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The type III effector PthG of *Pantoea agglomerans* pv. *gypsophilae* modifies host plant responses to auxin, cytokinin and light

Dan M. Weinthal · Sara Yablonski · Sima Singer · Isaac Barash · Shulamit Manulis-Sasson · Victor Gaba

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Abstract *Pantoea agglomerans* pvs. *gypsophilae* and *betae* are related gall-forming bacteria. While *P. agglomerans* pv. *beta* initiates gall formation on both beet and gypsophila, the gypsophila pathovar causes gall formation only on gypsophila. PthG is a type III effector determining host range of these pathogens, initiating the hypersensitivity response in beet, but is a virulence factor in gypsophila. The role of PthG and its mode of action in pathogenicity remain unclear. Transgenic *Nicotiana tabacum* plants expressing pthG were created. PthG over-expression was often lethal, and surviving *pthG*-bearing lines showed morphological and developmental abnormalities such as leaf deformation and abnormal vascular branching, dwarf stature, loss of apical dominance, seedling apical meristem loss, rapid germination, reduced fertility, plants which cease growth for several weeks later producing a new lateral shoot, and loss of endophyte resistance (bearing unusual saprophyte populations). Some transformants required light for seed germination and showed rapid seedling greening. In in vitro assays PthG expression modified responses to auxin and cytokinin, inhibiting root and shoot production but not callus formation. In vitro differentiation responses to light were modified by PthG expression. This effector may interfere in the plant auxin signalling pathways resulting in higher observed auxin and ethylene levels, and subsequent blockage of root and shoot development. Apparently PthG tunes the host response to high hormone levels, changing the developmental response. Since shoot and root development are delayed, we hypothesize that callus/gall formation is supported by this activity. However, interference by PthG with hormone and light signalling does not explain all the responses observed in *pthG*-bearing lines.

Keywords Shoot formation · Root formation · Callus · Tissue culture · Transgenic plants · Transgenic tobacco

Abbreviations

BA	Benzyl adenine
CT	Cycle threshold

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D. M. Weinthal · S. Yablonski · S. Singer · S. Manulis-Sasson · V. Gaba (✉)
Department of Plant Pathology and Weed Science,
ARO Volcani Center,
POB 6, Bet Dagan 50250, Israel
e-mail: vpgaba@volcani.agri.gov.il

D. M. Weinthal · I. Barash
Department of Plant Sciences, Faculty of Life Sciences,
University of Tel Aviv,
Tel Aviv 69978, Israel

Present Address:
D. M. Weinthal
Department of Molecular,
Cellular and Developmental Biology,
The University of Michigan,
Ann Arbor, MI 48109, USA

GA_3	Gibberellic acid
HR	Hypersensitive response
hrc	*hrp* conserved
hrp	Hypersensitive response and pathogenicity
IAA	Indole-3-acetic acid
LB	Luria Broth
MS	Murashige and Skoog (1962) medium
NAA	1-naphthaleneacetic acid
Pab	*P. agglomerans* pv. *betae*
Pag	*P. agglomerans* pv. *gypsophilae*
PDA	Potato dextrose agar
qRT-PCR	Quantitative reverse transcriptase (Real Time) PCR
STS	Silver thiosulphate
T3SS	Type III secretion system
TC	Transgenic ("empty vector") control

Introduction

Biotrophic bacterial pathogens multiply parasitically on living tissues, may induce delayed plant symptoms, and must suppress the host basal defences to multiply effectively within the host and acquire nutrients in the apoplast (Barash and Manulis-Sasson 2007, 2009). Gram negative pathogenic bacteria of animals and plants have developed the T3SS, injecting virulence proteins into host cells, to suppress defences (Navarro et al. 2006). The presence of *hrp* and *hrc* genes, mainly the T3SS protein complex and its effector proteins, is essential for pathogenicity of most Gram-negative phytopathogenic bacteria (Büttner and Bonas 2006). T3SS effectors are necessary for bacterial multiplication within the host apoplast.

The bacterium *Pantoea agglomerans* (previously *Erwinia herbicola*) is a frequent epi- and endophyte, but rarely pathogenic (Barash and Manulis-Sasson 2009). However, isolates of this bacterium are tumorigenic pathogens on gypsophila (*Gypsophila paniculata*), beet (*Beta vulgaris*) (see Barash and Manulis-Sasson 2009), and a few other species. Gall formation on gypsophila and beet is caused by related pathovars: *Pag* is pathogenic only on gypsophila but elicits a hypersensitive response on beet (Ezra et al. 2000, 2004), whereas *Pab* is pathogenic on both beet and gypsophila (Barash and Manulis-Sasson 2009).

Pathogenicity of these pathovars is determined by the presence of indigenous plasmids, designated $pPATH_{Pag}$ and $pPATH_{Pab}$ for *Pag* and *Pab*, respectively (Barash and Manulis-Sasson 2009). pPATH contains a pathogenicity island harbouring a *hrp*-gene cluster, T3SS effectors, a gene cluster encoding for auxin and cytokinin synthesis amongst others (Barash and Manulis-Sasson 2007).

Plant gall formation as a reaction to parasitic attack is based on plant cell hyperplasia and hypertrophy, induced mainly by increases in auxin and cytokinin levels (Morris 1986), generally attributed to pathogen-secreted phytohormones. However, inactivation of the *Pag* IAA and cytokinin biosynthesis pathways only reduced gall size by 50% (see Barash and Manulis-Sasson 2009). By contrast, any mutation in the T3SS-encoding *hrp* genes, or in the regulatory cascade that activates the *hrp* regulon, completely abolished gall initiation (see Barash and Manulis-Sasson 2009). These results suggest that gall initiation relies on T3SS effectors, rather than phytohormones produced by *Pag* or *Pab*.

The $pPATH_{Pag}$-borne pathogenicity island accommodates nine putative T3SS effectors; mutagenesis of six of them almost completely or significantly reduced gall formation (Barash and Manulis-Sasson 2007). Both *Pag* and *Pab* contain the T3SS effectors HsvG and HsvB which enable infection of gypsophila and beet, respectively. The T3SS effector differentiating between *Pag* and *Pab* (Ezra et al. 2000) is PthG. PthG exhibits dual functions: a virulence effector in gypsophila but a *Betae* genus T3SS effector (Ezra et al. 2004). Mobilizing *pthG* into *Pab* caused this pathovar to induce HR on beet, while retaining full pathogenicity on gypsophila. Moreover, a marker exchange mutant of *pthG* in *Pag* (*Pag*824-1 Mx27) expanded its host range to beet. This mutant substantially reduced gall formation in gypsophila (Ezra et al. 2000) suggesting that PthG is a virulence factor on gypsophila. Notably, a functional *pthG* is present in $pPATH_{Pag}$, whereas only remnants of this gene could be detected in $pPATH_{Pab}$ (Barash and Manulis-Sasson 2007).

The role of the PthG in determining *P. agglomerans* host range is clear, but its role in virulence is unclear. The goal of this work is to explore the function of PthG as a virulence factor using transgenic tobacco plants expressing low levels of PthG. We demonstrate that such tissues have altered sensitivities to auxin, cytokinins and light which modify plant development and morphology. Such alterations permit bacterial gall formation at high phytohormone concentrations.

Materials and methods

Bacterial strains, growth conditions and plasmids

The *Agrobacterium tumefaciens* strain EHA105 (Hood 1993) bearing the pJIC Sa_Rep plasmid (Hellens et al. 2000) was used for tobacco transformation. *E. coli* strain DH5α (Invitrogen, Carlsbad, CA, USA) was used for molecular manipulation. Plasmids used were pGreenII0029 (Hellens et al. 2000), pGreen*pthG* (a full length *pthG* in 35S-*pthG*-nos cassette in pGreenII0029) and pGreen*204* (35S-204-nos cassette in pGreenII0029) (Ezra et al. 2004). pGreen-*pthG204* contained a *pthG* remnant (from *Pab*) encoding a non-functional two-thirds length *pthG* gene, due to deletion and frame shift mutation (Ezra et al. 2000).

E. coli and *A. tumefaciens* were cultured on LB medium. Solid LB medium with 1.5% agar was used for temporary strain maintenance. *E. coli* were grown at 37°C and *A. tumefaciens* at 28°C. Fungal endophytes were detected by plant sample incubation on PDA at 25°C for 5 days.

Gene expression analysis by quantitative real-time PCR (qRT-PCR)

Total plant RNA was extracted using MasterPure RNA Purification Kit (Epicentre, Madison, WI, USA). Reverse transcription was carried out using Reverse-iT Max RTase Blend (ABgene, Epsom, UK) following the manufacturer's instructions. The resultant cDNAs were subjected to PCR. The primers used for amplification were designed on the basis of the *pthG* sequence and 18S rRNA primers (Beno-Moualem et al. 2004). Real time detection was performed with ABsolute QPCR SYBR Gr. Mix (ABgene, Epsom, UK) in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) and results analyzed with Rotor-Gene 6 software. A mixture of all cDNAs used for all the treatments was employed as template for calibration curves designed for each pair of primers. Relative quantification was calculated on the basis of ΔΔCT. The ΔCT value was determined by subtracting the CT results for the target gene from the endogenous control gene (18S) CT and then normalizing (ΔΔCT). Each experiment was conducted 3 times. For qRT-PCR of *pthG* the forward primer was 5′AGTCCGCTGAGTCGATCAGG3′ and the reverse primer used was 5′GGGATTGAAAAGCTCTGAGCCT3′. For qRT-PCR of 18S the forward primer was 5′ GCGACGCATCATTCATTCAAATTTC3′ and the reverse primer was 5′TCCGGAATCGAACCCTAATTC3′.

Plant transformation and hormone assays

The preparation of *pthG*-transformed tobacco plants was similar to Maldonado-Mendoza et al. (1996) using *A. tumefaciens* strain EHA105 (Hood 1993) containing the pSoup helper (Hellens et al. 2000), and pGreen-*pthG*, pGreen-*pthG204* (Ezra et al. 2004) or transgenic "empty vector" control. T_1 seedlings of *pthG* transformants were grown in a greenhouse or on Murashige and Skoog (1962) basal medium in a growth room (25°C, 16 h photoperiod, 30 μmol photons $m^{-2}s^{-1}$ cool white fluorescent light).

Seed germination

Seeds were sterilized in 70% ethanol for 1 min, followed by 30% commercial bleach (ca. 1% active chlorine final concentration) for 20 min, washed 3 times with sterile distilled water, plated on MS basal medium containing 100 mgl^{-1} kanamycin, and incubated in darkness (aluminum foil covered) or light as above for 2–3 weeks. To rescue seedlings without apical buds, cotyledon explants were placed on MS basal medium with 0.5 μM NAA and 0.5 μM BA. All lines were maintained in vitro on MS basal medium.

Phytohormone measurements

Auxin measurements were performed on 1 g of leaf tissue as described by Sagee et al. (1990). Fifty μl of the extract from 1 g tissue was incubated with 50 μl met-(^{3}H) IAA, 300 μl RIA buffer and 50 μl polyclonal anti met-IAA. After 1 h incubation at 37°C the sample was precipitated with 750 μl 1 M ammonium sulfate and redissolved in 50 μl methanol plus 1 ml scintillation buffer. The radioactivity was measured in a 1600 TR Liquid Scintillation counter (Packard, Meriden, CT, USA).

Ethylene measurements were performed on 3 week-old tobacco plants grown in 50 ml culture tubes on 10 ml of MS basal medium. The culture tubes were tightly sealed for 3 h with Suba•Seal caps (Sigma, St. Louis, MO, USA), and incubated in the light (as above). Air samples of 10 ml were taken for ethylene analysis

by gas chromatograph (Chalupowicz et al. 2006), (Model 3300, Varian, Sugarland, TX, USA).

Regeneration assay on transgenic tobacco

Tobacco leaf explants (3 mm×3 mm), 6–9 explants per plate, were transferred to MS basal medium in 90 mm diameter Petri dishes with 0, 0.5 and 5 μM NAA and/or BA, forming a 9 treatment matrix. Four matrixes were prepared per line per experiment; two each incubated in darkness and light as above. The following morphological responses were recorded after 4 weeks for each explant: survival, growth, and differentiation into callus, roots and shoots. In some treatments explants browned and died. With other treatments explants failed to grow but stayed green. Each experiment was repeated at least 5 times i.e. a minimum of 10 plates (60–90 explants) per line per treatment, in darkness and in light.

Ethylene action blocking assays on transgenic tobacco

The ethylene receptor blocker STS was prepared (Perl et al. 1988), filter sterilized and added to cooling MS basal medium (1 or 2 mgl^{-1} STS). After 30 days in culture, plant shoot fresh weight and leaf number were measured.

Effect of GA_3 on growth

GA_3 was dissolved in methanol and added to cooling MS basal medium at final concentrations of 0.1 or 1 mgl^{-1}. After 3 weeks culture, when the plants had reached the top of the tubes in the higher GA_3 concentration, shoot length and fresh weight were measured.

Visualization of leaf vasculature

Leaves were treated with dimethyl formamide until the chlorophyll was removed, and then soaked in a 0.1% solution of brilliant blue in water to visualize the vascular system.

Statistical analysis

Statistical analysis was performed using the SAS program on the Volcani mainframe computer, performing a GLM assay or the Jump5 statistical program using one way ANOVA Tukey HSD test at $\alpha<0.05$ or lower. Both tests are analysis of variance for independent samples comparing all possible pairs of means to propose significantly different samples. Each experiment was repeated at least three times before analysis by the Statistical Unit, ARO Volcani Center. Details of the statistical tests used are given for each experiment.

Results

Production of transgenic plants

Leaf explants of *N. tabacum* cvs. Samsun NN and Hicks were inoculated with *A. tumefaciens* EHA105 containing pGreen-*pthG*, pGreen-*pthG204* or the empty vector. Transformed tobacco plants bearing the *pthG* regenerated poorly in vitro. Eventually, we selected six Samsun NN *pthG*-transgenic tobacco lines (44732, 44733, 44735, 44742, 44751 and 44765) with stable phenotypes that produced adequate seeds for further analysis. Several transgenic plants of cv. Hicks were generated but did not bear seeds. Attempts to transform *Arabidopsis thaliana* with *pthG* using the floral dip method (Harrison et al. 2006) were unsuccessful. Expression of *pthG* in leaves of the T_1 generation of putative Samsun NN transgenic plants was confirmed by qRT-PCR. Relative expression levels between 2 to 25 were detected, with the lowest and highest level in lines 44735 and 44765, respectively. No correlation was observed between relative *pthG* expression levels and phenotype (data not shown). Multiple control lines were produced bearing the *pthG204* (Ezra et al. 2004) with no visible phenotype. A single *pthG204*-bearing line was used as a control.

Phenotypic characterization of *pthG*-transgenic tobacco plants

pthG-transgenic tobacco plants showed several phenotypic differences compared with plants transformed with *pthG204* (line 44633) or the transgenic "empty vector" control (TC)(Fig. 1, Table 1). All *pthG*-bearing lines displayed some degree of leaf deformation (Fig. 1a–d, Table 1), ranging from constant severe deformation (line 44733), to occasional deformation

(lines 44751 and 44765). Interestingly, leaf shape deformations occurred in a repetitive radial order of 4–6 leaves. Examination of the vascular system suggested disturbed branching and greater arching in the *pthG*-bearing lines than in the transgenic control (Fig. 1e, f).

All *pthG* lines had lower stature than the transgenic controls, and several *pthG* lines had a dwarf phenotype (Fig. 1g; Table 1). Several of the *pthG*-bearing lines (44742, 44751, 44735) appeared to "segregate" in tissue culture. That is, at each subculture routinely only 25–33% of the plants would lengthen "normally" (for that line), the remainder failing to elongate. Only the tall phenotypes were subcultured at each passage. This phenomenon may be connected to the radial order of leaf shape production (above), suggesting that only third or quarter of lateral buds are "normal".

Three *pthG*-bearing transgenic lines (44733, 44735, 44742) required light for germination. Additionally, line 44735 (and sometimes 44733 and 44742) germinated without producing a first true leaf or a visible apical bud (Fig. 1h). Line 44735 was rescued by transfer of excised cotyledons to MS medium containing 0.5 μM NAA and 0.5 μM BA, and subsequent shoot regeneration. Following rescue line 44735 was only maintained in vitro. Seeds of all *pthG*-bearing lines germinated more rapidly than all controls, and the seedlings greened more rapidly in the light than controls (data not shown). All *pthG*-bearing lines branched more than controls (Fig. 1i) suggesting a reduction in apical dominance.

Some *pthG*-bearing transgenic lines (44733, 44735, 44742, 44765) ceased development for a prolonged period i.e. a month-old plant would cease growth, and after two further months produce a new lateral shoot (Fig. 1j) (a "quiescent" phenotype). Control lines flowered in the greenhouse about 3 months after germination, produced fruit and seeds, and died after 4–5 months. However, the *pthG*-bearing transgenic lines flowered after a minimum of 9 months in the greenhouse and most lines (44733, 44735, 44742, 44765) did not complete their life cycle 4 years after planting. These plants flowered, and in few cases produced seed, only after severe cutting back.

Some lines bearing the *pthG* gene (44733, 44735, 44742) were of reduced fertility: not flowering, or bearing infertile flowers or seedless fruits (Fig. 1k, l). Difficulty in obtaining fertile seeds of cvs Samsun NN and Hicks *pthG* lines (or *Arabidopsis*) may indicate that PthG expression can be lethal.

Most experiments were performed in vitro because of the difficulty in obtaining seeds. Severe contamination unique to the *pthG*-bearing lines (especially 44733) was detected, leading to the conclusion that these lines are more susceptible to internal colonization by micro-organisms. Fungi did not grow from the transgenic control and mutant *pthG* line 44633, while the majority of the *pthG* lines suffered from constant contamination (Table 1). This is exemplified in an experiment in which explants were excised from apparently healthy *pthG*-bearing plants of line 44733, surface sterilized and sown on PDA (Supplementary Fig. 1). *Alternaria alternata*, *Aspergillus*, *Penicillium* and yeasts were isolated from line 44733, while little internal contamination was found in the transgenic control (Supplementary Fig. 1).

Differences in the response to light between the *pthG*-bearing transgenic lines and the controls were noted in seed germination, greening and differential responses to light and darkness during in vitro regeneration induced by plant growth regulators (Table 2).

The phenotype of each *pthG*-bearing transgenic line is summarized in Table 1. Such phenotypes suggested a hormone or hormone-response deficiency interacting with photomorphogenic responses. To verify this hypothesis we performed a number of assays to examine the status of, and responses to, growth regulators in the *pthG*-bearing lines.

Auxin and ethylene levels in *pthG*-transgenic lines

The leaf deformation phenotype, especially the alterations in vascular branching, suggest auxin deficiency (Gallavotti et al. 2008). Auxin levels in the transgenic tobacco plants were measured by radio-immuno assay (Sagee et al. 1990): auxin levels in three different *pthG*-bearing lines with different degrees of leaf deformation (severe [44733], moderate [44742] and weak [44765]) are significantly higher (statistically different at $\alpha<0.05$ from the transgenic control (TC=100%) (Fig. 2).

Ethylene affects plant stature (Achard et al. 2003) and ethylene levels are known to rise during leaf senescence, resulting in increased pathogen sensitivity (Robert-Seilaniantz et al. 2007). To determine whether the dwarf phenotype and the susceptibility of the *pthG*

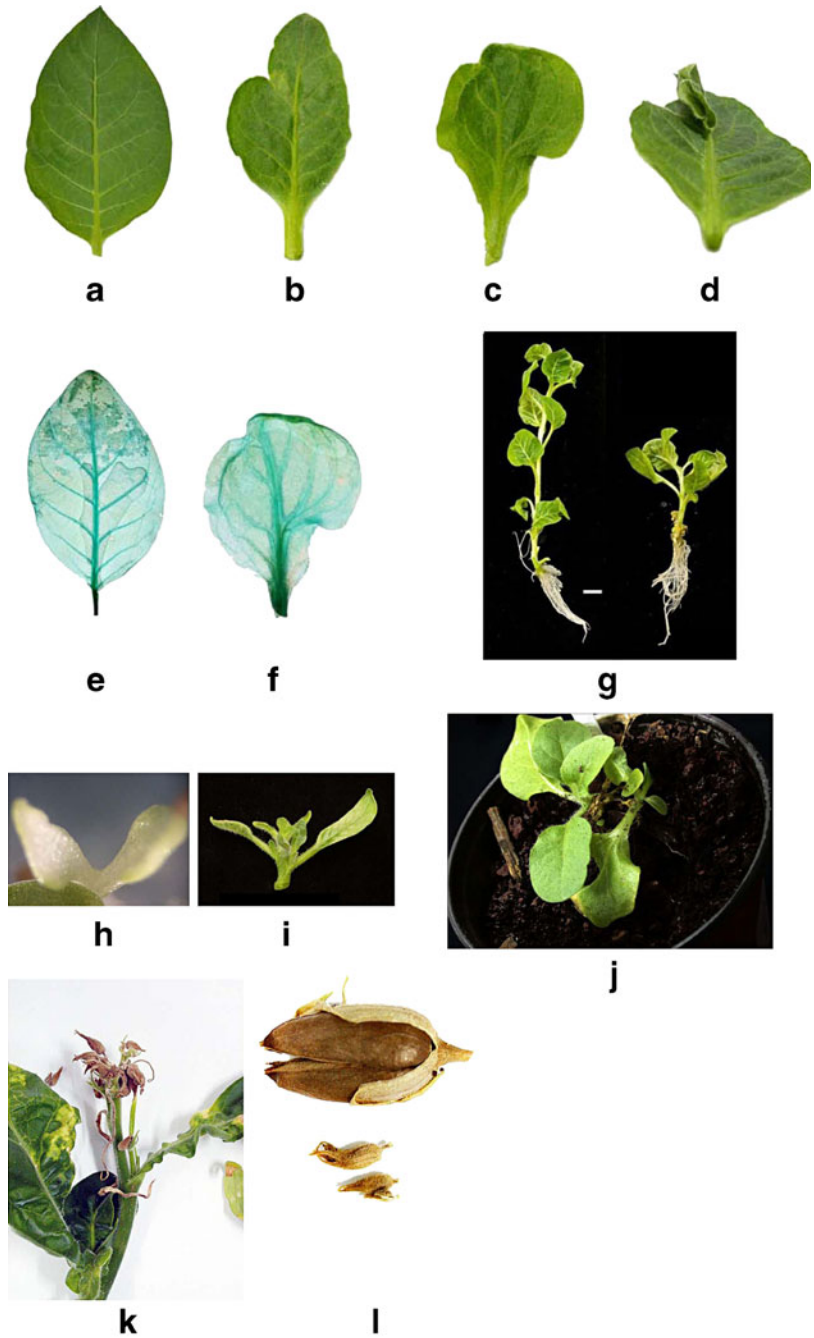

Fig. 1 Phenotypic characteristics of *pthG*-transgenic tobacco Samsun NN plants. Leaf deformation in *pthG*-bearing lines: leaf abnormalities in various lines differ from control (**a**) by severe deformation (**b**–**d**). Transgenic control leaf vasculature has regular branching (**e**), whereas the vasculature of leaves from plants bearing the *pthG* gene was more branched and more and irregularly arched (**f**), after visualization with brilliant blue. All *pthG*-bearing lines had a shorter or dwarf phenotype, as exemplified by line 44742 (*right*), compared to the transgenic control (TC) (*left*) after a month in tissue culture (bar=1 cm) (**g**). Loss of apical dominance in *pthG*-bearing plants: germination without apical meristem (**h**). *pthG*-bearing transgenic lines show greater branching than controls (**i**) and some quiescent plants (**j**) grew a new lateral shoot instead of developing the original stem 3 months after planting. *pthG*-bearing lines have reduced fertility resulted in reduced flowering, infertile flowers or seedless fruits: flowering in *pthG*-expressing line (**k**) and fruits from *pthG*-expressing lines bear no seeds (**l**). The largest upper fruit is the wild type tobacco control (**l**)

Table 1 Phenotypic characterization of *pthG*-bearing Samsun NN tobacco lines

Phenotype	Line								
	pthG-bearing						Controls		
	44732	44733	44735[a]	44742	44751	44765	44633[b]	TC[c]	WT[d]
Leaf deformation	+	+	+	+	+	+	−	−	−
Dwarf stature	−	+	+	+	−	+\−	−	−	−
Loss of apical dominance	−	+	+	+\−	−	−	−	−	−
Absent seedling apical meristem	−	+\−	+	+\−	−	−	−	−	−
Dark germination	+	+\−	−	+\−	+	+	+	+	+
Reduced fertility	−	+	+	−	−	−	−	−	−
Quiescent plants	+	+	+	+	+	+	−	−	−
Altered vascular branching and arching	+	+	+	+	+	+	−	−	−
Rapid germination	+	+	+	+	+	+	−	−	−
Rapid seedling greening in light	+	+	+	+	+	+	−	−	−
Loss of endophyte resistance	+	+	+	+	−	−	−	−	NT[e]
"Segregating" phenotype	−	−	+	+	+	−	−	−	−

[a] Line 44735 was rescued from T1 seeds on MS medium with 0.5 μM each NAA and BA and maintained only in vitro; no plants were maintained in the greenhouse

[b] Line 44633 is a transgenic line with a mutant (partially deleted) *pthG* (pGreen*204*)

[c] TC: transgenic "empty vector" control

[d] WT: wild type Samsun NN

[e] not tested

lines to endogenous endophytes might be due to higher ethylene levels we measured the ethylene production of all *pthG*-bearing lines in vitro. The mean values for ethylene production of all *pthG*-bearing lines were higher than the transgenic (empty vector) control. However, in only two lines was ethylene production significantly different from the control ($\alpha<0.05$) (Fig. 3). Lines 44732 and 44742 show the highest ethylene production levels (group a) while the transgenic control produced the least ethylene, forming group b with lines 44735 and 44733. Lines 44751 and 44765 are located in both groups.

In vitro regeneration of transgenic tobacco lines

The phenotypes of the *pthG*-bearing lines, together with our findings of differences in auxin and ethylene levels between the *pthG*-bearing lines and the transgenic control (TC), suggest that PthG is involved in manipulation of host plant responses to phytohormones. Therefore we conducted assays to examine the responses of *pthG*-bearing tissues to exogenously applied auxin and cytokinin in a developmental assay—in vitro regeneration.

The in vitro regeneration assay was performed on all *pthG*-bearing lines, the transgenic (empty vector) control and *pthG204*-bearing line 44633. Leaf explants regenerated in vitro on 0, 0.5 and 5 μM of NAA and/or BA, forming a matrix of nine different growth regulator concentrations, examined in dark and light. Typical regeneration responses in the matrix are presented in Fig. 4. Strong differences are seen between the *PthG*-bearing lines (especially 44742 and 44765) and the transgenic empty vector control (Fig. 4). For lines 44742 and 44765 regeneration in the light is abolished or confined to intermediate auxin concentrations (Fig. 4). Line 44733 was more similar to the control than the other *pthG*-bearing lines, except at low BA and NAA concentrations (Fig. 4). The response of the *pthG*-bearing lines was more similar to the control in darkness (Fig. 4), although high auxin concentrations produced callus rather than roots (lines 44733 and 44742), and line 44765 was unresponsive in darkness in most growth regulator combinations (Fig. 4).

Table 2 Analysis of in vitro regeneration assays of transgenic Samsun NN tobacco lines

Physiological response	Growth regulator[a] (μM)	Control lines[b] mean (%)	*pthG* lines[c] mean (%)
Explant survival			
BA	0	89a	69b
	0.5	92a	92a
	5	97a	96a
Explant growth			
NAA	0	91a	69b
	0.5	90a	88a
	5	91a	88a
BA	0	82a	64b
	0.5	93a	87a
	5	96a	95a
Root development			
Light[d] Dark		42a	15b
Light		19b	10b
BA	0	51a	23b
	0.5	31b	12c
	5	6d	2d
NAA	0	11c	2d
	0.5	24b	12c
	5	46a	23b
Stem development			
BA	0	1d	2d
	0.5	41c	40c
	5	76a	56b

Results were calculated for each separate treatment in each experiment, and converted to % response (i.e. % of explants responding). Means for each treatment/line were aggregated for at least 5 different experiments (replicates) (mean % response). Results are only shown for treatments in which statistical differences were observed greater than $\alpha<0.05$. Different letters represent statistical groups as determined by GLS assay, statistically different at $\alpha<0.05$

[a] Applied concentration of plant growth regulator

[b] The sum of the transgenic control (TC) and *pthG204*-bearing 44633

[c] The sum of all *pthG*-transgenic lines (44732, 44733, 44735, 44742, 44751, 44765)

[d] All treatments in light were compared with all those in darkness

Statistically significant differences were found in a comparison of all *pthG*-bearing lines versus transgenic (empty vector) control and *pthG204*-bearing 44633 lines (Table 2). Explant survival of the *pthG*-bearing explants without BA (69%) increased to 92–96% at 0.5 μM and 5 μM (the control level). The % of explants of *pthG*-bearing lines able to grow in the presence of NAA or BA was significantly higher (87–95%) than without these phytohormones (69% and 64%, respectively). The addition of NAA and/or BA increased % of explants growing of the *pthG*-bearing lines to the control level (87–95%).

Explant survival and growth represent non-developmental responses to NAA and BA, while root and shoot regeneration indicate developmental responses to phytohormones (Table 2). Root development is affected by light and BA and NAA concentrations. Explants from *pthG*-bearing lines developed roots similarly in both dark and light (10–15%), whereas control explants formed significantly more roots in darkness (42%) than in light (19%). It is well known that cytokinin (BA) initiates shoot formation and inhibits root development, while auxin (NAA) causes root formation and inhibits shoot development. Here, increasing BA concentration caused a decrease in root formation in both *pthG*-bearing and control lines (Table 2). Addition of NAA resulted in increased root development, although with significant differences between the controls and *pthG*-bearing lines (Table 2). Nevertheless, fewer explants of the *pthG*-bearing lines overall develop roots than control explants in the absence of BA and at 0.5 μM BA. This may indicate that explants of the *pthG*-bearing lines are less responsive to auxin and more responsive to cytokinin.

Increasing BA concentration resulted in an increase in stem development in both *pthG* and control lines. However, at the highest BA concentration stem development in the *pthG*-bearing lines was significantly lower than in the controls (56% versus 76%). Explants from *pthG*-bearing lines produced more callus, but were not statistically different at $\alpha<0.05$ (data not shown).

Effect of ethylene receptor blocking on the growth of *pthG*-bearing plants

Ethylene production by the *pthG*-bearing lines was higher than in the controls (Fig. 3) and the responses to exogenous auxin and cytokinin differ from the controls (Table 2). Therefore we asked whether the stature of the *pthG*-lines is a response to endogenously produced ethylene. The effect of the ethylene receptor blocker STS (0, 1 or 2 mgl^{-1}) was tested in vitro on

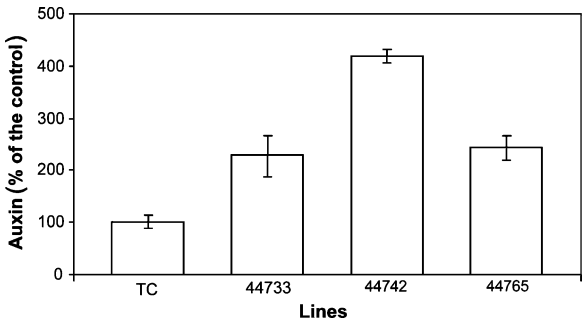

Fig. 2 Auxin levels in *pthG*-bearing cv. Samsun NN plants. Endogenous IAA levels of leaf tissues were measured in the transgenic empty vector control (TC) and selected *pthG*-bearing transgenic lines (44733, 44742, 44765). Analysis by JMP 5 of Tukey HSD (5%) demonstrates that these *pthG*-bearing lines all contain higher IAA levels than the control. The control value was 8.3±0.8 μg $IAAg^{-1}$ fresh weight. *Bars* represent the standard deviation. Four separate plants were analyzed per line, repeated thrice

"non-segregating" *pthG*-bearing lines (44732, 44733, 44765), covering the range of *pthG*-induced phenotypes, from a line similar in height to the control (44732) to dwarf (44733). "Non-segregating" lines nearly always produced plants that grew normally for that genotype. No significant differences in changes of plant fresh weight or leaf number caused by STS treatment were found between the *pthG*-bearing lines and the control (data not shown).

Effect of gibberellic acid on the growth of transgenic tobacco plants

Gibberellic acid (GA_3) is a well known phytohormone; defects in the gibberellin synthesis pathway are a common cause of dwarf stature in plants (Achard et al. 2003). Growing the short or dwarf *pthG*-bearing lines on GA_3 may complement a *pthG*-induced phenotype. We tested the effect of GA_3 in vitro on non-segregating *pthG*-bearing lines (Table 3). In the absence of exogenous gibberellin, significant differences in plant elongation were found: line 44732 is not different from the transgenic empty vector control, while the elongation of lines 44765 and 44733 is statistically different (Table 3). Following treatment with 0.1 mgl^{-1} GA_3, the control and line 44732 are still statistically different ($\alpha<0.05$) from lines 44733 and 44765. A similar result was obtained with 1 mgl^{-1} GA_3 (a concentration which saturated the growth stimulation), suggesting that addition of GA_3 could not restore the elongation of the *pthG*-bearing short/dwarf phenotypes to the control level. There were no statistically different growth (or relative % growth) stimulations compared to the controls (data not shown).

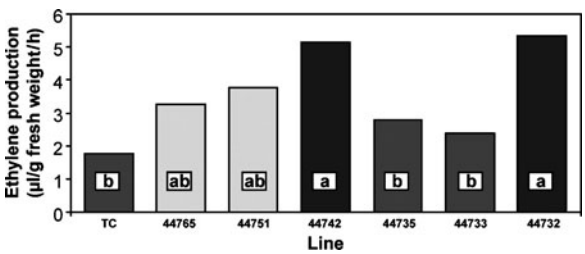

Fig. 3 Ethylene production by *pthG*-bearing cv. Samsun NN lines. The production of ethylene was measured, the means of 3 separate experiments were aggregated for each line (10–12 plants per line per experiment) and analyzed by the Tukey HSD assay. Statistically different groups at $\alpha<0.05$ are represented by different letters. Transgenic empty vector control (TC)

Challenge with fungal pathogen

A. alternata is a saprophyte and opportunistic pathogen on many crops (Rotem 1994), recovered in vitro from line 44733 (above). In vitro-grown leaves of all *pthG*-transgenic and control lines (Samsun NN wild type, transgenic empty vector control, line 44633-*pthG204*) were inoculated with *A. alternata* (see "Supplementary electronic material" for technical details). The kinetics of emergence of *A. alternata* onto PDA medium did not significantly vary between any of the *pthG*-transgenic lines and any of the control lines (data not shown). Additionally, there was no statistical difference between the grouped results for the *pthG*-transgenic lines and the grouped control lines data (data not shown).

Discussion

We have shown that transgenic tobacco plants bearing a bacterial pathogenicity effector, the *pthG* gene, have altered responses to the phytohormones auxin and cytokinin, and light, but not to the phytohormones gibberellin or ethylene. Phytohormones are known to be important in plant defence against microorganisms (Remans et al. 2006). Notably, bacterial oncogenes (Casanova et al. 2003) and several *Arabidopsis* mutants with impaired auxin metabolism or signaling confer similar phenotypes to those

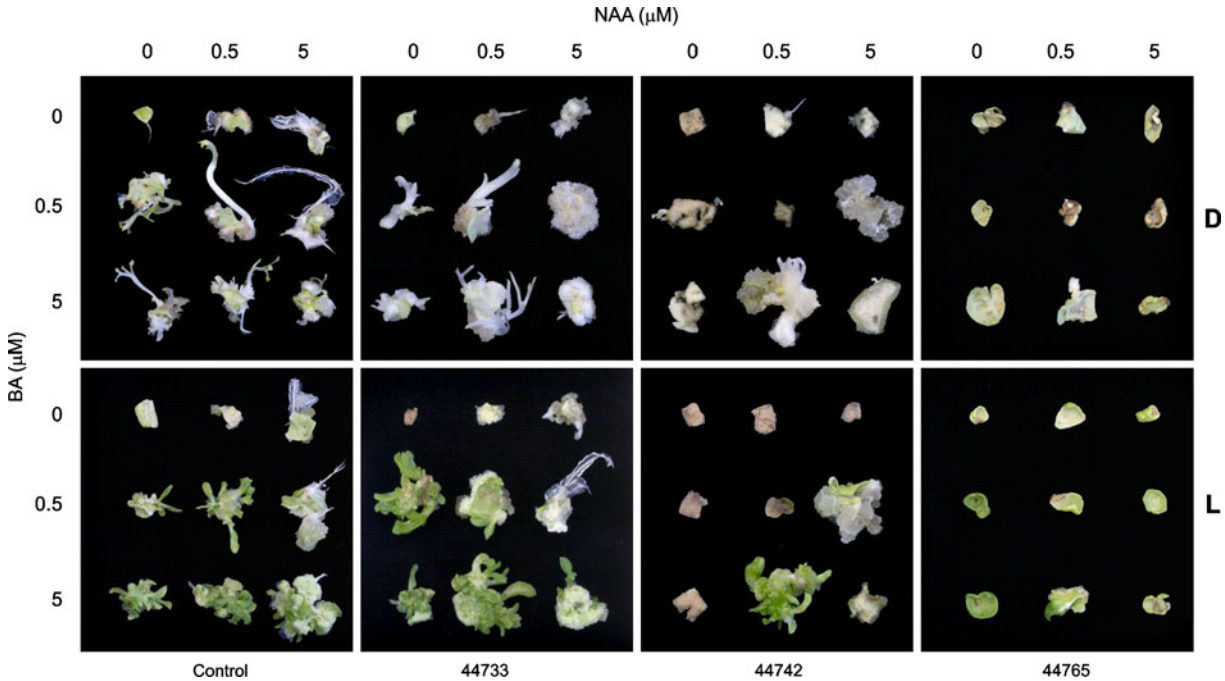

Fig. 4 In vitro regeneration assay of *pthG*-bearing Samsun NN tobacco lines. Explants from four-week-old *pthG*-transgenic plants were placed on media containing 0, 0.5 and 5 μM of NAA and BA forming a matrix of 9 different plant growth regulator treatments. Each matrix was examined in dark (D) and light (L). Control is transgenic empty vector control

observed here (Salmon et al. 2008). Although *pthG* expression could only be detected by reverse transcriptase-PCR or qRT-PCR, the *pthG*-transgenic lines were significantly altered compared to "empty vector" controls and multiple control lines bearing *pthG204* (Ezra et al. 2004), demonstrating the role of the PthG protein in the responses observed.

The *pthG*-bearing lines are less responsive to auxin and cytokinin, and responses were developmentally abnormal (Tables 1 and 2). PthG may function in an analogous manner to *Agrobacterium* genes modifying auxin responses i.e. *A. tumefaciens* T-DNA gene 5 (Korber et al. 1991), *A. rhizogenes rolB* and *rolC* oncogenes (Casanova et al. 2003). Analysis of auxin-overproducing *Arabidopsis* indicated that apical dominance and leaf epinasty are primarily controlled by auxin rather than ethylene (Ongaro et al. 2008). However, high levels of endogenous ethylene were partially responsible for the inhibition of stem elongation observed in auxin-overproducing tobacco (Romano et al. 1993).

There is often a close relationship between bacterial phytohormone production (especially auxin) and pathogenicity. Several bacteria cause gall formation in plants. Tissue hyperplasia was observed following

Table 3 Effect of GA_3 on growth of *pthG*-bearing lines. *pthG*-bearing Samsun NN "non-segregating" lines (44732, 44733, 44765) and the transgenic empty vector control (TC) were grown in tissue culture with the addition of 0, 0.1 or 1 mgl^{-1} GA_3. Plant growth was measured after 3 weeks and mean values calculated. The means of 3 separate experiments were aggregated for each treatment (20–25 plants per treatment per experiment), and analyzed by the Tukey HSD assay. Statistically different groups at $\alpha<0.05$ are represented by different letters, for each GA_3 concentration

GA_3 (mgl^{-1})	Plant line	Mean growth (mm)
0	TC	27a
	44732	25ab
	44765	19bc
	44733	13c
0.1	TC	73a
	44732	72a
	44765	53b
	44733	39b
1	TC	80a
	44732	80a
	44765	60b
	44733	57b

Xanthomonas citri infection (Brunings and Gabriel 2003). *A. tumefaciens* causes crown gall disease on a wide host-range by genetic transformation with T-DNA oncogenes encoding genes involved in synthesis of IAA and cytokinins (Zambryski et al. 1992; Zupan et al. 2000). The virulence of *Pseudomonas syringae* subsp. *savastanoi*, which causes galls on olive, was associated mainly with phytohormone secretion; yet the presence of *hrp/hrc* genes suggests that, as with *Pag*, T3SS effectors play a key role in pathogenicity (Sisto et al. 2004). Other *P. agglomerans*, genetically dissimilar to *Pag* (Weinthal et al. 2007), such as the wisteria (*P. agglomerans* pv. *milletiae*) (Goto et al. 1980) or the cranberry pathovars (*P. agglomerans* pv. cranberry) (Vasanthakumar et al. 2004) also form galls. Additionally, Gram-positive *Rhodococcus fascians*, which causes wide host range gall formation (Nester et al. 1984), secretes auxin (Vandeputte et al. 2005).

The *pthG*-phenotype is similar (dwarf and quiescent plants) to Auxin Response Factor (ARF)-mediated signalling mutants (Salmon et al. 2008), indicating that PthG interferes with auxin signalling. Auxin response factor-mediated signalling conveys positional information during organogenesis (Salmon et al. 2008). Leaf deformation and loss of apical dominance (excessive branching) and vascular arching were related to low or high auxin levels (Castellano and Vioque 2002).

PthG reduction of sensitivity to auxin also results in phenotypes reminiscent of *MONOPTEROS* (*MP*)/*Auxin response factor 5* gene mutants, which carry severe patterning defects (Vidaurre et al. 2007). *mp* mutant cotyledons may be variably fused and the hypocotyl/root axis replaced by an unorganized basal peg (Hardtke and Berleth 1998). This may explain the "segregation" phenotype of several *pthG*-lines in vitro and the leaf shapes observed, as each leaf primordium develops under a different auxin regime influenced by preceding primordia (Campanoni et al. 2003). We may hypothesize that the change in responses to auxin and cytokinin lead to increased auxin levels (Fig. 2) as with the *MONOPTEROS* mutant (Hardtke and Berleth 1998), where reduced sensitivity to auxin leads to higher endogenous auxin levels, in turn leading to increased ethylene levels.

Dark green leaves and rapid seedling greening may indicate high endogenous cytokinin concentrations (Inoue et al. 2001). The *pthG*-bearing lines that germinate without production of a shoot apex (Table 1) are reminiscent of the *Arabidopsis SHOOT MERISTEMLESS* mutant lacking a shoot apical meristem (Long et al. 1996). These phenomena together may explain the leaf deformation, dwarf and quiescent phenotypes, and the mild excess ethylene production displayed by the *pthG*-bearing lines. However, possibly all the responses noted are due to changes in auxin transport, and therefore the only conclusion possible is that *pthG* causes a severe disturbance in auxin signalling.

Pathogenicity genes from other phytopathogenic bacteria have been observed to interfere with auxin signalling. IAA levels increase following infection of *Arabidopsis* by *Pseudomonas syringae* pv. *tomato* DC3000 (Schmelz et al. 2003) or *Xanthomonas campestris* pv. *campestris* (O'Donnell et al. 2003). The T3SS effector AvrRpt2 from *Pseudomonas syringae* pv. *tomato* DC3000 triggered *Arabidopsis* to produce more auxin and to be more responsive to auxin (Chen et al. 2007). Additionally, the TENGU protein, a phytoplasma pathogenicity factor (Hoshi et al. 2009) causes small malformed leaves, stunted growth, severe dwarfism, excessive branching and shortened internodes. Notably, auxin-related genes are down-regulated in *tengu*-transgenic plants (Hoshi et al. 2009). *Pseudomonas syringae* effectors also target other plant hormone-mediated defenses such as the abscisic acid signalling pathway (de Torres-Zabala et al. 2007).

Dwarf plants and high microbial susceptibility are phenotypes related to high endogenous ethylene levels (Yang et al. 2008). Auxin levels in *pthG*-bearing tobacco were significantly higher than in controls (Fig. 2). The statistical difference between ethylene production of the transgenic *pthG*-bearing lines and the control was less significant than those differences for endogenous auxin content, suggesting that the high ethylene levels are primarily due to the high auxin levels (Romano et al. 1993). We were unable to establish in our preliminary experiment that *pthG*-transformants were more vulnerable to pathogens than controls. This surprising result might be due to the choice of micro-organism or experimental technique, and remains to be tested further.

Increased auxin/ethylene levels explain the higher susceptibility of the *pthG* transgenic lines to microbial infection (Navarro et al. 2006). Auxin can modify plant microRNA expression leading to susceptibility

or higher tolerance to biotrophic pathogens (Navarro et al. 2006) while ethylene is involved in plant defence responses (Robert-Seilaniantz et al. 2007) and plant senescence. Our results suggest that the *pthG*-bearing lines produce more ethylene and respond normally to the elevated ethylene levels. Ethylene suppresses gibberellin responses (Aloni et al. 1998), resulting in xylem vessel diameter reduction, reducing water flow to aerial organs, although with normal water flow to the gall. Root-synthesized cytokinin is carried in the transpiration flow. With excessive water loss (i.e. usually from leaves), solutes (and cytokinins) concentrate (Aloni et al. 2005). A larger cuticleless gall surface results in more dehydration leading to increased cytokinin concentration, which with increased auxin concentration causes callus formation. In the "green island phenomenon" nutrient flow is initiated towards the infected area of higher cytokinin concentration (Walters and McRoberts 2006).

The phenotype of all *pthG*-lines is light-regulated (Fig. 4, Tables 1 and 2); the mechanism of interaction between PthG and light is unclear. Phytohormone signalling pathways reveal a tight connection between light and auxin responses (Benjamins and Scheres 2008) through several pathways involving the *pin* genes (controlling auxin transport thereby regulating cell division and differentiation), COP9 signalosome (a conserved regulator of higher eukaryote development) and the ubiquitin activation pathway, all connected to defence responses (Vogel 2006). Moreover, light influences specific defence responses e.g. systemic resistance via the salicylic acid pathway (Zeier et al. 2004). The biotrophic pathogen strategy is based on coexistence (Spoel et al. 2007), and light-controlled mechanisms may tune the host to satisfy the pathogen while keeping the host alive.

However, interference of the PthG with plant auxin and light signalling does not explain all the physiological activities of this pathogenicity factor. For example, lines 44733 and 44765, which have similar endogenous IAA content (Fig. 2) and ethylene production (Fig. 3), have distinctly different responses in the in vitro regeneration assay (Fig. 4), in both dark and light. The intermediate response of line 44742 in vitro regeneration assay (Fig. 4), belies the fact that this line has the highest endogenous IAA content (Fig. 2) and ethylene production (Fig. 3). We are unable to explain these differences.

We hypothesize that PthG interferes in auxin signalling, leading the plant to respond only to certain auxin levels, resulting in increased host auxin and ethylene production required for bigger galls and host biotrophic pathogen susceptibility. Either blocking auxin transport or inoculation by *Pag* mutated in *pthG* at high auxin and cytokinin concentrations, cause failure to form galls (unpublished data). Additionally, bacterial phytohormones are not essential for gall formation by *Pag* (Barash and Manulis-Sasson 2009). The regulation of bacterial gene expression by auxin (Remans et al. 2006) indicates a role for bacterial auxin as a bacterial signalling factor. This leads us to suggest that by injecting PthG into host cells the pathogen tunes the host to respond to endogenous phytohormones (mainly auxin) leading to gall formation.

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