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RESEARCH PAPER

Insect peptide metchnikowin confers on barley a selective capacity for resistance to fungal ascomycetes pathogens

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Abstract

The potential of metchnikowin, a 26-amino acid residue proline-rich antimicrobial peptide synthesized in the fat body of *Drosophila melanogaster* was explored to engineer disease resistance in barley against devastating fungal plant pathogens. The synthetic peptide caused strong *in vitro* growth inhibition (IC_{50} value ~1 μM) of the pathogenic fungus *Fusarium graminearum.* Transgenic barley expressing the *metchnikowin* gene in its 52-amino acid pre-pro-peptide form under the control of the inducible *mannopine synthase* (*mas*) gene promoter from the T_i plasmid of *Agrobacterium tumefaciens* displayed enhanced resistance to powdery mildew as well as *Fusarium* head blight and root rot. In response to these pathogens, metchnikowin accumulated in plant apoplastic space, specifying that the insect signal peptide is functional in monocotyledons. *In vitro* and *in vivo* tests revealed that the peptide is markedly effective against fungal pathogens of the phylum Ascomycota but, clearly, less active against Basidiomycota fungi. Importantly, germination of the mutualistic basidiomycete mycorrhizal fungus *Piriformospora indica* was affected only at concentrations beyond 50 μM. These results suggest that antifungal peptides from insects are a valuable source for crop plant improvements and their differential activities toward different phyla of fungi denote a capacity for insect peptides to be used as selective measures on specific plant diseases.

Key words: Antimicrobial peptides, ascomycete fungi, barley, disease resistance, metchnikowin.

Introduction

Plant pathogens and pests account for severe and increasing crop losses worldwide, amounting to $30–$50 billion annually (Osusky *et al.*, 2000; Cook, 2006). One of the crucial problems in modern plant production is the rapid adaptation of fungal pathogens to fungicides that, increasingly, imposes costs of chemical plant protection. On the other hand, there is growing public concern about the hazards of agrochemicals on environment and human health. Consequently, there is an urgent need to explore innovative approaches for plant protection (Moffat, 2001). Tuning of plant defence responses to microbial pathogens for rendering them disease-resistant has been recognized as a superior strategy in sustainable agriculture (Kogel and Langen, 2005).

Plants, insects, amphibians, and mammals including human defend themselves against pathogenic microorganisms using peptides or small proteins (Zasloff, 2002; Brogden, 2005). Numerous experiments have provided examples for improving the disease resistance of crops by incorporating antimicrobial peptides, thus providing novel potential traits for further breeding efforts (Marcos *et al.*, 2008). Among these, transgenic expression of antimicrobial peptides from insects has emerged as a successful tool to create crops resistant to biotic stress (Vilcinskas and Gross, 2005). Antimicrobial peptides usually fall into two groups, distinguished by their primary and secondary structures. One group contains basic proteins, called defensins, with a structure containing an α-helix and two to five disulphide

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bonds connecting the helix to β-strands (Mygind *et al.*, 2005; Selsted and Ouellette, 2005; Langen *et al.*, 2006). Many of the defensin type antimicrobial peptides seem to interact with the plasma membrane and cause its permeabilization by either forming pores or blocking Ca^{2+} channels and, thus, disturbing the Ca^{2+} gradient essential for cellular activities. The other group of antimicrobial peptides is represented by the 52-amino acid residue proline-rich peptide metchnikowin (Mtk), identified as an immune inducible peptide in the fruit fly *Drosophila melanogaster* (Levashina *et al.*, 1995), Pyrrhocoricin, and Drosocin with a less well-defined straight chain secondary structure (Brogden, 2005).

In a previous communication, it was described that tobacco plants transgenic for gallerimycin, an antifungal peptide from the greater wax moth *Galleria mellonella*, which shares structural similarity with plant defensins (Schuhmann *et al.*, 2003), showed resistance to fungal pathogens *Golovinomyces cichoracearum* and *Sclerotinia minor* (Langen *et al.*, 2006). In the present study, the potential of the proline-rich antifungal peptide Metchnikowin (Mtk) has been explored to engineer, in barley, disease resistance against microbial plant pathogens. Mtk was chosen, because the synthetic peptide could inhibit *in vitro* growth of the agronomically important pathogenic fungi *Fusarium graminearum* and *F. culmorum*, at low concentrations. These fungi not only cause considerable crop losses, but also affect crop quality by the secretion of mycotoxins into infected grains of barley, wheat, and other cereals. By means of *in vitro* and *in vivo* assays, it is shown here that metchnikowin possesses selective antimicrobial activities against various pathogenic fungi including the biotrophic *Blumeria graminis*, which causes powdery mildew, and the necrotrophic *Fusarium graminearum* that causes root rot and heat blight; whilst the basidiomycete fungi *Piriformospora indica* (Schäfer and Kogel, 2009) and *Rhizoctonia solani* are hardly impaired by this insect peptide at physiological concentrations.

Materials and methods

Fungal materials

Blumeria graminis f. sp. *hordei* (*Bgh*) race A6 (Wiberg, 1974) was propagated on barley cv. Golden Promise. *Fusarium graminearum* strain 8/1 (Miedaner *et al.*, 2000) was grown on Nirenberg Synthetic Nutrient Agar (SNA) medium at 18 °C exposed to near-UV and white light (TLD 36 W-08, TL 40 W-33 RS, Philips, Hamburg, Germany) with a 12-h light photoperiod for 1–2 weeks (Babaeizad *et al.*, 2009). *Piriformospora indica* (DSMZ 11827 from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was propagated as described by Waller *et al.* (2005). *Rhizoctonia solani* isolate AG8 was kindly provided by Dr. Timothy Paulitz, Department of Plant Pathology, Washington State University, USA and maintained on plates of potato dextrose agar (PDA, Roth, Germany).

Generation of transgenic barley plants

Transformation was carried out with spring barley (*Hordeum vulgare* L.) cv. Golden Promise grown in a climate chamber at 18/14 °C (light/dark) with 60% relative humidity (RH) and a 16 h photoperiod and a photosynthetic photon flux density of 240 μmol m^{-2} s^{-1}. To introduce the metchnikowin encoding sequence into the barley genome, the 156 bp *Mtk* intron-less gene (GenBank Accession no. AY226419; Lazzaro and Clark, 2003) was amplified from genomic DNA of wild-type *D. melanogaster* strain OregonR using *Mtk* specific primers (Mtk-Fwd 5′-GGATCCATGCAACTTA-ATCTTGGA-3′ and Mtk-Rev 5′-GTCGACTTAATAAA-TTGGACCCGGT-3′) flanked by *Bam*HI/*Sal*I restriction sites. The resulting amplicon was ligated into the same sites of the formerly manipulated expression vector p35S-BAM (Schmidt, DNA Cloning Service, Hamburg, Germany) for substitution of *mas* promoter (cut from pGE2-*mas::gallerimycin*, Langen *et al.*, 2006) instead of CaMV *35S*. The expression cassette of *mas::metchnikowin::nos-T* (in p*Mas::Mtk* plasmid), cut by *Sfi*I, was cloned into the *Sfi*I-sites of binary vector pLH6000 (DNA Cloning Service, Hamburg, Germany; see Supplementary Fig. S1B at *JXB* online). For barley transformation, pLH6000::*Mas::Mtk* was introduced into the *Agrobacterium* strain AGL-1 (Lazo *et al.*, 1991) through electroporation (*E. coli* Pulser, Bio-Rad, Munich, Germany). *Agrobacterium*-mediated transformation, selection, and regeneration of transformants were performed as described by Tingay *et al.* (1997). T_0 plants were transferred into a greenhouse with temperatures ranging from 20–28 °C and uncontrolled humidity. Transformants were selected based on transgene integration test via genomic PCR with *Mtk* specific primers.

Bioassays for plant resistance

For the powdery mildew assay, barley seeds (surface-sterilized by 70% ethanol for 1 min, and a sodium hypochlorite solution containing 6% active chlorine for 2 h) were germinated on filter papers for 3 d, then transplanted into soil (Frühstorfer Erde, Hawita Gruppe, Vechta, Germany), and maintained in a climate chamber (Percival Scientific, Boone, Iowa, USA) with a 16 h photoperiod, 22/18 °C (light/dark) and 60% RH. After 7 d, first leaf segments were placed on 0.5% (w/v) water agar medium supplemented with 20 mg l^{-1} benzimidazole (Merck-Schuchardt, Munich, Germany) and, subsequently, inoculated with *Bgh* (5 conidia mm^{-2} density) using air current dispersal in an inoculation pillar. The plates were preserved in the same climate chamber for 5 d to, eventually, count *Bgh* colonies.

For the *Fusarium* root rot assay, roots of 3-d-old seedling were gently shake-incubated in *F. graminearum* inoculum (5×10^4 conidia ml^{-1}; Deshmukh and Kogel, 2007) and, in parallel, in a 0.05% Tween-20 aqueous solution as a mock

treatment for 2 h. Subsequently, seedlings were transplanted in a 2:1 mixture of expanded clay (Seramis; Asterfoods, Verden, Germany) and Oil Dri (Damolin, Mettmann, Germany) and maintained in a climate chamber with 60% RH and a 16 h photoperiod, 22/18 °C (light/dark), with 240 μmol m^{-2} s^{-1} photon flux density for 2 weeks. Later, shoot biomass was measured and root samples for quantitative measurement of fungal biomass were subjected to DNA isolation.

To perform the *Fusarium* head blight assay, spikes of 16-week-old plants were sprayed with *F. graminearum* inoculum (2×10^5 conidia) as described by McCallum and Tekauz (2002). Inoculated spikes were kept in plastic bags for 48 h for providing the required high humidity. After 3 weeks, the seeds were harvested and surface-sterilized by 70% ethanol and sodium hypochlorite solution containing 3% active chlorine for 1 min and 20 min, respectively, to eliminate any superficial contamination. Sterilized seeds were placed on PDA medium for 3–5 d. *Fusarium* resistance was scored based on *Fusarium* contamination outgrowing from the endosperm. The resistance assay was replicated twice for each transgenic line.

Effects of Mtk on P. indica*–barley symbiosis*

To assess the effect of Mtk on *P. indica*, surface-sterilized barley seeds were germinated for 3 d on filter paper. Roots of seedling were immersed and gently shake-incubated in an aqueous solution of 0.05% Tween-20 containing 5×10^5 *P. indica* chlamydospores ml^{-1} for 2 h (Deshmukh *et al.*, 2006). Inoculated seedlings were transplanted in Seramis:OilDri (2:1) as described above. Shoot biomass was measured after 21 d. Colonization of plant roots by *P. indica* was detected by microscopy using wheat germ agglutinin stain conjugated with Alexa Fluor 488 (WGA-AF 488, Molecular Probes, Karlsruhe, Germany; Deshmukh *et al.*, 2006).

In vitro *antifungal assay*

Fungicidal activity of a synthetic, active 26-amino acid Mtk peptide (purity more than 80%, Seqlab, Göttingen, Germany) and *Mtk* expressed in barley were determined using an inhibition assay (Cavallarin *et al.*, 1998). Intercellular washing fluids (IWFs) of transgenic plants were extracted from leaves by vacuum infiltration of 10 mM phosphate buffer (pH 7.2). Infiltrated leaves were centrifuged (700 *g*, 5 min) and IWFs were obtained. For the *in vitro* antifungal activity assay, *F. graminearum* conidia (1×10^4 conidia) were incubated in IWF at room temperature for 24 h. Fungal growth was monitored on an inverse microscope (Olympus, Japan). Quantitative assay for inhibitory activity on pathogen development was performed after 24 h via staining the cultures in microtitre plate wells with Trypan blue (10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml distilled water, and 0.02 g of Trypan blue, Sigma-Aldrich Chemie, Steinheim, Germany). Absorbance (OD_{595nm}) was measured using spectrophotometer (Tecan Deutschland GmbH, Crailsheim, Germany).

Pathogen/auxin inducibility of the mannopine synthase *(*mas*) gene promoter*

To analyse the inducibility of *A. tumefaciens mas* promoter by pathogens, transgene expression was monitored at 12, 24, and 48 h after *Bgh* (50 conidia mm^{-2}) and 2, 4, and 6 d after *F. graminearum* (5×10^4 conidia ml^{-1}) inoculations. Mock treatments were provided for each time point. To test auxin inducibility of the *mas* promoter, *Mtk* plants were treated with 1 mM indole-3-acetic acid (IAA) (Sigma-Aldrich Chemie) or water as the foliar spray. Transgene expression levels were checked at 0, 2, 4, 12, 18, and 24 h after spray. RNA extraction and RT-PCR were performed for each plant material as outlined below.

Determination of fungal biomass

The extents of root colonization by *F. graminearum* and *P. indica* were determined using quantitative PCR. Genomic DNA was isolated from 100 mg of root tissue by means of DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). To perform quantitative PCR, 5 ng of total DNA were used. Amplifications were performed in 5 μl of 2× SYBR green JumpStart mix (Sigma–Aldrich Chemie) with 10 pmol of the respective oligonucleotides using an Mx3000P thermal cycler (Stratagene, La Jolla, CA, USA). Relevant melting curves were determined at the end of cycling to ensure the amplification of just one PCR product. Cycle of threshold (*Ct*) values were ascertained with Mx3000P V2. *Ct* values were generated by deducting the raw *Ct* values of the *F. graminearum Fg16N* (Nicholson *et al.*, 1998) and the *P. indica β-tubulin* gene (Serfling *et al.*, 2007) from the respective raw *Ct* values of the plant-specific *ubiquitin* gene and relative fungal DNA abundance was determined as $2^{-\Delta\Delta Ct}$.

Transcript analyses

For quantitative two-step RT-PCR, 2 μg of total RNA, extracted from 100 mg of plant material using RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Fermentas, Sankt Leon-Rot, Germany) was reverse transcribed to first-strand cDNA using SuperScript™ II Reverse Trascriptase (Invitrogen, Carlsbad, CA). Five ng of first-strand cDNA were used for each quantitative PCR with gene-specific primers. Comparative expression levels ($2^{-\Delta\Delta Ct}$; Livak and Schmittgen, 2001) were then calculated. Expression levels are shown relative to the level of *ubiquitin* expression steady in all RNA samples. Values are the means of four samples of two biological experiments assayed through quantitative PCR. Specific oligonucleotide pairs were used for *ubiquitin* (Accession no. M60175), *Pathogenesis-related-1b* (Accession no. Z21494), and *Pathogenesis-related-5* (Accession no. AY839293) (Deshmukh and Kogel, 2007).

Histochemical analyses

To detect defence responses of barley plants to *Bgh* attack in epidermal cells, an endogenous peroxidase-dependent

in situ staining procedure was performed using 3, 3-diaminobenzidine (DAB; Sigma-Aldrich Chemie, Steinheim, Germany; Thordal-Christensen *et al.*, 1997) at 36 hai on the first leaves of 7-d-old plants detached and placed on 0.5% (w/v) water agar containing 20 mg l^{-1} benzimidazole. Leaf storage, fungal staining, and microscopy were done as described by Hückelhoven and Kogel (1998).

In vitro *analysis of Mtk on protoplasts of fungi*

Mycelia were produced by inoculating 100 ml modified *Aspergillus* minimal medium (Waller *et al.*, 2005) with multiple agar plugs of 7-d-old *R. solani* and *F. graminearum* as well as 30-d-old *P. indica* cultures. The cultures were shake-incubated at RT for 7 d (100 rpm). Subsequently, mycelia were crushed using a blender and transferred to 100 ml of fresh CM medium (Waller *et al.*, 2005). After 3 d, mycelia were harvested by filtration using sterile Miracloth filter and washed with 50 ml of 0.9% NaCl solution. Washed mycelia were incubated for 1 h at 37 °C in a cell wall-degrading enzyme mixture (0.2 g 10 ml^{-1} lysing enzymes from *Trichoderma harzianum*, Sigma). The reaction product was then filtered through sterile Miracloth and washed with 10 ml STC (1 M sorbitol, 10 mM TRIS-HCl, pH 7.5, 50 mM $CaCl_2$). The eluted protoplasts were settled down by centrifugation at 4 °C for 10 min at 3000 rcf. The pellet was resuspended in 1 ml of STC (4 °C) and transferred to a microcentrifuge tube. Protoplasts were collected by centrifugation at 4 °C for 5 min at 3000 rcf. The pellet was washed in 1 ml STC and the number of protoplasts was determined using a Fuchs Rosenthal counting chamber. To determine the antifungal activity of Mtk, 400 protoplasts from each fungus were incubated in different concentrations of Mtk in microtitre plates at RT for 24 h (four replicates each). Protoplast numbers were recorded using an inverted contrasting microscope (Leica DM IL, Germany).

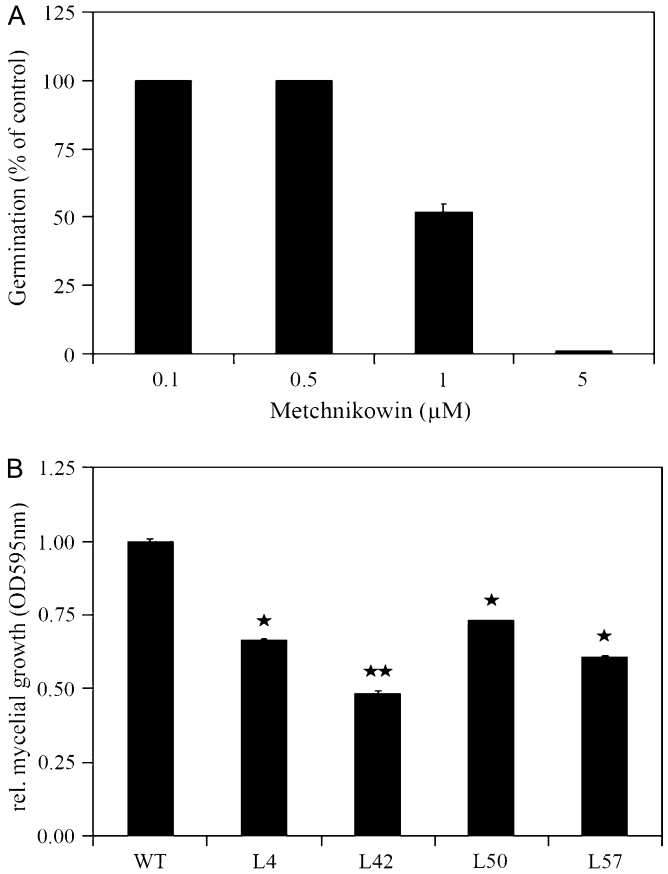

Fig. 1. Inhibition of *Fusarium graminearum* macroconidia by metchnikowin (Mtk). (A) Inhibition of germination with increasing concentrations of Mtk in distilled water with IC_{50} of 1 μM. (B) Inhibition of mycelium growth by intercellular washing fluids (IWFs) extracted from transgenic barley *Mtk* lines L4, L42, L50, and L57 compared with that from the wild type (WT) Golden Promise. Conidia were incubated in IWF for 24 h. Growth of mycelia was determined by measuring absorbance at 595 nm after Trypan blue staining. Values are means of two replications and error bars show the standard errors of the experiments. *, ** Significant differences from the control at $P < 0.05$ and $P < 0.01$, respectively (*t* test).

Results

Metchnikowin expressing barley shows resistance to pathogenic fungi

Metchnikowin is a 26-amino acid residue proline-rich peptide synthesized in the fat body of *Drosophila melanogaster* as a 52 amino acid pre-pro-peptide upon microbial challenges. The Mtk amino acid sequence is shown in Supplementary Fig. S1A at *JXB* online. According to initial studies, the peptide inhibits the growth of Gram-positive bacteria and the ascomycete fungus *Neurospora crassa* (Levashina *et al.*, 1995). Custom synthesized 26-amino acid Mtk also inhibited the germination of conidia of the plant pathogenic fungus *Fusarium graminearum* with IC_{50} value of 1 μM (Fig. 1A). Encouraged by these results, transgenic barley plants expressing the *Mtk* gene encoding the pre-pro-peptide were produced. *Agrobacterium*-mediated transformation of immature embryos of the barley cv. Golden Promise (GP) was carried out with a plasmid (see Supplementary Fig. S1B at *JXB* online), containing the coding region of the *Mtk* gene under the control of the *mannopine synthase* (*mas*) gene promoter from T_i plasmid of *Agrobacterium tumefaciens* (Velten and Schell, 1985; Langridge *et al.*, 1989). This promoter has proved to be responsive to wounding, plant growth hormones, as well as fungal infection in carrot (Imani *et al.*, 2002) and tobacco (Langen *et al.*, 2006). Integration of *Mtk* into the barley genome was verified by PCR using *Mtk* specific primers. One hundred transgenic lines with no visible abnormality in growth were regenerated from immature embryos. Out of these lines, 13 lines were analysed in more detail to confirm the level of *Mtk* transcription using RT-PCR. Four lines, namely, L4, L42, L50, and L57 showed particularly high transcription of the *Mtk* gene (see Supplementary Fig. S1C at *JXB* online) and were targeted for further studies. These lines were cultivated to obtain the T_1 and T_2 generations for subsequent bioassays. Intercellular washing fluids (IWFs) from

leaves of *Mtk*-containing plants showed inhibitory effects on the growth of *F. graminearum* mycelia when compared with IWF from wild-type GP barley (Fig. 1B). This demonstrates the functionality of the insect origin pre-sequence for targeting the Mtk into the plant apoplast.

Mannopine synthase (mas) gene promoter is inducible by auxin and pathogens in monocots

It was shown earlier that the *mas* promoter from *A. tumefaciens* is auxin and pathogen responsive in dicotyledonous plants (Langridge *et al.*, 1989; Imani *et al.*, 2002; Langen *et al.*, 2006). To test auxin inducibility of the *mas* promoter in the monocotyledonous barley, transcription of *Mtk* in transgenic line L4 was assessed by quantitative RT-PCR after foliar spray of synthetic Indol-3-acetic acid (IAA) and fungal inoculation (see Supplementary Table S1 at *JXB* online). As a result, the level of *Mtk* transcription increased 3-fold with a maximum at 2 h after treatment with 1 mM IAA. Upon inoculation of line L4 plants with conidia of the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*), *Mtk* mRNA abundance was amplified over 3-fold with a climax at 24 h after inoculation (hai) and levelled off, thereafter. Similarly, *Mtk* mRNA abundance in the roots of L4 plants was raised beyond 3-fold in response to necrotrophic *F. graminearum* at 144 hai. Overall, these observations demonstrate significant, albeit, sparse activity and inducibility of the bacterial *mas* promoter in different tissues of a monocotyledonous plant upon infection by fungi with both biotrophic and necrotrophic life styles.

Mtk plants show improved resistance to Fusarium *root rot and head blight*

To evaluate the effect of Mtk in barley plants on necrotrophic *Fusarium* species, *Mtk* plants were infected with *F. graminearum* that causes root rot (FRR) and head blight (FHB). Roots of 3-d-old seedlings of wild-type GP and *Mtk* expressing plants of line L4 were dip-inoculated into a solution of *F. graminearum* conidia for 2 h, transferred into pots and evaluated for disease symptoms 2 weeks later. At that stage, infected seedlings of the wild-type GP were retarded in growth compared with non-inoculated individuals, while those of transgenic line L4 were almost as tall and vigorous as the corresponding non-infected plants (see Supplementary Fig. S2 at *JXB* online). Shoot biomass of the four sets of plants is shown in Fig. 2A. The transgenic line L4 showed only a 10% cutback in biomass compared with 50% reduction in wild-type GP plants. The seed remnant and root bases of infected GP displayed discoloration, a typical symptom of *F. graminearum* root rot, whereas the inoculated *Mtk* plants developed a non-symptomatic root system (not shown). In order to quantify the infection more accurately, fungal biomass in roots was determined by fungal DNA using quantitative PCR and *F. graminearum*-specific primers. In agreement with the observed FRR symptoms, fungal DNA abundance was reduced by 80% in roots of line L4 plants compared with that in wild type (Fig. 2B).

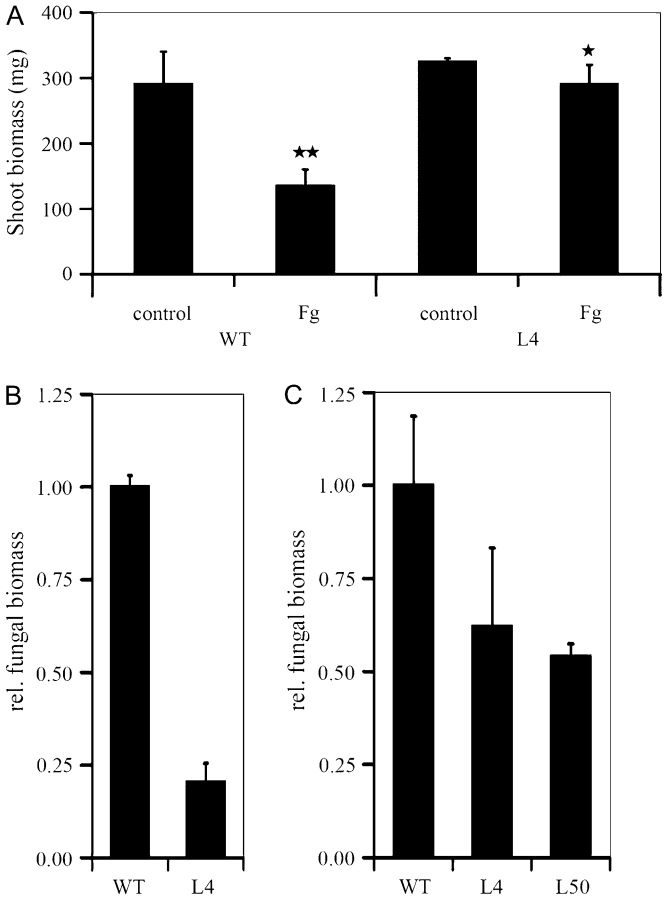

Fig. 2. Transgenic *Mtk* barley responses to inoculation with *Fusarium graminearum* (*Fg*). (A) Upon inoculation of the roots of 3-d-old wild type (WT) Golden Promise seedlings with *Fg* conidia, WT biomass production measured 14 d after inoculation was reduced, while seedlings of transgenic line L4 developed almost like the non-inoculated control individuals. The experiment was replicated once and the bars indicate the standard errors of two separate experiments. *, ** Differences from the control at $P < 0.05$ and $P < 0.01$, respectively (*t* test). (B, C) Quantitative measurement of fungal DNA abundance in roots affected by *Fusarium* root rot (FRR, B) and in kernels affected by *Fusarium* head blight (FHB, C), respectively. Fungal DNA was sampled from roots of *Fusarium* infected WT and L4 plants as well as from seeds of WT, L4, and L50 plants 14 d after root and 21 d after spike inoculation, respectively. Results were normalized using the plant *ubiquitin* gene. Data are the means of values from two independent experiments and error bars indicate the corresponding standard errors.

To test for resistance to head blight caused by *F. graminearum* (FHB), developing barley spikes of GP and transgenic *Mtk* plants were sprayed with 2×10^5 conidia inoculum and covered with plastic bags for 48 h for retaining high humidity for macroconidia germination. After 3 weeks, grains were harvested, surface-sterilized, placed on potato dextrose agar (PDA) medium, and assayed for the presence of fungus outgrowing from the endosperm. In *Mtk*-expressing lines L4 and L50, FHB severity was judged to be reduced to about 20% as

compared to GP in two independent experiments. Fungal biomass in infected endosperm was also determined by quantitative PCR. Consistently, declines of fungal infection in grains by up to 38% and 46% were observed in respective transgenic lines L4 and L50 (Fig. 2C).

Mtk-producing plants demonstrate improved resistance to powdery mildew fungus

The development of powdery mildew fungus race *Bgh-A6*, which undergoes a compatible interaction with Golden Promise was examined on *Mtk*-expressing barley. Detached first leaves of 7-d-old wild type and *Mtk* plants were inoculated with fungal conidia and evaluated 5 d later for interaction phenotypes. All *Mtk*-containing lines showed a strong reduction (60% on average) in the frequency of the *Bgh* colony as well as in colony size compared with GP (Fig. 3A), which shows the enhanced resistance of *Mtk* plants to powdery mildew. Next, the effect of *Mtk* expression on the defence reactions typically exhibited by barley leaves in response to *Bgh* inoculation was determined. Microscopic inspection revealed no effect on fungal germination on barley leaves. However, the number of germinated conidia that penetrated successfully into epidermal cells of the transgenic lines was reduced significantly (Fig. 3B). Less than 10% of germinated conidia penetrated an epidermal cell, formed a functional haustorium, and elongated secondary hyphae. By contrast, more than 50% of germinated conidia produced functional haustoria in GP. As observed, the failure of haustorium formation in *Mtk* plants was associated with augmented frequencies of effective cell wall apposition (CWA) underneath the attempted penetration sites. Moreover, the incidence of the hypersensitive response (HR) resulting in the collapse of the attacked host cells was also higher (Fig. 3C). In order to test whether *Mtk* plants show an overall enhanced defence status, the transcript levels of *pathogenesis-related* (*PR*) genes were measured in transgenic and GP plants. Basal expression levels of genes *PR-1b* and osmotin (*PR-5*) in leaves from non-challenged *Mtk* and GP plants was indistinguishable (data not shown), suggesting that *Mtk* does not induce stress responses in barley. However, consistent with earlier reports, both genes were up-regulated upon inoculation with virulent *Bgh* (Fig. 4). At 24 hai, expression of *PR-1b* was induced 12-fold in GP and 35-fold in transgenic line L42. Correspondingly, *PR-5* was induced 4-fold in GP compared with 6-fold in line L42.

Metchnikowin shows differential activities against ascomycete and basidiomycete fungi

The effect of *Mtk* expression on *P. indica*, a beneficial basidiomycete colonizing roots of a broad plant spectrum including barley (Kogel *et al.*, 2006), was analysed as well. *P. indica* belongs to the order Sebacinales that represents a huge range of symbiotically living, mycorrhizal fungi (except the arbuscular fungi of Glomeromycota) with major ecological relevance (Waller *et al.*, 2005).

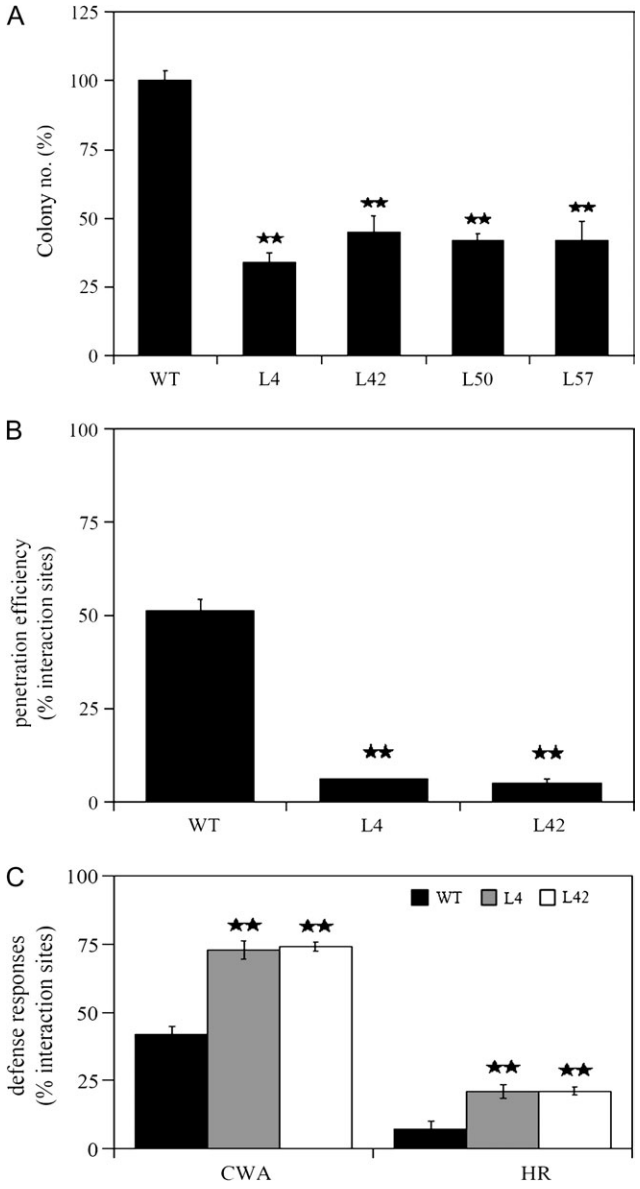

Fig. 3. Resistance of *Mtk* barley against *Blumeria graminis* f. sp. *hordei*. (A) The frequencies of *Bgh* colony formation on *Mtk* lines L4, L42, L50, and L57 at 5 dai are reduced, significantly, in comparison with that in wild-type Golden Promise (WT). Data are the means of values from 20 individuals per line. Error bars indicate standard errors. (B) Penetration efficiency of *Bgh* on WT, L4, and L42. Data were obtained via microscopic evaluation using DAB staining of leaf segments and are the means of successful penetration events associated with mature haustoria and the formation of elongated secondary hyphae in a total of 100 interaction sites in 12 individuals per line at 36 hai. Error bars represent standard errors. (C) Defence responses of barley to *Bgh*; frequencies of cells showing a non-penetrated cell wall apposition (CWA) or a hypersensitive response (HR) in response to *Bgh* attack are higher in *Mtk* lines L4 and L42 as compared with Golden Promise (WT). Data were obtained as described in section (B). Error bars show the standard errors. ** The difference from the control at $P < 0.01$ (*t* test).

Unexpectedly, *in vitro* tests revealed a lower sensitivity of *P. indica* to Mtk. No significant inhibition of chlamydospore germination was observed by up to 50 μM Mtk (Fig. 5A). This analysis reveals the tolerance of *P. indica* chlamydospores to high doses of Mtk, which is ten times stronger than the completely lethal dose to conidia of *F. graminearum*. Consistently, colonization of *Mtk* line L4 roots by *P. indica* was not impaired. As evidenced by quantitative PCR, fungal biomass in line L4 roots was even somewhat higher when compared with wild-type GP (Fig. 5B). Microscopic analysis corroborated an unrestricted colonization of transgenic barley roots by *P. indica* (see Supplementary Fig. S3 at *JXB* online). Furthermore, growth promotion elicited by *P. indica* in line L4 was in the same range as in wild-type GP (Fig. 5C), together indicating that the mutualistic potential of the fungus has not been disturbed by *Mtk* expression.

In order to substantiate the differential toxicity of Mtk, protoplast viability of various fungi was tested in the presence of the insect peptide. To this end, protoplasts isolated from axenic mycelia of the ascomycete *F. graminearum* as well as the basidiomycetes *P. indica* and *Rhizoctonia solani* were incubated in increasing concentrations of Mtk (2–50 μM). A reduced survival rate and, thus, a higher sensitivity of *F. graminearum* protoplasts (approximate IC_{50} of 1 μM) was found compared with those of *P. indica* and *R. solani* (IC_{50}: ~20 μM; Fig. 6), which supports the hypothesis that the differential sensitivities of the fungi might be owing to the structural and/or biochemical differences in higher fungi.

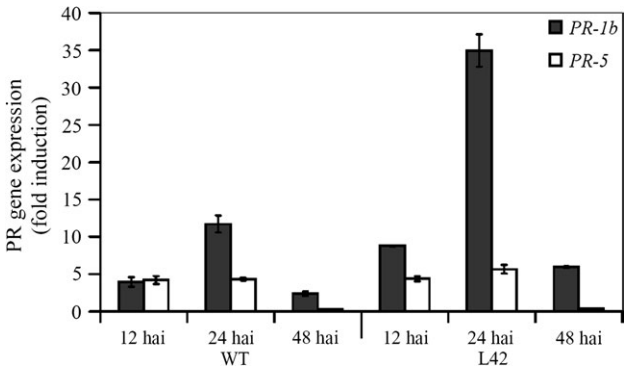

Fig. 4. Induction of *PR* genes in *Mtk* plants and Golden Promise (WT) in response to *Blumeria graminis* f.sp. *hordei* (*Bgh*). *PR-1b* and *PR-5* gene expression was monitored in 7-d-old *Mtk* plants of line L42 and in WT at 12, 24, and 48 h after *Bgh* inoculation. RNAs were extracted from first leaves of three plants for each time point and transcript levels were quantified using RT-PCR. Values were normalized with constitutively expressed *ubiquitin* as the internal control and are presented relative to the expression level of the zero time point. Data are the means of values from two independent experiments and error bars show the standard errors.

Discussion

In this study, the potential of an antifungal peptide from *Drosophila melanogaster* was explored to engineer, in barley, disease resistance against devastating microbial plant pathogens. The proline-rich antifungal metchnikowin was chosen, because the peptide caused strong *in vitro* growth inhibition of pathogenic fungi of the genus *Fusarium* that are of high agronomic relevance in many crops.

As the instability of insect antimicrobial peptides in transgenic plants has been attributed to post-translational degradation by intracellular proteases (Mills *et al.*, 1994), the sequence encoding the Mtk pre-pro-peptide was cloned into the plant transformation binary vector to target the mature peptide into the plant apoplastic space. This strategy has been successfully employed to confer resistance against fungal pathogens on tobacco by transgenic expression of the antifungal peptide gallerimycin from *Galleria mellonella* (Langen *et al.*, 2006). Intercellular washing fluid of *Mtk*-expressing Golden Promise strongly inhibited the growth of *F. graminearum in vitro*, demonstrating that these transgenic plants produce a functional signal sequence, which is able to target the

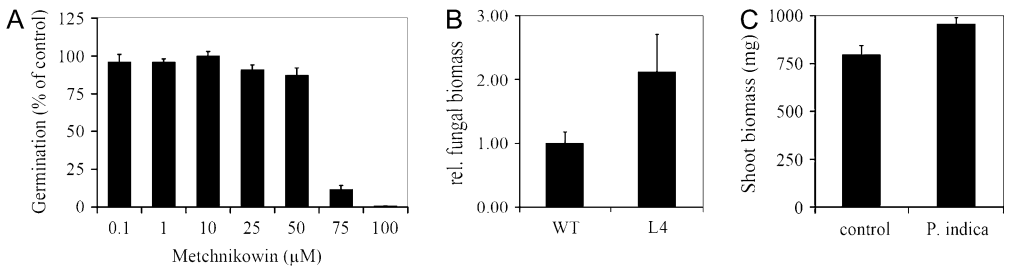

Fig. 5. The effect of Mtk on *Piriformospora indica* development. (A) *In vitro* assay for Mtk antifungal activity on *P. indica* chlamydospore germination. Chlamydospores were incubated in different concentrations of synthetic Mtk and the percentage of chlamydospore germination was measured after 24 h. (B) Relative fungal biomass in *P. indica*-colonized roots of WT and transgenic plants of line L4. Values were normalized by the plant *ubiquitin* gene. Error bars illustrate the standard errors of two independent experiments. (C) Growth promoting effect elicited by *P. indica*. Shoot weight of transgenic line L4 plants was measured 21 d after root colonization by *P. indica*, and compared with that of corresponding non-colonized plants. Data are the means of values out of two independent experiments and 25 plants each. Error bars show the standard errors.

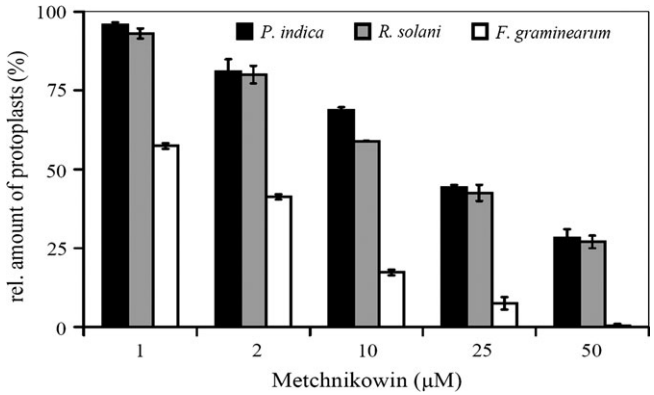

Fig. 6. Detrimental effect of synthetic Mtk peptide on fungal protoplast. Protoplasts of *Fusarium graminearum*, *Rhizoctonia solani*, and *Piriformospora indica* were incubated for 24 h in increasing concentrations of Mtk. Subsequently, the numbers of surviving protoplasts were determined by microscopy.

mature peptide into the apoplast correctly (Fig. 1B). *Mtk* was expressed under the control of the *Agrobacterium tumefaciens mannopine synthase* (*mas*) promoter that had been shown earlier to be responsive to phytohormones, wounding, and fungal infection in dicot plants (Langridge *et al.*, 1989; Imani *et al.*, 2002; Langen *et al.*, 2006). It is shown here that the *mas* promoter is also inducible in leaves and roots of barley by IAA as well as fungal pathogens *B. graminis* and *F. graminearum*, which show biotrophic and necrotrophic life-styles, respectively. The overall advantage of the use of the *mas* promoter is its pathogen inducibility along with the fact that its basal activity in barley is much lower compared with the frequently used CaMV *35S* or maize *ubiquitin* promoters (see Supplementary Table S1 at *JXB* online). Consistently, negative side-effects of Mtk were not detected in the transgenic plants. Interestingly, and in contrast to barley, the basal activity of the *mas* promoter in maize is much higher and similar to that of the CaMV *35S* or maize *ubiquitin* promoters (Lee *et al.*, 2007).

Assessment of Mtk effects on powdery mildew fungus during its interaction with *Mtk* barley provided evidence that the antifungal peptide impedes establishment of a functional haustorium, whose formation is a prerequisite for the commencement of the biotrophic phase. Microscopic analysis revealed that germination of conidia is not affected, and higher plant resistance is associated with higher frequencies of typical defence responses (Hückelhoven and Kogel, 2003), for example, hypersensitive response (HR) of attacked cells and the development of cell wall apposition (CWA) underneath attempted penetration sites. Thus, antifungal Mtk intensifies plant defence in epidermal cells, which leads to a diminished number of functional haustoria. Increased HR frequencies and accompanying cell death of attacked epidermal cells could be initiated by locally elevated Mtk levels reaching a toxic threshold for the plant cell. Although this cannot be excluded, it seems unlikely as *Mtk* transgenic plants never showed distinguishable detrimental effects on plant organs. Alternatively, and more likely, antifungal Mtk in the leaf apoplastic space may weaken the fitness of the attacking fungus and, in that way, disturb the pathogenic fungus' essential capability actively to suppress the plant defence. Since defence suppression is brought about by effector molecules secreted by fungal microbes (Birch *et al.*, 2009), it is anticipated that the fungal secretory pathway is indirectly affected by Mtk. Notably, active suppression of plant defence is a prerequisite for a successful fungal infection, a compatible interaction, and, eventually, pathogenesis (Kogel *et al.*, 2006).

To elucidate in depth the impact of *Mtk* on the defence system, the expression of *pathogenesis-related* genes *PR-1b* and *PR-5* were analysed. Without *Bgh* challenge, *Mtk* and GP plants showed equally low basal levels of *PR* genes expression. Upon fungal inoculation, *PR* genes expression significantly increased in GP and in *Mtk* plants (Fig. 4), which further supports the hypothesis that *Mtk* plants show a higher defence status upon challenges with powdery mildew compared with non-transgenic plants. Importantly, low *PR* gene expression in non-challenged *Mtk* plants suggests a low general stress status of the transgenic plants. Hence, molecular evidence is provided that expression of antifungal Mtk does not set off high fitness costs expected to affect yield in various other resistance strategies (Heil and Baldwin, 2002; Tian *et al.*, 2003).

Consistent with *in vitro* inhibition of *Fusarium* spp. by synthetic Mtk, expression of *Mtk* in barley strikingly decreased *Fusarium* head blight and root rot damage (Fig. 2). Bioassays for disease resistance revealed that pathogen development in both roots and kernels was hampered. Thus, the lines generated in the present study might provide a promising source for breeding programmes aimed at improving the resistance to diseases caused by *Fusarium* spp.

There are a number of reports in which antimicrobial peptides of insect (Osusky *et al.*, 2000; Langen *et al.*, 2006), frog (Chakraborti *et al.*, 2003; Yevtushenko and Misra, 2007) or human (Zakharchenko *et al.*, 2005; Aerts *et al.*, 2007) have been used to render the transformed plants more resistant to fungal pathogens. Beside the evaluation of Mtk effects on the development of pathogenic fungi, its possible adverse impact on the beneficial root endophyte *Piriformospora indica* (Waller *et al.*, 2005) has been investigated, too. The results show that Mtk does not inhibit growth of this fungus in roots of transgenic plants. In order to substantiate the differential toxicity of Mtk, and since the ascomycetes *Blumeria* and *Fusarium* were affected by Mtk, the hypothesis was tested whether Mtk exerts differential activities on ascomycete and basidiomycete fungi. The results demonstrate that protoplasts isolated from axenic mycelia of ascomycete *F. graminearum* were much more sensitive to Mtk than those from the basidiomycetes *P. indica* and *Rhizoctonia solani* (Fig. 6), suggesting that the differential sensitivity of the fungi might be attributed to structural and/or biochemical differences of ascomycetes and basidiomycetes.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. (A) Whole amino acid sequence of native metchnikowin peptide transformation; (B) T-DNA construct used for plant transformation; (C) relative quantification of *Mtk* expression in lines L4, L42, L50, and L57 by quantitative RT-PCR with cDNA obtained from total RNA as template.

Supplementary Fig. S2. Growth response of Golden Promise and *Mtk* seedlings L4 to *Fusarium graminearum* inoculation.

Supplementary Fig. S3. Microscopy of root colonization by *P. indica* in Golden Promise (WT) and transgenic barley L4.

Supplementary Table S1. Activity of *mas* promoter in barley after auxin treatment and during challenges with different pathogens.

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