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The Arabidopsis defense response mutant *esa1* as a model to discover novel resistance traits against *Fusarium* diseases

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Abstract

The *Arabidopsis thaliana* mutant *esa1* was previously shown to exhibit enhanced susceptibility to the necrotrophic fungal pathogens *Alternaria brassicicola*, *Botrytis cinerea* and *Plectosphaerella cucumerina*. In this work, we tried to elaborate on this susceptibility by investigating whether the *esa1* phenotype can be extended to *Fusarium* species, a genus that includes several economically relevant pathogens. We show that the *esa1* mutant exhibits increased susceptibility to several *Fusarium* species, including *Fusarium oxysporum* f. sp. *matthiolae*, *F. solani*, and *F. culmorum*. Furthermore, we show that the causal agent of the Panama disease on banana, *F. oxysporum* f. sp. *cubense*, a pathogen for which wild-type *A. thaliana* shows non-host resistance, causes enhanced lesion formation on *esa1* as compared to wild-type plants, suggesting that *esa1* is more sensitive to *F. oxysporum* f. sp. *cubense*. In addition, we were able to show that the *A. thaliana* wild-type resistance phenotype towards the latter pathogen can be partially restored by expression of the pathogenesis-related proteins PR1 or PR5 from tobacco in *esa1*, suggesting that *PR1* and/or *PR5* expression may be useful traits to obtain enhanced resistance to *F. oxysporum* f. sp. *cubense* in banana. As such, *esa1* proves to be an ideal model system for research on the plant's defense response against fungal pathogens in general and *Fusarium* species in particular.
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Keywords: *Arabidopsis*; *Fusarium* spp.; *Fusarium oxysporum* f. sp. *cubense*; Disease resistance; *esa1*

1. Introduction

The genus *Fusarium* contains over 20 species of filamentous fungi widely distributed on plants and in the soil [1]. Most *Fusarium* species are found in (sub)tropical areas, but some species inhabit soils in cold climates. *Fusarium* is one of the most important genera of fungal plant pathogens, causing devastating diseases like *Fusarium* wilt and *Fusarium* root/stem rot in numerous economically important crops [1–3]. One of the most common *Fusarium* pathogens, *Fusarium solani*, is a broad spectrum soil pathogen which is the causal agent of root and stem rot diseases of various vegetables [1,4,5]. An important disease caused by this pathogen leading to substantial yield reduction, is the 'sudden death syndrome' of soybean [1,4]. In addition, *Fusarium* root and stem rot diseases also cause enormous yield losses and quality reductions of cereals [2,6]. The total estimated losses in the US between 1991 and 1997 due to *Fusarium* head blight or Scab, caused by *F. graminearum* and *F. culmorum*, amounted to $1.3 billion [7]. In addition, these *Fusarium* species also produce mycotoxins that are harmful to human and animal health [8,9]. Such mycotoxins, including zearalenone and trichothecenes produced in *Fusarium*-infected cereals and legumes, have been proven to cause acute disease symptoms (mycotoxicoses) on humans and animals after ingestion. Even more seriously might be chronic effects like immune-suppression and cancer caused by subacute dosages of these mycotoxins in food and feed [1,10–12].

On the other hand, most of the *Fusarium* wilt diseases are caused by *Fusarium* fungi belonging to the species *Fusarium*

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oxysporum. For example, *Fusarium* wilt on tomato, caused by *F. oxysporum* f. sp. *lycopersici*, is one of the most damaging diseases of tomato [1]. Besides dicotylous plants, also monocotylous plant families can be affected by *F. oxysporum* strains. The Panama disease of banana is a vascular wilt disease caused by the fungal pathogen *F. oxysporum* f. sp. *cubense*. It ranks as one of the most destructive plant diseases of all time and is still a serious threat to subsistence production [3,13,14].

Since many of the *Fusarium* pathogens are soil-born that, in the field, cannot be controlled by means of fungicides, new strategies to reduce the impact of *Fusarium* diseases are needed. A possible solution is the development of resistant cultivars based on the introduction of resistance genes [15–19]. In order to unravel the plant's defense response against *Fusarium* species and to identify novel resistance traits, we make use of the model plant *Arabidopsis thaliana* [20]. *A. thaliana* is a flowering plant belonging to the *Brassicaceae* that are susceptible to some *Fusarium* species. Epple et al. [21] documented that wild-type *in vitro* grown Arabidopsis plants developed disease symptoms after inoculation with *F. oxysporum* f. sp. *matthiolae*. Even the wheat-attacking fungal pathogens, *F. graminearum* and *F. culmorum* were shown to develop analogous disease symptoms on wild-type Arabidopsis plants after flower-inoculation in a similar fashion as on cereals [22]. Recently, the Arabidopsis *esa1* mutant (*e*nhanced *s*usceptibility to *Alternaria brassicicola*) that displays enhanced susceptibility towards a number of necrotrophic fungal pathogens, was isolated [23]. Although the *ESA1* gene could not yet be identified, this mutant can be used as a model system to study *Fusarium* disease resistance mechanisms and to select (heterologous) resistance traits. In this work we show that the *esa1* mutant exhibits increased susceptibility to several *Fusarium* pathogens and that this susceptibility can be partially restored by the overexpression of specific pathogenesis-related (PR) genes.

2. Methods

2.1. Growth of plant material

Seeds of *A. thaliana* wild-type Columbia (Col0) were provided by the Nottingham Arabidopsis Stock Center (NASC). *Esa1* seeds are M5 progeny from EMS mutated Col0 received from Lehle Seeds and are available at the Centre for Microbial and Plant Genetics. Seeds of both lines were sown in DCM 'Zaai-en Stekgrond' (Sint-Kathelijne-Waver, Belgium) and were placed in phytotrons with a temperature of 21 °C, 75% humidity and a 12 h day–light cycle with a light intensity of approximately 120 μmol m^{-2} s^{-1}.

2.2. Fusarium strains

The following *Fusarium* strains were used: *F. oxysporum* f. sp. *cubense* race 1 (IMI, 141109), race 2 (IMI, 141113), race 4 (kindly provided by Dr. Ching-Yan Tang (TBRI, Taiwan)), *F. oxysporum* f. sp. *matthiolae* (CBS, 247.61), *F. oxysporum* f. sp. *raphani* (CBS, 488.76), *F. graminearum* (PGS, F35; MUCL, 30161), *F. culmorum* (PGS, F15; IMI, 180420) and *F. solani* (kindly provided by Bart Lievens (Scientiae Terrae, Sint-Kathelijne-Waver, Belgium)). *F. solani* and all *F. oxysporum* strains were grown on half-strength potato dextrose broth (PDB) agar plates at 22 °C. *F. graminearum* and *F. culmorum* were grown on solid cereal agar (CA)-medium at 22 °C. After 2 weeks of growth, spore harvesting of the fungi was done as described previously [24].

2.3. Plant inoculations

Leaf and root dip inoculations were performed on 4-week-old soil-grown Arabidopsis plants, while flower inoculations were done on 6-week-old flowering plants. For flower-inoculations flowers and buds of flowering Arabidopsis wild-type and *esa1* mutant plants were spray inoculated with a suspension of 5×10^5 spores/ml water [22]. Leaf inoculation assays were done by drop-inoculation, thereby spotting one 5 μl drop containing 5×10^5 spores/ml water on each leaf. Root dip inoculation assays were done as follows. Plants were pulled out of the soil, their roots were rinsed in water and subsequently dipped in a solution of the respective spore suspension (5×10^5 spores/ml water) for 10 s and replanted into fresh soil. After inoculation, plants were kept at 100% relative humidity throughout the experiment (leaf and flower inoculations).

Application of fusaric acid (commercially available, Sigma–Aldrich) was done by drop inoculation, applied as 5 μl drops of the toxin (at 5 μM) on all leaves of Arabidopsis plants. For the application with the culture filtrate of *F. oxysporum* f. sp. *cubense* race 4, one Erlenmeyer flask with 100 ml Czapek-Dox culture medium, prepared as described [25], was inoculated with one mycelium PDB agar disk and statically incubated at 28 °C for 4 weeks. The liquid culture medium was filtered over miracloth and centrifuged (8000 rpm, 20 min). The supernatant was passed through a membrane filter (22 μm pore ∅) to remove remaining fungal structures. The culture filtrate was concentrated to 20% of the original volume by evaporation and applied on leaves as 5 μl drops of the concentrated filtrate as described [25,26].

2.4. Disease assessments on Arabidopsis

Disease symptoms were scored either by visual or digital analysis techniques. Visual analysis of the developing symptoms was performed by assessing the percentage of infected branches, infected leaves or the average diameter of the developing lesions at specific time points after inoculation and depending on whether floral or foliar inoculations were performed. To quantify the amount of disease symptoms visible on Arabidopsis plants after flower inoculation, a numerical scoring system developed by Urban et al. [22] was used with few modifications. No distinction was made between new and old siliques. To determine the degree of disease development, the infection process was divided into two parts, flower infection (F) and silique infection (S). The final *Fusarium*-Arabidopsis disease (FAD) value was calculated by addition of

the two component scores. FAD value = (F + S)/# branches. The diameter of the lesions appearing after leaf inoculation assays was measured by a high precision vernier calliper. For root inoculation assays, plants were scored for chlorosis on, and necrosis of the leaves.

For digital analysis of the disease symptoms image analysis software for plant disease quantification ('ASSESS', by Lamari, APS Press) was used. This software determines the percentage of lesion area on each leaf starting from digital images generated with a digital camera (DSC-S70 camera, Sony).

2.5. DNA or RNA isolation from leaves and cDNA synthesis

For real-time PCR purposes, DNA was extracted out of 100 mg grinded powder from entire leaf canopy of two infected plants, frozen in liquid nitrogen, in triplicate. For PCR analysis of primary transformants, DNA was extracted from 1 leaf of 10 individual plants from each population. DNA isolation was done using Qiagen DNeasy Plant kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.

For RT-PCR analyses, total RNA was isolated from 1 leaf of 10 individual plants each using Concert plant RNA reagent (Invitrogen, Paisley, UK), according to the manufacturers instructions. The dry pellet was dissolved in 50 μl RNase free water and stored at −80 °C. Prior to cDNA synthesis, the RNA samples were subjected to DNaseI treatment (New England Biolabs, Beverly, MI), to remove residual DNA, according to the manufacturers instructions. For the synthesis of cDNA, up to 1 μg of total RNA was subjected to a reaction with M-MuLV reverse transcriptase (New England Biolabs, Beverly, MI) in a volume of 30 μl, according to the manufacturer's instructions. The cDNA was used immediately in RT-PCR reactions.

2.6. Fungal biomass quantification

For the determination of fungal biomass, a serial DNA dilution extracted from the *in vitro* grown respective pathogen was used as a standard, as described in Ref. [27]. The following primers were used: primers AFP305 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and AFP346 (5′-GGTATGTTCACAG GGTTGATG-3′) for the identification of the fungal biomass of *F. solani*, AFP14 (5′-AGTATTCTGGCGGGCATGCCTGT-3′) and AFP15 (5′-ACAAATTACAACTCGGGC CCGAGA-3′) for the identification of fungal biomass from the different *F. oxysporum* strains. For each PCR reaction, samples contained 1 μl DNA extract, 1 μl of both primers (10 μM), 4 μl of master mix (containing FastStart *Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye and $MgCl_2$; Roche Diagnostics, Basel, Switzerland) and 14 μl water. Forty-five cycles of amplification (95 °C denaturation for 10 s, annealing for 5 s at appropriate temperature and 72 °C polymerization for appropriate times) were carried out in sealed LightCycler Capillaries in a LightCycler (Roche Diagnostics, Basel, Switzerland). Annealing was performed at 60, 68, 68 °C for the amplification of *F. solani*, *F. oxysporum* f. sp. *matthiolae* and *F. oxysporum* f. sp. *cubense*, respectively. Polymerization was done during 6, 15, 15 s for the amplification of *F. solani*, *F. oxysporum* f. sp. *matthiolae* and *F. oxysporum* f. sp. *cubense* DNA, respectively. For each pathogen, the fungal biomass on each plant line was calculated as the average detected biomass on three samples per plant line containing each a mix of two inoculated plants.

2.7. Construction of plasmids

The coding regions of the genes encoding the pathogenesis-related proteins PR1 and PR5 were isolated from tobacco [28,29] by means of PCR-analysis. The respective coding regions were cloned in the plasmid pFAJ3551 containing the constitutive [u*OCS*]p*MAS*-promotor and the terminator of the mannopine synthase gene from *Agrobacterium tumefaciens* [30]. Subsequently, the expression cassettes were excised from these vectors with the endonucleases *Pme*I and *Eco*RI (New England Biolabs, Beverly, MI) and introduced in the plant transformation vector pFAJ3481 containing the coding sequence of the phoshino-acetyltransferase gene as selection marker. The resulting plant transformation vectors were each introduced by electroporation in *A. tumefaciens* GV3101, containing the pMP90 helper plasmid.

2.8. Transformation of A. thaliana

The *Agrobacterium*-mediated floral dip method was used to transform flowering *A. thaliana esa1* mutant plants [30,31]. Primary transformants were selected based on resistance against phosphinotricin (5 mg/l, Basta; Bayer, Leverkusen, Germany) and used for further analysis.

2.9. (RT-)PCR-analysis on genetic transformants

PCR-analysis was done on the primary transformants to confirm the insertion of the respective transgenes. Primers amplifying the coding region of the gene were chosen: OWB461 (5′-CTCGCTCGCCCACCATGGGATACTCCACA-ACATTAG-3′) and OWB462 (5′-CTGGTTCGGCCCAGAGC-TCTTCAGTAGGGACGTTGTCCTCTCC-3′) for *PR1*, OWB467 (5′-CTCGCTCGCCCACCATGGGCAACTTGA-GATCTTC-3′) and OWB468 (5′-CTGGTTCGGCCCA-GAGCTCTTCACTTAGCCACTTCATCACT-3′) for *PR5*.

For the study of transgene expression of *PR1* and *PR5* in primary transformants, RT-PCR was performed on undiluted cDNA. The Arabidopsis housekeeping gene *EF1α* was used as an internal control. The following primers were used: AFP498 (5′-TGAGCACGCTCTTCTT GCTTTCA-3′) and AFP499 (5′-GGTGGTGGCATCCATCTTGTTACA-3′) for the amplification of *EF*1α, OWB461 and OWB462 for the amplification of *PR1* and OWB467 and OWB468 for the amplification of *PR5*.

Each (RT-)PCR sample contained 1 μl DNA template or 8 μl cDNA template, respectively, in a total volume of 50 μl. The *Taq* DNA polymerase (New England Biolabs, Ipswich, MA, UK) of *Thermus aquaticus* was used. Thirty-five cycles of amplification (95 °C denaturation for 5 min, 57 °C annealing for 30 s and 72 °C polymerization for 50 s) were carried out in a

P × 2 Thermal cycler (Thermo Electron corporation, Milford, MA, USA). For *PR1* amplification, the PCR amplification reaction was additionally preceded by five PCR cycles with an annealing temperature of 47 °C.

2.10. Statistical analysis

All tests were performed at significance level 0.05 using SAS software. To compare the susceptibility levels of wild-type and *esa1* plants towards *Fusarium* pathogens and the differences in expression levels or fungal biomass, a Kruskall–Wallis ANOVA test was performed. Additionally, to compare the susceptibility levels of wild-type and *esa1* plants towards different inoculums, Proc Mixed analysis was performed on the different data sets.

3. Results

The *esa1* mutant was previously isolated in a screen of EMS-mutagenised (M2) Arabidopsis wild-type (Col0) plants for enhanced susceptibility to the necrotrophic foliar pathogen *A. brassicicola* [23]. Initial disease spectrum characterization of the *esa1* mutant pointed out that this mutant is also more susceptible to the necrotrophic pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*, but exhibits wild-type levels of resistance towards the biotrophic pathogens *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica*. The susceptibility phenotype was found to correlate with a delayed induction of both phytoalexin accumulation and the gene encoding plant defensin PDF1.2 upon inoculation with pathogens [23]. To determine whether the *esa1* mutant can be used as a model system to elucidate disease response mechanisms towards *Fusarium* species, we challenged this mutant with several economically important *Fusarium* pathogens.

3.1. The esa1 mutant shows enhanced susceptibility to the cereal pathogen F. culmorum

Previous experiments pointed out that wild-type Arabidopsis plants are susceptible towards two important cereal pathogens *F. graminearum* and *F. culmorum* [22]. We tested whether the susceptibility of the *esa1* mutant towards these two pathogens was altered as compared to wild-type plants. Inoculations with these pathogens were done on flowering Arabidopsis plants as described [22]. At different time points after inoculation, plants were scored according to the numerical scoring system described by Urban et al. [22] with some modifications (see Section 2). Three days after inoculation of *esa1* and wild-type plants with *F. graminearum*, disease symptoms appeared on flowers of both plant lines. On wild-type plants, mycelium of the pathogen was visible on numerous flowers. The severest symptoms, including desiccation of flowers and stem constriction of flower heads, appeared more abundant on *esa1* plants, a trend that was still visible 9 days after inoculation. At that time point in the development of the disease, more severe symptoms appeared on *esa1* plants represented by a higher FAD value for *esa1* plants compared to wild-type plants (Fig. 1A). On the other hand, wild-type plants had more branches with few disease symptoms but none of these differences in disease symptoms were statistically significant with regard to the mean FAD value (Fig. 1A).

Similar observations were done after inoculation with *F. culmorum*. Few disease symptoms were visible on wild-type and *esa1* plants 3 days after inoculation. Only about 1 and 7% of the branches for wild-type and *esa1* plants, respectively, showed drying of the flowers (data not shown). Nine days after inoculation there was a significant difference in disease symptoms visible on wild-type and *esa1* plants as calculated by the mean FAD value (0.46 and 5.65, respectively, Fig. 1B). On nearly all branches of the *esa1* plants, drying of flowers was detected and about 34% of the branches exhibited stem restriction of flower heads. On the other hand, drying of flowers and stem constriction of the flower heads appeared merely on 10 and 0%, respectively, of the branches of wild-type plants.

These results confirm that wild-type Arabidopsis is susceptible to the cereal pathogens *F. graminearum* and *F. culmorum* as previously observed [22], and additionally indicate that the *esa1* mutant is clearly more susceptible to the cereal pathogen *F. culmorum* but not significantly to *F. graminearum*.

3.2. The esa1 mutant exhibits enhanced susceptibility to the broad-spectrum pathogen F. solani and to the Brassicaceae pathogen F. oxysporum f. sp. matthiolae

To further elucidate the susceptibility spectrum towards other *Fusarium* species, the *esa1* mutant was also challenged

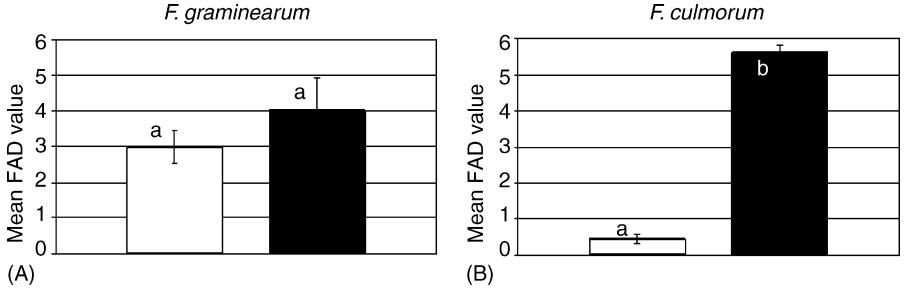

Fig. 1. Percentage of affected plant branches 9 days after flower inoculation of Arabidopsis wild-type and *esa1* plants with *F. graminearum* (A) and *F. culmorum* (B). White bars indicate wild-type plants and black bars *esa1* plants. Disease scoring was done by determining mean FAD values. (A and B) Average disease progression with standard errors (±S.E.) of three independent experiments in the greenhouse on 12 plants each. Different letters indicate that the corresponding data are different ($P < 0.05$) according to Kruskal–Wallis test.

with *F. solani* (broad-spectrum pathogen), *F. oxysporum* f. sp. *matthiolae* and *F. oxysporum* f. sp. *raphani* (both pathogens of *Brassicaceae*).

Although the life cycle of most *Fusarium* pathogens largely revolves in the soil, we chose to perform leaf inoculation assays for reason of ease, reproducibility and comparability. This type of *Fusarium* inoculation assays was also previously documented for Arabidopsis by Epple et al. [21,32]. Both the percentage of infected leaves and diameter of necrotic lesions on leaves were scored at specific days post-inoculation. In general, disease symptoms started to develop on the infected tissues about 6 days after inoculation with the different pathogens, which is comparable to earlier described infection progression with the *Fusarium* leaf inoculation assay [32].

Six days after inoculation with the broad spectrum pathogen *F. solani*, clear symptoms appeared both on the wild-type and *esa1* leaves, which enlarged during the infection process. Twelve days after inoculation, 45% of the inoculated *esa1* leaves and 22% of the inoculated wild-type leaves clearly showed necrotic lesions (Fig. 2A). The average diameter of lesions on the *esa1* plants 12 days after inoculation was about two-fold larger than on wild-type plants (*P*-value: 9.6×10^{-4}; Fig. 2B). This lesion increase indicates that *esa1* exhibits increased susceptibility towards *F. solani* as compared to the wild-type plants.

Similarly, the lesions caused by *F. oxysporum* f. sp. *matthiolae* gradually enlarged and developed on average two-fold larger necrotic spots on *esa1* leaves than on infected wild-type leaves at 12 days after inoculation (Fig. 2B). At this time point, about 40% of the inoculated *esa1* leaves showed symptoms, whereas on only 25% of the inoculated wild-type leaves lesions developed (Fig. 2A). The lesion diameter on *esa1* was statistically significantly different from the lesions on wild-type plants (*P*-value: 0.013), suggesting *esa1* shows enhanced susceptibility to this pathogen as well.

In contrast, however, the average lesion diameter on wild-type plants inoculated with *F. oxysporum* f. sp. *raphani* enlarged up until day 12 after inoculation but no clear increase in average lesion diameter was detected on *esa1* leaves from 6 to 12 days after inoculation (data not shown). Twelve days after inoculation only 14 and 4% of the *esa1* and wild-type leaves, respectively, developed necrotic lesions (Fig. 2A) while lesion diameter visually appeared larger on *esa1* plants than on wild-type plants (Fig. 2B). This was also observed at 6 and 9 dpi (results not shown), but the difference was never statistically significant, suggesting that the susceptibility of *esa1* to *F. oxysporum* f. sp. *raphani* is not significantly altered as compared to wild-type plants.

To increase objectivity and reproducibility of the disease assessment, image analysis software for plant disease quantification (“ASSESS”, Lamari, American Phytopathological Society) was applied on digital pictures of leaves with lesions to determine the percentage of infected leaf area. Exemplary, Fig. 3 shows the disease symptoms on *esa1* and wild-type plants 12 days after inoculation with *F. oxysporum* f. sp. *matthiolae* (Fig. 3A) and the calculation of infected leaf area by ASSESS (Fig. 3B). Challenging *esa1* plants with this pathogen resulted in a two-fold increase of infected leaf area compared to wild-type plants (*P*-value: 3.9×10^{-2}; Fig. 3B), confirming our conclusions from the visual disease assessment stating that the mutant *esa1* displays larger lesions in response to *F. oxysporum* f. sp. *matthiolae* as compared to wild-type plants. Similar results were obtained for *F. solani* (data not shown).

In addition, to study the difference between wild-type and *esa1* at the level of pathogen proliferation on the leaves, we estimated the relative biomass of *F. solani* and *F. oxysporum* f. sp. *matthiolae*, at 6 days post-inoculation using real-time PCR as described [27]. The results in Fig. 4A show that significantly more *F. solani* pathogen biomass is present on the *esa1* plants as compared to wild-type plants. Similar results were obtained for *F. oxysporum* f. sp. *matthiolae* (data not shown). These data demonstrate that both pathogens are able to proliferate better on *esa1* as on wild-type plants and suggest that the larger necrotic

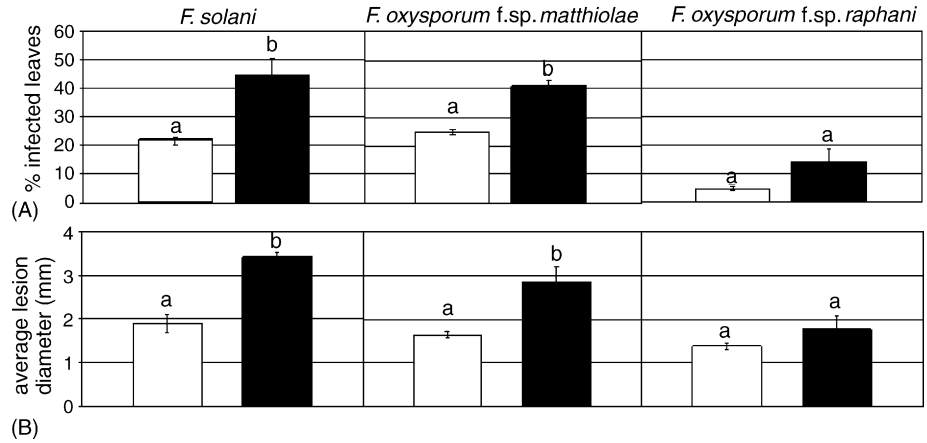

Fig. 2. Quantification of disease development in *Arabidopsis thaliana* wild-type and *esa1*, inoculated with different *Fusarium* pathogens. *F. solani*, *F. oxysporum* f. sp. *matthiolae* and *F. oxysporum* f. sp. *raphani*. Data represent percentage of infected leaves (A) and average diameter of lesions (B) formed 12 days post-inoculation with standard errors (±S.E.) based on three independent experiments in phytotron on twelve plants each. White bars indicate wild-type plants and black bars *esa1* plants. Different letters indicate that the corresponding data are different ($P < 0.05$) according to Kruskal–Wallis test.

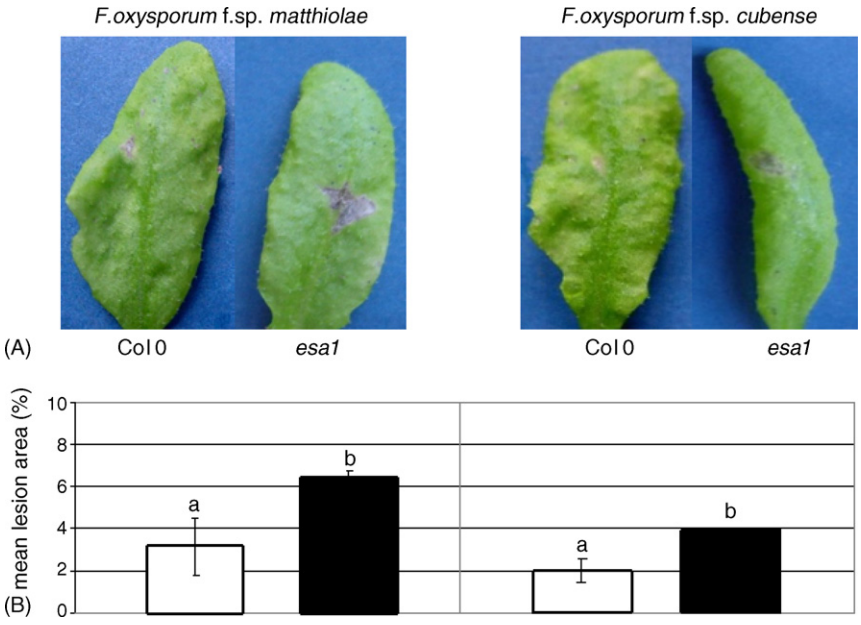

Fig. 3. Lesion development on *esa1* as compared to wild-type after challenging the plants with *F. oxysporum* f. sp. *matthiolae* and *F. oxysporum* f. sp. *cubense* race 4. (A) Disease symptoms developed on wild-type (Col0) and *esa1* 12 days after inoculation with *F. oxysporum* f. sp. *matthiolae* and *F. oxysporum* f. sp. *cubense* race 4. (B) Quantification of infected leaf area on wild-type (Col0) and *esa1* by means of ASSESS 12 days after inoculation with *F. oxysporum* f. sp. *matthiolae* and *F. oxysporum* f. sp. *cubense* race 4. Data represent average disease progression of three independent experiments on six leaves each; standard error bars (±S.E.) are indicated. White bars indicate wild-type plants and black bars *esa1* plants. Different letters indicate that the corresponding data are different ($P < 0.05$) according to Kruskal–Wallis test.

lesions on *esa1* leaves are indeed caused by an increased susceptibility phenotype of the mutant to the respective pathogens.

In conclusion, *esa1* is more susceptible to *F. solani* and *F. oxysporum* f. sp. *matthiolae* as compared to wild-type.

3.3. *esa1* exhibits enhanced lesion progression upon inoculation with the heterologous (banana) pathogen *F. oxysporum f. sp. cubense*

In general, a given plant species is host for only a minor subset of micro-organisms. Resistance to micro-organisms outside this subset is termed non-host resistance and potentially phytopathogenic micro-organisms incapable of infecting any cultivar of a given plant species are referred to as heterologous pathogens [33,34]. In addition to the observed increased susceptibility of *esa1* to several pathogens of the genus *Fusarium*, we therefore investigated the possible susceptibility of *esa1* to the heterologous pathogen *F. oxysporum* f. sp. *cubense*, causal agent of the Panama disease on *Musa* spp. (banana). Strains of this pathogen have been classified into four different races. This subdivision is based on their ability to cause disease on different types of host plants in the field. Races 1, 2 and 4 are pathogenic on respectively 'Gros Michel', 'Bluggoe' and 'Cavendish' banana cultivars while race 3 is pathogenic on *Helioconia* spp. [35]. In this study we specifically focussed on the *Fusarium* races (1, 2 and 4) of banana.

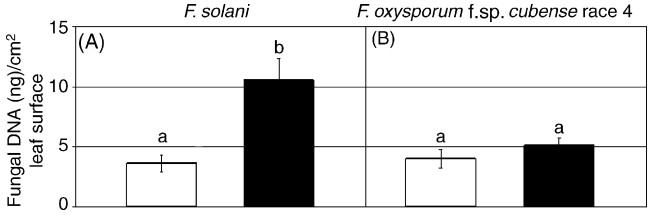

Fig. 4. Quantification of *Fusarium* biomass on wild-type and *esa1* infected leaves. Data represent relative fungal biomass of *F. solani* (A) and *F. oxysporum* f. sp. *cubense* (B) on wild-type and *esa1* leaves 6 days after drop inoculation, estimated by calculating the amount of pathogen DNA via real-time PCR. Data are averages of three independent experiments on eight leaf disks of 2 cm^2; standard error bars (±S.E.) are indicated. Values are based on the quantification of a standard dilution series of DNA extracted from the respective *in vitro* grown *Fusarium* species. Different letters indicate significant differences according to the Kruskal–Wallis test ($P < 0.05$).

To do so, we first performed a root dip inoculation assay with *F. oxysporum* f. sp. *cubense* race 4 on both *esa1* and wild-type plants. However, no symptoms appeared on any of the inoculated plants (results not shown). Therefore, leaf inoculation assays were performed similarly to the assays described in the previous section. Twelve days post-inoculation on average 14–26% of the *esa1* leaves and only 4–7% of the wild-type leaves showed yellow-brown necrotic lesions when inoculated with the different *F. oxysporum* f. sp. *cubense* races (Fig. 5A). The average size of lesions varied from 1.5 to 3 mm for *esa1*, dependent on the pathogen race (Fig. 5B), while lesions on wild-type plants, if occurring, were non-spreading with an average diameter of less than 1 mm (Fig. 5B). These differences in average spot diameter between *esa1* and wild-type were significant for the three pathogens (*P*-value is 2.6×10^{-3}; 3.2×10^{-2} and 7.8×10^{-3}, respectively), suggesting *esa1* is more susceptible to *F. oxysporum* f. sp. *cubense*.

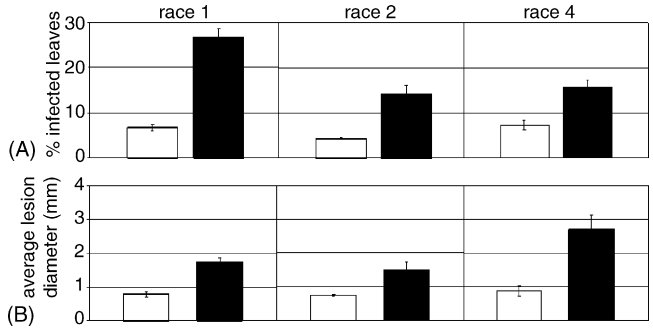

Fig. 5. Quantification of disease development in *A. thaliana* wild-type and *esa1*, inoculated with three different races of the heterologous pathogen *F. oxysporum* f. sp. *cubense*. Data represent percentage of infected leaves (A) and average diameter of lesions (B) formed 12 days post-inoculation of respectively races 1, 2 and 4, based on three independent experiments in phytotron on 12 plants each, standard error bars (±S.E.) are indicated. Grey bars indicate wild-type plants and black bars *esa1* plants. Different letters indicate that the corresponding data are different ($P < 0.05$) according to Kruskal–Wallis test.

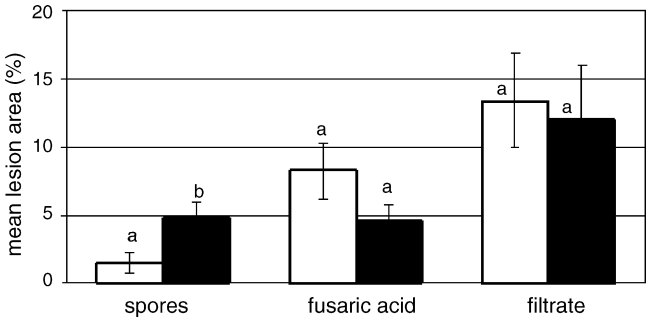

Fig. 6. Symptom development on wild-type and *esa1* plants after inoculation with *F. oxysporum* f. sp. *cubense* race 4 spores, fusaric acid or with filtered *F. oxysporum* f. sp. *cubense* race 4 growth medium. Data represent average percentage of lesion area as measured with ASSESS software with standard error bars (±S.E.) of leaves drop inocula (5 μl) with a spore suspension (5×10^5 spores/ml) of *F. oxysporum* f. sp. *cubense* race 4, with fusaric acid (at 5 μM) or with a culture filtrate of *F. oxysporum* f. sp. *cubense* race 4 of six leaves of each treatment. Different letters indicate that the corresponding data of the same treatment are significantly different ($P < 0.05$) according to Proc Mixed analysis.

Fig. 3 (right panels) shows *esa1* and wild-type plants 12 days after inoculation with the non-host pathogen *F. oxysporum* f. sp. *cubense* race 4 as well as the respective percentage of lesion area on the leaves as calculated by means of ASSESS-software. Twelve days after inoculation with *F. oxysporum* f. sp. *cubense*, necrotic leaf area was 4.0 and 2.0% on *esa1* and wild-type, respectively (*P*-value: 0.055, Fig. 3B), confirming the visual established difference between the *esa1* mutant and wild-type plants. Additionally, relative fungal biomass was estimated for *F. oxysporum* f. sp. *cubense* race 4 using real-time PCR. In contrast to the visual and digital symptom assessment, however, Fig. 4B shows that no significantly increased fungal biomass can be detected on *esa1* plants at 6 days after inoculation with *F. oxysporum* f. sp. *cubense* as compared to wild-type plants. A similar result was obtained at 12 days post-inoculation. This indicates that the larger lesions on *esa1* are due to an increased sensitivity of the mutant to the pathogen and/or its virulence factors or, alternatively, to one of the pathogen's phytotoxic compounds, rather than to an increased susceptibility and disease development.

Taken together, our disease assays indicate that the *esa1* mutant is more sensitive to the heterologous banana pathogens *F. oxysporum* f. sp. *cubense* of races 1, 2 and 4, in contrast to wild-type *A. thaliana* plants. However, the nature of this sensitivity is not clear.

3.4. The esa1 mutant is sensitive to the F. oxysporum f. sp. cubense pathogen, rather than being sensitive to its phytotoxic compounds

Inoculation of *esa1* plants with *F. oxysporum* f. sp. *cubense* resulted in the development of larger grey-brown lesions surrounded by a yellow halo as compared to wild-type plants. As *Fusarium* pathogens produce many toxins, the development of these symptoms may be due to the presence of the toxins, rather than to an increased infection caused by enhanced pathogen proliferation. One well-identified non-specific *F. oxysporum* toxin is fusaric acid. Barna et al. [36] previously reported membrane damage of tobacco leaves caused by fusaric acid, indicating that this toxin can effectively cause disease symptoms on plants.

To study the possibility that the larger lesion phenotype in *esa1* plants is due to the production of toxins, we investigated the effect of application of fusaric acid on both *esa1* and wild-type plants as compared to fungal inoculation. Fig. 6 shows that fusaric acid indeed causes lesions on *A. thaliana* wild-type and *esa1* plants. However, the clear difference in susceptibility as observed between wild-type and *esa1* is not observed when the plants are treated with fusaric acid, as measured with ASSESS software at 10 days post-inoculation, suggesting that the sensitivity of the *esa1* mutant is not (solely) due to the phytotoxic action of fusaric acid.

Since fusaric acid alone did not cause a significant difference in disease symptoms, we tested whether a combination of several *F. oxysporum* f. sp. *cubense* exudates, instead of one specific toxin, can cause necrotic lesions of specific size on wild-type versus *esa1*. To this end, a culture filtrate of *F. oxysporum* f. sp. *cubense* race 4 was prepared according to Companioni et al. [25]. Applying a similar extract on banana leaves, Companioni et al. [25,26] described that significantly larger necrotic lesions developed on *Musa* cultivars susceptible to *F. oxysporum* f. sp. *cubense* as compared to a resistant cultivar, thereby clearly linking disease susceptibility with sensitivity to fungal components. Inoculation of *esa1* and wild-type leaves with the *F. oxysporum* f. sp. *cubense* race 4 culture filtrate resulted in the development of lesions, but no significant difference in lesion size developed on *esa1* versus wild-type could be observed, in contrast to leaves inoculated with fungal spores (Fig. 6).

These data suggest that the observed enlarged necrotic lesions on the *esa1* mutant are not solely related to the action of phytotoxic compounds produced by *F. oxysporum* f. sp. *cubense* race 4, but rather to fungal proliferation on the plant.

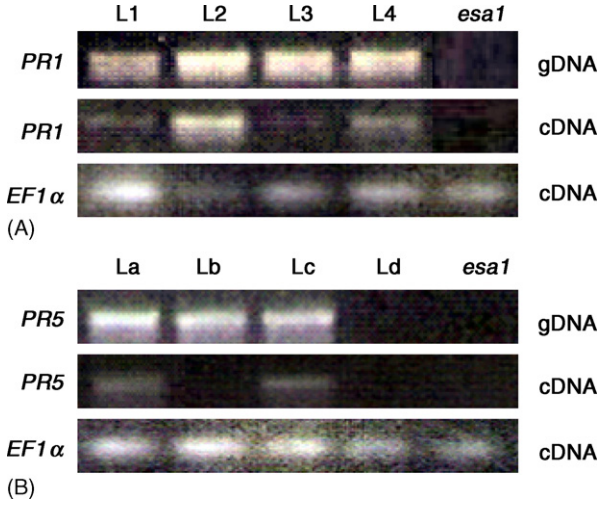

Fig. 7. Control analysis for the integration and gene expression of *PR1* and *PR5* transgenes in *esa1* primary transformants overexpressing the *PR1* (A) and *PR5* (B) transgenes. L1–L4 and La–Ld, four independent *esa1* transformants of either the *PR1* or *PR5* overexpressing population; *esa1*, non-transgenic control line; *PR1*, PCR fragment obtained with *PR1*-specific primers, approx. 400 bp; *PR5*, PCR fragment obtained with *PR5*-specific primers, approx. 600 bp; *EF1α*, PCR fragment obtained with *EF1α*-specific primers, approx. 100 bp; gDNA, PCR reaction performed on genomic DNA isolated from the respective lines; cDNA, RT-PCR reactions performed on cDNA synthesized from total RNA isolated from the respective lines.

3.5. Overexpression of specific PR-proteins in the esa1 mutant partially restores the wild-type phenotype upon inoculation with F. oxysporum f. sp. cubense race 4

Transgenic expression of specific PR proteins has been demonstrated in many studies to be a successful strategy to generate resistance to particular pathogens [37–40]. As we demonstrated, the *esa1* mutant is susceptible to several necrotrophic pathogens [23], including different *Fusarium* species [this study], as well as more sensitive to inoculation with the heterologous *F. oxysporum* f. sp. *cubense*. It thus appears that the *esa1* mutant is an ideal model system to evaluate novel and potentially interesting resistance traits for their effectiveness *in planta* against *Fusarium* diseases.

Based on the earlier observed induction of the endogenous *PR1*, *PR5*, *PR12* and *PR13* genes by *F. oxysporum* f. sp. *matthiolae* in Arabidopsis [21], we investigated whether genes encoding two of these specific classes of PR-proteins, the tobacco PR1 and PR5 proteins, could confer resistance to *F. oxysporum* f. sp. *cubense* when over-expressed in the *esa1* mutant. The presence and gene expression of the transgenic cassettes in the *esa1* plants were confirmed in several individual plants of populations of primary *esa1* transformants by PCR and RT-PCR, respectively (shown for four randomly chosen individuals in Fig. 7). These *esa1* transgenic populations expressing the individual *PR* transgenes were assayed for their sensitivity towards *F. oxysporum* f. sp. *cubense* race 4.

As can be seen in Fig. 8A, *PR1*- and *PR5*-expressing *esa1* plants show a reduced number of infected leaves during the infection process as compared to *esa1* plants. This reduction was statistically significant at 6 and 9 days post-inoculation. In addition, *PR1*- or *PR5*-expressing *esa1* plants exhibited on average smaller necrotic lesions as compared to non-transgenic *esa1* plants. This decrease in average lesion diameter was statistically significant at 6 and 9 days post-inoculation for *PR1*- and *PR5*-expressing plants, respectively (Fig. 8B).

In conclusion, *PR1*- and *PR5*-expressing plants showed an at least partially restored wild-type phenotype towards *F. oxysporum* f. sp. *cubense* race 4 as compared to control *esa1* and wild-type plants.

4. Discussion

We investigated the susceptibility of the *A. thaliana* defense response mutant *esa1* towards different pathogenic *Fusarium* species. The *esa1* mutant is EMS-generated and shows an increased susceptibility phenotype towards the necrotrophic fungal pathogens *A. brassicicola*, *B. cinerea* and *P. cucumerina* relative to wild-type *A. thaliana* [23]. The results presented here show that abolishment of the ESA1 defense response component seriously disturbs the plant's defense response against *Fusarium* pathogens of wild-type *A. thaliana* (*F. culmorum*, *F. solani* and *F. oxysporum* f. sp. *matthiolae*) as well as increases sensitivity to the heterologous pathogen *F. oxysporum* f. sp. *cubense*.

We previously demonstrated that the susceptibility of *esa1* to necrotrophs is dependent on reactive oxygen species (ROS) and is associated with a delayed production of the plant defensin

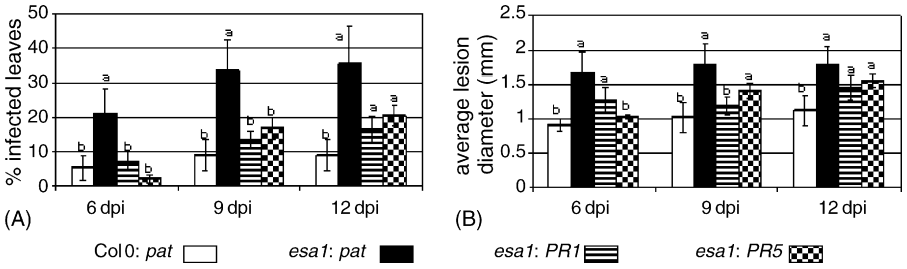

Fig. 8. Effect of the introduction of specific *PR*-transgenes on the lesion occurrence on *esa1* caused by *F. oxysporum* f. sp. *cubense* race 4. (A) Percentage of infected leaves at different time points after inoculation with *F. oxysporum* f. sp. *cubense* race 4. (B) Average lesion diameter at different time points after inoculation with *F. oxysporum* f. sp. *cubense* race 4. Data represent average disease progression of three different experiments on six plants each; standard error bars (±S.E.) are indicated. Different letters indicate that the corresponding data are different ($P < 0.05$) according to Kruskal–Wallis test. Col0: *pat*, *esa1*: *pat*, transgenic lines transformed with empty vector cassette (containing the *pat* gene for BASTA resistance); *esa1*: *PR1*, *esa1*: *PR5*, *esa1* transgenic line with [u*OCS*]p*MAS-PR1*-t*man* and [u*OCS*]p*MAS-PR5*-t*man* overexpression cassette [30], respectively.

PDF1.2a and the phytoalexin camalexin and a stronger production of the pathogenesis-related PR1 protein as compared to wild-type plants [23]. Different map-based cloning attempts to identify the mutated gene failed thus far, not preventing, however, *esa1* from being used as a valuable mutant plant for further defense response research, as shown here. Since the identity of the *ESA1* gene is as yet unknown, we can only speculate about the role of its gene product in pathogen defense in general and towards *Fusarium* species in particular. The mutation in *esa1* affects the resistance spectrum to a whole range of necrotrophic pathogens and is possibly located in a gene that plays a regulatory role in mounting pathogen-induced defense responses, rather than in a gene that has a direct effector-like action on pathogen containment. This conclusion is further supported by previous observations that *esa1* has pleiotropic effects, affecting at least three distinct defense response pathways [23,41]. Presumably, the delayed production of the defense response molecules PDF1.2a and camalexin and stronger production of PR1 upon pathogen challenging of *esa1* [23] are also responsible for the perceived susceptibility towards the different *Fusarium* species. Concomitantly, it was recently shown that Arabidopsis resistance to *F. oxysporum* requires ethylene-, jasmonate and salicylic acid-dependent pathways [42]. ESA1 might thus be involved in the signal transduction of such specific signals, in a way that in the *esa1* mutant the overall activation of plant disease responses is delayed or abolished. Recently, the *RFO1* gene (*r*esistance to *F. oxysporum*) was shown to contribute to *F. oxysporum* disease containment in Col0 relative to ecotype Taynuilt-0 [43]. The *RFO1* gene encodes the cell wall-associated kinase-like kinase 22 [43,44], a member of the WAK/WAKL family of cell-wall associated receptors proposed to provide communication between the plant cell wall and the cytoskeleton [44,45]. It has been suggested that the loosening of the cell wall structure during glycolytic digestion by a pathogen might be indirectly perceived via the cell wall-anchored WAK/WAKLs [45]. Whether *ESA1* encodes a similar pathogen-related receptor remains an interesting possibility.

Previously, Epple et al. [21,32] observed that the *Brassicaceae* fungal pathogen *F. oxysporum* f. sp. *matthiolae* was able to infect wild-type Arabidopsis plants after foliar inoculation [21,32]. We extended this procedure to a subset of agronomically important *Fusarium* species that are pathogens of *A. thaliana* as well. In addition, foliar inoculation with the heterologous vascular pathogen *F. oxysporum* f. sp. *cubense* on *esa1* also leads to visible necrotic lesions, similar to the symptoms caused by the other tested pathogens. This is remarkable, since *F. oxysporum* f. sp. *cubense* is incapable of infecting *A. thaliana* wild-type plants, probably due to what is called non-host resistance of the plant [33,34]. In general, a pathogen needs to comply with several barriers during the processes leading to disease. If a pathogen fails to overcome one of these, it is often referred to as a non-host or heterologous pathogen. The same obstacles can also be serious challenges for host pathogens and often reduce their success rate significantly [33]. Such defense shields can be present constitutively or specifically induced in response to attack by both host and non-host pathogens and these responses can be independent of the genotype of the individual pathogen. With this and previous work [23], we showed that ESA1 is involved in both the defense response against necrotrophic host pathogens (*P. cucumerina*, *B. cinerea*, *F. graminearum*, *F. culmorum*, *F. oxysporum* f. sp. *matthiole*, *F. solani*) and also towards non-host pathogens (*A. brassicicola* and possibly also to *F. oxysporum* f. sp. *cubense*), suggesting ESA1 regulates or is part of a common resistance barrier.

However, our observation that no increased *F. oxysporum* f. sp. *cubense* fungal biomass is detected on *esa1* plants as compared to wild-type plants, indicates that this pathogen cannot proliferate on *esa1* plants (at least not more than on wild-type plants), which would be consistent with the non-host resistance towards *F. oxysporum* f. sp. *cubense* not being suppressed in *esa1*. As such, the observed disease symptoms on the *esa1* mutant may be due to enhanced sensitivity to the pathogen (e.g. to its toxin production or to virulence factors) rather than to penetration and fungal colonization of the plants by the *Fusarium* pathogen. For example, for *Nicotiana* sp. it was reported that several ecotypes show sensitivity towards the fungal toxin AAL from *A. alternate* f. sp. *lycopersici*, while they remain resistant towards the pathogen itself [46]. In contrast, several Arabidopsis ecotypes are susceptible to victorin, a toxin produced by the oat necrotrophic pathogen *Cochliobolus victoriae*, and these sensitive ecotypes displayed also susceptibility towards pathogen infection [47]. *Fusarium* species produce several phytotoxins, like fusaric acid and fumonisins. However, in the case of the *esa1* mutant, inoculations with either (i) a fusaric acid or (ii) a *F. oxysporum* f. sp. *cubense* race 1 culture filtrate, reported to be phytotoxic on *F. oxysporum* f. sp. *cubense* susceptible *Musa* cultivars, revealed that the observed enhanced susceptibility is not (solely) due to the action of *Fusarium* toxic compounds produced *in vitro*. Based on these results, we can postulate that the observed enhanced sensitivity to *F. oxysporum* f. sp. *cubense* fungal inoculation is due to the action of the fungus growing on the plant (e.g. to the action of specific plant-induced fungal toxins). Alternatively, a significantly higher proliferation of *F. oxysporum* f. sp. *cubense* race 4 on *esa1* plants may still occur, but could eventually not be detected by the applied real-time PCR assay. In that case, a detailed microscopic analysis of *F. oxysporum* f. sp. *cubense* inoculated *esa1* versus wild-type plants is recommended to conclude on a possible higher proliferation of the fungus in *esa1*.

In addition to the foliar inoculation assays, we performed a root dip inoculation assay with *F. oxysporum* f. sp. *cubense* race 4, which is the most devastating *cubense* race. However, in contrast to the foliar inoculation assay, no disease symptoms were observed upon root inoculation. This could mean that the banana pathogen is not able to penetrate the roots of the *A. thaliana esa1* mutant but can nevertheless induce necrotic lesions when it is inoculated on *esa1* leaves. Possibly, entrance of a non-host plant through stomata, opened because of the high humidity in the inoculation trays, facilitates the infection process.

From our observations, it additionally became clear that the enhanced lesion progression on the *esa1* mutant towards *F. oxysporum* f. sp. *cubense* race 4 can be partially restored to wild-type levels by the introduction and heterologous expression of either the *PR1*- or the *PR5*-type gene in *esa1*. Involvement of PR1 and PR5 proteins in plant disease resistance in general has been documented before [40,48]. Our results on PR5 in *esa1* are consistent with the reported involvement of PR5 homologous proteins in disease resistance to *Fusarium* species in wheat [49]. In addition and also consistent with our data, the Arabidopsis mutant *npr1-1* (non-expressor of PR1) exhibits increased susceptibility to *F. oxysporum* [42]. The fact that both PR1 and PR5, which are inducible by the salicylic acid pathway and the ethylene-jasmonate pathway, respectively [41], are involved in wild-type phenotype restoration of *esa1* against *F. oxysporum* f. sp. *cubense*, is consistent with previous reports on the positive cooperation of different defense response signaling pathways upon infection with *F. oxysporum* [42]. It has also been shown that overexpression of the *A. thaliana* PR13 (thionin) protein enhances resistance against *F. oxysporum*, not only in *A. thaliana* but also in tomato [32,50], indicating that this gene would also be a good candidate to obtain restored wild-type phenotype against *F. oxysporum* f. sp. *cubense* in *esa1* and, as an extension, against Panama disease by introducing it in banana. We originally also included an Arabidopsis *PR13*-overexpressing construct for transformation of *esa1* plants. We however failed in testing this due to the low regeneration frequency of the *A. thaliana PR13*-expressing *esa1* plants, while we obtained a normal regeneration frequency with the other similar plasmids. Whether overexpression of the endogenous *PR13* gene is toxic for the plants is unclear since successful overexpression of this gene in Arabidopsis plants has been reported before [32]. Although it has been shown that, next to *PR1*, *PR5* and *PR13*, also the endogenous *PR12* gene is induced upon inoculation of *A. thaliana* with *F. oxysporum* [21,32], a *PR12*-type transgene expressing *esa1* population was not found to exhibit a restored wild-type phenotype towards *F. oxysporum* f. sp. *cubense* (Van Hemelrijck, unpublished data).

Since pathogenesis-related proteins like PR1 and PR5 seem to be able to restore resistance to *F. oxysporum* f. sp. *cubense* and given that they are presumed to directly act on the pathogen and its proliferation [40,49], our data additionally suggest that there is fungal growth on the *esa1* leaves, in contrast to our fungal biomass data. This would be consistent with the *esa1* mutant showing enhanced susceptibility towards *F. oxysporum* f. sp. *cubense*.

Our *in planta* disease assays not only confirm that *esa1* is an ideal model system for research on the plant's defense response against fungal pathogens in general and *Fusarium* species in particular, but additionally suggests that *PR1*- or *PR5*-expression may be useful to obtain enhanced resistance to the Panama disease pathogen *F. oxysporum* f. sp. *cubense* race 4.

The *esa1* defense response mutant can thus be used as a tool to unravel disease resistance mechanisms in Arabidopsis. Further mutagenization of *esa1* by means of T-DNA, transposon or activation tagging [51] can lead to the identification of novel components involved in the disease resistance against *Fusarium* species. This research is currently in progress in our lab.

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