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RESEARCH PAPER

Ntann12 annexin expression is induced by auxin in tobacco roots

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Abstract

***Ntann12*, encoding a polypeptide homologous to annexins, was found previously to be induced upon infection of tobacco with the bacterium *Rhodococcus fascians*. In this study, Ntann12 is shown to bind negatively charged phospholipids in a Ca^{2+}-dependent manner. In plants growing in light conditions, *Ntann12* is principally expressed in roots and the corresponding protein was mainly immunolocalized in the nucleus. *Ntann12* expression was inhibited following plant transfer to darkness and in plants lacking the aerial part. However, an auxin (indole-3-acetic acid) treatment restored the expression of *Ntann12* in the root system in dark conditions. Conversely, polar auxin transport inhibitors such as 1-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) inhibited *Ntann12* expression in light condition. These results indicate that the expression of *Ntann12* in the root is linked to the perception of a signal in the aerial part of the plant that is transmitted to the root via polar auxin transport.**

Key words: Annexin, auxin, Ca^{2+}-binding protein, light, nucleus, polar auxin transport, root.

Introduction

Annexins are proteins found in most eukaryotes but are absent from yeasts and prokaryotes (Moss and Morgan, 2004). These proteins contain generally four repeats of a 70 amino acid domain (Gerke and Moss, 1997). Several differences exist between plant and animal annexins. The type II Ca^{2+}-binding site provided by the endonexin fold referred to as the consensus sequence KGXGT-(38 residues)-D/E (Geisow *et al.*, 1986) is highly conserved in 3–4 repeats of animal annexins, whereas the consensus sequence is strictly conserved only in the first repeat in plants (Hofmann, 2004). Furthermore, animal annexins have a long and variable N-terminal domain which is the site for post-translational modifications and interaction with membranes and other proteins (Gerke *et al.*, 2005), whereas plant annexins have a short N-terminal sequence that is involved in the function of the protein (Hofmann *et al.*, 2000; Dabitz *et al.*, 2005). Annexins are characterized by their ability to bind negatively charged phospholipids in a Ca^{2+}-dependent manner (Boustead *et al.*, 1989). Both animal and plant annexins have also been shown to bind phospholipids in a Ca^{2+}-independent manner, suggesting that they can be associated with or inserted into membranes (Lim *et al.*, 1998; Gerke and Moss, 2002; Dabitz *et al.*, 2005; Gorecka *et al.*, 2007).

Animal annexins have been demonstrated to be involved in Ca^{2+} signalling and transport, membrane dynamics and trafficking, cell proliferation, exocytosis, and endocytosis

(reviewed by Gerke *et al.*, 2005). In plants, annexins are described as multifunctional components of growth and adaptation, being associated with diverse cellular processes, and expressed throughout the plant life cycle (reviewed by Mortimer *et al.*, 2008). These proteins have diverse functional domains, including a Ca^{2+}-binding site, a potential F-acting-binding motif, a potential haem-binding domain, and an ATP/GTP-binding domain (reviewed by Mortimer *et al.*, 2008). However, their precise role/function in plants is not clearly understood (Hofmann, 2004; Konopka-Postupolska, 2007). *In vitro* studies revealed biochemical properties for plant annexins, including nucleotide phosphodiesterase activity (Calvert *et al.*, 1996; Shin and Brown, 1999), peroxidase activity (Gorecka *et al.*, 2005; Laohavisit *et al.*, 2009), callose synthase regulation activity (Andrawis *et al.*, 1993), and F-actin binding activity (Calvert *et al.*, 1996; Hu *et al.*, 2000; Hoshino *et al.*, 2004). In addition, plant annexins have been shown to mediate channel-like Ca^{2+} transport (Hofmann *et al.*, 2000; Laohavisit *et al.*, 2009) possibly involved in reacive oxygen species signalling (Laohavisit *et al.*, 2010).

Various studies have shown that plant annexins are expressed in tissues/cells associated with secretion and polarized growth, such as pollen tube tips (Blackbourn *et al.*, 1992) and root caps (Clark *et al.*, 1992; Carroll *et al.*, 1998), or differential growth during gravitropism (Clark *et al.*, 2005*a*). In addition, plant annexin expression is differentially regulated by exposure to salt, gravity, abscisic acid (ABA), drought, high or low temperatures, hydrogen peroxide, phosphate deprivation, and heavy metals (Kovács *et al.*, 1998; Breton *et al.*, 2000; Clark *et al.*, 2001; Cantero *et al.*, 2006; Jami *et al.*, 2009; Konopka-Postupolska *et al.*, 2009; Huh *et al.*, 2010).

The overexpression of *AnnAt1* in *Arabidopsis* has been shown to protect cells against drought stress (Konopka-Postupolska *et al.*, 2009) and oxidative stress (Gorecka *et al.*, 2005), and to play a role in osmotic stress and ABA signalling (Lee *et al.*, 2004). In accordance with this, transgenic cotton and tobacco plants expressing mustard annexin *AnnBj1*, a homologue to *AnnAt1*, were shown to be more tolerant towards different abiotic stress treatments (Jami *et al.*, 2008; Divya *et al.*, 2010). In addition, AnnAt1 has been found to rescue the *Escherichia coli ΔoxyR* mutant from H_2O_2 stress (Gidrol *et al.*, 1996) and mammalian cells from oxidative stress (Kush and Sabapathy, 2001). Moreover, *annAt1* and *annAt4*, but not *annAt2* mutants, were shown to be hypersensitive to ABA and osmotic stress during germination and early seedling growth (Lee *et al.*, 2004). Seedlings of *annAt1* and *annAt2* mutants grown in the dark showed inhibited root and hypocotyl growth, respectively (Clark *et al.*, 2005*b*). Recently, Huh *et al.* (2010) demonstrated that, under long day conditions, the sensitivity to abiotic stress of double *annat1 annat4* mutants was lower compared with single mutants and this effect was reversed in transgenic *35S:AnnAt4*, suggesting that AnnAt1 and AnnAt4 act as regulators of abiotic stress such as drought and salt.

The isolation and the preliminary characterization of the tobacco *Ntann12* gene, encoding a putative annexin whose expression was found to be induced in tobacco BY-2 cells following infection by the phytopathogenic bacterium *Rhodoccocus fascians*, were previously reported (Vandeputte *et al.*, 2007). The closest homologues to Ntann12 are the annexin-like protein RJ4 (65% identity and 81% similarity) that has been identified to be expressed predominantly in developing and ripening fruit (Wilkinson *et al.*, 1995), and the *Medicago truncatula* MtAnn1 (58% identity and 77% similarity) that has been shown to be induced during symbotic interactions and suggested to be involved in the Ca^{2+} response signal elicited by symbiotic signals from rhizobia and mycorrhizal fungi (de Carvalho Niebel *et al.*, 1998; de Carvalho-Niebel *et al.*, 2002). The closest *Arabidopsis* homologue to Ntann12 is AnnAt8 (57% identity and 78% similarity), which was found to be induced mainly by dehydration and NaCl treatment (Cantero *et al.*, 2006).

In this study, biochemical investigations indicate that Ntann12 binds to negatively charged phospholipids in a Ca^{2+}-dependent manner. Moreover, Ca^{2+} concentration affects *in vitro* Ntann12 translocation from cytosolic to membrane-enriched fractions. *Ntann12* is highly expressed in root cells and the protein was mainly immunolocalized in the nucleus. *Ntann12* expression in the root system was found to be regulated by a light-induced signal coming from plant aerial parts, and polar auxin transport seems to be required for *Ntann12* expression in root cells. Taken together, the data presented in this study show the role of light and polar auxin transport in the regulation of the expression of the *Ntann12* annexin in tobacco roots.

Materials and methods

Plant materials and growth conditions

Non-transgenic and transgenic tobacco plants (*Nicotiana tabacum* cv. Havana) were grown aseptically on Murashige and Skoog (MS) medium (Micro and 1/2 concentration Macro elements including vitamins; Duchefa) supplemented with 200 mg l^{-1} kanamycin (Duchefa) when needed and were grown at 23 °C under a 16 h light photoperiod (70 μmol m^{-2} s^{-1}, cool white fluorescent lamp, Osram). Sown seeds, or acclimatized plants, were cultivated on soil in a growth chamber at 25 °C under a 16 h light photoperiod.

Production of the recombinant Ntann12 protein in Escherichia coli

The pBAD-DEST49 expression system (Invitrogen, Merelbeke, Belgium) was used to produce a recombinant Ntann12 protein with His-Patch (HP) thioredoxin as an N-terminal fusion partner of the cloned gene product and a hexahistidine tag as a C-terminal fusion partner. According to the manufacturer, a fusion partner with HP thioredoxin may improve the translation and the solubility of the fusion protein. The *Ntann12* cDNA (Vandeputte *et al.*, 2007) was flanked by *attB1* and *attB2* recombination sites by two successive PCRs, the first one using the primers F 5′-AAAAAGCAGGCTATGGCTACAATCAATTA-3′ and R 5′-AGAAAGCTGGGTTTAGTTATCATTTCCC-3′ and the second with the primers containing *attB1* and *attB2* sites for Gateway cloning by recombination (Invitrogen). After the generation of the entry clone (BPNtann12) in plasmid pDONR-221 (Invitrogen), a second recombination reaction was performed with pBAD-DEST49 according to the manufacturer's instructions and cloned into *E. coli* TOP10 (Invitrogen).

Production of recombinant proteins in TOP10 cells was induced by the addition of 0.2% L-(+)-arabinose to cultures at an optical density at 600 nm of ~0.8, and cultivation was continued for an additional 6–7 h at 37 °C. Cells were harvested by centrifugation and cell pellets were frozen at –80 °C. Subsequently, cells were extracted using a Qproteome™ Bacterial Protein Prepkit (Qiagen, Hilden, Germany), containing lysozyme and benzonase (Qiagen) supplemented with protease inhibitor cocktail (Sigma). Lysates were centrifuged at 16 000 *g* for 30 min at 4 °C and the supernatant (soluble fraction of the bacterial proteins) was collected and used immediately.

Protein analysis

Tobacco seedlings were grown for 4 weeks in solid MS medium. Roots and leaves were harvested separately, immediately frozen and ground to a fine powder in liquid nitrogen using a mortar and pestle, and stored at –80 °C until required. The powder was homogenized and incubated in extraction buffer [50 mM TRIS, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol (DTT), 2% benzonase, and protease inhibitor cocktail for native conditions or 100 mM TRIS-HCl, pH 7.5, 10 mM EDTA, 100 mM $LiCl_2$, 1% SDS, and protease inhibitor cocktail for non-native [conditions](1 g powder ml^{-1} extraction buffer), and centrifuged at 3220 *g* (Eppendorf 5810R, rotor A-4-81) for 30 min at 4 °C. To assess the Ca^{2+} response of Ntann12 proteins in plant cells, the total protein extract was treated with either $CaCl_2$ or EDTA before separation of membrane and soluble protein fractions by ultracentrifugation at 125 000 *g* (Beckman Optima™ LE-80K, rotor SW60) for 1 h at 4 °C. After centrifugation, the supernatant (cytosolic fraction) was recovered, and the pellet (membrane-enriched fraction) was resuspended in an appropriate volume of extraction buffer (Lee *et al.*, 2004). The crude protein samples, and the cytosolic and the membrane-enriched fractions were divided into aliquots and preferably used immediately, or frozen at –80 °C. Protein concentrations were measured using Dye reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Preparation of anti-Ntann12 antibodies

A polyclonal antibody was raised in rabbit against a mix of two Ntann12-specific peptides (ATINYPENPSPVADAC and DPQKYYEKVIRYAI) (Eurogentec Inc., Belgium) and was named anti-Ntann12. Non-purified antibodies were used for western blotting. Affinity-purified antibodies using the synthesized peptides (TOYOPEARL AF-Amino-650M, Eurogentec Inc.) were utilized for immunolocalization. An enzyme-linked immunosorbent assay (ELISA) was performed to demonstrate the specificity of the anti-Ntann12 antibodies for the synthesized peptides as well as the absence of cross-reactivity with the pre-immune serum (Eurogentec). The protein concentration of the purified antibody is 0.25 μg $μl^{-1}$.

Electrophoresis and immunoblotting

Analysis by SDS–PAGE was performed on 10% acrylamide gels (Invitrogen) with molecular mass standards from Invitrogen (SeeBlue Plus 2), for 50 min at 200 V in 5% (v/v) running buffer (Invitrogen), 0.5% (v/v) antioxidant (Invitrogen). Proteins separated by SDS–PAGE were stained with Coomassie blue (Invitrogen), or were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) for 1 h at 30 V in 10% (v/v) methanol, 5% (v/v) transfer buffer (Invitrogen), and 0.5% (v/v) antioxidant (Invitrogen). The PVDF membrane was processed for immunoblotting by incubating for 1 h at 20 °C in phosphate-buffered saline–Tween (PBS-T) (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween, pH 7.4) containing 5% (w/v) low fat milk. The blot was incubated overnight at 4 °C in the same buffer containing antiserum raised against Ntann12 at a dilution of 1:5000, washed three times in PBS-T, and then incubated with protein A–peroxidase (Sigma) at a dilution of 1:50 000 for 4 h at 4 °C. Following three washes in PBS-T, peroxidase activity was made visible by incubating the blot with Lumigen™ solutions (GE Healthcare) for 5 min and then scanning (Typhoon 9200). Ntann12 proteins from plants and from bacteria were detected using the above protocol. Western blot using pre-immune serum was performed at a similar dilution, and no cross-reactive band was observed (data not shown).

Phospholipid binding assay

Liposomes were prepared using a 2:1 mixture (mol/mol) of L-α-phosphatidylcholine (PC) and L-α-phosphatidyl-L-serine (PS) (Sigma). Large unilamellar vesicles (100–200 nm) were prepared as follows. Lipids (30 mg) were dissolved in chloroform. A dried film was obtained by evaporation of chloroform under a flow of nitrogen, followed by overnight drying under vacuum. The lipid film was vortexed in 7.5 ml of HEPES buffer (100 mM KCl, 2 mM $MgCl_2$, 25 mM HEPES pH 7.5) at 37 °C, frozen in liquid nitrogen, and thawed (five cycles). Finally, the suspension was extruded 10 times through two stacked 100 nm (pore diameter) polycarbonate filters (Nuclepore, Costar, Cambridge, MA, USA) in a thermobarrel extruder (Lipex Biomembranes, Vancouver, Canada) at 37 °C. Proteins (50 μg) were incubated for 30 min at 20 °C with liposomes (400 μg of phospholipids) in 1 ml of HEPES buffer containing 0 or 2 mM Ca^{2+}. After centrifugation at 86 000 *g* (Beckman Optima™ LE-80K, rotor SW60) for 1 h at 4 °C, the supernatant containing liposomes was fractionated over a discontinuous sucrose gradient (35–30–25–20%–Sample). Sucrose was diluted in HEPES buffer containing 0 or 2 mM Ca^{2+}. Following centrifugation overnight at 86 000 *g* (Beckman, rotor SW60) and 4 °C, liposomes were harvested in the upper layer and in the 20% sucrose layer. Liposomes were washed twice with HEPES buffer containing 0 or 2 mM Ca^{2+} by centrifuging for 1 h at 86 000 *g* and 4 °C, and suspended in an appropriate volume of HEPES buffer containing 0 or 2 mM Ca^{2+}. The liposomal fractions were analysed by 10% SDS–PAGE and gel blotting with the anti-Ntann12 antibody.

Immunolabelling

Roots were fixed with 4% paraformaldehyde and 0.3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 h at room temperature and incubated overnight at 4 °C. Samples were then serially dehydrated in increasing concentrations of methanol, embedded in Lowicryl K4M (Electron Microscopy Sciences) at –20 °C, and left to polymerize for 2 d at –20 °C under low wavelength UV light. Ultrathin sections (50–70 nm thick) were collected (i) on glass slides and processed for immunofluorescence (see below) and (ii) on formvar carbon-coated nickel grids that were subsequently floated on drops of 1% bovine serum albumin (BSA) in PBS buffer for 30 min at room temperature. After overnight incubation at 4 °C with anti-Ntann12 rabbit antibodies (diluted 100 times in PBS/1% BSA), grids were washed with PBS and treated for 2 h at room temperature with goat anti-rabbit antibodies conjugated to 5 nm colloidal gold particles (diluted 100 times) (British BioCell). Finally, grids were washed in PBS and then stained with uranyl acetate and lead citrate. Observations were made on a Tecnai 10 electron microscope and images were captured with a MegaView II camera and processed with AnalySIS and Adobe Photoshop softwares. For immunofluorescence, the same protocol was used, except that goat anti-rabbit antibodies conjugated to AlexaFluor 488 (Invitrogen) (diluted 500 times) were used. Samples were observed in a Zeiss fluorescence microscope (Axioscope) coupled to an Axiocam CCD camera. Captured images were processed by Axio and Photoshop software. Appropriate controls using either pre-immune serum or no first antibody were performed and no signal was observed (data not shown).

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA from leaves of 6-week-old plants was prepared using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) then treated with DNase I (DNA-*free*™ from Ambion, Austin, TX, USA). RNA quality and quantity were assessed with a Bioanalyzer 2100 (Agilent). Single-stranded cDNA was synthesized from 1 μg of mRNA using the Reverse Transcription System (Promega, Madison, WI, USA).

qRT-PCR analysis was performed in an ABI 7900 system (Applied Biosystems) using 1 μl of each cDNA. Transcriptional changes were calculated based on the comparative $\Delta\Delta C_T$ method as described by Livak and Schmittgen (2001) and are reported as ratios between expression in roots and in leaves of wild-type plants. The C_T value of each gene was normalized to the C_T value of the reference gene *EF1α*. The expression of each gene was investigated in three biological replicates and six technical repeats. Expression levels of *Ntann12* and *EF1α* were assessed using the primers F 5′-CTTCTCTGCCCTTGTAACTAT-3′ and R 5′-CAACCGCTACAAGGGTGATTA-3′ for *Ntann12* and F 5′-TGCTACCACCCCCAAGTACTC-3′ and R 5′-TAAAGCTGGCAGCACCCTTAG-3′ for *EF1α*. Conditions for qRT-PCR 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 a 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.

pNtann12-GUS *expression*

The amplification of the *Ntann12* promoter region, the construction of *pNtann12-GUS* construct, and the plant transformation were described previously (Vandeputte *et al.*, 2007). Plants were cultured in solid or liquid MS medium with 50 μM indole-3-acetic acid (IAA), 50 μM 1-naphthylphthalamic acid (NPA), or 50 μM 2,3,5-triiodobenzoic acid (TIBA) (Sigma). β-Glucuronidase (GUS) staining was perfomed as described by Hemerly *et al.* (1993).

Results

Recombinant tobacco Ntann12 is a Ca^{2+}-dependent phospholipid-binding protein

The phospolipid binding property of Ntann12 was investigated using the recombinant Ntann12 protein produced in *E. coli*. Effective expression of the recombinant Ntann12 protein was monitored by SDS–PAGE and western blot analysis. As shown in Fig. 1A, anti-Ntann12 polyclonal antibodies clearly cross-react with a polypeptide of ~54 kDa, corresponding to the expected molecular weight of the fusion protein obtained in the pBAD-Dest49 destination vector based on 36 kDa for the native Ntann12 plus 14 kDa for the N-terminal His-Patch thioredoxin plus 4 kDa for the C-terminal tag. Subsequently, recombinant Ntann12 proteins were incubated with liposomes either in the presence or in the absence of Ca^{2+}. Following sucrose gradient centrifugation, liposome fractions were analysed by immunoblot with the anti-Ntann12 antibody. As shown in Fig. 1B, in the absence of Ca^{2+}, no association of the proteins with liposomes was detected, whereas in the presence of 2 mM Ca^{2+}, Ntann12 proteins were detected in the liposome fraction, indicating that recombinant Ntann12 is a Ca^{2+}-dependent phospholipid-binding protein.

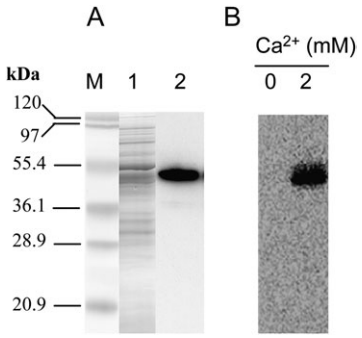

Fig. 1 Production of recombinant Ntann12 and its Ca^{2+}-dependent phospholipid binding property. (A) Western blot analysis of recombinant proteins produced in *E. coli* using anti-Ntann12 antibodies. Lane 1, gel migration of 0.1 mg of total bacterial extract stained with Coomassie; lane 2, gel migration of 5 ng of bacterial extract revealed by anti-Ntann12 antibodies. (B) Phospholipid binding property of recombinant Ntann12. Proteins were incubated with liposomes (2:1 PC/PS) in buffer containing 0 or 2 mM Ca^{2+}. Proteins (40 μg) were analysed by western blot using the anti-Ntann12 antibodies. M, molecular marker.

Ntann12 *is highly expressed in the root system*

Ntann12 expression was investigated by histochemical GUS staining in *pNtann12-GUS* transgenic plants and by qRT-PCR in wild-type plants. Protein levels were compared in both aerial parts and in roots by western blot using anti-Ntann12 antibodies. As shown in Fig. 2A, *Ntann12* is highly expressed in the root system of 1-month-old *pNtann12-GUS* transgenic plants but not in the aerial part. A close-up picture of the root extremity shows that the GUS activity is highly visible within the root maturation zone but not in the root cap, or in the root apical meristem and the elongation zone (Fig. 2B). In longitudinal cross-sections, the GUS activity is detected mainly in the cortex cells of the roots (Fig. 2C). As determined by qRT-PCR analysis, the *Ntann12* expression level was ~250 times more abundant in the roots than in the aerial parts of 1-month old plants (Fig. 2D). To investigate whether the *Ntann12* expression level was correlated with the abundance of the protein in the different plant parts, a western blot analysis was performed. The anti-Ntann12 antibodies cross-react with a protein of ~36 kDa in roots, but not in leaves, confirming that Ntann12 is more abundant in roots than in leaves (Fig. 2E).

Subcellular distribution of native Ntann12 is modulated by Ca^{2+} concentration

In order to determine the intracellular distribution of Ntann12 in roots and to evaluate whether its distribution is regulated by Ca^{2+} concentration, immunoblots were made using both cytosolic and membrane-enriched fractions from tobacco root extracts. Total native protein extracts were incubated with either Ca^{2+} or EDTA before separation of the cytosolic fraction from the microsomal fraction. As shown in Fig. 3, a band corresponding to the molecular

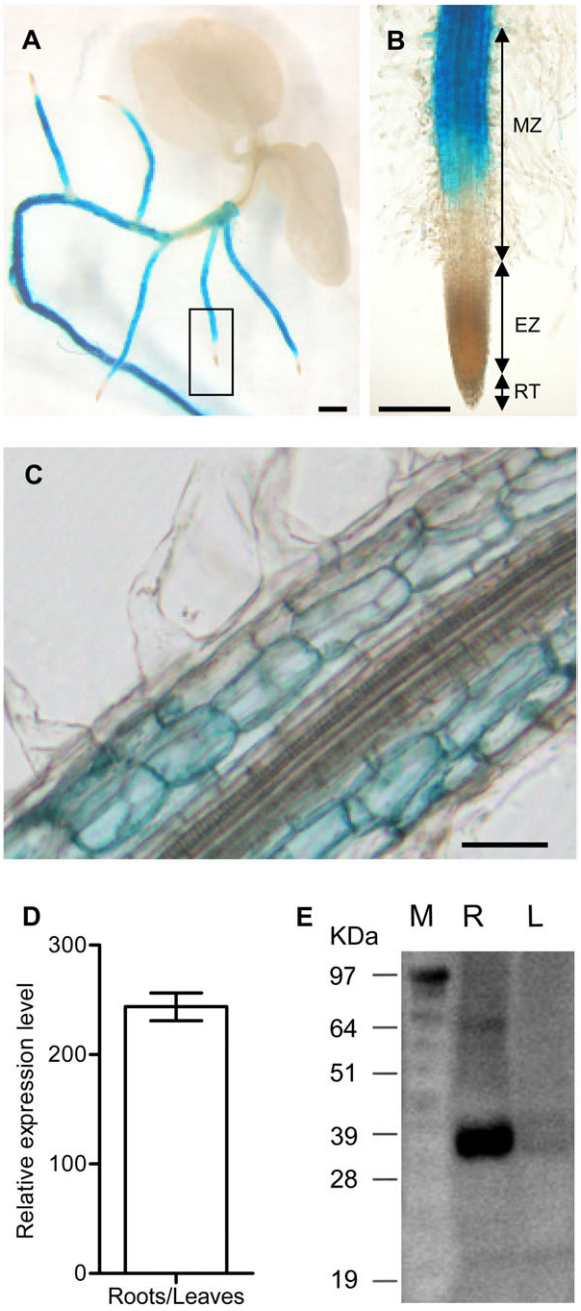

Fig. 2 *Ntann12* expression in 4-week-old plants. (A) *pNtann12-GUS* expression in whole tobacco plant; bar=1 mm. (B) Close-up of (A) showing *pNtann12-GUS* expression in the root tip; bar=0.5 mm. (C) GUS staining in a longitudinal section of the root; bar=100 μm. (D) Relative expression of *Ntann12* in roots and in aerial parts of plants, as determined by qRT-PCR analysis. (E) Western blot analysis of total protein extracts (10 μg) from roots and leaves using anti-Ntann12 antibodies. EZ, elongation zone; L, leaf; M, molecular marker; MZ, maturation zone; RT, root tip; R, root.

weight of Ntann12 was detected in both the cytosolic (Fig. 3A) and membrane-enriched fractions (Fig. 3B) at all tested Ca^{2+} or EDTA concentrations.

Ntann12 was detectable in the membrane-enriched fraction even when no Ca^{2+} was added, indicating that Ntann12

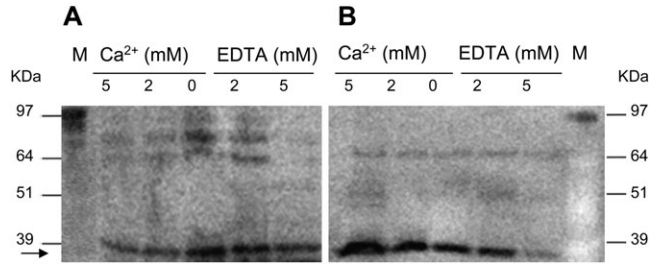

Fig. 3 Subcellular distribution and Ca^{2+} response of native Ntann12 proteins. The total protein extract from roots of 4-week-old tobacco plants was treated with either $CaCl_2$ or EDTA before separation of the membrane-enriched and cytosolic fractions and western blot analysis using the anti-Ntann12 antibodies. (A) Cytosolic fraction. (B) Membrane-enriched fraction. For the analysis, 40 μg of membrane-enriched fraction proteins and 60 μg of cytosolic fraction proteins were loaded into gels. M, molecular marker. The arrow denotes the position of the expected size for Ntann12.

is associated to some extent with the membrane in a Ca^{2+}-independent manner (Fig. 3B). The amount of Ntann12 bound to or associated with the membrane-enriched fraction increased with increasing Ca^{2+} concentration, whereas the Ntann12 level decreased when EDTA was added, becoming almost undetectable in 5 mM EDTA buffer, supporting the fact that Ntann12 phospholipid binding is Ca^{2+} dependent. An opposite effect was noticed in the cytosolic fraction where the Ntann12 level decreased with increasing Ca^{2+} concentration (Fig. 3A). This result is consistent with a Ca^{2+}-mediated translocation of Ntann12 from the cytosolic to the membrane-enriched fraction.

Ntann12 is localized in the nucleus of root cells

To investigate the subcellular localization of Ntann12 in roots, immunohistochemical localization experiments were performed using both fluorescence and electron microscopy. Fig. 4 shows the results of an immunolabelling experiment on 100 μm cross- (Fig. 4A–D) or longitudinal (Fig. 4E–H) sections as well as on 50–70 nm (Fig. 4I–L) sections through 4-week-old tobacco roots. Both cell walls and the vascular system display a background signal due to autofluorescence (Fig. 4M–O). The co-localization of both 4',6-diamidino-2-phenylindole (DAPI) and fluorescent signals indicate that Ntann12 was present in the nucleus of almost all cell types within the root (Fig. 4A–H). However, due to the intensive background fluorescence (Fig. 4O), it cannot be excluded that Ntann12 might be present in the cell periphery (cytoplasm and plasma membrane). To localize the Ntann12 signal more precisely within the nucleus, 50 nm cross-sections were labelled in the same conditions. The labelling was found to be localized as discrete spots in the nuclei (Fig. 4I–L). Subsequently, immunolabelling was investigated by immunotransmission electron microscopy. As shown in Fig. 5, and Supplementary Fig. S2 available at *JXB* online, within root cells, gold particles were detected in both the nucleus and the

cytoplasm. The labelling was present in the whole nucleus and might be associated with the nucleoli, euchromatin, and heterochromatin.

In conclusion, the data presented in Figs 3–5 clearly indicate that Ntann12 is present in the nucleus (Fig. 4) but also in the cytoplasm (Fig. 5 and Supplementary Fig. S2), and can be associated with the membrane system according to the physiological conditions (Fig. 3).

Light and polar auxin transport regulate Ntann12 *expression in the tobacco root system*

In silico analysis of *pNtann12* using PlantPAN (Chang *et al.*, 2008) indicates the occurrence of nine *cis*-element sequences possibly implicated in light responses: three GT1 consensus sequences and six GATABOX sequences (data not shown). Therefore, 1-month-old *pNtann12-GUS* transgenic plants were transferred for 48 h to darkness. As compared with the control kept in light conditions (16 h/8 h photoperiod) (Fig. 6A), GUS activity was no longer detected in the roots of the plants transferred to darkness (Fig. 6B). In accordance with this, Ntann12 proteins were not detected in roots of plants exposed for 48 h to darkness as compared with roots exposed to light conditions (Fig. 7). In addition, *Ntann12* expression was no longer detected in roots of *pNtann12-GUS* transgenic plants 48 h after removal of the aerial part either in light or in dark conditions (data not shown). To check whether light was the signal activating the expression of *Ntann12* in the roots, two treatments were carried out on plants grown in a greenhouse. The first treatment consisted of covering the soil with aluminium foil to ensure complete darkness for the root

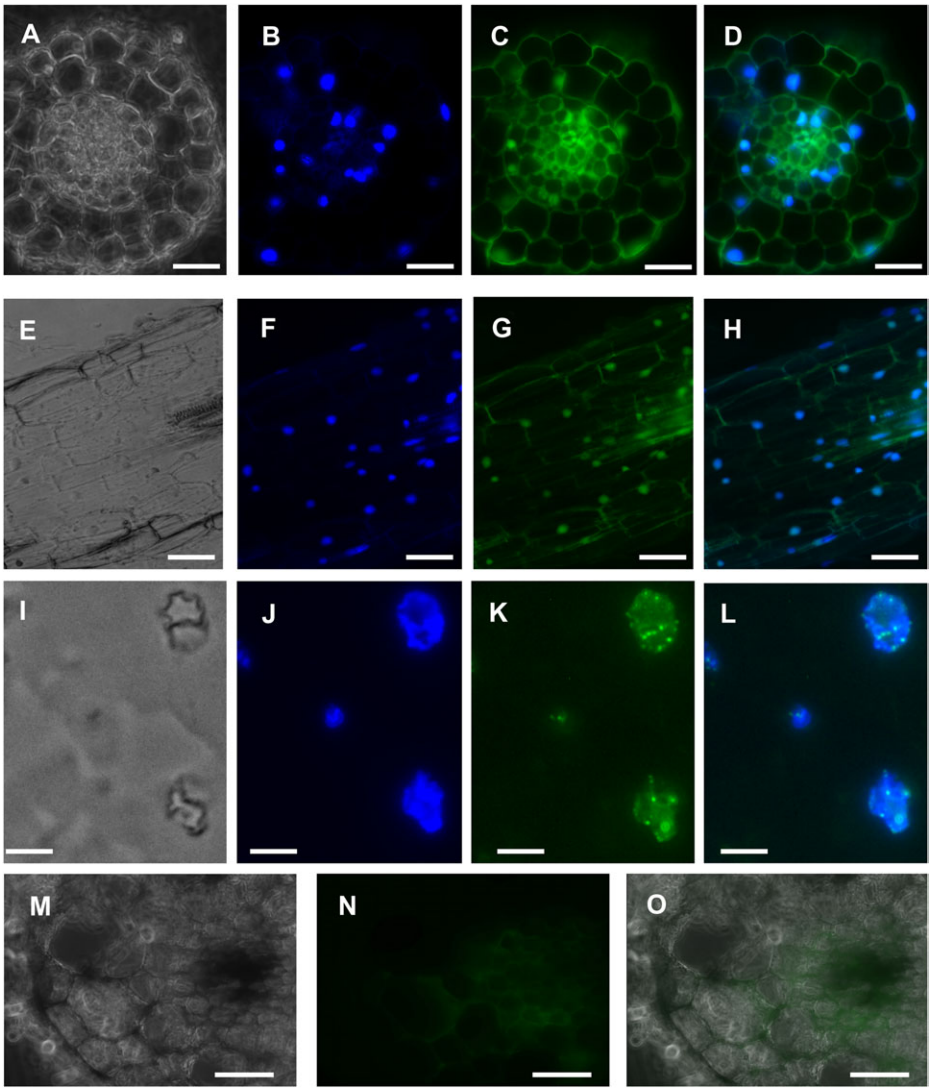

Fig. 4 Ntann12 immunolocalization in tobacco root sections visualized by fluorescence microscopy. (A–D) A 100μm cross-section. (E–H) A 100 μm longitudinal section. (I–L) A 50–70 nm cross-section. (M–O) A 100μm cross-section. (A), (E), (I), (M) Phase contrast. (B), (F), (J) Staining with 4',6-diamidine 2-phenyl indole (DAPI). (C), (G), (K) Immunostaining with affinity-purified antiserum against Ntann12. (D), (H), (L) Superposition of DAPI staining and immunostaining with affinity-purified antiserum against Ntann12. The green fluorescence is indicative of the presence of Ntann12, whereas DNA is represented by blue colour resulting from DAPI staining. (N), (O) The green fluorescence shows the background autofluorescence without immunostaining. Bar=50 μm (A–H and M–O), 5 μm (I–L).

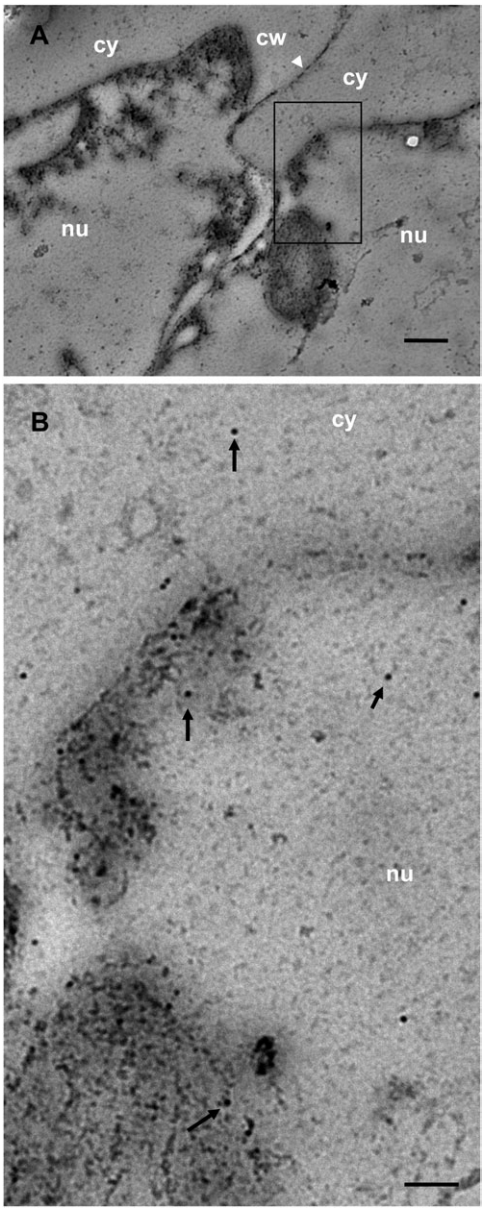

Fig. 5 Transmission electron micrographs of Ntann12 immunogold labelling in a section of root cells. (A) A section in two root cells. (B) Detail of (A) showing labelling in the cytoplasm and in the nucleus (see arrows). Cw, cell wall; cy, cytoplasm; nu, nucleus. Bars=400 nm (A), 50 nm (B).

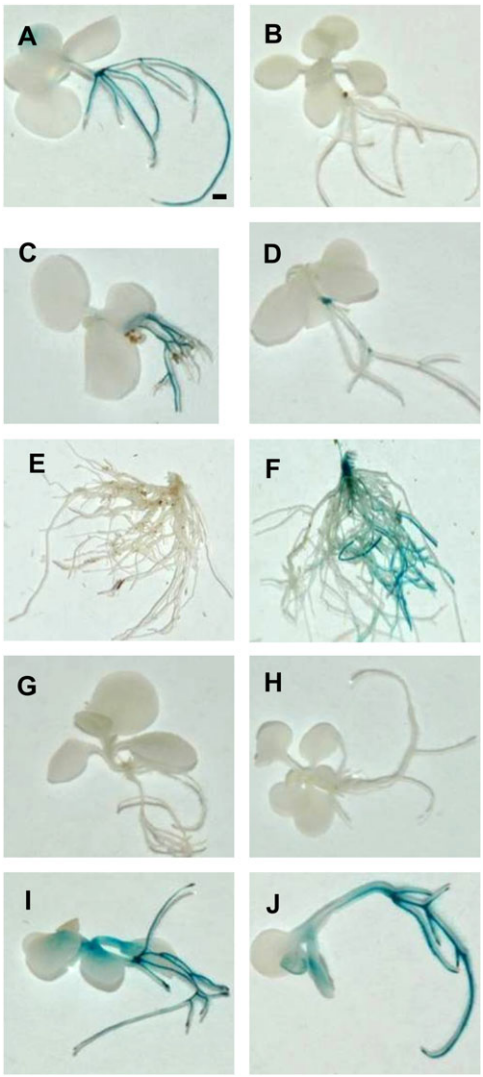

Fig. 6 *pNtann12* responses to light, darkness, IAA, and NPA. (A) A whole plant exposed to light. (B) A whole plant maintained for 48 h in darkness. (C) Aerial part exposed to light and roots to darkness. (D) A whole plant exposed to darkness. (E) A water-containing agar block fixed on the stem section of an aerial part-free plant. (F) An IAA-containing agar block fixed on the stem section of an aerial part-free plant. (G) A plant incubated for 48 h with 50 μM NPA and exposed to light. (H) A plant incubated for 48 h with 50 μM NPA in the darkness. (I) Whole plants treated with 50 μM IAA and exposed to light. (J) Whole plants treated with 50 μM IAA in darkness. (A–D) and (G–J) Two-week-old plants. (E-F) Roots of 6-week-old plants. Bar=1 mm.

system and the second was to place aluminium foil on the whole aerial part of the plant to ensure complete darkness. As shown in Fig. 6C, when the leaves were submitted to light and the roots to darkness, the expression pattern was similar to that observed in *in vitro* conditions (Fig. 6A). In contrast, when the leaves were placed in darkness for 48 h (Fig. 6D), the GUS activity in the root system dramatically decreased, indicating that the expression of *Ntann12* is regulated by a light-activated messenger in the aerial part of the plant.

As auxin has been associated with several light-regulated processes (reviewed by Swarup *et al.*, 2002), the possibility that auxin could be linked to the signal coming from aerial parts exposed to light was investigated. The aerial parts of *pNtann12-GUS* plants grown in soil were cut off, small agar blocks containing either IAA or water were fixed to the cut stems section, and the soil was covered with aluminium foil. As shown in Fig. 6E, after 48 h treatment, no GUS activity was detected in the roots when the cut stem section was covered with the agar block containing water. In contrast, when IAA was applied to the cut stem section, a strong GUS signal was detected in the whole root system (Fig. 6F). Consequently, 4-week-old *pNtann12-GUS* transgenic plants

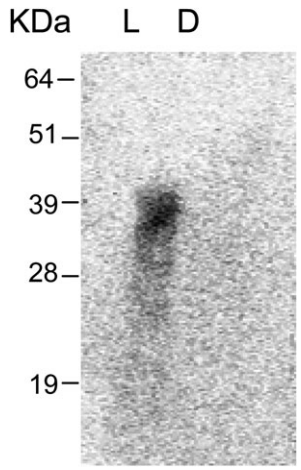

Fig. 7 Western blot analysis of Ntann12 expression in roots of 4-week-old-plants grown in solid MS and exposed to light (L) or to darkness (D) for 48 h. Total protein extracts (10 μg) of roots were separated by SDS–PAGE and western blot analysis with the anti-Ntann12 antibodies.

were subcultured in liquid MS medium containing the polar auxin transport inhibitor NPA. No GUS activity was detected in plants treated with NPA either in light (Fig. 6G) or in dark (Fig. 6H) conditions. These results indicate that polar auxin transport is a limiting factor for *Ntann12* expression in the root system under light conditions. Similar results were obtained with TIBA (data not shown). Moreover, plants treated with IAA in the light (Fig. 6I) or in darkness (Fig. 6J) showed GUS staining in all parts, clearly indicating that *Ntann12* is induced by auxin.

Discussion

In this report, it is shown that the recombinant Ntann12 is a Ca^{2+}-dependent phospholipid-binding protein, confirming that it has the biochemical property of annexins (Fig. 1). Furthermore, the distribution of native Ntann12 in the cytosol or in the cell membrane fraction is demonstrated to be modulated by the Ca^{2+} concentration (Fig. 3). Similarly to Ntann12, *Arabidopsis* AnnAt1 and AnnAt2 have been found to be localized in both the cytosol and microsome compartments (Clark *et al.*, 2005*b*). In addition, the translocation of plant annexins from the cytosol to membranes has been shown to occur in response to specific stimuli (for a review, see Mortimer *et al.*, 2008), such as touch (Thonat *et al.*, 1997), cold (Breton *et al.*, 2000), and salinity (Lee *et al.*, 2004), suggesting a role for annexins as signalling molecules associated with multifunctional components of plant growth and adaptation to environmental stimuli.

Although most plant annexins are found in the cytosol, several have also been shown to be located in various compartments, such as the cell wall (Kwon *et al.*, 2005), the plasma membrane (Santoni *et al.*, 1998), the vacuole (Seals and Randall, 1997), the chloroplast envelope membranes (Seigneurin-Berny *et al.*, 2000), or the endoplasmic reticulum (Huh *et al.*, 2010), or to be extracellular (Laohavisit *et al.*, 2009). Similarly to other plant annexins, by means of immunolocalization, Ntann12 was shown to be mainly present in the nucleus (Figs 4, 5). For instance, P35 annexin from pea has been immunolocalized in the nucleus (Clark *et al.*, 1998). As shown by these authors, a band was detected by immunoblot in fractions corresponding to purified nuclei, nuclear envelope matrix, nucleoli, and chromatin. Futher studies showed that *M. sativa* ANNMS2 annexin was immunolocalized in the nucleoli (Kovács *et al.*, 1998). As far as is known, no nuclear localization signal has been found for any of the annexin localized in the nucleus, including Ntann12, and the mechanism of their nuclear import has not been elucidated yet. The recruitment of a specific annexin in a particular membrane has been proposed to be dependent on the cell type, the Ca^{2+} concentration, the pH, by post-translational modification, the lipid composition, and membrane oxidation, but the targeting mechanisms are unknown (reviewed by Gerke and Moss, 2002: Laohavisit and Davies, 2011).

Several mammal annexins have been shown to be localized in the nucleus. For instance, ANXA2 has been functionally involved in DNA replication (Liu and Vishwanatha, 2007). ANX11 has been shown to translocate from the nucleus to the spindle poles in metaphase and to the spindle midzone in anaphase, and to be recruited to the midbody in late telophase (Tomas *et al.*, 2004). In addition, these authors showed that cells silenced for *Anx11* fail to establish a functional midbody, suggesting a fundamental role for this annexin in the terminal phase of cytokinesis. Finally, suppression of *AnxA3* by RNA interference (RNAi) in primary cultured parenchymal rat hepatocytes has been shown to inhibit DNA synthesis (Niimi *et al.*, 2005). Therefore, as suggested for human annexins (Rick *et al.*, 2005), it may be assumed that plant nuclear annexins, including Ntann12, participate in a nuclear response to cell stimulation or to Ca^{2+} transient signalling, presumably by regulating DNA replication or transcription.

As shown by qRT-PCR, immunoblot, and promoter–GUS analyses carried out in 1-month-old tobacco plants grown *in vitro* or *in vivo*, *Ntann12* is highly expressed in roots but almost not detected in the aerial parts (Fig. 2). Within the root system, GUS staining indicated that *Ntann12* is mainly expressed within the root maturation zone, but not in the root cap or in the root elongation zone. Several other plant annexins have been shown to be expressed in root tissues, such as AnnAt1 (Lee *et al.*, 2004). By *in situ* mRNA hybridization studies, *AnnAt1* was found to be expressed in all cells of the root, except at the root tip where the expression is restricted to the root cap, and *AnnAt2* was expressed in endodermal cells near the root hypocotyl junction (Clark *et al.*, 2001). *MtAnn1* was shown to be expressed in cortical and endodermal cell layers of the roots (de Carvalho-Niebel *et al.*, 2002) and the annexin Gh1 was found to be one of the major proteins in the root proteome of *Gossypium hirsutum* and *G. arboreum* (Mavlonov *et al.*, 2009). As suggested by Huh *et al.* (2010), the annexin expression patterns may depend on both culture conditions and developmental stage.

The present study also indicates that the expression of *Ntann12* in the root system is regulated by a signal produced in the aerial parts of the plant under light conditions, a signal that is possibly auxin. Actually, GUS staining almost disappeared in the roots of *in vitro pNtann12-GUS* transgenic plants that were placed in darkness for 48 h (Figs 6B, 7) and when the aerial part was removed from plants grown in soil (Fig. 6E). However, GUS staining in the roots was restored when auxin was applied to a stem section of an aerial part-free root system (Fig. 6F) or to dark-growing or light-growing plants (Fig. 6I, J). The involvement of IAA in the regulation of *Ntann12* is supported by the observation that the auxin efflux carrier inhibitor NPA repressed *Ntann12* in both light and dark conditions (Fig. 6G, H), indicating that the expression level of Ntann12 in roots is regulated by polar auxin transport. According to the literature, light affects the expression of several *Arabidopsis* annexins (Cantero *et al.*, 2006; Huh *et al.*, 2010). Moreover, a role in nyctinastic movement has been suggested for the p35 *Mimosa pudica* annexin, which accumulated in the cytosol at night but was redistributed to the outermost periphery of the motor cells in the pulvinus during the daytime (Hoshino *et al.*, 2004).

Molecular links between light and auxin signalling pathways are well documented since light-regulated transcription factors have been shown to affect auxin responses, and mutations in Aux/IAA genes affect light signalling (reviewed by Tian and Reed, 2001). More basically, several results suggest that light regulates the amount of auxin or polar auxin transport, and this leads to quantitative changes in local auxin concentrations that correlate with local tissue growth rates and/or particular physiological processes. For instance, dim red light increased auxin transport in cucumber hypocotyls relative to dark-grown hypocotyls (Shinkle *et al.*, 1998). Additional facts suggest that auxin has a central role in shoot to root relationships (reviewed by Sachs, 2005). Indeed, lateral root development has been shown to be modulated by shoot-localized light signalling and requires shoot-derived transport of auxin (Reed *et al.*, 1998; Bhalerao *et al.*, 2002). Auxin formed in leaves is transported to the roots, both by phloem and by polar transport (Friml, 2003). In this latter case, IAA is directionally transported by plasma membrane-localized auxin influx and efflux carriers in transporting cells.

To gain knowledge of the function of Ntann12, transgenic tobacco plants overexpressing or down-regulating *Ntann12* were produced (Supplementary data S3 at *JXB* online). Overall, no phenotypic differences between transgenic and wild-type plants were observed during germination and growth, or after biotic (*R. fascians* infection) and abiotic stress (NaCl, light) or hormonal treatment (auxin) (data not shown). As discussed by Lee *et al.* (2004), members of the annexin family may function at a particular developmental stage or exhibit functional redundancy, possibly explaining the absence of phenotype. Further investigations on multiple mutants are required to shed light on the biological function of the diversity of plant annexins.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Ntann12 immunolocalization in tobacco root 100 μm cross-sections visualized by fluorescence microscopy.

Figure S2. Transmission electron micrographs of Ntann12 immunogold labelling in a section of several root cells.

Supplementary data S3. Characterization of T_2 transgenic tobacco progeny overexpressing and down-regulating *Ntann12*.

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