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Short Communication

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The Antimicrobial Peptide Thanatin Reduces Fungal Infections in *Arabidopsis*

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Abstract

We explored the antifungal activity of thanatin, a 21 amino acid synthetic peptide from the hemipteran spined soldier bug *Podisus maculiventris*, against the mycotoxin-producing plant pathogenic ascomycete *Fusarium graminearum*. *In vitro* germination assays showed complete inhibition of macroconidia germination and mycelia growth by >10 μM thanatin. Moreover, detached leaves of thanatin-expressing *Arabidopsis thaliana* plants displayed enhanced resistance towards colonization with *F. graminearum*. Consistent with this, the plants showed also enhanced resistance of detached leaves to colonization with *Botrytis cinerea*. The results demonstrate a potential of thanatin for use in plant protection.

Introduction

The use of antimicrobial peptides (AMPs) has emerged as an alternative strategy for plant protection because it bears the potential to reduce microbial adaptation, and at the same time avoids, 'cost for resistance' that usually occurs once the plant's own defence is activated (Vilcinskas and Gross 2005; Marcos et al. 2008). Most of the AMPs display a broad-spectrum activity against Gram-positive and Gram-negative bacteria as well as fungi (Guani-Guerra et al. 2010). Antimicrobial peptides especially of insect origin represent a promising reservoir for engineering disease resistant crop plants (Gao et al. 2000; Langen et al. 2006; Rahnamaeian et al. 2009). Insect AMPs are typically cationic and often consist of <100 amino acid (aa) residues (Bulet and Stöcklin 2005). Peptides characterized by an even number of cysteine residues display the largest and widely distributed categories, classified into three main groups: (i) peptides containing an α-helix and two to four disulphide bonds connecting the helix to ß-strand (e.g. defensins), (ii) peptides with triple-stranded antiparallel ß-sheet, and (iii) peptides forming a hairpin-like ß-sheet structure (e.g. thanatin).

Thanatin is produced by the hemipteran spined soldier bug *Podisus maculiventris* where it confers an activity against both Gram-positive and Gram-negative bacteria, as well as filamentous fungi and yeast at μM concentrations. It is one of the smallest AMPs containing 21 aa residues (molecular weight 2.4 kDa), including two cysteine residues that form a disulphide bridge, and has a ß-hairpin structure (Fehlbaum et al. 1996). The peptide shares no sequence similarities to other insect AMPs but shows some similarity to brevinins, a family of AMPs isolated from frog skin secretions (Bulet and Stöcklin 2005). While the exact mechanism of interaction with microbial targets is still unknown, it is evident that there is a correlation between the hydrophobicity of the ß-hairpin structure and the antimicrobial activity (Orikasa et al. 2009).

Fusarium head blight (FHB) represents an agronomic important disease of cereal plants. Mechanistic research on plant resistance against this disease is hampered by the long time needed for the production of genetically transformed cereals such as barley and wheat. Identification of alternative host models to study *Fusarium* interactions resulted in the establishment of an artificial yet useful *Arabidopsis thaliana* model system (Makandar et al. 2010). In the present study, we explored the potential of thanatin in the *Arabidopsis* model system.

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Materials and Methods

For *in vitro* assays, a spore suspension (4×10^3 conidia/ml) of *F. graminearum* strain IFA 65 (Steiner et al. 2009) was incubated with 0.1–100 μM of synthetic thanatin (Biochem Ltd., Shanghai, China) at room temperature (Jansen et al. 2005). Furthermore, a suspension of *B. cinerea* (2.5×10^4 conidia/ml; strain B05.10, Rui and Hahn 2007) was incubated with thanatin concentrations ranging from 0.05 to 10 μM. Microscopic images were taken 24 h after inoculation (hai) using an inverse stereo microscope (Leica DM IL, Leica, Germany). To evaluate the antifungal activity of thanatin in *Arabidopsis* plants, T3 transgenic plants expressing thanatin and transgenic control plants (no. 14) were used for *in vitro* assay. The antifungal activity of leaf protein extracts from 5-week-old *Arabidopsis* plants was assessed against *B. cinerea*. Protein extracts were prepared according to Wang and Constabel (2004). *B. cinerea* was maintained on HA-agar medium (1% Malt extract, 0.4% Glucose and 0.4% Yeast extract), and then, agar blocks with fungal mycelium were incubated in leaf extracts at 22°C for 24 h. Subsequently, agar blocks were transferred to fresh HA-agar plates, and outgrowth of the mycelium was measured 24 h later.

Intercellular washing fluids (IWFs) were obtained from *Arabidopsis* transgenic plants as well as non-transgenic Col-0 (5-week-old) by centrifugation according to Lohaus et al. (2001). The amount of IWF obtained from 1 g of tissue (fresh weight) was 0.2–0.3 ml. *B. cinerea* conidia (2×10^4 conidia/ml) were incubated in 20 μl IWF from each transgenic line as well as non-transgenic plants in microtiter plates at RT for 24 h. For *in planta* expression, *Arabidopsis* ecotype Col-0 was transformed with a construct encoding the mature thanatin peptide (GenBank 6730068), complemented with the signal peptide (SP) of chitinase 26 from *Hordeum vulgare* (*HvChi26*, GenBank L34210) for secretion into the apoplast, under control of cauliflower mosaic virus (CaMV) 35S promoter. The entire cassette (35s::SP-Thanatin-NosT) was cloned into binary vector pLH6000 (DNA Cloning Service, Germany, GenBank: AY234328.1). Both pLH6000 (no. 14, empty) and pLH6000 35s::SP-Thanatin-NosT (see Fig. 2) were introduced into the *Agrobacterium tumefaciens* strain AGL1 by electroporation (Lazo et al. 1991). Plant transformation and regeneration was performed as described by Bechtold et al. (1993). Homozygous lines for each gene were used for phenotypic characterization.

Thanatin expression was quantified in 5-week-old *Arabidopsis* leaves. qRT-PCR analysis was performed using an Mx3000p thermocycler (Stratagene Research, La Jolla, CA, USA). Transcript expression analysis was performed using the FullVelocity® SYBR® Green QRT-PCR Master Mix kit, 1-Step (Stratagene). Aliquots of 10 ng cDNA were used to amplify thanatin with

Table 1
Primers sequences used in this study for QPCR and vector generation

Primers	Sequence	AT (°C)
*BamH*1-Chi-fwd	**GGATCC**ATGAGATC GCTCGCGGT	60
*Sal*I-than-stop	**GTCGAC**TCACATGCGCTGGCACTT	
UBQ5-fwd	CCAAGCCGAAGAAGATCAAG	60
NP_191784.1		
UBQ5-rev	ACTCCTTCCTCAAACGCTGA	
Thanatin-for	TAA TAC GAC TCA CTA TAG GG	55
Thanatin-rev	ATC CGC ATA TAG TTC CTC CTT TC	

Incorporated restriction enzyme sites are shown in bold at the 5′-end of primers. AT, Annealing temperature.

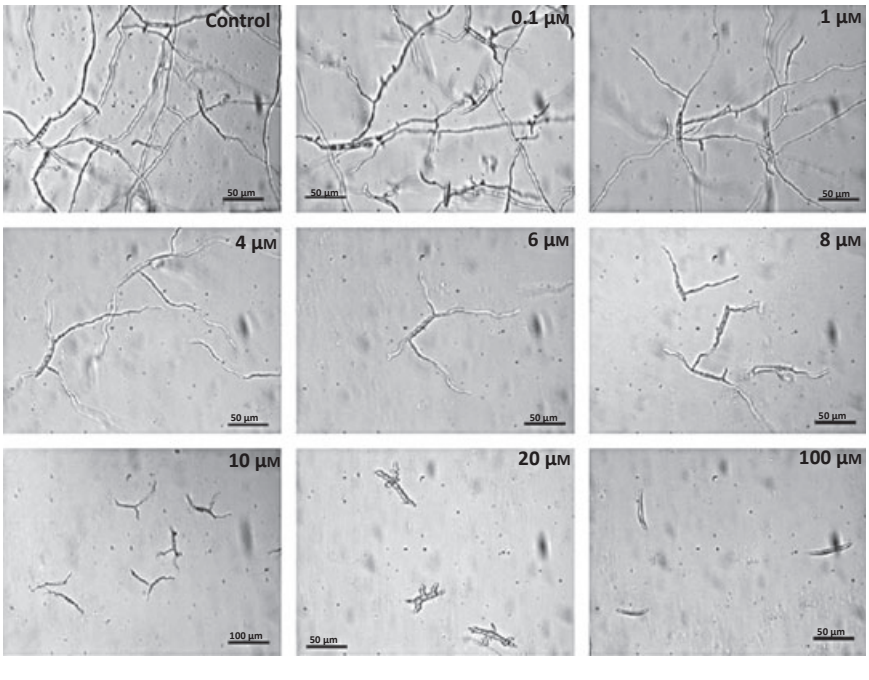

Fig. 1 *In vitro* inhibition of macroconidia germination in axenic *F. graminearum* culture with increasing concentrations of thanatin

the primers thanatin-for and thanatin-rev (Table 1). For relative quantification of thanatin expression, ubiquitin-5 (UBQ5) was measured using primers UBQ5-for and UBQ5-rev (Eurofins MWG OPERON, Germany, Table 1).

Transformants were examined by inoculation of leaves with conidia of *F. graminearum* or *B. cinerea*. Thirty rosette leaves of 10 transgenic plants per transgenic line were inoculated with 5 µl droplets of *Fusarium* or *Botrytis* inoculum. For assessing the progression of disease symptoms, the lesion size (in cm) was measured 5 days after infection (dai) from the digital images using the free software ImageJ program (http://rsb.info.nih.gov/ij/index.html). The percentage of leaf area showing chlorosis relative to the non-inoculated leaf was calculated.

Results and Discussion

To assess the antimicrobial potential of thanatin, we tested various concentrations of the synthetic peptide for *in vitro* inhibitory activity against *Fusarium graminearum*. After 24 h of incubation with macroconidia, increasing concentrations of thanatin resulted in a marked reduction of the length of emerging germ tubes and hyphae (Fig. 1). Thanatin concentration of 1 µM and above caused growth retardation and concentrations above 10 µM additionally caused abnormalities in fungal germ tube morphology, resulting in swelling, shortening and an increase in hyphal wall thickening of hyphal wall. Thanatin concentration above 20 µM inhibited fungal germination completely. We previously showed antifungal activity of metchnikowin (Mtk), an AMP from *Drosophila melanogaster*, against various *Fusarium* species. In the previous study, 5 µM synthetic Mtk caused 100% *in vitro* growth inhibition against *F. graminearum* (Rahnamaeian et al. 2009). Moreover, expression of thanatin in rice conferred partial resistance to rice blast disease caused by *Magnaporthe oryzae* with half-maximal inhibition of *in vitro* appressorium formation by approximately 30 µM of synthetic peptide (Imamura et al. 2009). The finding that concentrations < 10 µM thanatin cause fungal growth inhibition confirms the data known from the literature showing MICs (minimal inhibitory concentrations) of thanatin ranging from < 5 µM against *Neurospora crassa*, *Nectria haematococca*, *Trichoderma viride*, *Alternaria brassicola*, *Fusarium culmorum* to < 20 µM against *Ascochyta pisi* and *Fusarium oxysporum* (Fehlbaum et al. 1996).

To approve the *in vitro* data, we generated *A. thaliana* plants expressing thanatin targeted to the apoplast (Fig. 2 and Methods). Integration of the thanatin gene into the *Arabidopsis* genome was confirmed by PCR with primers designed to amplify the promoter-transgene region for each construct (data not shown). To quantify the level of thanatin transcripts, five thanatin-expressing transgenic lines were selected and analysed using qRT-PCR. Based on a comparison with the housekeeping gene UBiQ5, the two transgenic lines 410 and 411 exhibited distinctly high thanatin transcript levels (Fig. 3) and were used for subsequent bioassays. To assess the degree of resistance conferred by thanatin, detached leaves from 5-week-old plants were inoculated with *F. graminearum* macroconidia. Plants containing empty vector constructs (controls) devel-

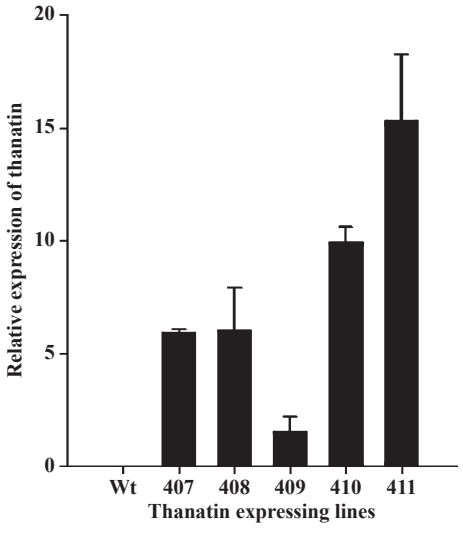

Fig. 3 qRT-PCR of thanatin expression in T1 transgenic *Arabidopsis* lines 407–411. Thanatin expression level varies in independent transgenic lines. No specific amplification product could be detected in transgenic control plant. Each value represents the mean of three replicates ± SE

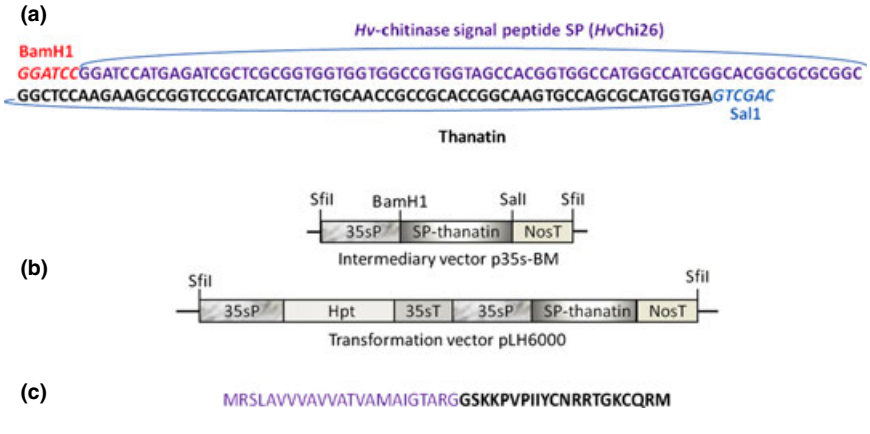

Fig. 2 Schematic diagram of the T-DNA construct used for transformation of *Arabidopsis* with thanatin. (a) Nucleotide sequence of the HvChi26-thanatin open reading frame, with the thanatin sequence underlined. (b) Cassette of the pLH6000-thanatin transformation vector for thanatin expression. (c) Whole aa sequence of SP HvChi26-thanatin, with the mature thanatin sequence in bold. SP, signal peptide

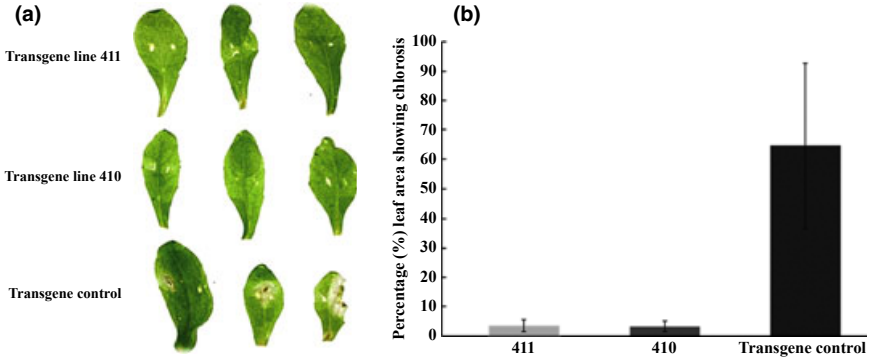

Fig. 4 (a) Disease symptoms caused by 5×10^4 macroconidia/ml droplets of *F. graminearum* on transgenic *Arabidopsis* lines 410 and 411 as compared to transgenic empty vector plants (control) 3 dai. (b) Percentage of leaf area with chlorosis relative to the non-inoculated leaves. The results are from one representative of two experiments and are averages of six leaves from six plants per line

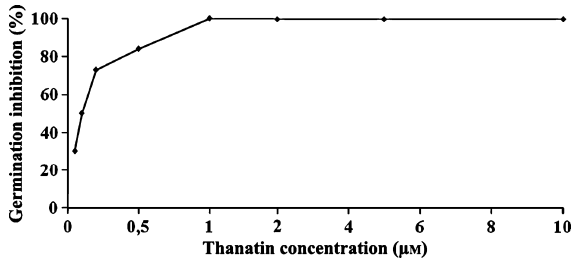

Fig. 5 Dose-effect relationship of synthetic thanatin on spore germination of *B. cinerea in vitro*

oped disease symptoms by 3 dai showing typical necrotic lesions and yellowing to different degrees on all the inoculated leaves (Fig. 4a,b). In contrast, lines 410 and 411 exhibited almost no or very small chlorotic and necrotic lesions which did not further increase in diameter after 5 dai (data not shown). These observations are consistent with the results of the *in vitro* germination assay.

Although the *Arabidopsis–Fusarium graminearum* interaction is artificial, use of *Arabidopsis* for our assessments substantially reduced the work load and costs of testing antimicrobial peptides *in planta*. This strategy is especially applicable for high throughput *in vivo* pre-screening of AMPs in T1 generation of transgenic plants.

We extended our study to analyse the effect of thanatin on the necrotrophic fungal pathogen *Botrytis cinerea*. As depicted in Fig. 5, thanatin also prevented *in vitro* germination of *B. cinerea* conidia by a half-maximal inhibitory concentration of 0.1 µM, which further support and extend the data known from the literature showing MICs of thanatin < 5 µM against *B. cinerea* (Fehlbaum et al. 1996). Consistent with this, mycelial growth of the fungus was also reduced by incubating the mycelium with leaf extracts (protein extracts) from 5-week-old *Arabidopsis* plants expressing thanatin (Figure S1A,B). When detached leaves from 5-week-old *Arabidopsis* lines 410 and 411 were inoculated with *B. cinerea*, they showed reduced infections as compared to control plants (Figure S2) alike their response to *F. graminearum* (Fig. 4). In additional agreement with these findings, intercellular washing fluids (IWFs) isolated from line 410 and 411 showed antifungal activity against *B. cinerea* when tested in axenic germination assays (Figure S3). This demonstrates that the ectopically expressed thanatin is secreted into the apoplastic space as expected from the presence of the HvChi26 signal peptide.

Consistent with earlier reports, the resistant properties of thanatin are not sufficient to render plants completely resistant to a fungal microbe. Thus, it is proposed that thanatin might be tested in the future in combinations with other antimicrobial factors such as AMPs with different mode of actions or pathogenesis-related proteins.

Acknowledgements

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Conflict of Interest

No conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *In vitro* antifungal activity of leaf extracts from *Arabidopsis* transgenic control plants no. 14 (a) and thanatin transgenic lines (b) against *B. cinerea* (A).

Figure S2. Disease symptoms of *B. cinerea* (2×10^4 conidiospores/ml) evaluated 4 days after the inoculation on non-transgenic control *Arabidopsis* Col-0 (Wt) and representative transgenic lines expressing thanatin (lines 410 and 411).

Figure S3. Representative micrographs of *B. cinerea* conidia after 24 h incubation with IWFs (20 μg/μl) from non-transgenic plants Col-0 (A–C) and thanatin transgenic plants (D–F).

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