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The tobacco *Ntann12* gene, encoding an annexin, is induced upon *Rhodoccocus fascians* infection and during leafy gall development

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SUMMARY

Annexins are calcium-binding proteins that have been associated in plants with different biological processes such as responses to abiotic stress and early nodulation stages. Until now, the implication of annexins during plant–pathogen interactions has not been reported. Here, a novel plant annexin gene induced in tobacco BY-2 cell suspension cultures infected with the phytopathogenic bacterium *Rhodococcus fascians* (strain D188) has been identified. Expression of this gene, called *Ntann12*, is also induced, but to a lower extent, by a strain (D188-5) that is unable to induce leafy gall formation. This gene was also induced in BY-2 cells infected with *Pseudomonas syringae* but not in cells infected with *Agrobacterium tumefaciens* or *Escherichia coli. Ntann12* expression was also found to be stimulated by abiotic stress, including NaCl and abscissic acid, confirming a putative role in stress signal transduction pathways. In addition, promoter-*GUS* analyses using homozygous transgenic tobacco seedlings showed that the developmentally controlled expression of *Ntann12* is altered upon *R. fascians* infection. Finally, up-regulation of *Ntann12* during leafy gall ontogenesis was confirmed by RT-qPCR. Discussion is focused on the potential role of *Ntann12* in biotic and abiotic stress responses and in plant development, both processes that may involve Ca^{2+}-dependent signalling.

INTRODUCTION

The phytopathogenic Gram-positive bacterium *Rhodococcus fascians* infects a wide range of monocotyledonous and dicotyledonous plants, causing several types of malformations (Goethals *et al.*, 2001). The most severe symptom is the leafy gall, a particular hyperplasia comprising small leaves and numerous buds that are inhibited in their further outgrowth (Vereecke *et al.*, 2000). Phenotypic characteristics of the leafy gall are reminiscent of cytokinins and auxin action and *R. fascians* has indeed been shown to synthesize several cytokinins (Eason *et al.*, 1996) and the auxin indole-3-acetic acid (IAA) (Vandeputte *et al.*, 2005). The pathogenicity of *R. fascians* has been linked to the presence of a linear plasmid, pFiD188, which carries several virulence genes including an *isopentenyl transferase* (*ipt*) homologue that is indispensable for leafy gall formation (Crespi *et al.*, 1992). Indeed, mutation in this *ipt* homologue and plasmid-free strains, such as the avirulent strain D188-5, are unable to induce leafy gall development (Crespi *et al.*, 1992, 1994).

Plant responses upon *R. fascians* infection involve noticeable phenolic pattern alterations and gene expression changes. For instance, the coumarin derivative 7-methyl esculin has been identified in leafy gall but was not detected in non-infected plant tissues (Vereecke *et al.*, 1997). Changes in the concentration of other phenolics, such as caffeic acid and a cinnamoyl analogue, have also been reported (Vereecke *et al.*, 1997) but the impact of these changes on leafy gall development remains unclear. Regarding gene expression, several studies have reported induced or repressed plant genes during the plant–*R. fascians* interaction. Several cell cycle marker genes, such as *CycB1;1* and *CycD3;2*, have been shown to be induced following infection of tobacco and Arabidopsis plants (Vereecke *et al.*, 2000; de O. Manes *et al.*, 2001). A recent survey of differential gene expression in tobacco axils led to the identification of four genes that were up-regulated following infection with *R. fascians* (Simón-Mateo *et al.*, 2006). Among these, a gibberellin 2-oxidase homologue and an Arabidopsis CYP707A family cytochrome P450 monooxygenase homologue have been identified and were suggested to play a role in gibberellin and abscissic acid (ABA) metabolism, respectively (Simón-Mateo *et al.*, 2006). In another study focusing on the late stages of the interaction in plants, it was shown that genes encoding a chitinase, a β-1,3-glucanase and a

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pathogenesis-related protein homologous to NtPRp27 were up-regulated in leafy galls induced on *Atropa belladonna* (Nouar *et al.*, 2003). By contrast, a multicystatin-, a miraculin- and a methallothionein-like gene were down-regulated in symptomatic tissues. Because their expression resumed after the elimination of the bacteria from infected tissues, a possible role for these latter genes in shoot development was proposed (Nouar *et al.*, 2003).

At present, no molecular clue regarding the recognition and/or signalling processes involved during the *R. fascians*–plant interaction has been reported. Studying these processes is hampered by the difficulty in localizing plant cells responding to *R. fascians* and by the unpredictable nature of leafy gall emergence (Simón-Mateo *et al.*, 2006). In that context, the use of a cell suspension model provides a suitable alternative to investigate molecular processes occurring during the early cross-talks between plants and *R. fascians*. Indeed, a more homogeneous plant cell response can be obtained due to the increased number of plant cells that are in contact with the bacteria in comparison with plants infected at localized sites where reacting cells are surrounded by numerous non-infected cells. Therefore, gene expression in non-infected and in *R. fascians*-infected BY-2 tobacco cell suspension cultures was compared using differential display. This transcript profiling resulted in the identification of *Ntann12*, a gene coding for a novel putative annexin in tobacco. Annexins are viewed as potential links between Ca^{2+} as an intracellular signal and the regulation of membrane functions (Gerke *et al.*, 2005). In plants, annexins have been implied in different biological processes, including exocytosis/Golgi-mediated vesicle secretion (Carroll *et al.*, 1998; Clark *et al.*, 1992, 2005; Okamoto *et al.*, 2004), responses to abiotic stress (Cantero *et al.*, 2006; Gidrol *et al.*, 1996; Kovács *et al.*, 1998; Kush and Sabapathy, 2001; Lee *et al.*, 2004) and early stages of nodulation in *Medicago truncatula* upon interaction with the symbiotic bacterium *Rhizobium meliloti* (de Carvalho-Niebel *et al.*, 1998, 2002; Manthey *et al.*, 2004), but have not been associated with plant–phytopathogen interactions. In this study, we focus on the analysis of *Ntann12* expression in BY-2 cells grown under biotic and abiotic stress conditions, and in tobacco plants during seedling development upon infection of tobacco seedlings with *R. fascians* and during leafy gall ontogenesis.

RESULTS

Identification of *Ntann12*, an annexin gene induced in BY-2 cell suspensions following *R. fascians* infection

Gene expression profiles of non-infected and *R. fascians* strain D188 infected BY-2 cell suspensions were compared using differential display. Among the cDNA fragments that showed a differential accumulation following BY-2 cell infection, we focused on one of them for which the expression was up-regulated after 2 days of co-culture with the bacteria (Fig. 1a). This result was confirmed in an independent differential display experiment (data not shown). After cloning and sequencing, a BLASTX analysis revealed that the 346-bp cDNA fragment was similar to the 3′ end of plant annexins. Following RACE PCR, a 1169-bp cDNA, including a 45-bp 5′ untranslated region (UTR) and a 173 bp 3′ UTR, was recovered. The 951-bp coding sequence of *Ntann12* is predicted to encode a 317-amino-acid polypeptide with a calculated molecular weight of 36 kDa and an isoelectric point of 6.6. Figure 2 shows the alignment of the amino acid sequence of *Ntann12* (AAX78199) with the four most similar predicted proteins. Sequence comparison revealed the occurrence in the predicted Ntann12 protein of the four conserved repeats of plant annexins (reviewed by Hofmann, 2004; Moss and Morgan, 2004). Repeat I contains the characteristic type II motif for binding Ca^{2+} with sequence GXGT(38)D/E and repeat IV is characterized by the conservation of GT nucleotides, as has been reported for other plant annexins (Delmer and Potikha, 1997; Hofmann, 2004).

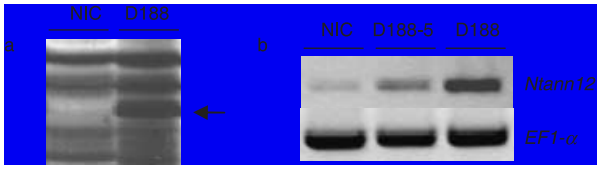

Fig. 1 *Ntann12* gene expression analysis in tobacco BY-2 cells co-cultured for 2 days with *R. fascians*. (a) Detail of a differential display gel showing the cDNA fragment (arrow) corresponding to the *Ntann12* gene that is up-regulated following infection by *R. fascians*. (b) RT-PCR analysis of *Ntann12* expression in BY-2 cells. NIC, non-infected cells; D188, cells infected by the virulent *R. fascians* strain D188; D188-5, cells infected by the avirulent *R. fascians* strain D188-5. The *EF1-α* gene was used as a loading control.

Ntann12 gene response is not specific to *R. fascians*

To examine whether the induction of *Ntann12* expression was associated with the virulence of the bacterium, RT-PCR analysis was carried out with cDNA prepared from BY-2 cells infected by either the *R. fascians* avirulent strain D188-5 or the virulent strain D188 using primers designed to amplify the entire coding sequence. As shown in Fig. 1b, after 2 days of co-culture, *Ntann12* was expressed slightly in non-infected cells and its expression was induced by both bacteria albeit to a lower extent for the avirulent strain D188-5.

In order to determine whether the induction of *Ntann12* expression was specific to *R. fascians*, BY-2 cells were infected with *R. fascians*, *Pseudomonas syringae* pv. *tabaci*, *Agrobacterium tumefaciens* or *Escherichia coli*. As shown in Fig. 3, RT-PCR analysis revealed that *Ntann12* expression is clearly induced after

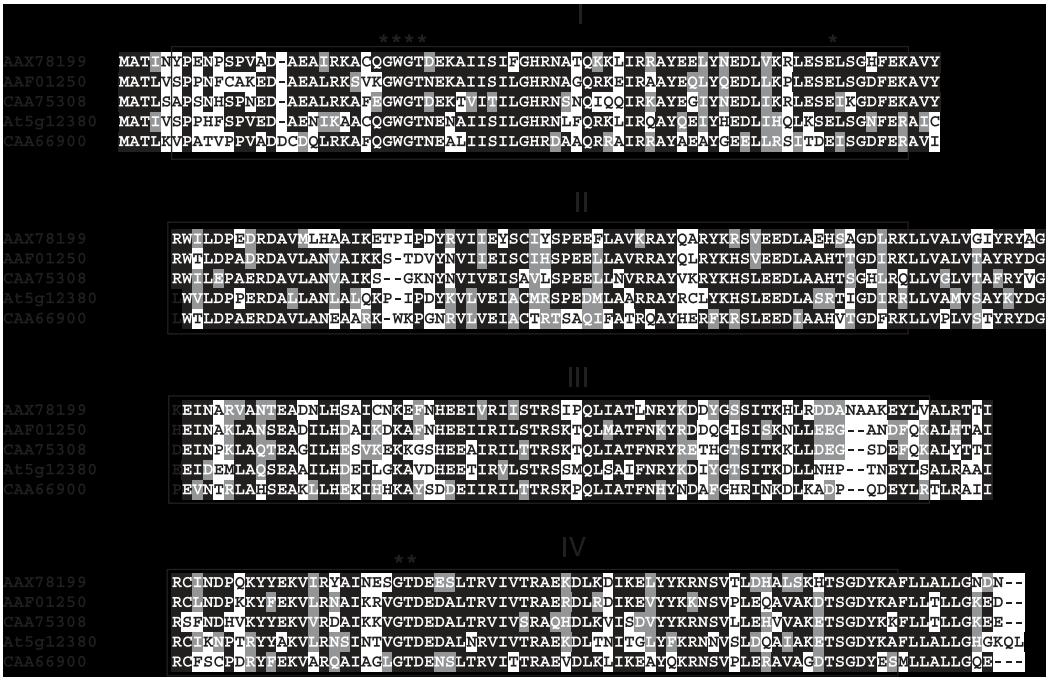

Fig. 2 Comparison of predicted amino acid sequence of Ntann12 (AAX78199) with those of *Fragaria* × *ananassa* (AAF01250), *Medicago truncatula* Mtann1 (CAA75308), *Arabidopsis thaliana* AnnAt8 (At5g12380) and *Zea mays* p33 (CAA66900). Asterisks indicate conserved amino acids for type II Ca^{2+} binding site GXGT(38)D/E (reviewed by Hofmann, 2004). Identical residues are shaded in black and similar residues are shaded in grey. I–IV indicate the four conserved repeats of annexins (reviewed by Moss and Morgan, 2004). The alignment was made by the CLUSTAL W program and page set-up by the BOXSHADE program.

2 days in cells co-cultured with *R. fascians* and *P. syringae*. *Ntann12* expression was not altered in BY-2 cells co-cultured with *A. tumefaciens* or *E. coli*. These data indicate that the *Ntann12* gene response is not totally associated with *R. fascians* plasmid-derived factors and that its expression can be induced by other pathogenic bacteria.

Ntann12 is induced by abiotic stress

Ntann12 expression was examined in response to abiotic stress conditions such as NaCl, ABA and H_2O_2. As shown in Fig. 4, RT-PCR analysis revealed that *Ntann12* expression is induced after 8 h upon NaCl treatment and after 24 h upon ABA treatment, whereas it seems to be unaffected by H_2O_2 treatment within 24 h. These data suggest that osmotic factors induce *Ntann12* expression but not oxidative stress.

Ntann12 is localized in the cytoplasm of BY-2 cells

The *Ntann12* open reading frame was fused either upstream, downstream or both to the *EGFP* coding sequence, and the resulting constructs were introduced into BY-2 suspension cells. EGFP-fluorescence detection was performed with stably transformed BY-2 cell cultures. As shown in Fig. 5, in interphase cells, fluorescence was localized in the cytoplasm and excluded from the nucleus. The localization pattern was similar for the three different Ntann12-EGFP fusion constructs tested. The cytoplasmic localization of Ntann12-EGFP is in agreement with the subcellular localization of other plant annexins that also reside in the cytosol and cell periphery (Breton *et al*., 2000; Clark *et al*., 2000; Thonat *et al*., 1997).

Ntann12 expression analysis in tobacco plants and in response to *R. fascians* infection

To monitor tissue and cell expression of *Ntann12* during development, a 542-bp DNA segment upstream of the ATG of the *Ntann12* gene was amplified by PCR and was fused to a *GUS* reporter gene. p*Ntann12-GUS* homozygous transgenic tobacco plants were generated and several of these plants were analysed during seedling development. As shown in Fig. 6, GUS activity was detected at the hypocotyl–root junction 3 and 4 days after sowing (Fig. 6a,b). Ten days post-germination, GUS activity was detected locally in the root system but not in the aerial parts of the seedlings (Fig. 6c). *Ntann12* expression increased gradually with plant age and was detected in almost all organs 24 days post-germination (Fig. 6d). This pattern is similar to those observed for two Arabidopsis annexin genes (*Annat1* and *Annat2*)

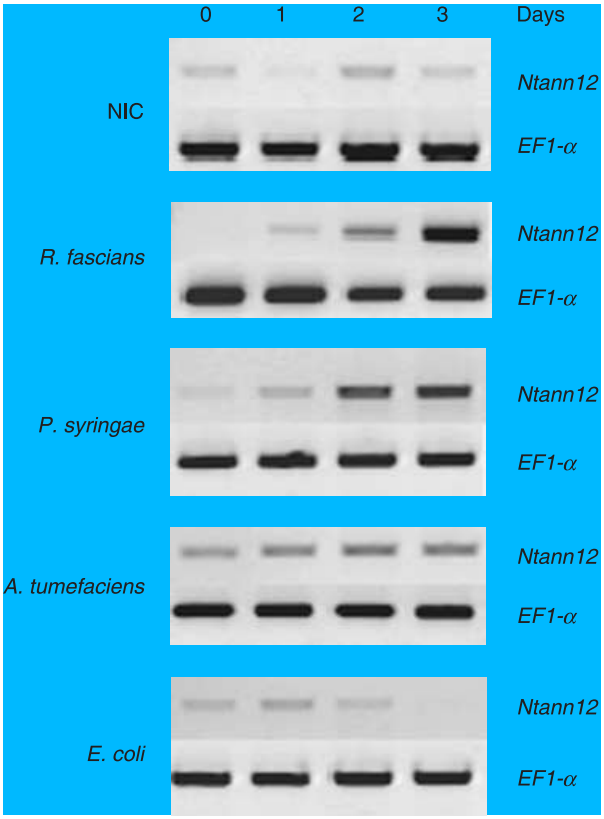

Fig. 3 Effects of biotic stresses on *Ntann12* expression. RT-PCR analysis of *Ntann12* gene expression following infection of BY-2 cells by different bacteria. The *EF1-α* gene was used as a loading control.

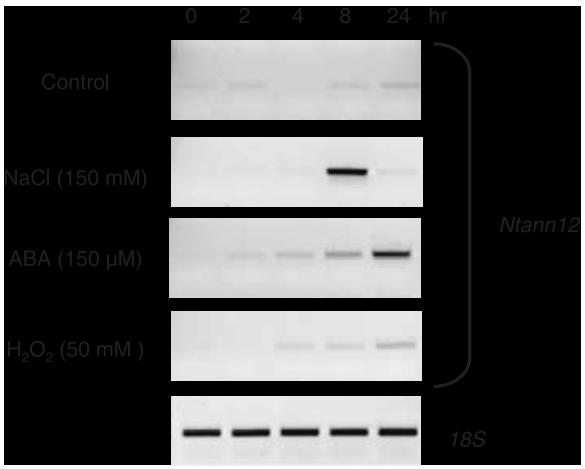

Fig. 4 RT-PCR analysis of *Ntann12* expression upon abiotic stresses in BY-2 cells. The *18S* gene was used as loading control.

by both *in situ* RNA localization (Clark *et al.*, 2001) and immunolocalization (Clark *et al.*, 2005).

In order to verify that the isolated promoter was responsive to *R. fascians*, 11-day-old p*Ntann12-GUS* transgenic plants cultivated

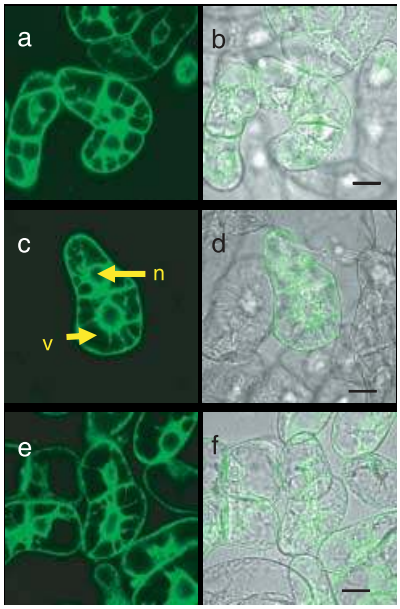

Fig. 5 Intracellular localization of Ntann12 fused to EGFP protein in tobacco BY-2 cells. (a,b) BY2-cells transformed with EGFP-Ntann12. (c,d) BY2-cells transformed with Ntann12-EGFP. (e,f) BY2-cells transformed with EGFP-Ntann12-EGFP. Images were taken by confocal microscopy (a, c and e) dark-field and (b, d and f) differential interference contrast. n, nucleus; v, vacuole. Scale bar = 100 µm.

in liquid medium were inoculated with *R. fascians*. GUS staining was applied 7 days post-infection. As shown in Fig. 7, a GUS staining specific to infected plants was detected on the surface of leaves, as revealed by the localized blue spots (Fig. 7b,d,f) that were not observed in non-infected transgenic plants (Fig. 7a,c,e). A closer observation of the blue-stained regions indicated that the *Ntann12* promoter response to *R. fascians* infection was pronounced in structures that could be associated with emerging adventitious meristems (Fig. 7f).

Ntann12 expression analysis during the leafy gall ontogenesis

Expression of *Ntann12* was evaluated by RT-qPCR at different developmental stages of leafy galls induced on tobacco plants following spot-inoculation of buds with *R. fascians* D188. Changes in *Ntann12* expression were also investigated in plants spot-inoculated with the avirulent *R. fascians* strain D188-5. Non-infected tobacco plants (i.e. spot-inoculated with YEB medium) were used as reference. As shown in Fig. 8, a significant increase in *Ntann12* expression occurred after 5 days following bud infection with strain D188. Similarly to the observations made for BY-2 cells (Fig. 1b), *Ntann12* expression was higher in D188-infected tissues than in those infected by D188-5, especially 7 days post-infection (Fig. 8).

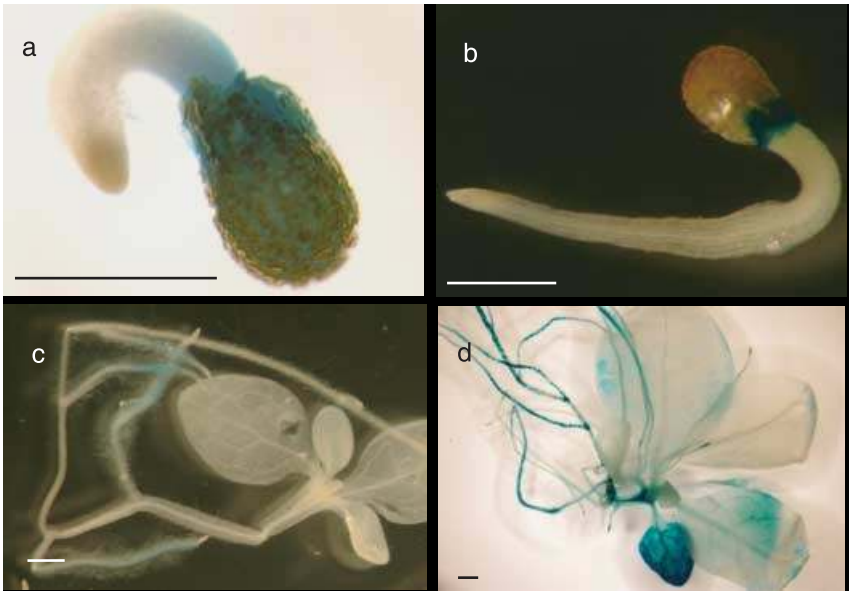

Fig. 6 Histochemical analysis of GUS activity during the development of tobacco seedlings transformed with the p*Ntann12-GUS* construct. (a) 3 days post sowing. (b) 4 days post sowing—at these stages, GUS activity was detected at the hypocotyl–root junction. (c) 10 days post sowing—GUS activity was detected in parts of the root system. (d) 24 days post sowing. Scale bar = 1 mm.

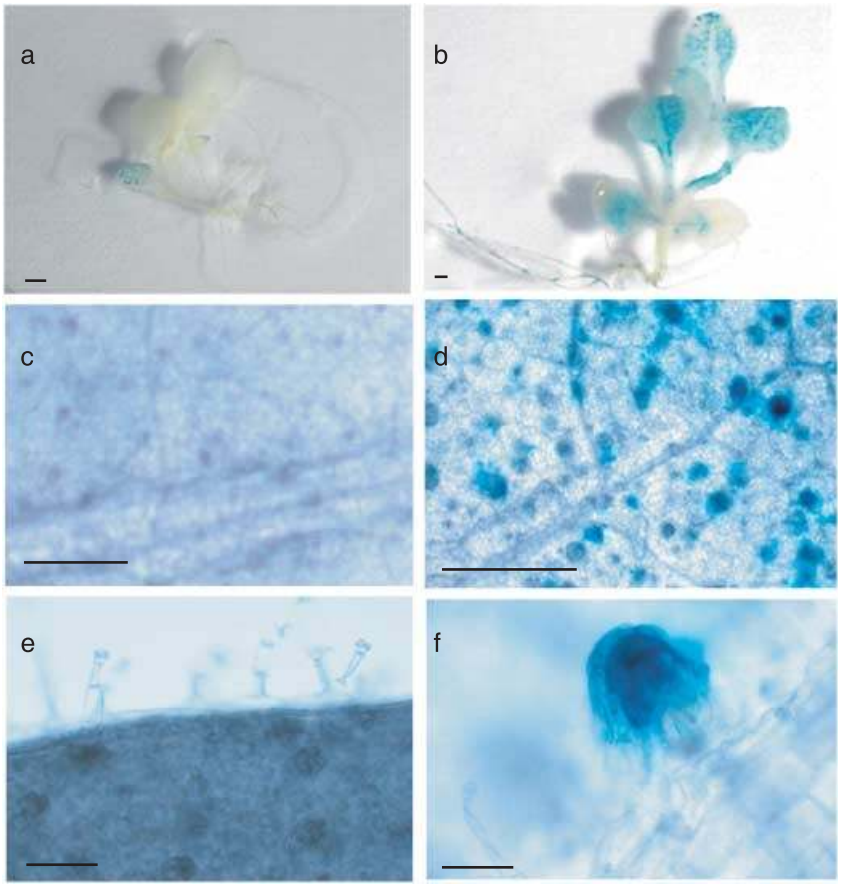

Fig. 7 *Ntann12* promoter response to *R. fascians* infection of tobacco plants. Eleven-day-old p*Ntann12-GUS* transgenic plants were inoculated with *R. fascians* in liquid medium. GUS staining was made 7 days post-infection. (a) Non-infected transgenic lines. (b) Transgenic plant infected with *R. fascians* D188. (c,e) Detail of (a). (d,f) Detail of (b). Scale bars = 1 mm (a–d), 0.2 mm (e) and 0.1 mm (f).

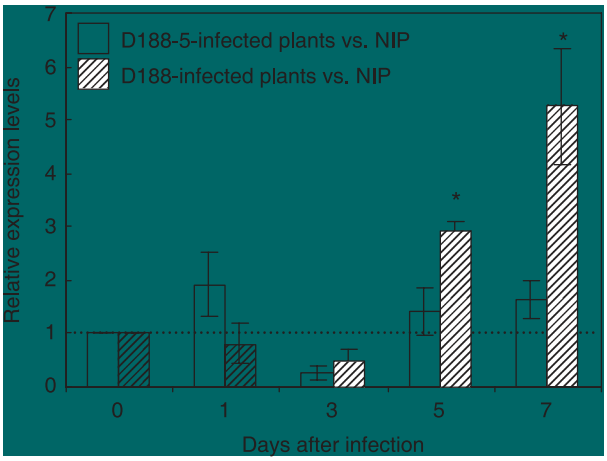

Fig. 8 RT-qPCR analysis of *Ntann12* expression during leafy gall ontogenesis. Four-week-old tobacco plants were either mock-infected, spot-inoculated with *R. fascians* strain D188-5 or with *R. fascians* strain D188. *EF1-α* was used as a template loading control. Relative expression levels are presented as ratios of expression in D188-5-infected plants vs. non-infected plants (NIP) (white bars) and in D188-infected plants vs. NIP (hatched bars). Bars indicate SE ($n = 3$). Asterisks indicate time points for which differences in expression were statistically significant ($P \leq 0.01$).

DISCUSSION

This work reports on the identification of a novel tobacco gene induced upon infection by the phytopathogenic actinomycete *R. fascians* (strain D188) (Fig. 1). This gene has been designated *Ntann12* and encodes a putative protein homologous to annexins (Fig. 2), a family of calcium- and phospholipid-binding proteins (Gerke *et al.*, 2005). We have shown that induction of *Ntann12* is not specific to *R. fascians* as infection of tobacco BY-2 cells with *P. syringae* pv. *tabaci*, a virulent pathogen causing chlorotic lesions on tobacco leaves (Anzai *et al.*, 1989), also led to an overexpression of *Ntann12* (Fig. 3). Additionally, *Ntann12* induction is probably restricted to infection with a certain range of bacteria given that *E. coli* did not trigger the expression of *Ntann12* in BY-2 cells. Similarly, co-culture of BY-2 cells with *A. tumefaciens* did not result in an increased accumulation of *Ntann12* transcript. This observation is in accordance with a recent study related to the response of *Arabidopsis thaliana* transcriptome to *A. tumefaciens* infection that did not highlight an increase in annexin gene expression (Ditt *et al.*, 2006).

Expression of plant annexin genes in response to non-pathogenic bacteria has already been reported. *Mtann1* and *Mtann2* have been shown to be associated with symbiotic interactions, in *Rhizobium* inoculated roots and in nodules of *M. truncatula* (de Carvalho-Niebel *et al.*, 1998, 2002; Manthey *et al.*, 2004). *Mtann1* has been proposed to be involved in the early stages of Nod factor signalling and in the cell cycle activation of cortical cells (de Carvalho-Niebel *et al.*, 1998, 2002). *Mtann2* was also found to be expressed in arbuscule-containing cells of mycorrhizal roots, and a role of the protein encoded by this gene in the membrane traffic was hypothesized (Manthey *et al.*, 2004). Whether *Ntann12* could play similar roles during the *R. fascians*–plant interaction remains to be determined.

We have observed that *Ntann12* was induced by the avirulent strain D188-5, although this induction was lower in infected BY-2 cells (Fig. 1b) or tobacco plants (Fig. 8) as compared with the induction by the virulent strain D188. This lower induction can result from a difference in plant infection efficiency between the two *R. fascians* strains and several lines of evidence support this hypothesis. Indeed, Cornelis *et al.* (2001) found that a lower proportion of BY-2 cells were infected by strain D188-5 than by strain D188. Although both *R. fascians* strains colonize tobacco and Arabidopsis plant surfaces equally well, the efficiency of strain D188-5 to penetrate plant tissues was reported to be lower than that of strain D188 (Cornelis *et al.*, 2001). In addition, both bacteria were shown to synthesize IAA, which was suggested to play a role in the colonization ability and in the growth and survival of *R. fascians* on its host plants, but IAA production was delayed in strain D188-5 (Vandeputte *et al.*, 2005). Finally, although strain D188-5 does not induce the formation of leafy galls, it has been shown to affect plant development by partially blocking root growth of infected Arabidopsis plants (Crespi *et al.*, 1992; Vereecke *et al.*, 2000). These observations indicate that plants respond to both *R. fascians* strains, at least at the recognition level.

Further characterization of *Ntann12* expression revealed that abiotic stresses also induced *Ntann12* expression. Indeed, exposure of BY-2 cells to NaCl or ABA resulted in an increase of *Ntann12* transcripts (Fig. 4). Expression of plant annexins, such as *AnnMs2* from *M. sativa* and several annexins from *A. thaliana*, is known to be induced upon various environmental signals, including ABA and NaCl (Hoshino *et al.*, 2004; Kovács *et al.*, 1998; Lee *et al.*, 2004; this study). Similarly to annexins of *M. sativa* (*AnnMs2*) (Kovács *et al.*, 1998) and *A. thaliana* (*AnnAt1*) (Lee *et al.*, 2004), *Ntann12* was induced within 24 h after application of ABA or NaCl. A role for annexins in the osmotic stress response is supported by the phenotype of *AnnAt1* and *AnnAt4* Arabidopsis T-DNA insertional mutants that were shown to be more sensitive to osmotic stress and ABA during germination and early seedling growth, as compared with wild-type plants (Lee *et al.*, 2004).

Several plant annexins have been proposed to play a role during oxidative stress. Indeed, hydrogen peroxide (H_2O_2) induces expression of annexins such as *oxy5*/*Annat1* in Arabidopsis (Gidrol *et al.*, 1996) and *AnnMs2* in *M. sativa* (Kovács *et al.*, 1998). In addition, heterologous expression of *AnnAt1* has been shown to rescue *E. coli* Δ*oxyR* mutants from H_2O_2 stress (Gidrol *et al.*, 1996) and to protect mammalian cells from H_2O_2-induced cell

death (Kush and Sabapathy, 2001). *Ntann12* was not induced by H_2O_2 treatment, suggesting that *Ntann12* is not implied in an oxidative stress response.

Like other plant annexins (Cantero *et al.*, 2006; Clark *et al.*, 2001, 2005; de Carvalho-Niebel *et al.*, 2002; Hoshino *et al.*, 2004; Shin and Brown, 1999), *Ntann12* expression is developmentally regulated (Fig. 6) but *R. fascians* infection results in an alternation of this expression. Ontogenesis of leafy gall involves a complex developmental alteration that begins with *de novo* cortical cell divisions at the sites of infection followed by the initiation and the formation of adventitious buds (de O. Manes *et al.*, 2001). During the early differentiation steps of these shoot primordia, several genes have been shown to be expressed, among which are genes involved in the cell cycle machinery (de O. Manes *et al.*, 2001; Vereecke *et al.*, 2000) and hormone homeostasis (Simón-Mateo *et al.*, 2006). *Ntann12* is also a gene whose expression is activated during leafy gall ontogenesis (Fig. 8) and at specific sites on tobacco leaves infected by *R. fascians* (Fig. 7). These specific infection sites probably correspond to actively dividing cells that will ultimately differentiate into the shoot primordia forming the leafy gall. The GUS staining pattern observed for *Ntann12* (Fig. 7) is similar to that of B-type cyclin gene expression on infected tobacco plants by *R. fascians* (D188) (de O. Manes *et al.*, 2001) and to the cytokinin oxidase gene expression following infection of pea plants with *R. fascians* (strain 602) (Gális *et al.*, 2005).

In conclusion, plant annexins have been implicated in a variety of physiological processes, most of them dealing with stress response functions (Hofmann, 2004). *NtAnn12* is indeed induced by various biotic and abiotic stresses and is also shown to be developmentally regulated. Annexins are proposed to be a link between Ca^{2+} signalling and membrane-related functions, such as membrane–cytoskeleton linkages, exocytic or endocytic processes and ion flux regulation (Gerke *et al.*, 2005). According to Hofmann (2004), annexins act as parts of the complex signalling network that organize stresses and/or developmental processes rather than reacting themselves to these plant cell responses. Further functional characterization of this gene will certainly contribute to a better understanding of its role in plant development/physiology and of the events occurring at the interface between the cellular envelopes of both actors of the *R. fascians*–plant interaction.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Non-transgenic and transgenic tobacco plants (*Nicotiana tabacum* cv. Havana) were grown aseptically on half-strength MS medium (Duchefa) supplemented with appropriate antibiotics when needed, or in a greenhouse at 25 °C with a 16/8-h light–dark photoperiod. Developing leafy galls used for RT-qPCR experiments were induced as described previously (de O. Manes *et al.*, 2001) on *in vitro* 4-week-old tobacco plants following spot-inoculation of axillary buds. Control plants were spot-inoculated with drops of the avirulent strain D188-5 (Crespi *et al.*, 1992; Desomer *et al.*, 1988) and non-infected plants (NIP) were mock-inoculated with drops of YEB medium. For RNA extraction, infected tissues were sampled at regular intervals and immediately frozen in liquid nitrogen.

Infection of BY-2 cell suspensions by *R. fascians* and other bacteria

N. tabacum L. cv. Bright Yellow 2 (BY-2) cell suspension was cultivated and maintained in modified liquid Linsmaier and Skoog (LS) medium (Nagata *et al.*, 1992) and incubated for 2 days in the dark at 28 °C under shaking (130 r.p.m.) before infection. For the differential display, the virulent strain D188 of *R. fascians* was used. After 2 days incubation in liquid YEB medium ($OD_{600nm} \sim 2$), 500 μL of bacterial suspension was inoculated into 100 mL of BY-2 cell suspension cultures. A YEB-inoculated cell suspension was used as control. Two days after bacterial inoculation, cell suspensions were filtered and frozen in liquid nitrogen. To confirm the differential expression by independent RT-PCR analysis, a similar procedure was performed using the virulent D188 or the avirulent D188-5 strain. For the expression analysis of *Ntann12* in response to different bacteria, BY-2 cell suspensions were inoculated with *R. fascians* (strain D188), *P. syringae* pv. *tabaci* LMG 5393 (obtained from the Belgian Coordinated Collection of Microorganisms—http://bccm.belspo.be/about/lmg.php), *E. coli* (strain DH5α) and *A. tumefaciens* (strain B6). In each case, bacteria were grown until an optical density at 600 nm of 2 was reached and 500 μL of bacterial culture was added to 2-day-old BY-2 cell suspension cultures. BY-2 cells were harvested 0, 24, 48 and 72 h after infection and frozen in liquid nitrogen.

Abiotic stress treatments

Two-day-old BY-2 cell suspension cultures were treated with NaCl (150 mM), ABA (150 μM) or H_2O_2 (50 mM). BY-2 cells were harvested 0, 2, 4, 8 and 24 h after treatment and frozen in liquid nitrogen.

RNA extraction and mRNA differential display

Total RNA was extracted from BY-2 cell suspension and from various tissues of tobacco plants using the RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of mRNA was performed using A, B, C and D RNAmap™ kits from GenHunter (Nashville, TN). In this work, the T12MG (where M = A,G,C) oligo-dT primer mix was used in combination with the 20 arbitrary primers (AP 1–20)

(GenHunter). Separation of cDNA fragments was made on 5% polyacrylamide denaturing gels and revealed by autoradiography. cDNA fragments were cut off from the gels, reamplified by the original primer combination, cloned in the pCR®4-TOPO® vector (Invitrogen) and sequenced.

Cloning of the *Ntann12* cDNA from BY2 cells and from tobacco plants

A 5′RACE system for rapid amplification of cDNA ends (Gibco BRL, MD) was used to amplify the 5′ part of the *Ntann12* cDNA on cDNA made from *R. fascians*-infected BY-2 cell suspension. The primers used were the forward (F) Poly G 5′-GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGGG-3′ furnished in the kit and the reverse (R) gene-specific primers 5′-CTTCTCTGCCCTTGTAACTAT-3′ and 5′-TTGTAGTAAAGCTCCTTGATA-3′. The amplified cDNA fragment was cloned in the pCR4®-TOPO® vector and sequenced. Subsequently, the entire length of the cDNA obtained for *Ntann12* was amplified using the primers F 5′-GAGAAGAAATTAAATCCATTGATTAGG-3′ and R 5′-CGACGGAAAAGAATATTGATAGC-5′ on cDNA obtained from BY-2 or from *N. tabacum* plantlets cultured *in vitro.* Both cDNAs were subsequently cloned in a pCR4®-TOPO® vector and sequenced. Protein sequences were aligned with CLUSTAL W (http://www.ebi.ac.uk/clustalw) (Thompson *et al.*, 1994), and page set-up was made with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Molecular weight was calculated with the software PEPSTATS (http://bioweb.pasteur.fr/seqanal/interfaces/pepstats.html).

RT-PCR analysis and real-time quantitative RT-PCR (RT-qPCR)

RT-PCR was performed using a reverse transcription system from Promega with primers amplifying 946 bp of the coding sequence of *Ntann12*, F 5′-ATGGCTACAATCAATTACCCT-3′ and R 5′-CATTTCCCAAAAGAGCGAGA-3′. EF1-α was amplified with primers F 5′-TGCTACCACCCCCAAGTACTC-3′ and R 5′-TAAAGCTGGCAGCACCCTTAG-3′ and 18S was amplified with primers F 5′-ATGGCCGTTCTTAGTTGGTG-3′ and R 5′-TGTCGGCCAAGGCTATAAAC-3′. RT-qPCR reactions were performed with primers F 5′-CTTCTCTGCCCTTGTAACTAT-3′ and R 5′-CAACCGCTACAAGGGTGATTA-3′. Conditions for RT-qPCR were as follows: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C. As a final step, a dissociation curve consisting of 15 s denaturation at 95 °C, 15 s at 60 °C and 15 s at 95 °C was performed to detect unwanted primer-dimers or PCR products that could interfere with the fluorescence data. Reactions were composed of 12.5 μL *iTaq SYBR Green* (Bio-Rad), 9.5 μL water, 2 μL primer mix (5 μM each) and 1 μL of cDNA (diluted four-fold). qPCRs were performed in an ABI 7900 system (Applied Biosystems). Expression of the *EF1-α* gene was used to normalize the samples. qPCR data were analysed by the $\Delta\Delta C_T$ method as described by Livak and Schmittgen (2001) and are reported as ratios between expression in plants infected with strains D188-5 or D188 vs. non-infected plants (NIP). To test whether *Ntann12* was expressed at a higher level following infection of plants by *R. fascians* and to test whether there was a difference of expression between D188-5- and D188-infected plants (the null hypothesis being that the expression of *Ntann12* was not altered in the different conditions), *t*-tests were conducted and a *P*-value was calculated and used to reject the null hypothesis. A *P*-value ≤ 0.01 was considered as being significant.

Amplification of the promoter region of *Ntann12*, construction of *Ntann12* promoter-GUS constructs and plant transformation

Genomic DNA was extracted from *N. tabacum* aerial parts using a DNeasy plant Mini kit (Qiagen). The Universal Genome Walker™ kit (Clontech) was used according to the manufacturer's instruction and allowed to amplify a 542-bp DNA fragment that was cloned in the pCR4®-TOPO® vector (Invitrogen) and sequenced. This fragment was flanked by *att*B1 and *att*B2 recombination sites by two PCR reactions. For the first PCR reaction primers F 5′-AAAAAGCAGGCTAAATACTCGGCGGTG-3′ and R 5′-AGAAAGCTGGGTCGTTAAACTCTCTTCC-3′ were used. A second PCR with primers F 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and R 5′-GGGGACCCTTTGTACAAGAAAGCTGGGT-3′ was performed, creating the entire *att*B1 and *att*B2 recombination sites (1 min at 94 °C, 1 min at 60 °C, 30 cycles of 1.5 min at 68 °C followed by 10 min at 68 °C). PCR reactions were performed using the Platinum Pfx Polymerase (Invitrogen). This PCR fragment was cloned into the Gateway™-compatible binary T-DNA destination vector pKGWFS7, allowing the fusion of *Ntann12* promoter with both *GFP* and *GUS* reporter genes (Karimi *et al.*, 2002) in reactions mediated by the Gateway™ BP and LR Clonase™ Enzyme Mix (Invitrogen). This plasmid was transferred into *A. tumefaciens* strain C58Rif^R (pGV2260). *N. tabacum* was transformed by the leaf disc protocol according to Deblaere *et al.* (1987) with slight modifications. Thidiazuron (1 mg/L) was used instead of benzylaminopurine. Transgenic plants were selected on half-strength MS medium (Duchefa) supplemented with 200 mg/L kanamycin at 25 °C under a 16-h/8-h light–dark photoperiod. T-DNA insertions were verified by PCR analysis and homozygous seedlings (T2) were used for histochemical GUS staining, which was performed as described by Hemerly *et al.* (1993).

Generation of EGFP fusion constructs and transformation of BY-2 cells

Gateway™ (Invitrogen) *att*B1 and *att*B2 sequence extensions were added for in-frame cloning into plant Gateway destination

vectors pK7WGF2, pK7WGF2 or pK7FWGF2 (Karimi *et al.*, 2002) to generate N-terminal, C-terminal or both N- and C-terminal EGFP-Ntann12 fusion products downstream of the *CaMV35S* promoter. Plasmids were transferred to *A. tumefaciens* strain LBA4404. Stable BY-2 transformation was carried out as described (Geelen and Inzé, 2001). Approximately ten transgenic BY-2 calli of 1 μm diameter were examined for fluorescence under a coverslip with an Axioskop (Zeiss) fluorescence microscope. EGFP-positive calli were analysed by confocal microscopy (Zeiss 100M, equipped with LSM510 software version 3.2). A 63× water-corrected objective (numerical aperture of 1.2) was used to scan the samples. The images were captured with the LSM510 image acquisition software (Zeiss).

Nucleotide sequence accession numbers

Sequences of *Ntann12* isolated from tobacco and BY-2 cDNAs are deposited in the GenBank nucleotide sequence database under accession numbers AY965682 and AY965683, respectively. The sequence of the *Ntann12* promoter is deposited in the GenBank nucleotide sequence database under accession number DQ900657.

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