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ORIGINAL ARTICLE

Gastrodia anti-fungal protein from the orchid *Gastrodia elata* confers disease resistance to root pathogens in transgenic tobacco

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Abstract Diseases of agricultural crops are caused by pathogens from several higher-order phylogenetic lineages including fungi, straminipila, eubacteria, and metazoa. These pathogens are commonly managed with pesticides due to the lack of broad-spectrum host resistance. *Gastrodia* anti-fungal protein (GAFP; gastrodianin) may provide a level of broad-spectrum resistance due to its documented anti-fungal activity in vitro and structural similarity to insecticidal lectins. We transformed tobacco (*Nicotiana tabacum* cv. Wisconsin 38) with *GAFP-1* and challenged transformants with agriculturally important plant pathogens from several higher-order lineages including *Rhizoctonia solani* (fungus), *Phytophthora nicotianae* (straminipile), *Ralstonia solanacearum* (eubacterium), and *Meloidogyne incognita* (metazoan). Quantitative real-time PCR and western blotting analysis indicated that *GAFP-1* was transcribed and translated in transgenic lines. When challenged by *R. solani* and *P. nicotianae*, *GAFP-1* expressing lines had reduced symptom development and improved plant vigor compared to non-transformed and empty vector control lines. These lines also exhibited reduced root galling when challenged by *M. incognita*. Against *R. solanacearum* expression of *GAFP-1* neither conferred resistance, nor exacerbated disease development. These results indicate that heterologous expression of *GAFP-1* can confer enhanced resistance to a diverse set of plant pathogens and may be a good candidate gene for the development of transgenic, root-disease-resistant crops.

Keywords Biotechnology · Meloidogyne · Nicotiana · Lectins · Phytophthora · Rhizoctonia

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Introduction

Managing root diseases of agriculturally important crops is a considerable challenge. Root diseases are caused primarily by pathogens from four higher-order lineages:[1] fungi, straminipila, metazoa, and eubacteria (Agrios 1997). These pathogens are difficult to control due to the protective environment of the soil and because reduced-risk pesticide chemistries are often only effective against organisms from a single phylum at best. Soil fumigants with broad spectrum, biocidal activity face increased regulation and, in the case of methyl-bromide, are scheduled for imminent phase out (Anonymous 2000). Several diseases, such as replant disorder on apple or peach tree short life (PSTL) on

[1] Due to debate surrounding the categorization of taxa above the phylum level, we will refer to such taxa as higher-order phylogenetic lineages instead of using the kingdom designation.

peach are caused by several factors and multiple pathogens from several higher-order lineages (Agrios 1997; Mazzola 1998; Beckman and Nyczepir 2004; Rumberger et al. 2004). PTSL, in particular, is caused by a combination of cold injury, the ring nematode *Mesocriconema xenoplax*, an ascomycete *Cytospora*, and a bacterium *Pseudomonas syringae* pv. *Syringae* (Agrios 1997; Beckman and Nyczepir 2004). In the absence of resistant rootstocks, these soil-borne pathogens cannot be managed with reduced-risk pesticides and producers must rely solely on cultural management strategies.

Plant lectins have been investigated for their ability to confer resistance to either insects, fungi, or nematodes, but none has been shown to provide enhanced resistance to organisms from multiple higher-order phylogenetic lineages (Etzler 1986; Chrispeels and Raikhel 1991). Moreover, plant lectins have been used as transgenes to provide insect and nematode resistance in plants, particularly the monocot mannose-binding lectins *Galanthus nivalis* agglutinin (GNA) and concanavalin A (ConA) (Burrows et al. 1998; Gatehouse et al. 1999; Setamou et al. 2002; Sun et al. 2002; Wu et al. 2002; Down et al. 2003; Ripoll et al. 2003), but only a few lectins have been shown to have anti-fungal properties. *Urtica dioica* agglutinin (UDA) from the stinging nettle (*U. dioica*) and hevein from the rubber tree (*Hevea brasiliensis*), for example, are low molecular weight, monomeric chitin-binding lectins that possess anti-fungal properties in vitro (Broekaert et al. 1989; Van Parijs et al. 1991; Does et al. 1999). For the most part, the effect of lectins on straminipila and phytopathogenic bacteria is largely unknown. Some mannose-binding lectins are known to affect rhizobacteria colonization of root hairs (Chrispeels and Raikhel 1991) and several recombinant plant lectins, including mannose-binding lectins (Bajaj et al. 2001), have been demonstrated to enhance nodulation in transgenic plants (Diaz et al. 1995; Eijsden et al. 1995; Rhijn et al. 1998), but the effects of plant lectins on disease caused by soil-borne phytopathogenic bacteria are undocumented.

A monocot mannose-binding lectin *Gastrodia* anti-fungal protein (GAFP, gastrodianin) was discovered in a myco-heterotrophic orchid, *Gastrodia elata* Bl. F. flavida S. Chow, that could impart increased disease resistance against fungi in transgenic plant systems (Xu et al. 1998; Wang et al. 2001). The lectin was purified from the terminal corm of *G. elata* and was documented to inhibit the growth of both ascomycete and basidiomycete fungal plant pathogens in vitro including *Valsa ambiens*, *Gibberella zeae*, *Botrytis cinerea*, *Armillaria mellea*, *Rhizoctonia solani*, and *Ganoderma lucidum* (Hu et al. 1988; Xu et al. 1998). Although demonstrating anti-fungal properties, GAFP is most similar in amino acid composition and three-dimensional structure to GNA. The mannose-binding sites of GAFP have an 88.9% amino acid homology with those of GNA (Lui et al. 2005), which is documented to have activity against metazoa, including insects and nematodes (Fitches et al. 2001; Wu et al. 2002; Down et al. 2003; Ripoll et al. 2003). The functional similarity of GAFP to anti-fungal lectins, and its structural similarity to GNA suggest that GAFP could provide resistance to diseases caused by pathogens of several higher-order lineages, and therefore may be a good candidate for the development of transgenic, disease resistant plants.

The objective of this study was to create and characterize tobacco plants expressing GAFP, and subsequently challenge the highest-expressing plant lines with representative pathogens from four higher-order lineages including *Phytophthora nicotianae* (straminipile), *R. solani* (basidiomycete fungus), *Ralstonia solanacearum* (eubacterium), and *Meloidogyne incognita* (nematode metazoan).

Materials and methods

In the experiments described below, plasmid pVNFbin, containing the VNF isoform of *GAFP-1*, was introduced into *Nicotiana tabacum* via *Agrobacterium*-mediated transformation. Transcription and translation of *GAFP-1*-VNF in the resulting transgenic tobacco lines was determined to verify successful expression of gastrodianin. Potential disease resistance of *GAFP-1* transgenic tobacco lines was assessed by challenging with straminipile, fungal, bacterial, and nematode pathogens under controlled conditions in the greenhouse.

Agrobacterium-mediated transformation

To construct the expression vector for *Agrobacterium*-mediated transformation, the nucleotide sequence of the *GAFP-1*-VNF isoform was inserted into the multiple cloning site of binary vector pAVAT1, a derivative of pTHW136 expression vector (Plant Genetic Systems N. V., Gent, Belgium), and was subsequently designated pAVNFbin (Wang et al. 2001). The *GAFP-1*-VNF encoding sequence was placed under control of the 35S promoter and the omega leader sequence.

Agrobacterium tumefaciens strain EHA 101 was transformed with pAVNFbin and pAVAT (empty vector control) expression plasmids, separately, using a

freeze-thaw protocol for enhanced transformant recovery (Chen et al. 1994). Both pAVNFbin and pAVAT transformed colonies were selected on Luria broth agar (pH 7.0; 1 l: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 18 g agar) amended with 50 mg/l kanamycin and 150 mg/l spectinomycin.

The presence of the *GAFP-1* in selected colonies was then confirmed by PCR using *GAFP-1* specific primers, primer 5 (5′-GGT ATT CCA CCT AGC CAT CAA GCA GCC-3′) and primer 7 (5′-TAT TCT CTT AGA CCG CTA GTA CAT GGA-3′) designed by Wang et al. (2001). Briefly, small amounts of individual *Agrobacterium* colonies were transferred to a PCR reaction cocktail of 50 μl total volume containing 20 μM of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (Promega, Madison, WI, USA), and 5.0 μl 10× reaction buffer (Promega, Madison, WI, USA). Cycling parameters were as follows: initial denaturation at 95°C for 2 min (to lyse the bacterial cell wall); 28 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 40 s; final elongation was 72°C for 10 min.

Nicotiana tabacum transformation

Agrobacterium tumefaciens strain EHA 101 containing pAVNFbin or pAVAT from above was used to subsequently transform tobacco (cv. Wisconsin 38) using a modified *Agrobacterium* co-cultivation method (Burow et al. 1990). Prior to transformation, *A. tumefaciens* colonies were streaked out onto Luria broth agar amended with 50 mg/l kanamycin and 150 mg/l spectinomycin for selection and grown at 28°C for 2 days in the dark. During co-cultivation, tobacco leaf tissue was sliced into approximately 3 cm square segments and punctured lightly with a scalpel to facilitate infection by *A. tumefaciens*. Generous amounts of transformed *A. tumefaciens* cells containing one of two plasmids, pAVNFbin or pAVAT, were smeared onto the leaf segments, and immediately placed onto co-cultivation media (Burow et al. 1990) for 6 days at 28°C. After co-cultivation, inoculated plant tissue was placed on selection media (Burow et al. 1990) with 100 mg/l kanamycin for selection and 500 mg/l carbenicillin to kill any residual *Agrobacterium* on the plant tissue. Subsequent regeneration of tobacco plants was conducted as previously described (Burow et al. 1990). Tobacco lines were clonally propagated as cuttings and maintained a climate-controlled Biosafety Level 2 (Richmond and McKinney 1993) greenhouse facility. Prior to formal experimentation, the presence of *GAFP-1* was confirmed in tobacco lines. Genomic DNA (gDNA) was extracted from root and leaf tissue independently using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and PCR was performed using *GAFP-1* specific primers, primer 5 and primer 7 (Wang et al. 2001) as described above.

Expression of *GAFP-1* in *Nicotiana tabacum*

The expression *GAFP-1* in the transgenic and control lines of *Nicotianae tabacum* (cv. Wisconsin 38) was characterized from both root and leaf tissue separately. Tissue samples were collected from 2-week-old rooted tobacco cuttings and RNA was first extracted according to the TRIzol method (Chomczynski 1993) using TRIzol® Reagent (Invitrogen Corporation, Carlsbad, CA, USA). RNA samples were then treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) to remove residual DNA and subsequently purified using the Qiagen RNeasy Mini kit for RNA purification (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. In preparation for transcription analysis, double stranded cDNA was synthesized from RNA samples using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions.

GAFP-1 transcription and relative transgene copy number for the transgenic tobacco lines was determined by performing quantitative real-time PCR (Q-RT-PCR) on the newly synthesized cDNA and gDNA, respectively. Q-RT-PCR was conducted using an iCycler iQ Real-Time PCR Detection System and the Optical System Software version 3.0a (Bio-Rad Laboratories, Hercules, CA, USA). Data were normalized with 18S ribosomal DNA expression using an 18S primer/competimer mix (QuantumRNA 18S Internal Standard; Ambion Inc., Austin, TX, USA). The iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect *GAFP-1* expression according to the manufacturer's instructions. The 30-μl reactions used to amplify a portion of the *GAFP-1* consisted of 15 μl of the iQ SYBR Green Supermix, 5 μl of tenfold diluted cDNA or gDNA template, and 6 mM of primers *gafpRT1-F* (5′-CAC AAG GCG GCT ACC TAT TC-3′) and *gafpRT1-R* (5′-CTT TCC GTT GGT TCC TGA TG-3′). The 30-μl reactions using the 18S primer/competimer mix contained 15 μl of the iQ SYBR Green Supermix, 2.4 μl of primer/competimers mix (4:6 ratio), and 5 μl of tenfold diluted cDNA template. The Q-RT-PCR cycling parameters were as follows: initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 30 s, and 55°C for 30 s; 72°C for 30 s, and 72°C for 10 min. Relative gene expression and copy number were calculated using the Comparative C_T method (Pfaffl 2001). *GAFP-1* and 18S rDNA amplicons in completed Q-RT-PCR reactions were verified using acrylamide gel electrophoresis.

GAFP-1 translation was verified using immunoblot analysis. Protein fractions from both root and leaf TRIzol extractions (see above) were purified according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Total cellular protein concentration was measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. SDS-PAGE was performed on 15 μg of purified total cellular protein using a Mini-Protean® 3 with 18% Tris–HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA, USA). Protein transfer to immuno-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) was accomplished using a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoretic transfer, membranes were air-dried for 2 h and re-wet in methanol, followed by water, and finally Towbin Buffer (25 mM Tris; 192 M glycine; 20% methanol; 1.0% SDS) according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Immunoblotting was conducted using rabbit anti-GAFP polyclonal antisera (1:10^4 dilution) developed by Zymed® Laboratories (Invitrogen, Carlsbad, CA, USA) and goat anti-rabbit alkaline phosphatase conjugated antibodies (1:1×10^7 dilution) (Promega Corp., Madison, WI, USA) according to standard methods (Gallagher et al. 1997). Detection was accomplished using Sigmafast™ BCIP/NBT tablets (Sigma Aldrich, St Louis, MO, USA). Gastrodianin signal bands from *GAFP-1* transgenic tobacco and purified *GAFP-1*-VNF standards from several blots were converted to numerical data and analyzed using Scion Image software v3.42 (Scion Corporation, Frederick, MD, USA) to provide a rough estimation of protein production. *GAFP-1*-VNF protein bands (12 kDa) from digitized blots were selected (signal threshold=108) and analyzed for mean intensity on an 8-bit scale (0–255) and signal area. Estimation *GAFP-1*-VNF of protein concentration was determined by comparing the ratio of mean signal intensity to signal area of 12 kDa protein bands from known amounts of purified recombinant gastrodianin (*GAFP-1*-VNF isoform) with those of protein bands from unknown samples using linear regression.

Disease resistance screening

A preliminary disease screening (not shown) using *P. nicotianae* was conducted to initially select the most promising *GAFP-1* transgenic tobacco lines. The three most tolerant lines were challenged individually with four pathogens of different eukaryotic lineages (Table 1). All pathogen species, isolates, or populations were isolated from or were known to infect tobacco and all inoculations were performed according to standard procedures (Table 1) (Hussey and Barker 1973; Rioger and Jeffers 1991; Csinos and Stephenson 1999; Hussey and Janssen 2002; Robertson et al. 2004).

Disease screening experiments were conducted on 4-week-old rooted tobacco cuttings clonally propagated from the initial transformants in a climate-controlled Biosafety Level 2 (Richmond and McKinney 1993) greenhouse facility. Symptom development was monitored daily for 60 days, upon which the experiments were terminated and final symptom levels were recorded. In *Phytophthora* and *Rhizoctonia* disease screening experiments, characteristic black shank and soreshin symptoms, respectively, were verified and symptom severity was measured as the percentage of stem height from the crown with necrosis. Similarly, bacterial wilt symptoms caused by *R. solanacearum* were verified and symptom severity was measured as the percentage of stem height with wilted leaves or shoots. In the *M. incognita* disease screening experiments, disease severity was measured as percent galling of the root system according to standard procedures (Hussey and Barker 1973; Hussey and Janssen 2002). Disease severity measures were calculated based on observations of final symptom development from ten plants for each tobacco line in each of three replications over time. Plants were arranged in a completely randomized design; three replications were blocks in the analysis of variance (SAS version 9.1; SAS Institute Inc., Cary, NC, USA). All analyses were

Table 1 Root-disease pathogens and corresponding methodologies used for inoculation and disease assessment

Species	Kingdom	Disease/pest common name	Isolate/race	Origin	Inoculation/disease assessment methodology
Phytopthora nicotianae	Straminipila	Black shank	011P-Tob	Clemson, SC	Rioger and Jeffers (1991)
Rhizoctonia solani	Fungi	Soreshin	023R-Tob	Tifton, GA	Csinos and Stephenson (1999)
Ralstonia solanacearum	Eubacteria	Southern wilt	NC132	Clemson, SC	Robertson et al. (2004)
Meloidogyne incognita	Metazoa	Root-knot nematode	Race 3	Blackville, SC	Hussey and Barker (1973), Hussey and Janssen (2002)

Fungal, straminipile, and bacterial pathogens were obtained from tobacco hosts

based on arcsine-square root-transformed percentage values. Main effects and interactions were considered statistically significant at α=0.05.

Results

Creation of transgenic *Nicotiana tabacum* containing the *GAFP-1*-VNF expression construct

Six transgenic explants of *N. tabacum* containing the *GAFP-1*-VNF gene and one explant containing the empty vector (pAVAT) were obtained via *A. tumefaciens* transformation. The *GAFP-1*-VNF expressing lines were designated pAVNF-1, 3, 4, 6, 7, and 10. All transformed lines were indistinguishable from the non-transformed line (NTC) in terms of their health and growth habit, and contained the gene after clonal propagation (Fig. 1). In a preliminary disease resistance screening against *P. nicotianae*, transgenic lines pAVNF-1, 3, and 10*GAFP-1* consistently had the lowest black shank severity (9.6, 23.7, and 18.5% stem necrosis) compared to lines pAVNF-4, 6, and 7 (72.7, 25.9, and 29.1% stem necrosis), and therefore, were selected for subsequent molecular and phenotypic analyses.

Expression of *GAFP-1*-VNF in *Nicotiana tabacum*

Real-time PCR on cDNA indicated that the pAVNF-1, 3, and 10 lines were transcribing the *GAFP-1*-VNF gene, while the empty vector (pAVAT) and NTC line did not (Fig. 2a). The level of *GAFP-1* transcription was not significantly different (*P*=0.980; *n*=9) between

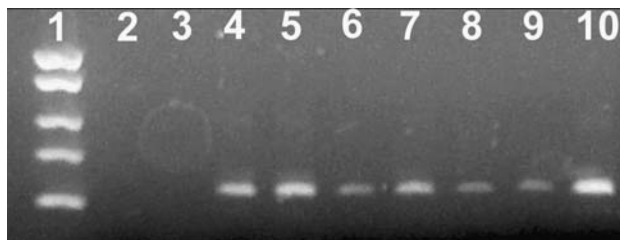


Fig. 1 Agarose gel (1%) illustrating the presence of *Gastrodia* anti-fungal protein (*GAFP-1*) amplicons obtained via PCR using *GAFP-1* specific primers, primer 5 and primer 7 (adapted from Wang et al. 2001) on genomic DNA from root tissue of tobacco prior to disease screening experiments. *Lane 1* contains 5 μl of exACTGene low range DNA ladder (Fisher Scientific International Inc., Ontario, Canada). *Lanes 2 and 3* contain reaction products from the empty vector and non-transformed control tobacco lines. *Lanes 4–9* contain an expected 661 bp band indicative of the full length *GAFP-1* amplicon from the transgenic tobacco lines pAVNF-1, 3, 4, 6, 7, and 10, respectively. *Lane 10* contains a 661 bp from a plasmid positive control pAVNFbin

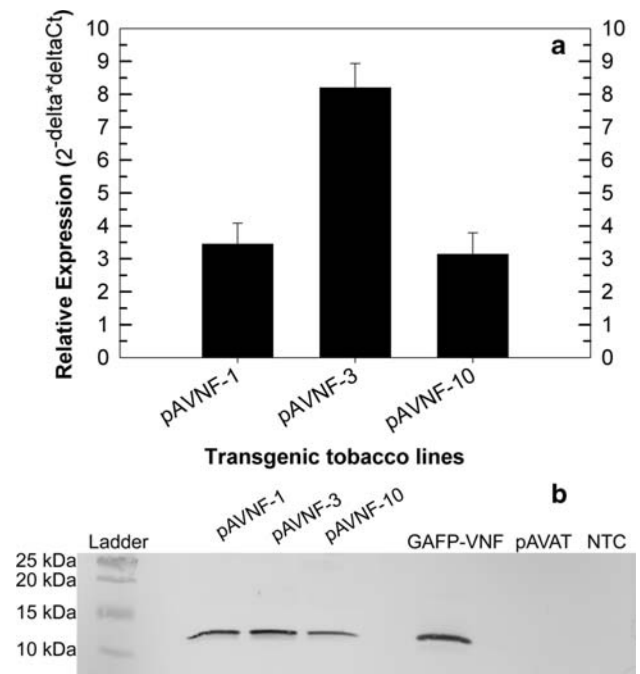


Fig. 2 *GAFP-1* transcription (**a**) and GAFP-1 protein synthesis (**b**) in tobacco lines. **a** Relative expression of *GAFP* determined by quantitative real-time PCR performed on cDNA displayed as $2^{-\mathrm{delta}\times\mathrm{delta(Ct)}}$ values. Values are means and standard deviations over root and shoot tissue for three plants. **b** Western blot using GAFP polyclonal anti-sera illustrating the presence of a 12 kDa protein band in 10 μg of total cellular protein from root tissue of *GAFP-1* transgenic tobacco lines (pAVNF-1, pAVNF-3, pAVNF-10) and the absence of a band in control lines (NTC = non-transformed control; pAVAT = empty vector). Purified recombinant gastrodianin (*GAFP-1*-VNF isoform) was applied at 0.5 μg and served as a standard

root and leaf tissue, hence the root and leaf data were combined to provide an estimate of the overall transgene transcription in the plant for each of the three lines (Fig. 2a). Overall, pAVNF-3 consistently had the highest level of gene transcription of the three lines (Fig. 2a). Q-RT-PCR reactions on gDNA indicated that the *GAFP-1* transgenic lines had the same *GAFP-1* copy number. There were no significant differences in $\Delta\Delta$Ct values (Pfaffl 2001) among lines (*P*=0.584; *n*=9) and mean $2^{\Delta\Delta\mathrm{Ct}}$ values were 1.40, 1.75, and 1.43 for tobacco lines pAVNF-1, 3, and 10, respectively.

Total cellular protein was successfully extracted and the translation of *GAFP-1*-VNF was verified for all *GAFP-1* transgenic lines by immunoblot analysis (Fig. 2b). The expected 12 kDa protein band for *GAFP-1*-VNF was detected by immunoblotting with polyclonal anti-sera in total cellular protein extracts of lines pAVNF-1, 3, and 10, and for purified recombinant gastrodianin. Putative 12 kDa gastrodianin bands were not detected in the pAVAT or the NTC lines. Based on image analysis of signal intensity of 12 kDa

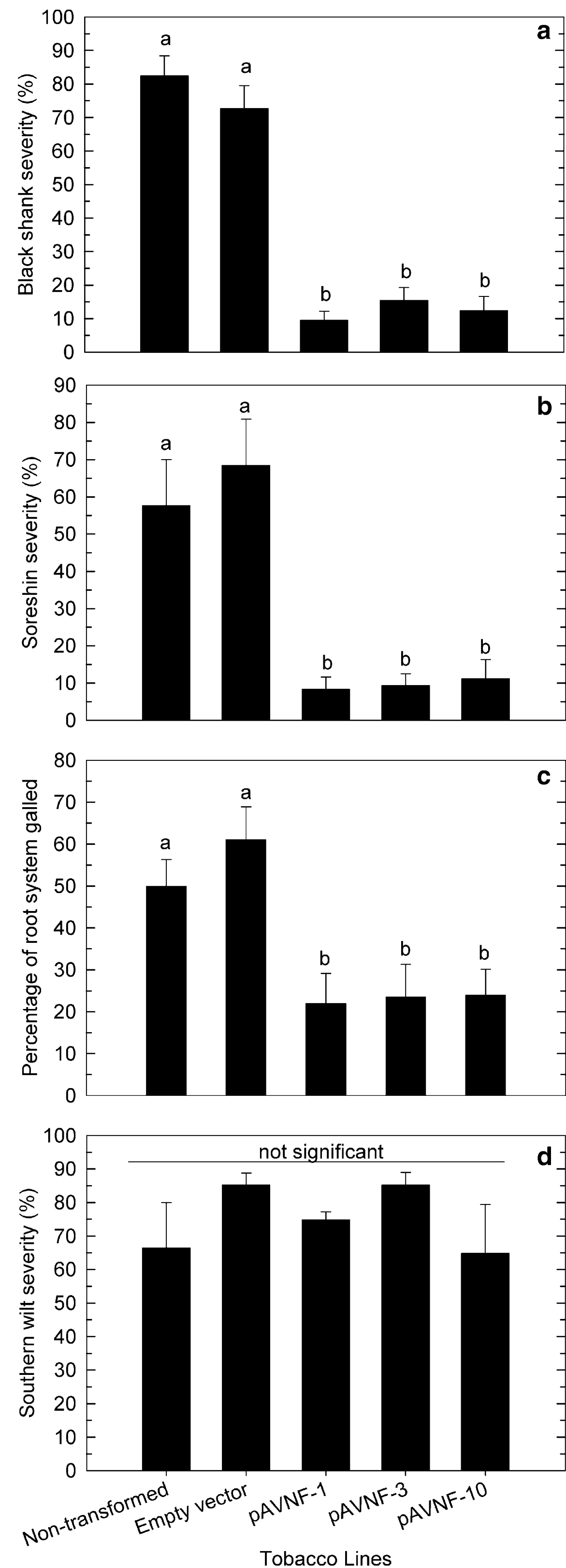
protein bands, *GAFP-1*-VNF production was similar among the three *GAFP-1* transgenic lines and estimated to be 0.019, 0.023, and 0.015 μg per μg of total cellular protein in lines pAVNF-1, 3, and 10, respectively.

Disease resistance screening

All tobacco lines developed characteristic disease symptoms when inoculated with each of the four pathogens used in this study (Table 1). Transgenic lines expressing *GAFP-1*-VNF had reduced symptom development and improved vigor against three of the four pathogens, indicating that these plants were partially resistant to infection (Figs. 3, 4). Control plants developed more severe characteristic disease symptoms and exhibited compromised vigor compared to the *GAFP-1*-VNF expressing lines (Figs. 3a–c, 4a–f).

When inoculated with *P. nicotianae*, all lines developed stem necrosis, pith discing, leaf chlorosis, and wilting symptoms, but the symptom severity of *GAFP-1*-VNF expressing lines was significantly (P<0.0001) lower than control lines. For example, *GAFP-1* transgenic lines had <16% stem necrosis compared with >73% for the two control lines (Fig. 3a). Pith discing, a characteristic black shank symptom, and ultimately death were observed for severely infected control plants but not in *GAFP-1*-VNF expressing lines. Among *GAFP-1*-VNF expressing lines, there were no significant (P=0.05) differences in symptom severity. Control lines were necrotic, wilted, and severely compromised in vigor (Fig. 4b), but *GAFP-1*-VNF expressing lines (not shown) were nearly as vigorous as their uninoculated controls and were typically indistinguishable when photographed (Fig. 4a).

All tobacco lines inoculated with the basidiomycete *R. solani* developed typical soreshin crown and stem necrosis along with browning of fine roots and reduction of root mass. Severely infected plants developed a more extensive stem necrosis shortly before or after damping off. There was significantly (P<0.0001) reduced soreshin necrosis severity for *GAFP-1*-VNF expressing lines, which had <11% stem necrosis compared with >59% for the control lines (Fig. 3b). There were no significant (P=0.05) differences in symptom severity among the *GAFP-1* transgenic lines. Control lines were typically necrotic, wilted, and compromised in vigor (Fig. 4c), but *GAFP-1*-VNF expressing lines (not shown) were typically indistinguishable from their uninoculated controls when photographed (Fig. 4a).



Plants from all tobacco lines developed root galls 60 days after root collar inoculation with the root-knot nematode *M. incognita*. Severely galled plants were chlorotic, stunted in shoot height, and had reduced root mass. The amount of root system galling was significantly (P<0.0001) lower in *GAFP-1*-VNF expressing

Fig. 3 Disease symptom development 50 days post-inoculation in *GAFP-1* transgenic tobacco lines (pAVNF-1, 3, and 10), the empty vector control line (pAVAT), and the non-transformed control line challenged individually with *Phytophthora nicotianae* causing black shank disease (**a**), *Rhizoctonia solani* causing soreshin (**b**), *Meloidogyne incognita* causing root galling (**c**), and *Ralstonia solanacearnum* causing southern wilt (**d**). Black shank (**a**), soreshin (**b**), and Southern wilt (**d**) severity are expressed as the percentage of the plant tissue displaying the disease symptom. Root-knot nematode symptoms (**c**) are expressed as percentage of the total root system displaying root galls according standard methods (adapted from Hussey and Barker 1973). Values are means and standard errors across three experiments with ten plants per tobacco line. Lines designated by the same letter are not significantly different according to Fisher's protected LSD test (α=0.05)

lines, which consistently had <24% of the root system galled compared with >50% for the two control lines (Fig. 3c). Among *GAFP-1* transgenic lines, the amount of root system galling was not significantly (P=0.05) different. The root systems of plants from control lines typically had high levels of galling (Fig. 4e), compared to typical plants from *GAFP-1* transgenic lines (Fig. 4f); however, there were some exceptions in both cases, which is reflected in the higher standard errors noted (Fig. 3).

All tobacco lines developed wilt symptoms when challenged with the bacterium *R. solanacearum*. Severely infected plants became entirely wilted and died. Overall, there were no significant (P=0.4065) differences in the severity of wilt symptoms between *GAFP-1* transgenic and control lines; wilt severity in all lines was >65% (Fig. 3d). Moreover, plants from all lines were equally wilted and compromised in vigor (Fig. 4d).

Discussion

This study provides the first comprehensive evaluation of a monocot mannose-binding lectin used as a transgene for resistance against diseases caused by several pathogens in a single herbaceous dicotyledonous host. These pathogens belong to several higher-order phylogenetic lineages including straminipila, fungi, eubacteria, and metazoa. Plant lectins have been investigated as transgenes for use in pest and disease management (Burrows et al. 1998; Does et al. 1999; Gatehouse et al. 1999; Setamou et al. 2002; Sun et al. 2002; Wu et al. 2002; Down et al. 2003; Ripoll et al. 2003; Wang et al. 2004), but the possibility of lectin transgenes having cross-taxa effects has not been investigated.

The *Agrobacterium*-mediated transformation used in the current study resulted ultimately in the regeneration of six transgenic lines, all physiologically indistinguishable from the background phenotype and expressing *GAFP-1*-VNF. *GAFP-1* transcription and translation in tobacco lines was confirmed, but there were no clear relationships between gene transcription and protein translation. The levels of protein production were similar among our *GAFP-1* transgenic lines and are comparable to what is reported for other transgenic systems expressing monocot mannose-binding lectins (Wang et al. 2001; Ripoll et al. 2003). Wang et al. (2001) estimated *GAFP-1* production in transgenic tobacco lines to be >0.3% of total cellular proteins, while Ripoll et al. (2003) estimated GNA production in transgenic *Arabidopsis* lines to be as high as 1.3% of total soluble protein. By comparison, *GAFP-1* production in the transgenic tobacco lines characterized in the current study was estimated to be between 1.5 and 2.3% of total cellular proteins.

In contrast to protein production, there was variation in the amount of *GAFP-1* transcription among the three lines selected for molecular characterization. For example, the level of *GAFP-1*-VNF transcripts was higher in line pAVNF-3, but there were no differences in *GAFP-1*-VNF copy number among the three lines determined by Q-RT-PCR on gDNA. In addition, there were neither considerable differences in the amount of recombinant *GAFP-1*-VNF produced, nor were there differences in disease resistance among the three lines. Other studies have investigated the transcription of lectin transgenes (Wang et al. 2001; Ripoll et al. 2003; Wang et al. 2004), but no information is reported on the accumulation of transcripts in relation to protein translation or level of disease resistance. In the current study, the fact that the *GAFP-1* transgenic lines had similar protein production and disease resistance but were variable in gene transcription could be due to the rate of transcription exceeding the translation capacity of the plant cells.

Heterologous expression of *GAFP-1*-VNF provided enhanced resistance to tobacco pathogens from the three eukaryotic lineages including straminipila, fungi, and metazoa. Firstly, *GAFP-1* expression conferred enhanced resistance to disease caused by the straminipilous pathogen, *P. nicotianae*. This was a surprising result, since UDA, a well known anti-fungal lectin, was shown to have no effect on the mycelial growth of *P. erythroseptica* in vitro (Broekaert et al. 1989). UDA, however, is known to have a chitin-binding mode of action, which would be ineffective against the achitinous straminipiles (Broekaert et al. 1989; Van Parijs et al. 1991). Secondly, constitutive expression of *GAFP-1*-VNF provided increased resistance to soreshin caused by *R. solani*. Although GAFP has been

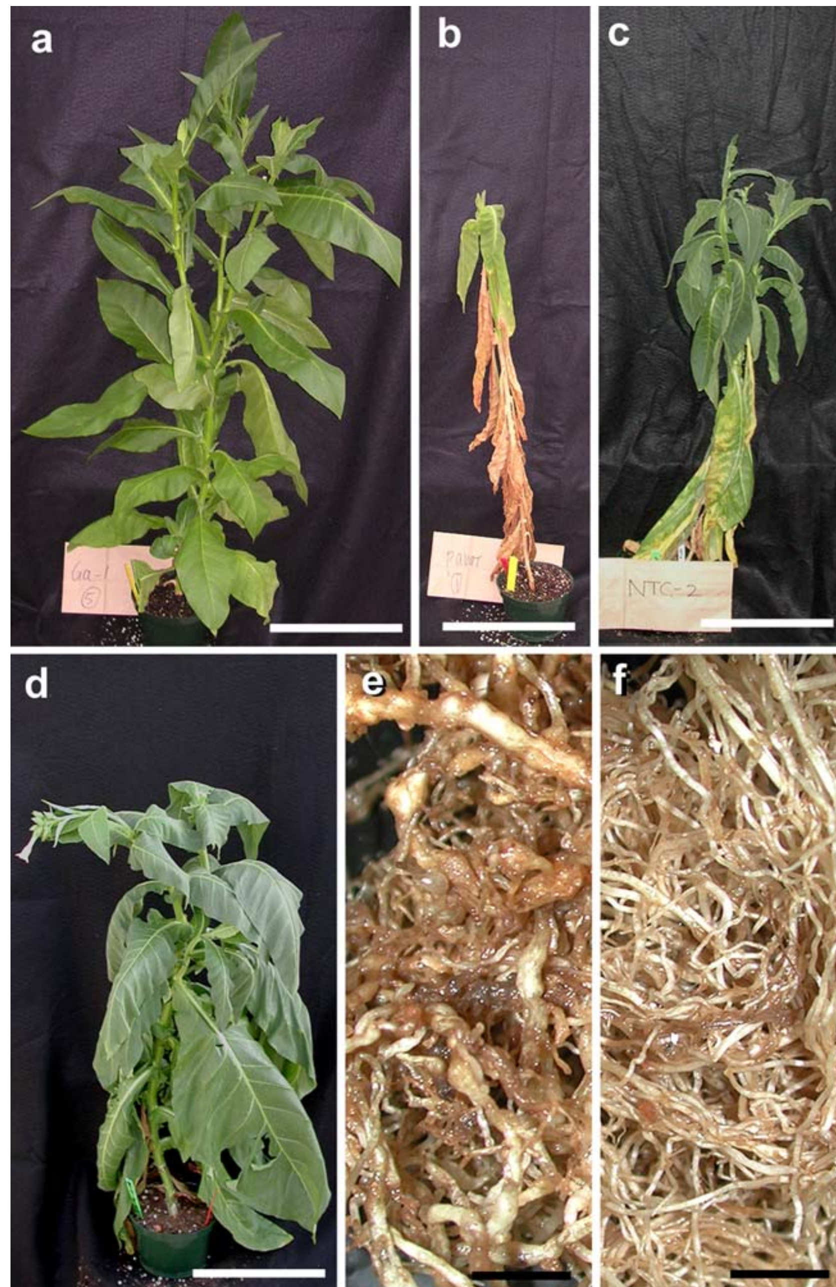


Fig. 4 Representative tobacco plants (**a–d**) illustrating typical plant vigor at the end of disease screening experiments. Uninoculated *GAFP-1* transgenic line pAVNF-1 (**a**) 60 days after planting. The control line pAVAT (**b**) 60 days after inoculation with *Phytopthora nicotianae*. The non-transformed control (NTC) line (**c**) 60 days after inoculation with *Rhizoctonia solani*, and the *GAFP-1* transgenic line pAVNF-10 (**d**) 60 days after inoculation with *Ralstonia solanacearum*. Tobacco root tissue illustrating representative high levels of nematode galling in control lines (**e**) and low levels of galling more frequently exhibited by *GAFP-1* transgenic lines (**f**). Scale bar is 25 cm in (**a–d**), and is 1 cm in (**e, f**)

shown to inhibit the growth of *R. solani* in vitro, this is the first instance of a lectin transgene enhancing resistance to a basidiomycete fungus. GAFP has also been shown to provide some resistance to the ascomycete *Verticillium dahliae*, which indicates the potential for cross-phylum activity (Wang et al. 2004) against fungi. Specifically, transgenic colored cotton (*Gossypium hirsutum*) expressing *GAFP-1* had reduced *Verticillium* wilt under field conditions, and this resistance remained stable in successive generations (Wang et al. 2004). Lastly, when challenged with the nematode *M. incognita*, GAFP expression consistently reduced root galling by 50% in *GAFP-1* transgenic lines compared to control lines. This level of resistance is consistent with an earlier study, which reported a 20–50% reduction in *M. incognita* root galling for transgenic *Arabidopsis thaliana* lines expressing the mannose-binding lectin GNA (Ripoll et al. 2003).

The activity of GAFP against diseases caused by pathogens from several distinct eukaryotic lineages reported in this study is likely a result of the lectin's mannose-binding properties. The specific mechanism of GAFP's anti-fungal activity is still speculated (Wang et al. 2003; Lui et al. 2005), but it is known that disruption of the mannose-binding domains of GAFP by site-directed mutagenesis prevents the lectin from

inhibiting fungal growth in vitro (Wang et al. 2003). Mannose binding may also be involved in GAFP's possible activity against nematodes. Both GNA and GAFP are strongly homologous in the structure and composition of their mannose-binding sites (Wang et al. 2003; Lui et al. 2005). Similar to what has been reported for transgenic *Arabidopsis* expressing GNA (Ripoll et al. 2003), we show that heterologous expression of *GAFP-1*-VNF also reduced nematode galling in our transgenic tobacco system (this study). Little is known about the specific mechanisms by which these plant lectins affect nematodes, but it is hypothesized that lectin binding interferes with either the nematode's chemosensory perception when attempting to establish feeding sites, or by binding glycoproteins in the gut causing a disruption in nematode feeding (Zuckerman 1983; Fitches et al. 2001; Ripoll et al. 2003). The mannose-binding lectin ConA is documented to bind to the glycocalyx, amphids, and cuticle of several *Meloidogyne* species (McClure and Stynes 1988; Lin and McClure 1996). In regards to straminipiles, oomycetes like *Phytopthora* are morphologically and physiologically similar to fungi in that they obtain nutrition via an absorptive mycelium. Hence, it is possible that mannose binding by GAFP may affect a mechanism common to the mycelial growth habit. Although the cell wall composition of *Phytopthora* is considerably different from fungi, it is known to contain mannose (Zentmyer 1980). One possibility is that mannose residues in oomycete hyphae may play an important role in hyphal elongation or cell wall stability. If this scenario were true, cell wall structure could become unstable, or hyphal elongation become disrupted when hyphae come in contact with high concentrations of the lectin. Another possibility is that the mannose residues in oomycete cell walls are involved in important host–pathogen interactions. If this was the case, lectin binding could discourage infection and invasion of the host tissue giving an apparent reduction in disease symptoms in the host. Overall, the mechanism of resistance to straminipile infection is unknown and merits further investigation.

In contrast to the observed resistance to diseases caused by the eukaryotic pathogens, no enhanced disease resistance was afforded by the *GAFP-1*-VNF transgene against the eubacterium *R. solanacearum*. At the same time the *GAFP-1* transgenic lines were no more severely impacted by *R. solanacearum* infection than the control lines. In a sense, the lack of resistance against bacteria provides indirect evidence for the transgene's involvement in the resistance to infection by the eukaryotic pathogens. Had increased resistance to all pathogens been observed, one might be left to wonder if a general defense reaction, such as systemic acquired resistance, had been induced by transgene insertion instead of the effects of *GAFP-1*-VNF expression.

The differential performance of the lectin transgene between eukaryote and prokaryote pathogens may simply be related to fundamental physiological and ecological differences between these two phylogenetic lineages. Lectins, including mannose-binding lectins, have been shown to enhance root nodulation by *Rhizobium* (Diaz et al. 1995; Eijsden et al. 1995; Rhijn et al. 1998; Rhijn et al. 2001). Since the underlying biochemical mechanisms for infection by *Rhizobia* and phytopathogenic bacteria are similar to a certain extent, it is possible that lectin transgenes could enhance infection by aggregation of phytopathogenic bacteria. In the current study, the levels of lectin production may have been insufficient to affect bacterial aggregation and, in turn, promote infection. Another possibility is that localization of the lectin within the plant prevented contact between *R. solanacearum* and *GAFP-1*-VNF. Unlike *Ralstonia solanacearnum* (Agrios 1997), the other three eukaryotic pathogens can initiate infection intracellularly, which would place them in direct contact with GAFP-1 during the initial infection process, provided that the lectin accumulated within the plant cells. The inability of *R. solanacearnum* to initiate infection intracellularly (Agrios 1997) may have prevented contact with the lectin.

In conclusion, the observed success of the current herbaceous system demonstrates the potential for using *GAFP-1* in the development of transgenic disease tolerant plants. Specifically, heterologous expression of *GAFP-1*-VNF can impart increased resistance against several eukaryotic pathogens from several higher-order taxa. Furthermore, the mode of action of these lectins is linked to saccharide binding rather than binding of host specific proteins, resulting in the disruption of different basic biochemical processes across different organisms, which in turn could reduce the ability of the pathogens to overcome the resistance confirmed by GAFP. Based on these considerations, *GAFP-1* could be a useful component of IPM programs for agriculturally important crops, including woody crops where the transgene could be incorporated into the rootstock. The use of a plant-derived gene for disease resistance along with the potential for rootstock confinement may increase the social acceptability of using this transgene.

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