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A defensin from tomato with dual function in defense and development

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Abstract Defensins are antimicrobial peptides that are part of the innate immune system, contributing to the first line of defense against invading pathogens. Defensins and defensin-like peptides are functionally diverse, disrupting microbial membranes and acting as ligands for cellular recognition and signaling. Here we show that the tomato defensin *DEF2* is expressed during early flower development. Defensin mRNA abundance, peptide expression and processing are differentially regulated in developing flowers. Antisense suppression or constitutive overexpression of DEF2 reduces pollen viability and seed production. Furthermore, overexpression of DEF2 pleiotropically alters the growth of various organs and enhances foliar resistance to the fungal pathogen *Botrytis cinerea*. Partially purified extracts from leaves of a DEF2-overexpressing line inhibited tip growth of *B. cinerea*. Besides providing insights into regulation of defensin expression, these data demonstrate that plant defensins, like their animal counterparts, can assume multiple functions related to defense and development.

Keywords Defensin · DEF2 · Transgenic tomato · Development

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Introduction

Although most animal and plant defensins kill or inhibit the growth of microbial pathogens directly (Schroeder 1999), some of these cationic peptides have been shown to activate the adaptive immune system (Yang et al. 1999) or mediate developmental events (Zhou et al. 2004). An epididymis-specific β-defensin proved to be important for the acquisition of sperm motility and initiation of sperm maturation (Zhou et al. 2004). Interaction between a defensin-like peptide secreted from pollen with its cognate *S*-locus receptor kinase on the stigma triggers a self incompatibility response to prevent self fertilization in *Brassica* (Takayama et al. 2001; Yang et al. 1999).

More than 300 defensin-like peptides exist in the *Arabidopsis* genome, 78% of which have a cysteine-stabilized α-helix β-sheet (CS$\alpha\beta$) motif common to plant and invertebrate defensins (Silverstein et al. 2005; Thomma et al. 2002). Defensin-like genes have been grouped into classical, seed- and nodule-specific defensins based on the number and positions of cysteine residues (Graham et al. 2008). Analysis of 1,100 expressed genes from 26 different species indicated that 60% of the defensin-like subgroups are restricted to single taxonomic clades. Taxonomic expansion was most striking in the case of nodule-specific defensin-like genes from legumes. It has been speculated

that an originally antimicrobial peptide was co-opted to function as a defensin-like peptide in self incompatibility (Nasrallah 2002). Additional functions in plant development are likely because defensins have been shown to inhibit the growth of roots and root hairs in *Arabidopsis thaliana* (Allan et al. 2008).

Like other plants, Solanaceae produce archetypal defensins [Supplemental Fig. S1A]. A subset of these defensins contains an acidic carboxyterminal domain following the mature peptide domain (Supplemental Fig. S1B). A prodefensin from tobacco has previously been shown to undergo processing (Lay et al. 2003), but the functional significance of removal of the C-terminal prodomain remains unknown. By comparison, processing is required for antimicrobial activity of mammalian α-defensins (Wilson et al. 1999). Most plant defensins are active against fungi (Thevissen et al. 2000) whereas others inhibit α-amylase activity from insects (Bloch and Richardson 1991) or protein synthesis (Mendez et al. 1990). Defensins are expressed in tissue-specific patterns (Ferrandon et al. 1998; Thomma and Broekaert 1998). In a study of flower development, a defensin from tomato was identified as one of six pistil-expressed genes (Milligan and Gasser 1995). This defensin, here referred to as *DEF1* (Supplemental Fig. S1B), is expressed in the outermost layers of leaf primordia and floral organs (Brandstadter et al. 1996). We report herein functional analysis of the closely related *DEF2* gene by altering its expression in transgenic tomato plants.

Materials and methods

Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicon*) cv. VF36 and cv. Zhongshu 5 were obtained from the Tomato Genetics Resource Center and Les Fuchigami (Wang et al. 2005), respectively. Plants were grown in 2 l pots containing Sunshine SB40 soil mix (SunGro Horticulture, Bellevue, WA) supplemented with osmocote fertilizer (15-9-12) under greenhouse conditions with supplemental lighting using high-pressure sodium lamps (400 W). Day and night time temperatures were 21 and 16°C, respectively.

Construction of bacterial expression vectors

Defensin clones TPP3 and cTOB11C9 were obtained from Charles Gasser (Department of Molecular and Cellular Biology, University of California, Davis) and Clemson University Genomics Institute, respectively. The pMAL-c2X vector (New England Biolabs, Beverly, MA) was used for bacterial expression of defensins. Primers 5′-CGGAATTCGGATCCCAAATTTGCAAAGCACCAAGC-3′ and 5′-GCTCTAGAGGATCCTTAACATGGCTTAGTGCATAGACACTTC-3′ were used for amplification of *DEF1* with Accuzyme (Bioline, Springfield, NY); primers 5′-CGGAATTCGGATCCCAGATGTGCAAATCAACAAGC-3′ and 5′-GCTCTAGAGGATCCTTAACAAACCTTAGTACATAGGCACTTTC-3′ were used for amplification of *DEF2*. The annealing temperature was 65°C. PCR products were directionally cloned into pMAL-c2X using *Eco*RI and *Xba*I and transformed into *E. coli* BL21. DNA inserts were sequenced. IPTG was used to induce expression. Amylose resin and maltose were used for protein purification and elution. Recombinant DEF1 and DEF2 peptides were cleaved with Factor Xa to release mature defensins.

Construction of plant expression vectors

pBinVec3 was obtained from Jeff Leonhard (Crop and Soil Science, Oregon State University, Corvallis). pBinVec3 is a derivative of pGPTV-KAN (Becker et al. 1992). The uidA::pAnos cassette of pGPTV-KAN was replaced with the pAg7::pmas::pAocs and pCaMV35S::pA35S cassettes of pPCV91 using *Eco*RI and *Hind*III sites (Martin et al. 2001; Strizhov et al. 1996). The resulting orientation of transcription from the quadruple CaMV 35S promoter is opposite to the direction of the *nptII* gene. Primers 5′-CGGGATCCCATGGCTCGTTCCATTTGCTTC-3′ and 5′-CGGGATCCTTACTCCATCACAATCTCTTCTTCAAG-3′ were used to amplify the complete open reading frame of *DEF2* with Accuzyme. The annealing temperature was 65°C. *DEF2* was inserted between the quadruple CaMV 35S promoter and the 35S terminator using a unique *Bam*HI cloning site. *Xcm*I was used to determine insert orientation. Plasmids containing *DEF2* in the sense or antisense orientation were transformed into the *Agrobacterium tumefaciens* EHA105. The DNA insert was sequenced prior to plant transformation.

Plant transformation

Tomato cv. Zhongshu 5 was transformed according to a published protocol (Wang et al. 2005), except that 500 mg l^{-1} cefotaxime was used to kill *Agrobacterium* after co-cultivation. Plantlets were transferred to soil, acclimated, and moved to the greenhouse. Transgenic T_0 lines were self-pollinated. Subsequent T_1 and T_2 generations were selected on ½ MS, 15 g l^{-1} sucrose, 100 mg l^{-1} kanamycin.

Analysis of plant genomic DNA

Genomic DNA was extracted from tomato leaves using cetyltrimethylammonium bromide (Fulton et al. 1995). For

Southern hybridization (Sambrook and Russell 2001), *DEF2* insert was excised with *Eco*RI and *Xho*I and used in conjunction with an AlkPhos Direct Labeling Kit (Amersham Biosciences, Piscataway, NJ) for detection of hybridizing bands.

The CaMV 35S terminator-specific primer 5′-TCTTA TATGCTCAACACATGAGCG-3′ was used in combination with a *DEF2*-specific forward 5′-ATCAACAAGCCA AACCTTCAAG-3′ or reverse primer 5′-GCTTCCCCAAC CAAAGTTGT-3′ to determine the orientation of sense and antisense constructs, respectively. Taq polymerase (GeneScript, Piscataway, NJ) was used for amplification with an annealing temperature of 55°C.

Reverse transcriptase (RT)-PCR and real-time PCR

Total RNA was extracted from leaves or floral buds and organs using the phenol-LiCl method (Sambrook and Russell 2001). cDNA was synthesized using 2 μg of RNA and a first-strand synthesis kit (GE Healthcare, Piscataway, NJ). Endogenous gene expression was traced using the forward primer for *DEF2* in combination with a primer 5′-AAAGCCCAATAACACGACATT-3′ specific to the 3'UTR of *DEF2*. Transgene expression was monitored using forward or reverse *DEF2* primer in combination with the CaMV 35S terminator-specific primer. Primers 5′-CG AGAGAAGATGACTCAGATC-3′ and 5′-AGGTGGGGC GACAACCTTG-3′ were used to amplify an intron-spanning piece of the actin *Tom52* (Moniz and Drouin 1996). Taq polymerase was used for amplification with an annealing temperature of 55°C. *DEF1* was amplified using primers 5′-CTCTTTGTTACCTATGAGGTAG-3′ and 5′-CTTCCTCACCCAAAGTTGCT-3′.

Primers and probes for real-time PCR of *DEF2* were 5′-GACCATGGCTCGTTCCATTT-3′, 5′-CAAAGAGCA CCATTGCCAAGA-3′, and 5′-6FAM-CCTCATGGCAC TTATG-MGBNFQ-3′. Primers and probes for the endogenous *DEF2* were 5′-GCTTGAAGAAGAGATTGTGAT GGA-3′, 5′-ACACGACATTAGAAGCTACCCTTTTAC-3′, and 5′-6FAM-TTGAGTGTCAAAATCA-MGBNFQ-3′. A VIC/MGB-labeled eukaryotic 18S endogenous control (Applied Biosystems, Foster City, CA) was used to determine relative mRNA abundance. Triplicate samples were analyzed. *DEF2* plasmid and salmon sperm DNA were used for calibration using the Relative Standard Curve Method, as recommended by the manufacturer.

Immunoblot analysis

A peptide-specific polyclonal antibody was generated against the surface-exposed defensin epitope QTFKGLaFTD, located between the first β-strand and the α-helix. An Ala was inserted at position 7 because the Cys residue is likely engaged in a disulfide bond and not accessible to the antibody. The peptide antigen and the antibody from rabbit were commercially generated (Biocarta, San Diego, CA).

Proteins were extracted in 130 mM acetic acid and subsequently incubated for 2 h on ice (Chipps et al. 2005; Schröder et al. 1992). An equal volume of ethanol was added to the extract and the mixture was stored in the freezer overnight. After centrifugation at 10,000*g* for 10 min, the pellet was resuspended in sample buffer (Laemmli 1970). The supernatant was concentrated in a Savant SpeedVac Concentrator (Thermo Scientific, Waltham, MA) and resuspended in 0.01% (v/v) acetic acid. Soluble proteins were quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Proteins extracted in sample buffer were quantified using the RC DC Protein assay (Bio-Rad, Hercules, CA).

Peptides were separated using a modified Tricine-based SDS-PAGE method (Schagger and von Jagow 1987; Titus 1990) and transferred to ProBlott membrane (Applied Biosystems, Foster City, CA). An ECL Western Blotting Analysis System (GE Healthcare, Piscataway, NJ) was used for antigen detection.

Phenotypic analysis

Plant height and fresh weight of ripening tomato fruits were measured. Numbers of seeds per fruit ($n \geq 3$) were counted and the age at which mature fruits were harvested was determined.

Plant inoculation

Foliar inoculation assays were performed as published (Guimaraes et al. 2004). Detached leaves were inoculated with 2 μl of a suspension containing 500 conidia of *Botrytis cinerea* strain B05.10. Conidia were preconditioned for 3 h in Gamborg's B5 medium containing 10 mM sucrose and 10 mM potassium phosphate, pH 6. The adaxial surface of each leaf was inoculated at 10 different locations. Leaves were inserted into moist florist foam and incubated in clear plastic boxes under saturating humidity and low light conditions (Guimaraes et al. 2004). Frequency and size of expanding lesions were recorded 2 and 3 days post-inoculation. Non-expanding lesions can form a dry black necrosis, whereas expanding lesions are brown and water-soaked (Guimaraes et al. 2004). A caliper was used to measure lesion diameters.

Antifungal assay

In vitro assays were carried out as previously described (Li et al. 2001; Osborn et al. 1995). Conidia were harvested

from sporulating cultures grown on potato dextrose agar (PDA) (Guimaraes et al. 2004). Conidia (5,000 ml^{-1}) were suspended in potato dextrose broth. Soluble fractions of acetic ethanol extracts were generated from leaves as described above. Aliquots in 0.01% (v/v) acetic acid (50 µl) were incubated with conidia (50 µl) in wells of microtiter plates for less than 24 h at ~20°C. Fungal germination and growth were visualized with an Axiovert S 100 microscope (Carl Zeiss Inc., North America). Partial purification of foliar extracts was achieved using 3 and 30 kD Microcon molecular cut-off filters (Millipore, Billerica, MA). Magainin 2 (American Peptide Company, Sunnyvale, CA) was used as a positive control.

Microscopy

Pollen viability was assayed as previously described (Li et al. 2004). Anthers were removed from flowers at anthesis and squashed into a droplet containing 5 µg ml^{-1} fluorescein diacetate and 50 µg ml^{-1} propidium iodide. An Axiovert S 100 (Carl Zeiss Inc., North America) was used for fluorescence microscopy in combination with FITC (excitation: 495.5 nm, emission: 531 nm) and Texas Red (excitation: 571 nm, emission: 627 nm) filter sets. Callose was visualized after staining squashed anthers from immature flowers with 0.05% (w/v) aniline blue in 0.1 M sodium phosphate, pH 8.5. A DAPI (excitation: 402.5 nm, emission: 462 nm) filter set was used for microscopy.

Statistical tests

Averages and frequencies were analyzed with the statistics software SAS (Cary, NC) using ANOVA and a generalized linear model (GENMOD), respectively. Least square means or contrasts were used to statistically determine separation ($\alpha = 0.05$). Homogeneity of variances was determined using Levene's test.

Results

Endogenous expression of defensin mRNAs and peptides

DEF2 was abundantly expressed in immature floral buds at the meiotic stage (Goldberg et al. 1993) but rapidly declined thereafter (Fig. 1a). *DEF2* was more abundant in petals and stamen than in sepals of 6–7 mm long floral buds. This pattern of expression was in stark contrast to *DEF1* (Fig. 1b), clearly illustrating specific regulation of defensin gene expression during flower development. Conversely to *DEF2*, expression of *DEF1* was low in stamen relative to petals and pistils, indicating that female and male reproductive organs preferentially express *DEF1* and *DEF2*, respectively.

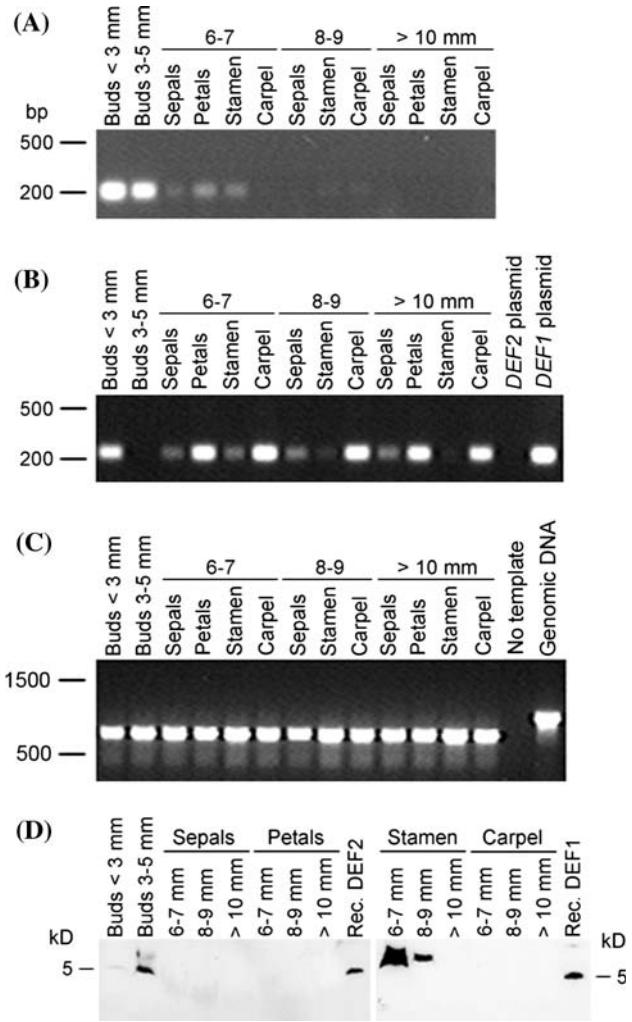

Fig. 1 Defensin expression in developing flowers. **a** *DEF2* mRNA levels in immature floral buds and organs of tomato cv. VF36, determined by RT-PCR. The gene was amplified for 30 cycles. Similar results were obtained after 20 or 25 cycles. Bud sizes define developmental stages. **b** *DEF1* mRNA levels in immature floral buds and organs of tomato cv. VF36, determined by RT-PCR. Bud sizes indicate developmental stages. Plasmids containing *DEF1* or *DEF2* were used to demonstrate gene-specific amplification. The gene was amplified for 30 cycles. Similar results were observed after 20 or 25 cycles. **c** *Actin* mRNA levels in immature floral buds and organs of tomato cv. VF36, determined by RT-PCR. Amplification of genomic DNA shows that cDNA preparations were not contaminated. The gene was amplified for 30 cycles. **d** Expression and processing of soluble defensin peptides in immature floral buds and organs of tomato cv. VF36, determined by immunoblot analysis. Extracted proteins (2.5 µg) or recombinant defensins (0.5 µg) were separated. Recombinant DEF1 and DEF2 fusion proteins, cleaved with Factor Xa, were used as size standards for mature defensin peptides

Mature peptides and pro-peptides of DEF1 and DEF2 are predicted to have molecular weights of 5.3 and 5.2 kD and 8.9 and 8.7 kD, respectively. As expected, mature forms of recombinant DEF1 and DEF2 peptides were

recognized by an anti-defensin epitope antibody in form of ~5 kD bands (Fig. 1d). Lack of discrimination between DEF1 and DEF2 peptides by the antibody maybe explained by the fact that only 2 out of 10 amino acids differed between DEF1 and DEF2 epitopes. This antibody recognized peptides of >5 kD only in immature floral buds and stamen of tomato cv. VF36 (Fig. 1d). A ~5 kD band indicative of mature defensins accumulated at the meiotic stage in <3 mm long floral buds (Fig. 1d). Two antibody-reactive bands were observed in 3–5 mm long floral buds, most likely indicating expression of prodefensin, presumably containing the C-terminal domain, and mature defensin. The putative defensin precursor was also expressed in stamen from 6–9 mm long floral buds because only a band of approximately 9 kD was observed (Fig. 1d). The antibody used for detection did not discriminate between recombinant DEF1 and DEF2 peptides (Fig. 1d). The finding that antibody-reactive bands of >5 kD were not detected in carpels (Fig. 1d), is surprising because this organ is known to strongly express *DEF1* mRNA (Milligan and Gasser 1995).

Effects of *DEF2* mRNA expression on fertility of T_0 and T_1 generations

To investigate the function of *DEF2*, tomato cv. Zhongshu 5 was transformed separately with sense and antisense constructs of this gene. Ten transgenic plants were recovered that expressed the antisense transcript, but just three plants overexpressed the transgene. The transformation efficiency for the *DEF2* sense construct was significantly lower, around 1% (data not shown), than the transformation efficiency for other genes, which is 30–40%. Endogenous *DEF2* mRNA was detected only in two of the overexpressors (Fig. 2), suggesting cosuppression (Jorgensen 1995) in the remaining transformant. This orientation-dependent difference in recovery of transformants provided evidence that ectopic expression of defensin interfered with plant regeneration ($\chi^2 = 5.33$, $P = 0.021$).

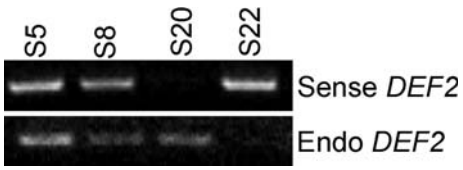

Fig. 2 *DEF2* mRNA levels in leaves of individual transgenic tomato plants cv. Zhongshu 5 of the T_0 generation as determined by RT-PCR. Sense plants are initialized S followed by numeric designators. Note that plant S20 does not express the transgene although it was confirmed as transformed by Southern blot hybridization. Plant S22 lacks detectable endogenous *DEF2* expression perhaps due to cosuppression

Transformant S5, one of the two plants ectopically expressing *DEF2* (Fig. 2), was lost because it never set seed. Line S22 was not analyzed further because endogenous *DEF2* mRNA expression was not detected (Fig. 2) and because no evidence of increased DEF2 protein expression was obtained by immunoblot analysis. Moreover, seed production and fruit weight was not significantly different from untransformed tomato (data not shown). The remaining line S8 and five antisense lines varying in *DEF2* expression were selected for further analysis (Fig. 3a). The sense line S8 produced fewer seeds than normal, but the defect was less severe compared to transgenic plant S5 (Fig. 3b). Analysis of the T_1 generation also yielded an inverse correlation between seed production and expression of antisense transcript (Fig. 3).

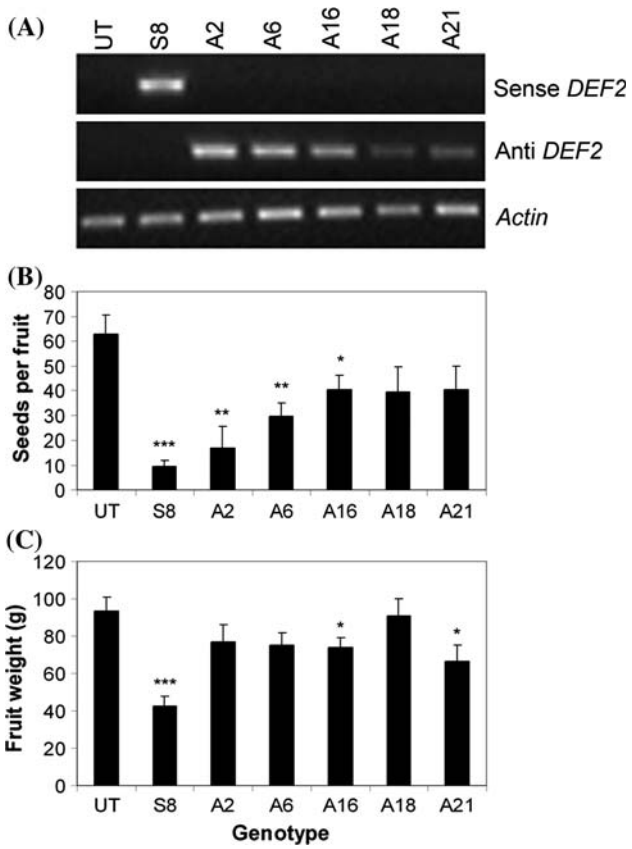

Fig. 3 Reduced seed set and fruit size as a function of altered *DEF2* expression. **a** Expression of *DEF2* transgene in immature floral buds of tomato cv. Zhongshu 5 containing sense or antisense constructs as determined by RT-PCR. Sense and antisense lines of the T_1 generation are initialized S and A, respectively, followed by numeric designators and are compared to untransformed (*UT*) plants. *Actin* was used as a loading control. Genes were amplified for 28 cycles. **b** Seed production of UT and transgenic tomato plants. *Error bars* indicate SEM ($n \geq 10$). *Asterisks* indicate statistically significant differences from UT at $P < 0.001$ (***), $P < 0.01$ (**), or $P < 0.05$ (*). **c** Fruit weights of UT and transgenic plants. *Error bars* indicate SEM ($n \geq 11$). *Asterisks* indicate statistically significant differences from UT at $P < 0.001$ (***) or $P < 0.05$ (*)

Fruit maturation was delayed by more than 4 weeks in line S8 compared to untransformed tomato (*t*-test, $P = 0.002$). Fruits from line S8 were also significantly smaller than fruits from untransformed plants whereas the effect of antisense expression on fruit size was relatively minor (Fig. 3c).

Expression of *DEF2* mRNA and defensin peptides during the T_2 generation

Transgene expression was monitored by real-time PCR in the selected sense and antisense lines S8 and A2 (Fig. 4a). Constitutive expression of *DEF2* driven by the cauliflower mosaic virus (CaMV) 35S promoter was observed during all floral stages analyzed. Constitutive expression of *DEF2* mRNA in line S8 was less than tenfold higher than the endogenous expression of this gene in ~3 mm long untransformed floral buds (Fig. 4a). Simultaneously, endogenous *DEF2* expression declined exponentially (Fig. 4a, b). No significant differences in endogenous *DEF2* expression were observed in lines S8 and A2 (Fig. 4b).

Mature DEF2 was expressed during all floral stages in line S8 (Fig. 4c). On the contrary, defensin was exclusively expressed in ~3 mm long floral buds of untransformed tomato cv. Zhongshu 5. Defensins, recovered from floral buds of untransformed plants and line S8, were insoluble at the meiotic stage. By comparison, defensin expression was absent from flowers of antisense line A2. *DEF2* mRNA and protein levels were therefore not correlated in line A2.

Effect of altered DEF2 peptide expression on male reproductive development

Based on fertility defects observed during T_0 and T_1 generations (Fig. 3), we hypothesized that altered DEF2 expression interferes with male development. We therefore tested pollen viability in homozygous sense and antisense plants of the T_2 generation (Fig. 5a and Supplemental Fig. S2). Pollen viability correlated with seed production in that

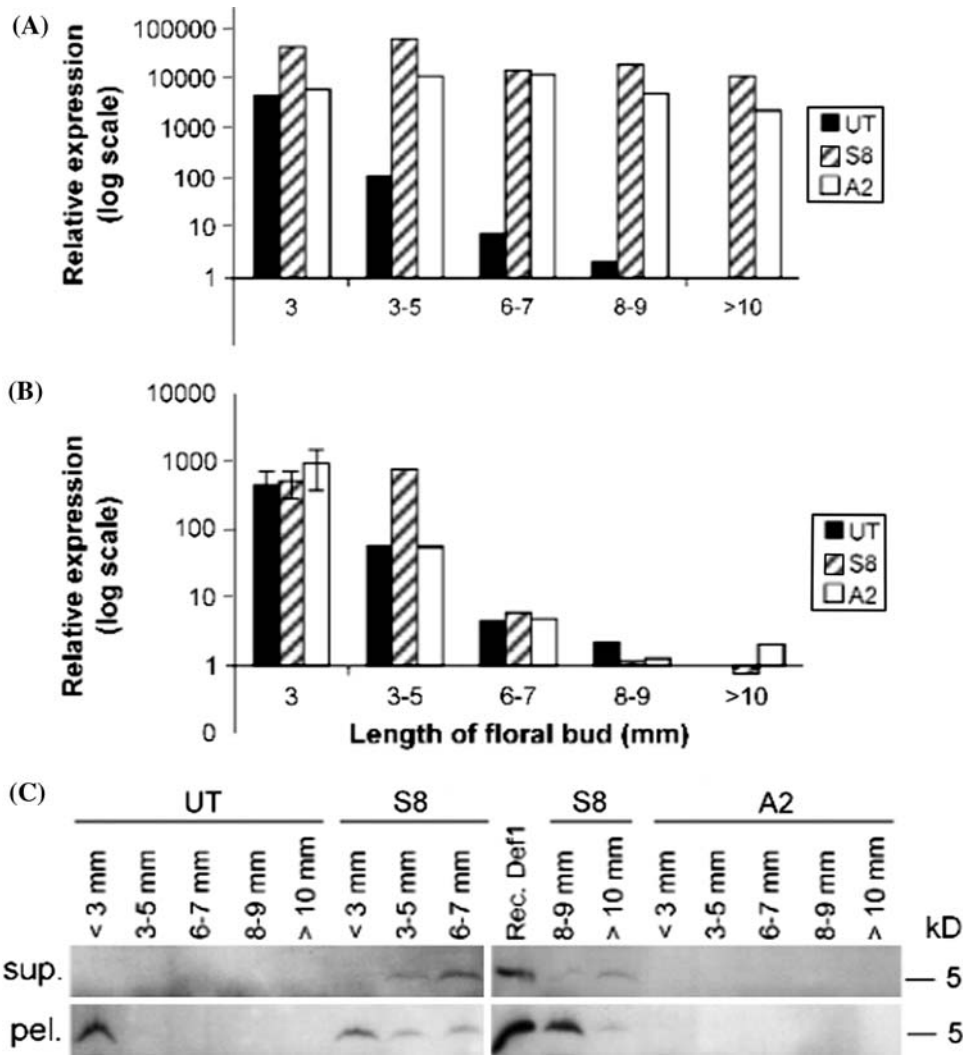

Fig. 4 *DEF2* mRNA and DEF2 protein expression in transgenic tomato plants of the T_2 generation. **a** Total *DEF2* mRNA levels in untransformed (*UT*) and homozygous transgenic lines (S8 and A2), determined by real-time PCR. Total *DEF2* mRNA levels in lines S8 and A2 primarily reflect transgene expression. Bud sizes define developmental stages. Results of an individual experiment are shown. **b** Endogenous *DEF2* mRNA levels, determined by real-time PCR. Averages of three experimental replicates are shown for the earliest developmental stage; *error bars* indicate s.e.m. Averages of two experiments are shown for all other stages. **c** Expression of DEF2 protein in supernatant and pellet fractions of extracts from UT, S8, and A2 plants, determined by immunoblot analysis. Recombinant (abbreviated Rec.) DEF1 (0.2 µg) was used as a size marker. Note that processed DEF2 peptide of the expected size is constitutively expressed in sense line S8. No DEF2 peptide expression is observed in antisense line A2

line S8 produced a significantly smaller percentage of viable pollen grains than line A2 (Fig. 5b). No significant differences in meiosis and tetrad formation were observed in immature anthers from line S8 and untransformed plants. The only aberration we observed in meiotic products of line S8 was an infrequent production of supernumerary microspores (Fig. 5c). As microscopic examination of cross sections through 3–5 mm long floral buds from line S8 revealed little evidence of aberrations, the defect that was observed in mature pollen grains must have expressed itself after microsporogenesis. The exact time point when the pollen viability defect occurred was not determined because we did not analyze later stages of microgametogenesis. Nevertheless, we hypothesize that continuous expression of mature DEF2 in developing flowers (Fig. 4c) interferes with further development and maturation of pollen grains.

The ratio of callose-containing pollen mother cells to tetrads was 6:1 for line A2 but 2:5 for untransformed tomato and line S8 when anther squashes from ~3 mm long floral buds were examined (Fig. 5c). Furthermore, two out of four flowers from line A2 were in meiosis or in the tetrad stage when cross-sections through developing anthers of ~3 mm long floral buds were examined, whereas none of four flowers from untransformed tomato were in meiosis but three flowers were in the tetrad stage. These observations indicate that silencing of defensin peptide expression (Fig. 4c) retards meiosis (Fisher's exact test, $P = 0.030$).

Alteration of plant growth by ectopic expression of DEF2 peptide

Ectopic expression of DEF2 had pleiotropic effects on plant growth. Although growth was initially retarded, homozygous S8 seedlings caught up and eventually surpassed the growth of untransformed tomato plants (Fig. 6a). The stature of S8 plants differed from

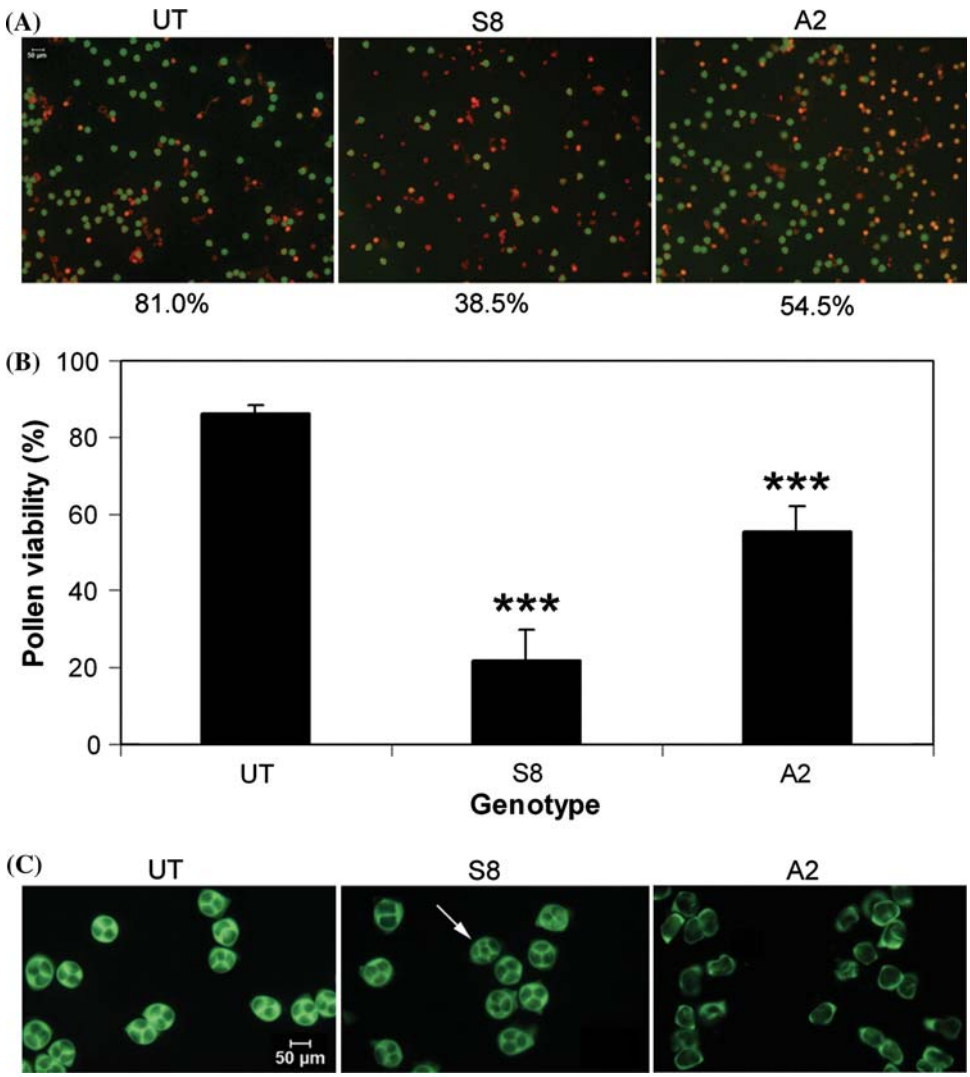

Fig. 5 Defects in male reproductive development occurring in *DEF2* transformants of the T_2 generation. **a** Staining of alive and dead pollen grains from untransformed (*UT*) and homozygous transgenic (S8 and A2) tomato cv. Zhongshu 5 with fluorescein diacetate (*green*) and propidium iodide (*red*), respectively. The percentage of viable pollen at anthesis is shown below the images. **b** Pollen viability as a function of genotype. *Error bars* indicate s.e.m. ($n = 7$). *Asterisks* indicate statistically significant differences from UT at $P < 0.001$. **c** Aniline blue staining of callose in tetrads (UT and S8) and pollen mother cells (A2). Production of supernumerary microspores is indicated (*arrow*)

untransformed plants in that the leaves were smaller and growth was more upright, resulting in a more open architecture (Fig. 6b). Sepals of line S8 were shorter, making flowers appear stubby (Fig. 6c). The style length of line S8 was significantly shorter relative to untransformed plants (Fig. 6d, Supplemental Fig. S3A). No genotypic differences in the size and numbers of ovules were apparent (Supplemental Fig. S3B).

Protection against *B. cinerea* by foliar expression of mature DEF2 peptide

Foliar expression of mature DEF2 (Fig. 7a) resulted in a significant decrease in disease incidence when tomato leaves were challenged with a conidial suspension of the pathogenic fungus *Botrytis cinerea* (Fig. 7b), consistent with the predicted antimicrobial activity of this peptide. Comparison of the relative antibody-reactive band intensities (Fig. 7a) suggests that DEF2 contributes to approximately 0.9% of the soluble protein in leaves of line S8, an expression level that is not dissimilar from the expression level of a radish defensin in transgenic tobacco (Terras et al. 1995). Despite this difference in disease incidence, the rate of lesion expansion was not altered. Noticeably, none of these changes in plant growth and defense affected line A2, illustrating a rather specific effect of antisense suppression on male reproductive development.

To further test the antifungal activity of DEF2, foliar extracts from transgenic lines or untransformed plants were incubated with conidia of *B. cinerea*. Soluble fractions inhibited hyphal growth (Fig. 8a). Surprisingly low protein concentrations were inhibitory. Foliar extract from the transgenic line S8 expressing DEF2 (Fig. 7a) was more active than those from the other two genotypes. Size fractionation was used to enrich for DEF2. Partial purification of molecules with a predicted size of 3–30 kD enhanced the genotype-dependent differences in antifungal activity (Fig. 8b). Whereas fractions from untransformed and antisense plants were not active, equivalent material from line S8 inhibited hyphal tip growth. It is likely that DEF2 is the active ingredient (i) because antifungal activity was only observed in size-fractionated material from line S8 and (ii) because DEF2 has a molecular mass of 5 kDa. Antifungal activity from line S8 differed from magainin, an antimicrobial peptide from the skin of *Xenopus levis* (Zasloff et al. 1988), in that the latter reduced the frequency of fungal germination, whereas the former disrupted fungal growth from the tip to the base. These effects were concentration-dependent. By comparison, fractions <3 kD did not contain antifungal activity (data not shown).

Discussion

Expression of defensin mRNAs and peptides

Our data suggest that different defensin genes are expressed in male or female organs (Fig. 1a, b). Surprisingly defensin was not found to be expressed in carpels of tomato cv. VF36 (Fig. 1d) although *DEF1* mRNA is abundantly

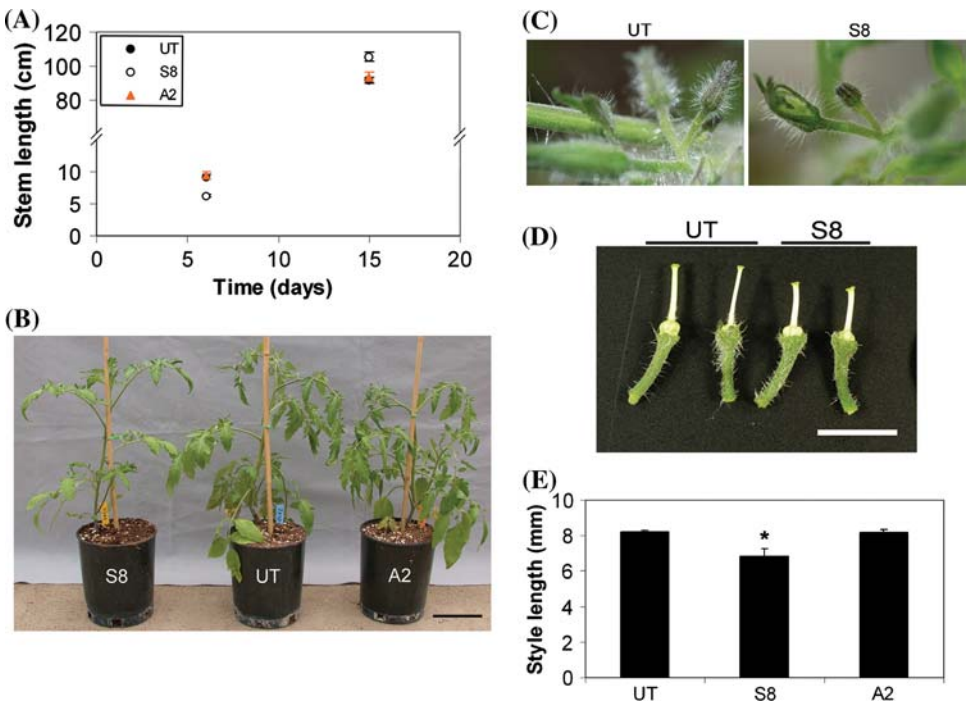

Fig. 6 Pleiotropic effects of ectopic *DEF2* expression on organ and plant growth. **a** Growth of untransformed (*UT*) and homozygous transgenic (S8 and A2) tomato cv. Zhongshu 5. *Error bars* indicate s.e.m. ($n = 7$). **b** Image of 8-week-old plants; *bar*, 10 cm. **c** Sepals of line S8 are shorter compared to UT tomato. **d** Image of carpels of flowers at anthesis; *bar*, 1 cm. **e** Style lengths as a function of genotype. UT, S8, and A2 tomato are compared. *Error bars* indicate s.e.m. ($n \geq 5$). *Asterisk* indicates statistically significant differences from UT at $P < 0.05$

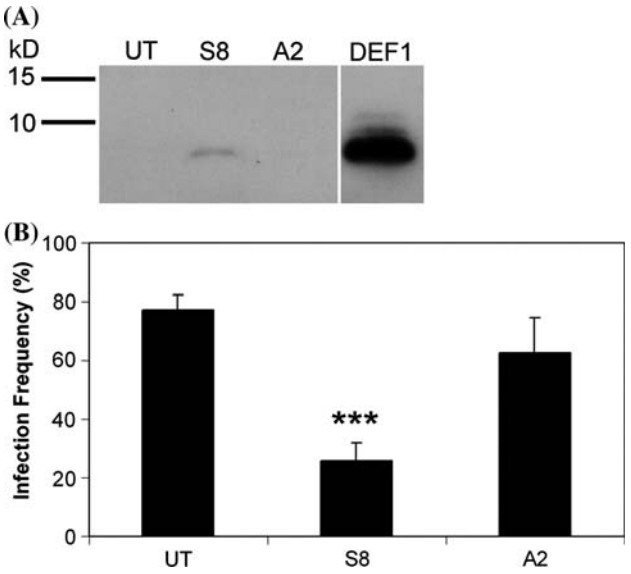

Fig. 7 Constitutive DEF2 expression protects against the fungal pathogen *B. cinerea*. **a** Expression of soluble defensin peptides in leaves of untransformed (UT) and homozygous transgenic lines (S8 and A2), determined by immunoblot analysis. Extracted proteins (2.5 µg) or recombinant DEF1 (0.9 µg) were separated. The latter was used as a size marker. **b** Frequency of foliar infection 3 days post-inoculation by *B. cinerea*. Leaves from seven plants of each genotype were tested. Homozygous transgenic plants of the T_2 generation (S8 and A2) were compared to untransformed (*UT*) plants ($n = 7$, means ± s.e.m.). *Asterisks* indicate statistically significant differences from UT at $P < 0.001$. The experiment was performed twice with similar results

expressed in this floral organ (Milligan and Gasser 1995). The most likely explanation is that DEF1 expression is regulated at translational or post-translational levels because the antibody used reacted with recombinant peptides of both DEF1 and DEF2 (Fig. 1d).

Defensin peptide expression apparently differed between the tomato cultivars that were studied. Whereas defensins were detectable at different stages of tomato cv. VF36 development (Fig. 1d), these peptides seemed to be present only in <3 mm long floral buds of tomato cv. Zhongshu 5 (Fig. 4c). However, floral buds from the latter cultivar were not dissected, thus obviating enrichment of defensins in particular floral organs. Antigenic bands indicative of prodefensin were only in material from tomato cv. VF36, but apparent differences in regulation of the processing enzyme between cultivars during later stages of anther development are expected to be inconsequential assuming that the precursor peptide is inactive. Nevertheless, mature defensin peptides accumulated during the meiotic phase of flower development in both cultivars.

Insoluble rather than soluble defensins were recovered from floral buds at the meiotic stage (Fig. 4c), indicating a possible association of these peptides with plant cell wall components. The pectin-rich middle lamella of pollen mother cells thickens about tenfold at this stage (Gorman and McCormick 1997). PSORT, a program for predicting subcellular localization, assigned DEF2 to the cell wall (Nakai and Horton 1999). Nevertheless, different plant defensins are localized in the cell wall (Gao et al. 2000; Terras et al. 1995) or in the vacuole (Lay et al. 2003). Thus, we cannot exclude possible targeting of tomato defensins to the vacuole. Association with the pellet may be an extraction artifact caused by binding to insoluble aggregates, such as negatively charged pectic polysaccharides, which are likely to interact with cationic peptides, or starch grains.

Antisense suppression of defensin peptides (Fig. 4c) in homozygous A2 plants was not paralleled by changes in *DEF2* mRNA expression (Fig. 4b) despite abundant expression of the transgene (Fig. 4a). Analysis of the primary transgenics also revealed that the majority of the antisense plants generated lacked detectable DEF2 peptide expression even though *DEF2* mRNA was expressed (Supplemental Fig. 4). Significant positive correlation between sense and antisense expression was recently documented in plants using abiotic stress conditions and a whole-genome tiling array (Matsui et al. 2008a, b). It is possible that *DEF2* antisense transcript blocks translation (Faghihi and Wahlestedt 2006). Alternatively, other types of regulation maybe involved as antisense RNAs participate in gene silencing, RNA stability, alternative splicing, RNA editing, RNA masking and methylation (Enerly et al. 2005; Hastings et al. 1997; Jen et al. 2005; Katiyar-Agarwal et al. 2006; Matsui et al. 2008a, b; Prescott and Proudfoot 2002; Tufarelli et al. 2003; Zubko and Meyer 2007).

Effects of altered *DEF2* expression on male reproduction

Our results illustrate that DEF2 functions in male reproductive development. Phenotypic analysis of DEF2-silenced tomato plants implies that this defensin promotes meiosis in wild-type plants. Retardation of meiosis in DEF2-silenced tomato (Fig. 5c) apparently translates into a pollen viability defect similarly to late-acting sporogenous male sterile mutants (Gorman and McCormick 1997). Thus, DEF2 appears to function in developmental signaling. The only defensin-like peptide known to contribute to developmental signaling is the sterility locus cysteine-rich protein, which is expressed in pollen, but which has not been investigated for antimicrobial activity to our knowledge.

Inactivation of DEF2 during later stages of flower development is required for survival and development of pollen grains as continuous production of this peptide in transgenic tomatoes leads to a defect in male fertility. Alternatively, pleiotropic effects of constitutive DEF2 expression on plant growth (Fig. 6) may indirectly result in

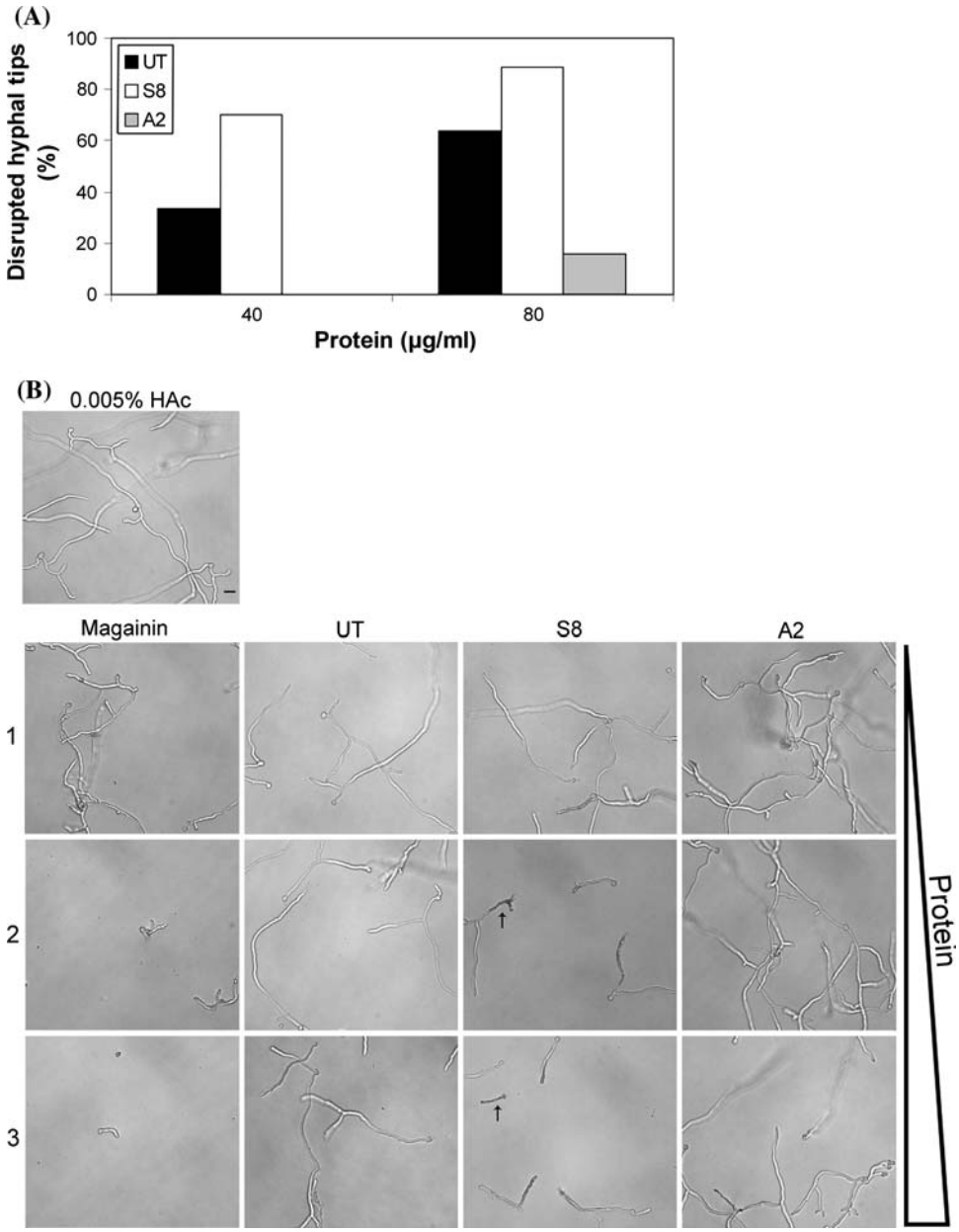

Fig. 8 DEF2-overexpressing tomato leaves contain activity that inhibits growth of *B. cinerea*. **a** Soluble fractions of foliar extracts from untransformed (UT) or transgenic plants (S8 and A2) inhibit fungal growth; percentages of disrupted hyphal tips were quantified microscopically. **b** Micrographs of conidia incubated in 0.5 × potato dextrose broth without (0.005% acetic acid) or with increasing concentrations of magainin (12.5, 25, or 50 µg ml^{-1}) or foliar extracts fractionated for molecular masses between 3 and 30 kD (0.75, 1.5, 3 OD_{280}); *bar*, 25 µm. Disruption of hyphal tip growth is indicated (*arrows*)

a pollen viability defect. However, the tapetum of plants ectopically expressing DEF2 appeared to be intact in anthers from 3–5 mm long floral buds (data not shown). We therefore favor the former possibility that ectopic expression of DEF2 has a direct detrimental effect on pollen grains during the later stages of anther development, including gametogenesis and pollen grain maturation. However, the CaMV 35S promoter used for constitutive expression of *DEF2* is not active in male gametophytes after the first mitotic division (Van Der Leede-Plegt et al. 1992). The tapetum also degenerates at this stage (Gorman and McCormick 1997). Thus, the detrimental effect of DEF2 would have to express itself previously or be the result of persistently active peptide.

Antifungal properties of the DEF2 peptide

Two transformants were generated that ectopically expressed *DEF2*. One of them was lost because it never set seeds. The remaining line S8 produced relatively few seeds, but fertility was sufficient to generate homozygous offspring. Plants ectopically expressing DEF2 were recovered significantly less frequently than antisense transgenics. Constitutive DEF2 expression had pleiotropic effects on plant growth. Mature DEF2 expressed in leaves (i) reduced the frequency of *B. cinerea* infection and (ii) inhibited hyphal tip growth in vitro. Based on the DEF2 expression levels in leaves from line S8 (Fig. 7a) and concentrations of protein required to inhibit hyphal growth

(Fig. 8), DEF2 appears to be as active as antifungal peptides from other plants (Osborn et al. 1995; Terras et al. 1995). Although the simplest explanation is that DEF2 itself is responsible for the observed antifungal activity, we cannot exclude the possibility that transgene expression induces other defence proteins or metabolites. Upregulation of other defence compounds could explain the relatively large effect of small DEF2 peptide levels (Fig. 7). Proteomics or transcriptomics would be a means to identify such induced defence pathways. Conclusive proof for the antifungal activity of DEF2 would require purification to homogeneity or analysis of a chemically synthesized and correctly folded peptide (Takayama et al. 2001). Nonetheless, our data clearly show that overexpression of DEF2 interferes with fungal invasion. Observations in line S8 may, however, not be entirely representative; recovery of two transgenics limited our ability to analyze constitutive DEF2 expression more broadly.

The mechanism of DEF2 action remains to be determined. However, certain plant defensins block L-type calcium channels (Spelbrink et al. 2004). Calcium dynamics have been studied during microsporogenesis and pollen development with highest levels of total Ca^{2+} during meiosis and the mature pollen stage (Tirlapur and Willemse 1992). Hyphal tip growth is dependent on a calcium gradient in the apical tip (Jackson and Heath 1993; Robson et al. 1991). While being speculative, our finding that partially purified extract from the transgenic line constitutively expressing DEF2 inhibits tip growth of *B. cinerea* is consistent with the idea that this defensin may alter Ca^{2+} transport. MsDef1 from *Medicago sativa* has been shown to disrupt Ca^{2+}-dependent localization of Rab GTPase to root hair tips (Allan et al. 2008). Conversely, an epididymis-specific β-defensin induces sperm motility by stimulating Ca^{2+} uptake (Zhou et al. 2004).

Conclusion

Table 1 summarizes the effects of altered *DEF2* expression on plant development and defence. Overexpression of *DEF2* had pleiotropic effects on plant growth. Inhibition of early seedling growth (Fig. 6a) may reflect the low recovery rate of *DEF2*-overexpressing transformants. Although the role of *DEF2* in development can only be tentative at this point because sense and antisense expression limited seed set (Fig. 3), these findings provide new insights into the regulation and functional diversification of antimicrobial plant defensins within the context of microsporogenesis, a developmental process that is still poorly understood (Yang et al. 2003).

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Table 1 Summary of effects of DEF2 transgene expression on plant development and defense

Phenotype	DEF2 overexpression	DEF2 antisense expression
Seed set[a]	Reduced	Reduced
Pollen viability[b]	Reduced	Reduced
Plant growth[b]	Changed	Unchanged
Style length[b]	Reduced	Unchanged
Disease resistance[b]	Increased	Unchanged

[a] Phenotype is based on two or more independent transgenic events

[b] Phenotypes is based on analysis of homozygous transgenic lines

References

Allan A, Snyder AK, Preuss M, NIelsen EE, Shah DM, Smith TJ (2008) Plant defensins and virally encoded fungal toxin KP4 inhibit plant root growth. Planta 227:331–339

Becker D, Kemper E, Schell J, Masterson R (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol Biol 20:1195–1197

Bloch C Jr, Richardson M (1991) A new family of small (5 kD) protein inhibitors of insect alpha-amylases from seeds of sorghum (*Sorghum bicolor* Moench) have sequence homologies with wheat gamma-purothionins. FEBS Lett 279:101–104

Brandstadter J, Rossbach C, Theres K (1996) Expression of genes for a defensin and a proteinase inhibitor in specific areas of the shoot apex and the developing flower in tomato. Mol Gen Genet 252:146–154

Chipps TJ, Gilmore B, Myers JR, Stotz HU (2005) Relationship between oxalate, oxalate oxidase activity, oxalate sensitivity, and white mold susceptibility in *Phaseolus coccineus*. Phytopathology 95:292–299

Enerly E, Sheng Z, Li KB (2005) Natural antisense as potential regulator of alternative initiation, splicing and termination. In Silico Biol 5:0033

Faghihi MA, Wahlestedt C (2006) RNA interference is not involved in natural antisense mediated regulation of gene expression in mammals. Genome Biol 7:R38

Ferrandon D, Jung AC, Criqui MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L, Reichhart JM, Hoffmann JA (1998) A drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the *Toll* pathway. EMBO J 17:1217–1227

Fulton TM, Chunwongse J, Tanksley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13:207–209

Gao A-G, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens CMT (2000) Fungal pathogen

protection in potato by expression of a plant defensin peptide. Nat Biotechnol 18:1307–1310

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell 5: 1217–1229

Gorman SW, McCormick S (1997) Male sterility in tomato. Crit Rev Plant Sci 16:31–53

Graham MA, Silverstein KAT, VandenBosch KA (2008) Defensin-like genes: genomic perspectives on a diverse superfamily in plants. Crop Sci 48:S3–S11

Guimaraes RL, Chetelat RT, Stotz HU (2004) Resistance to *Botrytis cinerea* in *Solanum lycopersicoides* is dominant in hybrids with tomato, and involves induced hyphal death. Eur J Plant Pathol 110:13–23

Hastings ML, Milcarek C, Martincic K, Peterson ML, Monroe SH (1997) Expression of the thyroid hormone receptor gene, erbAalpha, in B lymphocytes: alternative mRNA processing is independent of differentiation but correlates with antisense RNA levels. Nucleic Acids Res 25:4296–4300

Jackson SL, Heath IB (1993) Roles of calcium-ions in hyphal tip growth. Microbiol Rev 57:367–382

Jen CH, Michalopoulos I, Westhead DR, Meyer P (2005) Natural antisense transcripts with coding capacity in Arabidopsis may have a regulatory role that is not linked to double-stranded RNA degradation. Genome Biol 6:R51

Jorgensen RA (1995) Cosuppression, flower color patterns, and metastable gene expression states. Science 268:686–691

Katiyar-Agarwal S, Morgan R, Dahlbeck D, Borsani O, Villegas AJ, Zhu JK, Staskawicz BJ, Jin H (2006) A pathogen-inducible endogenous siRNA in plant immunity. Proc Natl Acad Sci USA 103:18002–18007

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lay FT, Brugliera F, Anderson MA (2003) Isolation and properties of floral defensins from ornamental tobacco and petunia. Plant Physiol 131:1283–1293

Li Q, Lawrence CB, Xing HY, Babbitt RA, Bass WT, Maiti IB, Everett NP (2001) Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco. Planta 212:635–639

Li L, Zhao YF, McCaig BC, Wingerd BA, Wang JH, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell 16:126 p. 783

Martin RC, Mok DWS, Smets R, Van Onckelen HA, Mok MC (2001) Development of transgenic tobacco harboring a zeatin O-glucosyltransferase gene from Phaseolus. In Vitro Cell Dev Biol Plant 37:354–360

Matsui A, Ishida J, Morosawa T, Mochizuki Y, Kaminuma E, Endo TA, Okamoto M, Nambara E, Nakajima M, Kawashima M, Satou M, Kim J-M, Kobayashi N, Toyoda T, Shinozaki K, Seki M (2008a) Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant Cell Physiol 49:1135–1149

Matsui K, Nishizawa M, Ozaki T, Kimura T, Hashimoto I, Yamada M, Kaibori M, Kamiyama Y, Ito S, Okumura T (2008b) Natural antisense transcript stabilizes inducible nitric oxide synthase messenger RNA in rat hepatocytes. Hepatology 47:686–697

Mendez E, Moreno A, Colilla F, Pelaez R, Limas GG, Mendez R, Soriano F, Salinas M, de Haro C (1990) Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, gamma-thionin, from barley endosperm. Eur J Biochem 194:533–539

Milligan SB, Gasser CS (1995) Nature and regulation of pistil-expressed genes in tomato. Plant Mol Biol 28:691–711

Moniz DSM, Drouin G (1996) Phylogeny and substitution rates of angiosperm actin genes. Mol Biol Evol 13:1198–1212

Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci 24:34–36

Nasrallah JB (2002) Recognition and rejection of self in plant reproduction. Science 296:305–308

Osborn RW, De Samblanx GW, Thevissen K, Goderis I, Torrekens S, Van Leuven F, Attenborough S, Rees SB, Broekaert WF (1995) Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae. FEBS Lett 368:257–262

Prescott EM, Proudfoot NJ (2002) Transcriptional collision between convergent genes in budding yeast. Proc Natl Acad Sci USA 99:8796–8801

Robson GD, Wiebe MG, Trinci APJ (1991) Involvement of Ca^{2+} in the regulation of hyphal extension and branching in fusarium-graminearum A-3/5. Exp Mycol 15:263–272

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379

Schröder J-M, Gregory H, Young J, Christophers E (1992) Neutrophil-activating proteins in psoriasis. J Invest Dermatol 98:241–247

Schroeder J-M (1999) Epithelial antimicrobial peptides: innate local host response elements. Cell Mol Life Sci 56:32–46

Silverstein KAT, Graham MA, Paape TD, VandenBosch KA (2005) Genome organization of more than 300 defensin-like genes in arabidopsis. Plant Physiol 138:600–610

Spelbrink RG, Dilmac N, Allen A, Smith TJ, Shah DM, Hockerman GH (2004) Differential antifungal and calcium channel-blocking activity among structurally related plant defensins. Plant Physiol 135:2055–2067

Strizhov N, Keller M, Mathur J, Koncz-Kalman Z, Bosch D, Prudovsky E, Schell J, Sneh B, Koncz C, Zilberstein A (1996) A synthetic cryIC gene, encoding a Bacillus thuringiensis delta-endotoxin, confers Spodoptera resistance in alfalfa and tobacco. Proc Natl Acad Sci USA 93:15012–15017

Takayama S, Shimosato H, Shiba H, Funato M, Che F-S, Watanabe M, Iwano M, Isogai A (2001) Direct ligand-receptor complex interaction controls Brassica self-incompatibility. Nature 413:534–538

Terras FRG, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Rees SB, Torrekens S, Van LF, Vanderleyden J, Cammue BPA, Broekaert WF (1995) Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell 7:573–588

Thevissen K, Cammue BPA, Lemaire K, Winderickx J, Dickson RC, Lester RL, Ferket KKA, Van Even F, Parret AHA, Broekaert WF (2000) A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of Saccharomyces cerevisiae to an antifungal plant defensin from dahlia (Dahlia merckii). Proc Natl Acad Sci USA 97:9531–9536

Thomma BPHJ, Broekaert WF (1998) Tissue-specific expression of plant defensin genes PDF2.1 and PDF2.2 in Arabidopsis thaliana. Plant Physiol Biochem 36:533–537

Thomma BPHJ, Cammue BPA, Thevissen K (2002) Plant defensins. Planta 216:193–202

Tirlapur UK, Willemse MTM (1992) Changes in calcium and calmodulin levels during microsporogenesis, pollen development and germination in gasteria-verrucosa (Mill) duval, H. Sex Plant Reprod 5:214–223

Titus D (1990) Probe-design peptide separation system technical manual. Promega Corporation, Madison

Tufarelli C, Stanley JA, Garrick D, Sharpel JA, Ayyub H, Wood WG, Higgs DR (2003) Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat Genet 34:157–165

Van Der Leede-Plegt LM, Van De Ven BCE, Bino RJ, Van Der Salm TPM, Van Tunen AJ (1992) Introduction and differential use of various promoters in pollen grains of nicotiana-glutinosa and lilium-longiflorum. Plant Cell Rep 11:20–24

Wang Y, Wisniewski M, Meilan R, Cui M, Webb R, Fuchigami L (2005) Overexpression of cytosolic ascorbate peroxidase in tomato confers tolerance to chilling and salt stress. J Am Soc Hortic Sci 130:167–173

Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC (1999) Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 286:113–117

Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schroeder JM, Wang JM, Howard OMZ, Oppenheim JJ (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286: 525–528

Yang S-L, Xie L-F, Mao H-Z, San Puah C, Yang W-C, Jiang L, Sundaresan V, Ye D (2003) *Tapetum Determinant1* is required for cell specialization in the Arabidopsis anther. Plant Cell 15:2792–2804

Zasloff M, Martin B, Chen H-C (1988) Antimicrobial activity of synthetic magainin peptides and several analogues. Proc Natl Acad Sci USA 85:910–913

Zhou CX, Zhang Y-L, Xiao L, Zheng M, Leung KM, Chan MY, Lo PS, Tsang LL, Wong HY, Ho LS, Chung YW, Hg Chan (2004) An epididymis-specific beta-defensin is important for the initiation of sperm maturation. Nat Cell Biol 6:458–464

Zubko E, Meyer P (2007) A natural antisense transcript of the Petunia hybrida Sho gene suggests a role for an antisense mechanism in cytokinin regulation. Plant J 52:1131–1139