aestivum*) genotypes with the virulent *Pt* race 77W × R by ascr#18, the major ascaroside secreted into the rhizosphere by plant-parasitic nematodes. Spraying the leaves with ascr#18 24 h before inoculation with fungal uredospores slowed disease development and resulted in a reduction of the number of rust pustules on treated compared to untreated leaves. Dose–response analysis over the nano- and micromolar range revealed a broad optimum concentration down to 0.01nM ascr#18. Microscopic analysis showed very early arrest of the fungus at the appressorial stage, with associated enhanced local accumulation of H_2O_2 and abortive stoma penetration. Similarly, ascr#18 also induced strong resistance to *Pt* race PKTTS, confirming its race-unspecific biocontrol activity. The results of this study are consistent with and extend previous research that has shown that ascr#18 activates plant immunity and thus protects plants from pathogens even at very low doses.

Keywords Ascaroside · Biocontrol · Crop protection · Induced resistance · *Puccinia triticina* · Triticum · Wheat

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Introduction

In the face of climate change, political crises, and a growing population, there is an urgent need to secure and even increase wheat production to ensure sustainable food security (Poore and Nemecek 2018; OECD 2020). Leaf rust caused by *Puccinia triticina* (*Pt*) is one of the most common diseases affecting wheat worldwide (Bolton et al. 2008; Huerta-Espino et al. 2011; Kolmer 2013) and is exacerbated by temperature increases (Helfer 2014; Junk et al. 2016; Caubel et al. 2017). Although widely successful in the past, control of leaf rust using conventional measures involving resistance genes (*R* genes) and/or synthetic pesticides becomes ineffective over time because of strong selection pressures on fungal populations in intensive agricultural production systems to overcome *R* gene function or develop resistance or tolerance to pesticides (Andersen et al. 2018; Hawkins et al. 2019; van Esse et al. 2020).

Modern integrative crop protection strategies include methods that rely on the plant's natural immune system (Galli et al. 2024). Plants, like animals, have evolved various innate and acquired immune strategies to combat microbial diseases (Sharrock and Sun 2020; Mermigka et al. 2020). Plants depend primarily on two interconnected layers of the innate immune system to recognize and respond to pathogens (Jones and Dangl 2006; Spoel and Dong 2012; Han 2019). Firstly, pattern-recognition receptors (PRRs) recognize microbe-associated molecular patterns (MAMPs) from a wide spectrum of microbes, resulting in pattern-triggered immunity (PTI). A second layer involves disease resistance (*R*) proteins that recognize pathogen effector molecules or their activities on host targets, resulting in effector-triggered immunity (ETI). In addition, plants can acquire disease resistance by previous encounters with microbes or natural compounds such as plant hormones resulting in systemic acquired resistance (SAR) or induced systemic resistance (ISR), depending on whether the salicylic acid or jasmonate defense pathway is activated (Ryals et al. 1996; Pieterse et al. 2014; Klessig et al. 2018). This knowledge has led to the commercial development of synthetic resistance inducers such as Benzothiadiazole (BTH) or Probenazole that mimic the activity of a natural inducer and help to protect plants against various diseases (Nakashita et al. 2002; Kogel et al. 1994; Görlach et al. 1996; Vlot et al. 2021). More recently, it was discovered that some synthetic resistance inducers and natural compounds such as salicylic acid, β-aminobutyric acid (BABA), or acyl-homoserine lactones (AHLs) at low concentrations trigger induced resistance via defense priming in plants leading to a physiological state that enables plants to respond more rapidly and/or more robustly to a challenge inoculation after exposure to biotic or abiotic stress (detailed reviews see Conrath et al. 2015; Balmer et al. 2015; Baccelli and Mauch-Mani 2016; Cooper and Ton 2022). An early response of primed plants is their production of reactive oxygen species (ROS) at the site of attack when they recognize a pathogen or pest, which is associated with the termination of the invasion or infestation (Balmer et al. 2015). Since a primed plant has only a very limited part of its defense system activated, priming could be an approach to protecting plants from diseases and pests while minimizing energy expenditure and thus yield losses (Westman et al. 2019; Schenck et al. 2014; Jung and Cecchini 2023).

Several recent studies have shown that a family of nematode-derived pheromones called ascarosides induces resistance in many plants against a broad spectrum of pathogens and pests by upregulating specific defense signaling pathways (Manosalva et al. 2015; Ali et al. 2018; Klessig et al. 2019; Ning et al. 2020). The term ascaroside originally referred to a distinct type of lipid first detected in parasitic roundworms of the family *Ascaridia* more than 100 years ago (Flury 1912). Ascarosides serve a wide range of biological functions which is facilitated by a great diversity of ascaroside chemical structures (Ludewig and Schroeder 2013). These are based on the sugar ascarylose, which is linked to fatty acid-like side chains of varying lengths and often decorated further with building blocks derived from amino acids, folate, and other primary metabolites. Plants can metabolize ascarosides and thereby change their chemical message, generating ascaroside mixtures that repel diseases and pests and reduce infection (Manohar et al. 2020; Yu et al. 2021).

Here, we report on the biocontrol effect of ascr#18, the most abundant ascaroside secreted by plant-parasitic nematodes into the plant rhizosphere (Manosalva et al. 2015), on *P. triticina* infections of various wheat (*T. aestivum*) genotypes. Importantly, ascr#18 was effective in the nano- and micromolar range, indicating a broad optimal concentration for controlling *Pt*. Our finding identifies a novel mode of ascr#18-induced resistance by triggering the accumulation of H_2O_2 at attacked stomata, a characteristic also observed with other resistance inducers (Schenck et al. 2015).

Materials and methods

Plant material, fungal inoculation, and ascr#18 treatment

The *P. triticina*-susceptible wheat varieties *T. aestivum* cv. Chinese Spring (spring wheat) and winter wheat Arina LR (both provided by the Julius Kuehn Institute (JKI) Kleinmachnow, Germany), Zentos, Chinofuz (provided by the JKI Quedlinburg, Germany), and Boolani (Seed

and Plant Improvement Institute, Karaj, Iran) were used. The leaf rust *Pt* race 77W × R (Serfling et al. 2013) was a gift of the JKI Quedlinburg. The virulence/avirulence profile of race 77W × R used in field trials and seedling test is found in Rollar et al. (2021). The wheat cv. Boolani is susceptible to *Pt* race PKTTS (Delfan et al. 2022). The profile of virulence/avirulence of race PKTTS is shown in Table S1.

The ascaroside ascr#18 was a gift from Ascribe Biosciences, 95 Brown Rd, Ithaca, NY, USA. For all experiments, wheat plants were grown in a pot containing fine-structured soil (Fruhstorfer Erde type T; HAWITA Gruppe GmbH, Vechta, Germany) in a growth chamber under controlled conditions with the temperature set to 18/20 °C (night/day), light period of 16 h, and 65% relative humidity. Ten-day-old seedlings were sprayed with ascr#18 in an aqueous solution containing 0.1% ethanol until run-off using a hand sprayer (Carl Roth, Germany); control plants were sprayed with 0.1% ethanol. After 24 h, leaves were inoculated by brushing with two-week-old *Pt* uredospores isolated from *T. aestivum* cv. Kanzler, using a mix of rust uredospores and talcum powder (Alliance Chemical, Germany) in a concentration of 1:4 (McIntosh et al. 1995). The inoculated seedlings were grown at 18/20 °C (night/day) with 16 h of photoperiod and 95% relative humidity for three days, followed by 65% relative humidity for seven days. The number of uredinia was evaluated on one leaf per plant in an area of 0.5 cm^2 after 10 days post-inoculation (dpi) by use of a binocular (Leica Microsystems GmbH, Wetzlar, Germany).

Test on direct toxicity of ascr#18

To test whether there is a direct effect of ascr#18 on the germination of *P. triticina*, 9-cm petri dish plates of water agar (3% w/v agar) were pretreated with 2 mL of 1 μM ascr#18 dispensed in 0.1% v/v ethanol using a sprayer (Preval, Art.-Nr. YC44.1). Subsequently, a suspension of *Pt* isolate 77W × R (5 mg of uredospores in 25 mL 0.1% w/v agar) was sprayed onto the agar plates either 15 min or 24 h after ascr#18 application. Inoculated plates were incubated in dark at 25 °C for 10 h at 100% relative humidity. Three plates were prepared for each treatment, and 100 uredospores were examined for germination on each plate. Uredospores were rated as germinated when germ tubes were visible and at least five times the size of the uredospore. Ethanol (0.1%) was used in the absence of ascr#18 as control. Water agar with 0.16 g/L prothioconazole (Proline©, Bayer CropScience) was used as positive control for inhibition of *Pt* germination.

Statistics

For statistical analysis, data were checked for normality. The *t* test for normalized data and Mann–Whitney test for unnormalized data were performed in experiments with two groups to compare. For dose effect experiments, the analysis was done by one-way ANOVA, and multiple comparisons were carried out using Tukey's post-hoc test ($p < 0.05$). All statistical analyses and graphs were done with GraphPad Prism 8 software. For the germination test, data were fitted to a linear model using the function aov (Chambers et al.1992) in R. Tukey honest significant difference test was conducted on the fitted model using the TukeyHSD function ($p < 0.05$; Miller 1981).

Microscopy

Pt-infected leaves (10 dpi) of ascr#18-treated and mock-treated (0.1% ethanol) control plants were fixed in 4% paraformaldehyde (in PBS buffer) or 0.15% trichloroacetic acid (in chloroform:ethanol 20:80, v/v). Fungal structures were visualized using chitin-specific staining with WGA-AF488 (wheat germ agglutinin; Molecular Probes, Karlsruhe, Germany). Leaves were investigated under an epifluorescence microscope (Axio Imager.A2, Carl Zeiss, Oberkochen, Germany) and a confocal laser scanning microscope (CLSM; TCS SP8, Leica Microsystems GmbH, Wetzlar, Germany) by use of ZEISS ZEN 3.8 and Leica LAS X software, respectively. WGA-AF488 was visualized at λ_{exc} 494 nm, λ_{em} 515, and fluorescence control settings were set to λ_{exc} 631 nm, λ_{em} 642. For H_2O_2 detection, *Pt*-infected leaves of ascr#18- and mock-treated plants were collected: 12 hpi, 24 hpi, 48 hpi, and 96 hpi, and samples were stained with 3,3′-diaminobenzidine (DAB)-tetrahydrochloride (Hückelhoven et al. 1999) and subsequently kept in 0.15% trichloroacetic acid (in chloroform:ethanol 20:80, v/v). To evaluate the DAB-stained area, the average size of precipitates on the attacked stomata was quantified using ImageJ free software (https://imagej.net/ij/).

Results

Ascr#18 induces resistance against *Puccinia triticina* in all tested wheat cultivars

The effect of ascr#18 on wheat against leaf rust was first tested with the four cultivars (cvs.): Zentos, Chinese Spring, Arina LR, and Chinofuz. Leaves of 10-day-old seedlings were sprayed with 1μM ascr#18 and, 24 h later,

inoculated with uredospores *Pt* race 77W × R. Ascr#18 significantly reduced the number of *Pt* uredinia on all four wheat genotypes as compared to mock treatment (*t* test for normalized data, Mann–Whitney test for unnormalized data; *p* < 0.05): Zentos (70%), Chinese Spring (71%), Arina LR (77%), and Chinofuz (81%) (Fig. 1; Supplementary Fig. 1). To exclude the possibility that the effect on *Pt* development was due to direct toxic effects of ascr#18, we exposed the fungus to 1 μM ascr#18 on water agar plates for 10 h. Consistent with previous reports on other fungal pathogens (Manosalva et al. 2015; Klessig et al. 2019), *Pt*'s germination rate was unaffected by ascr#18 and was comparable to the control treatments (Table 1). We concluded that ascr#18 induces resistance to leaf rust fungus in the wheat cultivars tested.
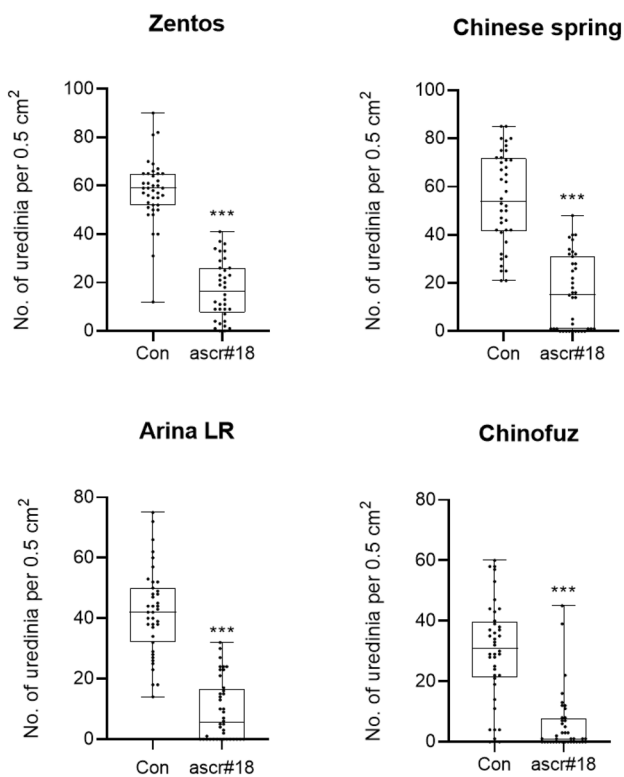
Fig. 1 The ascaroside ascr#18 reduces the number of uredinia in wheat leaves inoculated with the leaf rust fungus *Puccinia triticina*. Leaves of 10-day-old wheat cvs. Zentos, Chinese Spring, Arina LR, and Chinofuz were sprayed with 1 μM ascr#18 in 0.1% ethanol and, 24 h later, inoculated with uredospores of *Pt* race 77W × R. Controls (Con) were treated with 0.1% ethanol. The number of uredinia was counted at 10 dpi. Per experiment, 15 seedlings were treated, each represented by a single dot in the boxplot. The experiment was repeated twice. Minimum/maximum values are represented by whiskers, and center represents the median in the boxplot. Statistics was performed with *t* test for normalized data and Mann–Whitney test for unnormalized data (*p* < 0.05). Asterisks indicate significant differences to the control group (****p* ≤ 0.0001).

Ascr#18 induces resistance against *Puccinia triticina* in the nM range

Next, we conducted a dose–response experiment in the concentration range between 0.000001 and 10 μM ascr#18. Leaves of 10-day-old seedlings of cvs. Chinese Spring and Zentos were sprayed with the respective concentrations of ascr#18 and, 24 h later, inoculated with *Pt* race 77W × R. Ascr#18 significantly reduced the number of uredinia on both wheat genotypes down to a concentration of 0.01 nM (one-way ANOVA, Tukey's post-hoc test; *p* < 0.05) (Fig. 2).

To broaden the agronomic relevance, we extended our investigation to the wheat cv. Boolani, which is susceptible to *Pt* race PKTTS. Ten-day-old seedlings were sprayed with ascr#18, and the dose effect on the number of uredinia at 10 dpi was analyzed. As expected, the number of uredinia was greatly reduced over a wide range of concentrations, suggesting that the effect of ascr#18 on *Pt* is not race-specific (Supplementary Fig. 2).

Ascr#18 induces impaired appressorial stoma penetration

Next, we examined microscopically how fungal growth was inhibited in response to ascr#18 treatment. To this end, cv. Chinese Spring was inoculated with *Pt* race 77W × R and, 10 days later, stained with chitin-specific WGA-AF488 to detect fungal hyphae and infection structures. Fluorescence microscopy at low magnification showed that fungal mycelium formation was greatly reduced and the density of uredinia on the examined leaf section was consistently very low after treatment with ascr#18 (Fig. 3a, b). Moreover, hyphae on the leaf surface were much shorter and barely branched (Fig. 3c, d). Further analysis using confocal laser microscopy (CLSM) showed that penetration of the fungus from an appressorium into the substomatal cavity often failed. Examples of the penetration failure on ascr#18-treated leaves are shown in Fig. 3e–h. Either the fungus did not penetrate the leaf at all (Fig. 3e, f) or, in rare cases, was arrested at the stage of substomatal vesicle formation (Fig. 3g, h). In agreement with this, 3D analysis of leaves using CLSM at 10 dpi showed a strong fungal invasion in the mesophyll of the control plants (Fig. 3i), whereas hardly any fungal structures were found in the mesophyll after ascr#18 treatment (Fig. 3j).

Ascr#18-mediated resistance is associated with enhanced early H$_2$O$_2$ accumulation at stomata

A previous study on the priming activities of AHLs showed that *N*-3-oxo-tetradecanoyl-l-homoserine lactone (oxo-C14-HSL) primed plants for accumulation of phenolic compounds, lignification of cell walls and promoted closure

Table 1 *Pt* uredospore germination in percent (%) of all uredospores per treatment[1]

Treatment	Experiment	Rep 1	Rep 2	Rep 3	Mean	Significance groups[2]
[3]1μM ascr#18 in 0.1 EtOH	1	73.64	71.00	78.85	74.49	a
	2	72.82	71.00	72.17	72.00	
	3	70.87	76.00	65.55	70.81	
[4]1μM ascr#18 in 0.1 EtOH	1	86.51	84.91	78.51	83.31	ab
	2	77.00	73.58	70.30	73.63	
	3	77.00	73,58	71.00	73.86	
0.1% EtOH	1	72.07	77.88	75.70	75.22	ab
	2	77.45	78.64	75.00	77.03	
	3	74.07	77.00	74.00	75.02	
H_2O dest	1	82.20	76.47	78.10	78.92	ab
	2	74.00	76.00	76.00	75.33	
	3	79.00	76.00	71.15	75.38	
H_2O dest $+0.16$ g/L prothioconazol	1	0	0	0	0	c
	2	0	0	0	0	
	3	0	0	0	0	

[1]Evaluation, 10 h after inoculation with *Pt*; [2]treatments with the same letter did not have significantly different germination rates ($p < 0.05$). Application of ascr#18 at 24 h[3] and 15 min[4] before inoculation with *Pt*
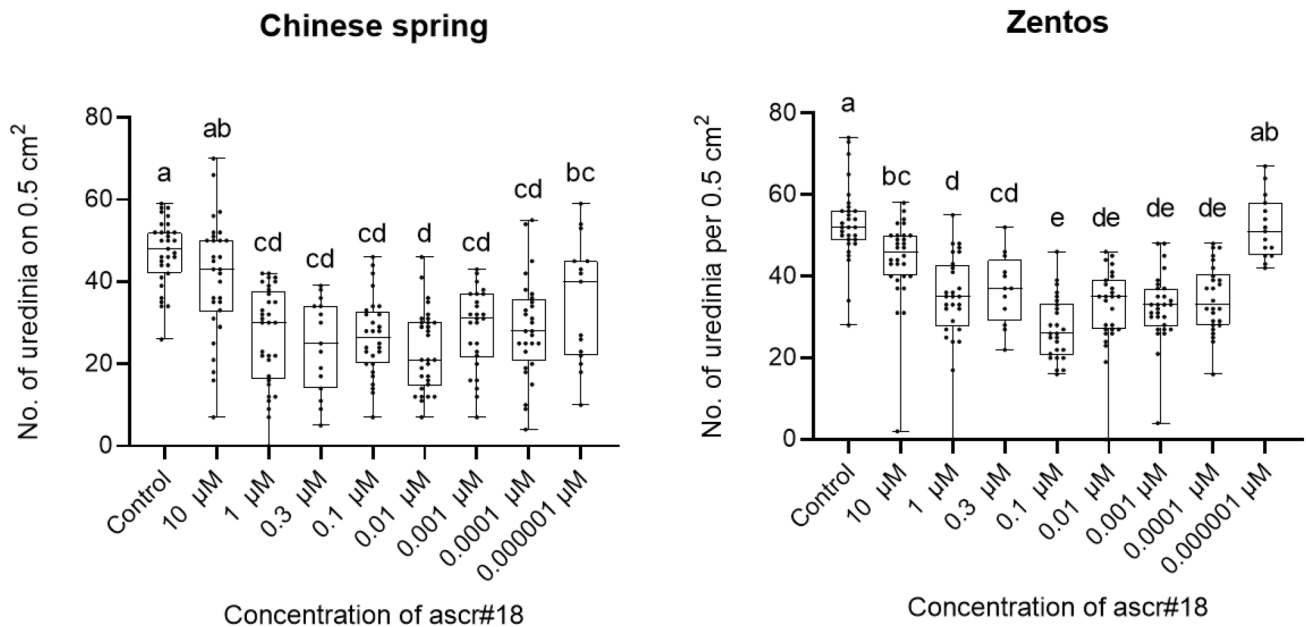
Fig. 2 Dose–response analysis of the effect of ascr#18 on the formation of uredinia in wheat. Leaves of 10-day-old seedlings of cvs. Chinese Spring and Zentos were sprayed with the indicated concentration of ascr#18 dissolved in 0.1% ethanol and, 24 h later, inoculated with uredospores of *Puccinia triticina* race 77W×R. Control seedlings were treated with 0.1% ethanol. The number of uredinia was counted at 10 dpi. Treatments were done on 15 seedlings, each represented by a single data point in the boxplot. The experiment was repeated twice. Minimum/maximum values are represented by whiskers, and center represents the median in the boxplot. Statistics was performed with one-way ANOVA, and boxplots with different letters are significantly different according to Tukey's post-hoc test ($p < 0.05$).
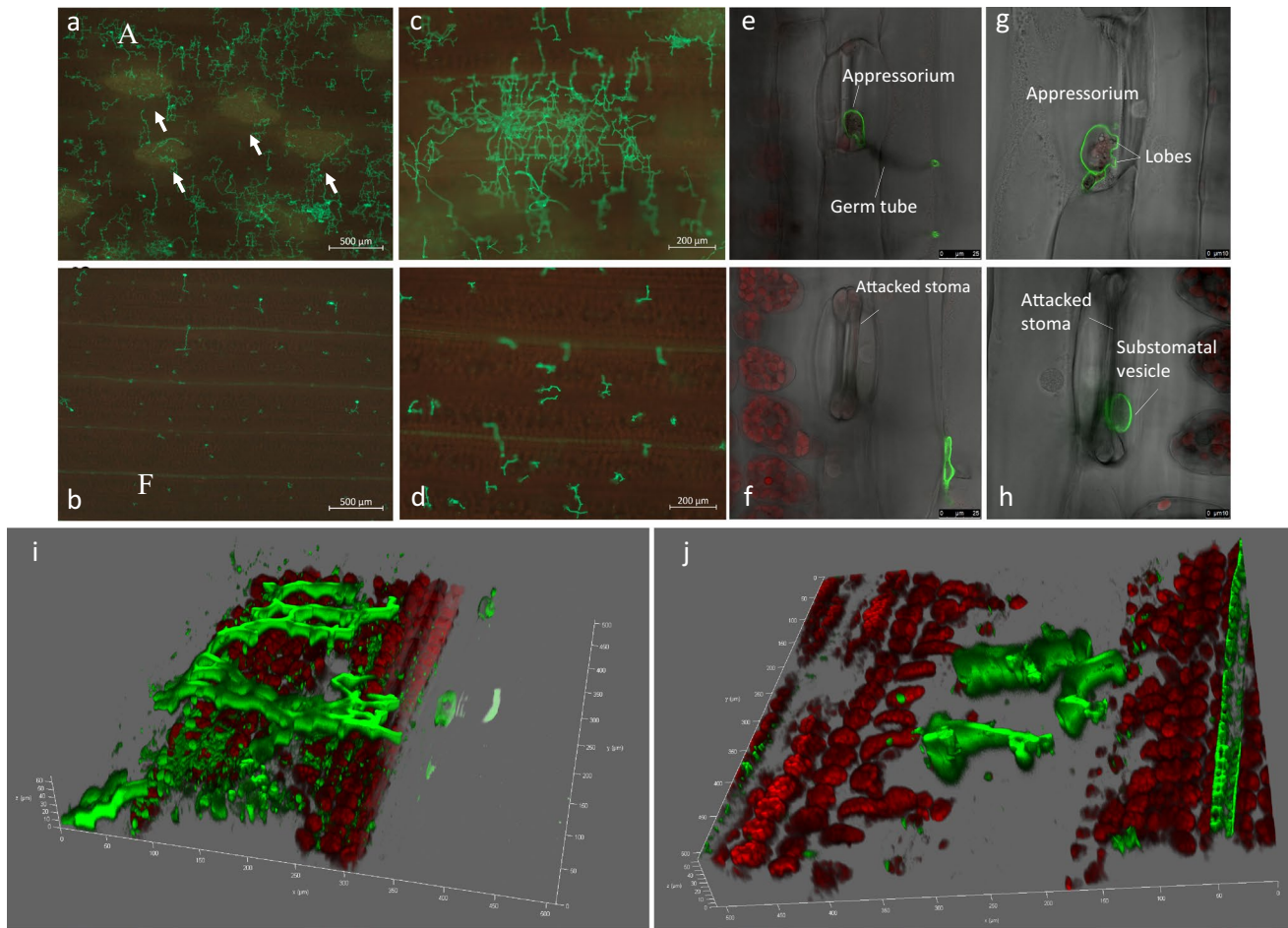
Fig. 3 *Pt* structures on leaves of wheat cv. Chinese Spring as visualized by WGA-AF488 staining. Plants were treated with 0.1% ethanol (**a**, **c**: control) or 1 μM ascr#18 in 0.1% ethanol (**b**, **d**, **e**, **g**, **f**, and **h**) and, 10 days later, harvested for microscopic investigation. On ascr#18-treated leaves, only a few or no uredinia were formed; hyphal development was impaired, and germ tubes were short and barely branched (**b**, **d**). Many appressoria in ascr#18-treated leaves were not able to penetrate the stomata as revealed by CLSM inspection of the fungal infection structures in different layers under the appressorium (**e**, **g**, **f**, **and h**). In the rare cases, penetration of stomata in ascr#18-treated leaves was successful; substomatal vesicles were visible in layers below the appressorium, but formation of primary infection hyphae was not seen (**g**, **h**). Three-dimensional analysis of infected leaves by CLSM revealed heavily infected mesophyll tissue in control plants (**i**), while in ascr#18-treated leaves, barely any fungal structures were found in the mesophyll tissue (**j**). White arrows: uredinia on control leaf.

of stomata in response to *Pseudomonas syringae* infection (Schenck et al. 2014). For wheat leaf rust, *R* gene-mediated prehaustorial resistance in *Triticum monococcum* was also reported to be associated with H_2O_2 accumulation at sites of attempted infection (Serfling et al. 2016). Since we did not detect a hypersensitive reaction (HR) of epidermal or mesophyll cells, nor papillae formation in ascr#18-treated leaves at sites of attempted stomata penetration, we tested the possibility that the arrest of the fungus at this early stage of infection is associated with enhanced H_2O_2. Indeed, DAB-stained leaf samples collected at different times after inoculation with race 77W×R uredospores showed much more H_2O_2 accumulation as revealed by brown precipitate at attacked stomata of plants treated with 1 μM ascr#18 than at stomata of control plants at 12, 24, and 48 hpi (Table 2). Notably, at later time points (96 hpi), little H_2O_2 was detected by DAB staining only, suggesting that the accumulation of hydrogen peroxide is only transient and limited to the site of attempted penetration (Fig. 4).

Discussion

Here, we demonstrate broad and highly efficient resistance-inducing activity of the currently best-studied and most active ascaroside, ascr#18, using a representative set of wheat cultivars and two *P. triticina* races with very different virulence spectra. Recording *Pt* infection on infected leaves

Table 2 Area of brown precipitation due to the accumulation of H₂O₂

Hours post-infection	Control		ascr#18		t test
	Mean area (μm^2)	SD	Mean area (μm^2)	SD	p value
12 h	363.62	412.87	1371.96***	597.74	4.8215E−06
24 h	780.20	1109.81	2039.48**	1080.48	0.00013
48 h	1130.82	920.19	1790.74	1309.43	0.0554
96 h	697.79	651.67	575.17	591.15	0.485

The mean area represents the average area measured from up to 15 infection spots of 7–8 leaves from different plants per repetition. The experiment was repeated twice. SD indicates standard deviation. Statistics was performed with t test for normalized data. Asterisks indicate significant differences to the control group (**$p \leq 0.001$, ***$p \leq 0.0001$)

showed that spray-pretreatment with ascr#18 significantly reduced the number of uredinia as compared to mock-pretreated *Pt*-inoculated plants. A dose–response analysis over the nano- and micromolar concentration range revealed a unusually broad optimum concentration down to 0.01 nM for the control of wheat leaf rust indicating that ascr#18 is a very potent resistance inducer. Moreover, microscopic analysis showed very early abortion of the fungus in the prepenetration stage. This was associated with local accumulation of H₂O₂ as visualized by DAB staining at attacked stomata. It is noteworthy that no papilla formation or HR of epidermal or mesophyll cells could be detected at the site of the attempted penetration. Instead, the fungus did not overcome the appressoric stage in many penetration attempts with the formation of substomatal vesicles in only rare cases. Overall, our results are consistent with the current view that H₂O₂ accumulation and the resulting strengthening of the cell wall and regulation of stomata play a key role in the very early defense responses of plants triggered by resistance inducers (Schenck et al. 2014; for review Balmer et al 2015).

Previous reports showed the strong resistance-inducing effect of ascr#18 in plant protection against a virus (Turnip Crinkle Virus), a bacterium (*P. syringae* pv. *tomato*), a fungus (e.g., *Blumeria graminis* f. sp. *hordei*), an oomycete (*Phytophthora infestans*), and two nematodes (*Heterodera schachtii* and *Meloidogyne incognita*) in four plant species (barley, potato, tomato, and *Arabidopsis*) (Manosalva et al. 2015). In another report, ascr#18 was shown to induce resistance to four crops (wheat, soybean, rice, and tomato) against eight pathogens/pests, including one virus, bacteria, fungi, an oomycete, and a nematode (Klessig et al. 2019), overall suggesting that ascarosides are effective tools that can be used in crop production. References Hoogkamp et al. (1998), Schabdach et al. (2014), Schmittgen and Livak (2008), Yandell (1997). are given in list but not cited in text. Please cite in text or delete them from list. corrected

There are only a few reports on the mode of action of resistance-inducing agents that are effective in controlling rust fungi on cereal crops. The bacterium *Ensifer* (syn. *Sinorhizobium*) *meliloti* induces resistance via priming against *Puccinia hordei* (Matros et al. 2023). Interestingly, the authors compared the priming activity of strain *E. meliloti expR + chthat* which produced large amounts of the AHL 3-*oxo-C14-HSL* with a transformed strain *E. meliloti attM* that does not accumulate AHL, suggesting an AHL-induced *P. hordei* resistance. Interestingly, oxo-C14-HSL in Arabidopsis can induce the oxylipin/SA signaling pathway and thus a stomata defense response and cell wall strengthening, preventing pathogen invasion (Schenck and Schikora 2015). This mode of action is similar to the effect of ascr#18 in our analysis, although a more detailed molecular investigation of the similarities and differences between AHL and ascr#18 is required.

7-oxo- and the 7-hydroxysterols also can induce resistance toward *Puccinia striiformis* and *P. hordei* in barley and wheat when sprayed onto primary leaves using 10^{-4} M in 1% ethanol (Schabbach et al. 2014) two days prior to challenge inoculation with the pathogen. It was suggested that the sterol derivatives selectively activate plant defense mechanisms that impair the development or differentiation of infection structures. Thus, changes in the morphology or chemistry of the cuticle that prevent the formation of appressoria at the stomata could suppress the fungus.

Induced resistance against *P. triticina* has also been achieved by treating wheat (cv. Arina) with the beneficial bacterium *Pseudomonas protegens* CHA0 (by seed coating) and the compound β-aminobutyric acid (BABA) (soil drenching) (Bellameche et al. 2021). BABA was tested at high concentrations (10–20 mM), and a dose-dependent reduction of pustule formation was observed with greatest protection at 20 mM. In light of these results, previous and our current work shows that ascr#18 acts at many orders of magnitude lower concentrations (Fig. 4; Manosalva et al. 2015; Klessig et al. 2019).

Similar to our study, accumulation of H₂O₂ in both CHA0- and BABA-treated plants was mostly detected in host guard cells at penetration sites, and both treatments reduced fungus penetration and haustorium formation. The authors suggested that during recognition or formation of appressoria, generation of H₂O₂ in guard cells is induced,
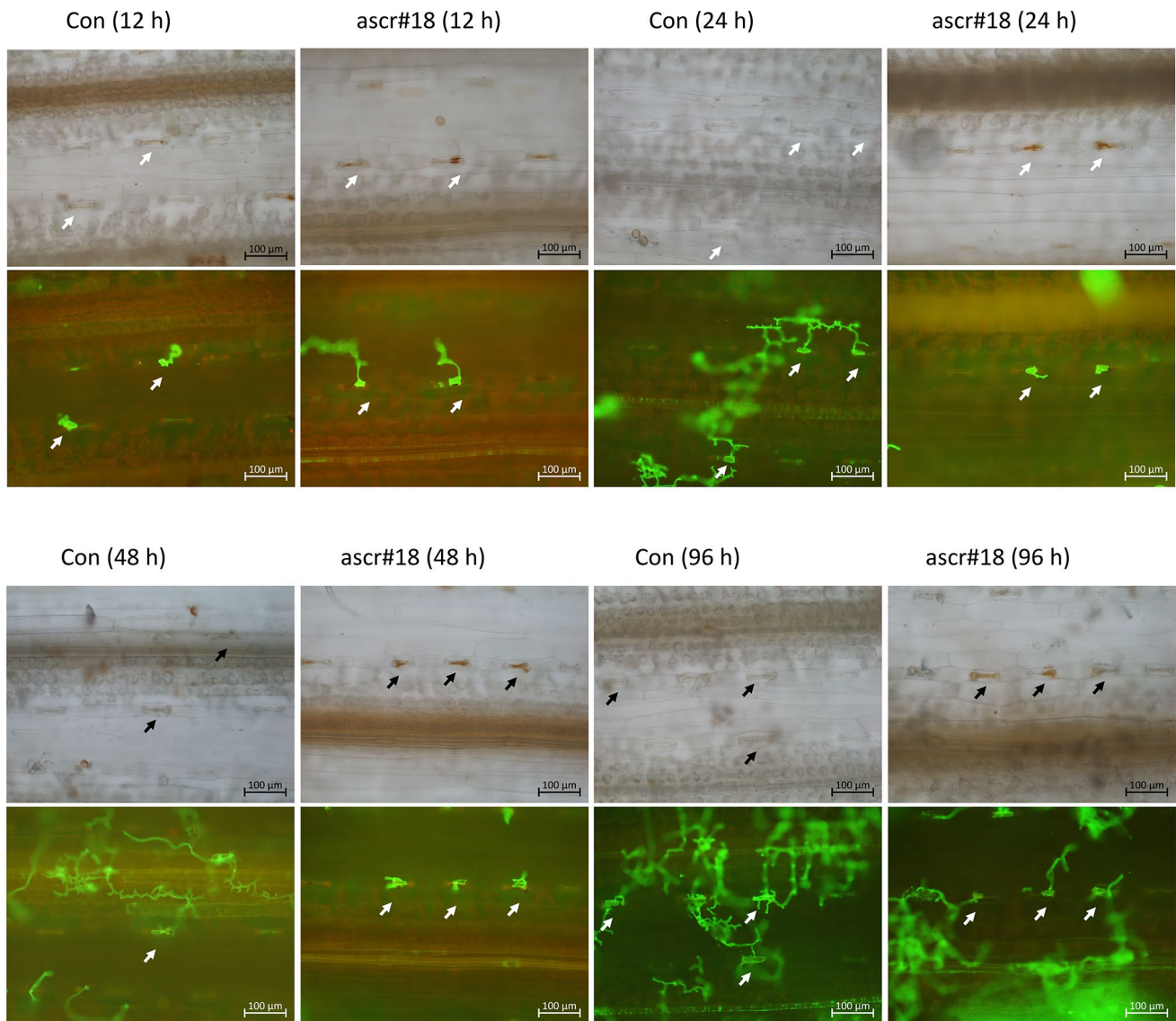
Fig. 4 DAB-mediated visualization of hydrogen peroxide accumulation at the sites of attempted penetration, indicated by a brown precipitate. *Pt* was stained with WGA-AF488. For each sample, brightfield images (top row) show the DAB-based brown precipitate, and fluorescence images (bottom row) show WGA-AF488 fluorescence of the fungal chitin structures. Plants were treated with 0.1% ethanol (Con) or 1 μM ascr#18 in 0.1% ethanol, inoculated 24 h later with *Pt* race 77W×R, and sampled at the time indicated in the image. After 12, 24, and 48 h, a higher accumulation of hydrogen peroxide was observed in the leaves treated with ascr#18 compared to the control samples. After 96 hpi, only little accumulation of hydrogen peroxide was detected in ascr#18-treated plants. White arrows indicate attacked stomata.

possibly following secretion of rust effectors, and mechanical forces during adhesion of appressoria over stomata may also elicit H_2O_2 generation in guard cells (Bellameche et al. 2021). In Arabidopsis, H_2O_2 accumulation in guard cells was involved in signal transduction during ABA-mediated stomatal closing (Sun et al. 2017). Similarly, appressorium formation of *P. triticina* also caused stoma closure in wheat leaves (Bolton et al. 2008).

In conclusion, ascr#18 enables induction and modulation of different signaling pathways to activate immune responses in plants at very low concentrations. Thus, ascr#18 has an interesting potential as biological control agent to reduce disease damage and increase sustainable food security.

WheatInterfere of the Bundesministerium für Bildung und Forschung (BMBF), Germany, to KHK.

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability Not applicable.

Declarations

Conflict of interest The research described in the manuscript was not funded by private partners or industry. Authors Akshita Kamboj (she), Jennifer Thielmann (she), Saba Delfan (she), and Karl-Heinz Koge (he) declare that they have no conflict of interest. Authors Murli Manohar, Frank Schroeder, and Daniel Klessig are co-founders of Ascribe Bioscience, a company that develops plant treatments based on small molecules from microbiota.

Consent for publication All authors declare consent of publication.

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