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Agrobacterium T-DNA-encoded protein Atu6002 interferes with the host auxin response

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SUMMARY

Several genes in the *Agrobacterium tumefaciens* transferred (T)-DNA encode proteins that are involved in developmental alterations, leading to the formation of tumours in infected plants. We investigated the role of the protein encoded by the *Atu6002* gene, the function of which is completely unknown. *Atu6002* expression occurs in *Agrobacterium*-induced tumours, and is also activated on activation of plant cell division by growth hormones. Within the expressing plant cells, the Atu6002 protein is targeted to the plasma membrane. Interestingly, constitutive ectopic expression of Atu6002 in transgenic tobacco plants leads to a severe developmental phenotype characterized by stunted growth, shorter internodes, lanceolate leaves, increased branching and modified flower morphology. These *Atu6002*-expressing plants also display impaired response to auxin. However, auxin cellular uptake and polar transport are not significantly inhibited in these plants, suggesting that Atu6002 interferes with auxin perception or signalling pathways.

INTRODUCTION

Members of the *Agrobacterium* genus are phytopathogenic bacteria with the rare ability to transfer and integrate DNA into the genome of their plant host (Gelvin, 2003). The naturally transferred bacterial genes are expressed in the host plant cells and induce uncontrolled cell proliferation (e.g. crown galls) and the production of opines, small molecules that provide a source of carbon and nitrogen for the bacteria (Escobar and Dandekar, 2003). These DNA sequences are located on a specialized plasmid, the Ti-plasmid (tumour-inducing plasmid), which contains a segment of DNA transferred to the host genome (T-DNA, transferred DNA) and is composed of 12–15 genes (Britton *et al*., 2008; Lacroix and Citovsky, 2013). The protein products of the T-DNA genes are involved in two main processes: the synthesis of opines and the production or regulation of sensitivity to phytohormones, resulting in the formation of crown galls; because crown galls represent neoplastic growths or tumours, these latter genes are also termed oncogenes.

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Although T-DNA oncogenes are central to tumour formation and development, their biological function has been characterized for only a few of their protein products (Britton *et al*., 2008). For example, the product of the gene *6b*, a nuclear protein that may act as a histone chaperone and interfere with the host microRNA (miRNA) pathway, stimulates plant cell division independently of growth regulators and induces the expression of various genes, including genes involved in cell division (Terakura *et al*., 2007; Wang *et al*., 2011). Several other T-DNA oncogenes, such as *iaaM* and *iaaH*, and *ipt*, are involved in the synthesis of auxin and cytokinin hormones, respectively (Schroder *et al*., 1984; Thomashow *et al*., 1986). Gene *5*, however, mediates the synthesis of an auxin antagonist (Körber *et al*., 1991), thus moderating auxin responsiveness and increasing the apparent cytokinin to auxin ratio.

The gene for Atu6002, initially termed the C-protein, is found only in the Ti-plasmid of *Agrobacterium vitis* and nopaline-specific *Agrobacterium tumefaciens* strains C58 or Sakura. The *Atu6002* gene is absent in the T-DNAs of all other *Agrobacterium* species and strains, i.e. *Agrobacterium rhizogenes* and octopine-specific *A. tumefaciens*, sequenced to date, as well as in closely related bacteria belonging to the Rhizobiales family. Initial studies have shown that an *Agrobacterium* C58 *a-acs* mutant, lacking seven T-DNA genes, including *Atu6002*, close to the left border, exhibits reduced shoot formation during the late stages of tumour development; co-inoculation of this mutant with a strain carrying only *Atu6002* in its T-DNA restored shoot formation, and thus the wild-type tumour phenotype (Otten *et al*., 1999). These observations suggest a role for Atu6002 in tumour development and plant growth and morphogenesis. Since these early experiments, the Atu6002 protein has remained unexplored. Here, we provide insights into the biological function of Atu6002, showing that, in plants, it is a plasma membrane protein that affects host responses to auxin.

RESULTS

Atu6002 expression in *Agrobacterium*-induced tumours and in infected plant tissues

For a better understanding of the role of *Atu6002*, it would be useful to determine the kinetics of expression of this oncogene during tumour formation. To this end, we first used reverse transcription-polymerase chain reaction (RT-PCR) to analyse the expression of *Atu6002* in tobacco leaf discs infected with the wild-type C58.C1 strain of *Agrobacterium*, and in tumours developing on these explants. Figure 1A shows that *Atu6002* expression was detectable at 7 days post-inoculation (dpi), and transcription levels increased notably at 28 dpi, when tumours were well developed (Fig. 1B). It should be noted that, at 0 and 7 dpi, there were no visible tumours (Fig. 1B), and the percentage of transformed cells in the tissue was presumably very low, which explains the low levels of T-DNA transcripts detected by RT-PCR analysis (Fig. 1A). The absence of the contaminating Ti-plasmid in the RT-PCRs was demonstrated by the inability to amplify a Ti-plasmid component, the *virD2* gene (Fig. 1A). Control experiments, using the constitutively expressed tobacco actin *Tac9* gene, confirmed the equal input of RNA and reaction efficiency (Fig. 1A).

Next, we visualized the activity of the *Atu6002* promoter during tumour formation. Transgenic tobacco plants were produced that carried an intron-containing reporter transgene encoding β-glucuronidase (GUS) under the control of the *Atu6002* promoter, corresponding to the entire intergenic region of *Atu6002*. Leaf

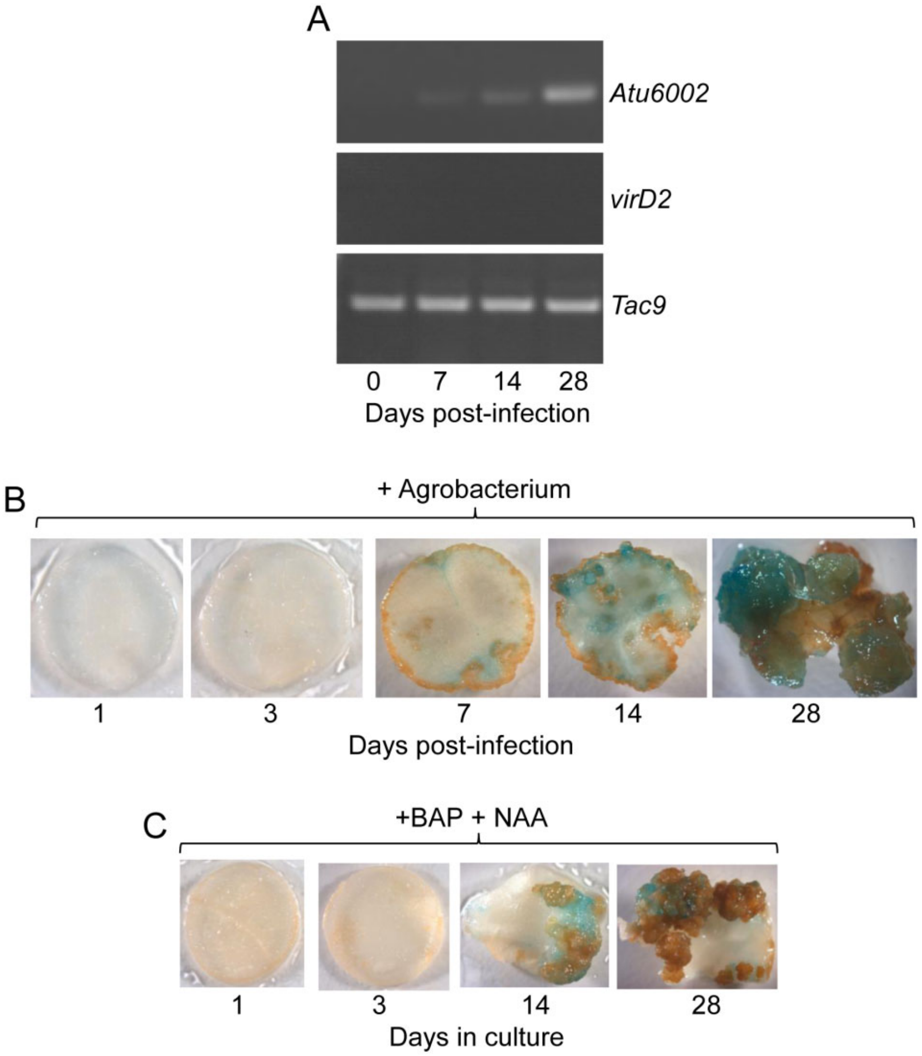

Fig. 1 Time course of expression of *Atu6002* in crown gall tumours and plant calli. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *Atu6002* expression in crown gall tumours. (B) Histochemical β-glucuronidase (GUS) assay for detection of *Atu6002* promoter activity in *Agrobacterium*-induced tumours on At6002pro-GUS transgenic leaf explants. (C) Histochemical GUS assay for the detection of *Atu6002* promoter activity in phytohormone-induced calli on At6002pro-GUS transgenic leaf explants. Blue staining indicates GUS expression. BAP, 6-benzylaminopurine; NAA, 1-naphthaleneacetic acid.

discs from these Atu6002pro-GUS plants were inoculated with the wild-type *Agrobacterium* C58.C1, and GUS enzymatic activity in the infected tissues was monitored by histochemical staining. Although no reporter gene expression was detected in untreated tissues (not shown), GUS was expressed in *Agrobacterium*-infected explants and in tumours formed in these tissues (Fig. 1B). Consistent with the RT-PCR data (Fig. 1A), *Atu6002* promoter-driven GUS expression was detectible at 7 dpi, and its level increased as the tumours increased in size at 14 and 28 dpi (Fig. 1B). The activity of the *Atu6002* promoter was not detected at earlier stages of infection, i.e. at 1 and 3 dpi, when no tumours were visible (Fig. 1B). Interestingly, GUS activity was restricted to the tumours and their immediate vicinity and was not observed in the intervening areas of the plant explants (Fig. 1B), suggesting that the *Atu6002* promoter responds to bona fide tumour formation, rather than to a global reaction of the plant to pathogen challenge, such as the immune response.

Potentially, the *Atu6002* promoter may be activated when cells proliferate, such as during neoplastic growth. Thus, we tested promoter activation on induction of cell proliferation by exogenous growth hormones instead of *Agrobacterium*-induced oncogenesis. Figure 1C shows that the application of synthetic auxin and cytokinin hormones, 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), respectively, promoted GUS reporter activity with kinetics similar to those observed during tumour formation. That no GUS activity was detected in the first 2 days after hormone application suggests that the Atu6002 promoter does not respond to the hormone itself; instead, it is activated after sustained cell division, when calli become visible (Fig. 1C). Similar to the expression in *Agrobacterium*-induced tumours (Fig. 1B), the expression of GUS activity was restricted to areas of callus formation (Fig. 1C). Therefore, the *Atu6002* promoter is probably activated during cell division induced either by the combined effect of T-DNA oncogenes or by exogenously added growth hormones. It should be noted that the conditions of the crown gall tumour appeared to be more conducive to *Atu6002* promoter induction because they supported higher levels of GUS expression in virtually all tumours, whereas only some of the hormone-induced calli expressed the reporter (compare Fig. 1B and Fig. 1C).

Atu6002 is an integral plasma membrane protein in plant cells

Subcellular localization is an important characteristic of a protein and its potential biological function. Thus, we tagged Atu6002 with green fluorescent protein (GFP) and transiently expressed it in the epidermis of *Nicotiana benthamiana* leaves and onion scales. Figure 2A shows that, in *N. benthamiana*, Atu6002-GFP accumulated along the cell periphery of the cell, potentially at the plasma membrane or the cell wall. The expression of Atu6002-GFP in onion scales yielded similar results (Fig. 2B). It should be noted that, because Atu6002-GFP is constitutively expressed in the transformed cells, some GFP fluorescence was also observed in the cytoplasm, where this protein is synthesized before being targeted to the plasma membrane. We distinguished plasma membrane targeting from cell wall localization by plasmolysis assay when, during plasmolysis, the cell wall components retain their original localization pattern, whereas the plasma membrane becomes displaced following physical shrinkage of the plasmolysed cells (Lacroix and Citovsky, 2011; Tian *et al*., 2004). This detachment was best visible when the fluorescence data were superimposed over the phase images of the whole cells in Fig. 2C, which shows that the Atu6002-GFP fluorescent signal associated with the detached plasma membrane, but not with the cell wall, in plasmolysed cells.

These observations support the annotation of Atu6002 as an integral plasma membrane protein based on the PSORT algorithm for the prediction of protein localization in cells (http://psort.hgc.jp/). The most likely topology of Atu6002, which comprises six transmembrane domains with a cytoplasmic amino-terminus, as predicted by the TMpred algorithm (http://www.ch.embnet.org/software/TMPRED_form.html), is shown in Fig. 2D. These six domains are positioned in alternating cytoplasm-to-extracellular space and extracellular space-to-cytoplasm orientations (Fig. 2D, red- and green-coloured sequences, respectively).

Expression of Atu6002 in tobacco results in a severe developmental phenotype

How does the presence of Atu6002 affect plant growth and morphogenesis? To address this question, we generated transgenic tobacco plants that constitutively expressed *Atu6002* under the control of the strong and constitutive cauliflower mosaic virus (CaMV) 35S promoter. RT-PCR analysis identified four independent transgenic lines with high levels of *Atu6002* gene expression (Fig. 3, lanes 2–5). As expected, control experiments did not detect *Atu6002* transcripts in the parental, wild-type plants (Fig. 3, lane 1), and confirmed equal RNA input and RT-PCR efficiency using the constitutively expressed *Tac9* gene (Fig. 3, lanes 1–5). All four transgenic lines produced a severely altered developmental phenotype, whereas transgenic lines with weak or undetectable *Atu6002* transgene expression did not develop these alterations (not shown).

One of the high-expresser Atu6002 transgenic lines, line 2 (Fig. 3), was selected for further analyses. These plants were stunted compared with wild-type plants (Fig. 4A,B); this difference in plant height was caused by a significant reduction in the length of the internodes (Fig. 4B). Additional phenotypic changes in Atu6002 plants included smaller, lanceolate leaves (Fig. 4C) and partial loss of apical dominance, which manifested as enhanced lateral shoot formation (Fig. 4B). The flower morphology of Atu6002 plants was also altered; the total length of Atu6002

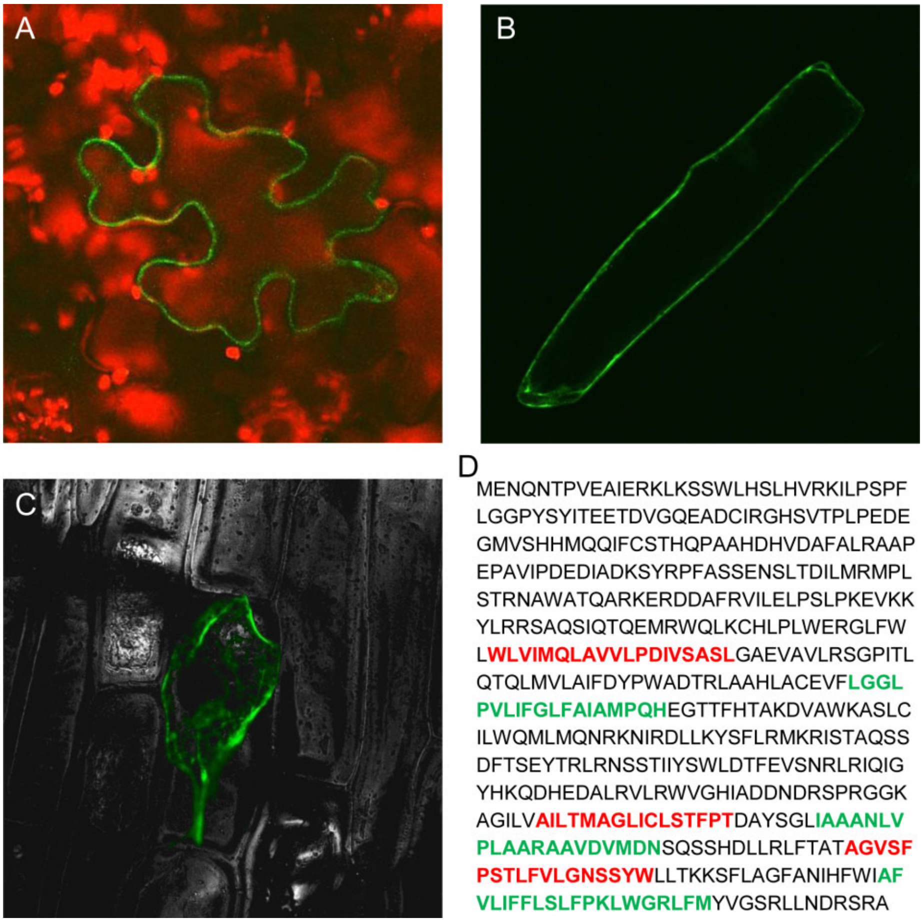

Fig. 2 Subcellular localization of Atu6002-GFP. (A) Atu6002-GFP expressed in leaf epidermis of *Nicotiana benthamiana*. Green fluorescent protein (GFP) fluorescence is in green and plastid autofluorescence is in red. (B) Atu6002-GFP expressed in the epidermis of onion scales. GFP fluorescence is in green. (C) Atu6002-GFP expressed in plasmolysed epidermis of onion scales. GFP fluorescence is in green, and over-imposed phase image is in grey. All images are single confocal sections. (D) Amino acid sequence of Atu6002. The predicted transmembrane domains in the cytoplasm-to-extracellular space orientation are highlighted in red, and in the extracellular space-to-cytoplasm orientation are highlighted in green.

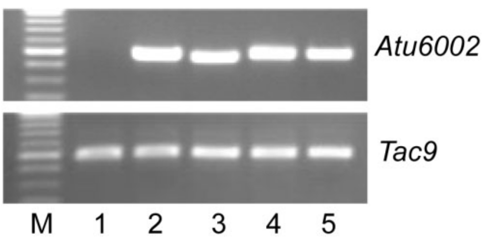

Fig. 3 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *Atu6002* expression in independent Atu6002 transgenic tobacco plant lines. Lane M, molecular size markers; lane 1, wild-type plants; lanes 2–5, transgenic lines 1–4, respectively.

flowers was about one-half that of wild-type flowers (Fig. 4F), and their stamens were shorter, with anthers carrying apparently much less or no pollen (Fig. 4G,H). In contrast, the overall root morphology of Atu6002 plants was not detectably affected (Fig. 4D). Although Atu6002 plants were largely infertile, we were able to obtain seeds by manually pollinating them with wild-type pollen. In these experiments, the transgenic Atu6002 phenotype was retained in the progeny plants (e.g. Fig. 4E).

Atu6002 interferes with auxin signalling

Interestingly, the overall phenotype of the Atu6002 tobacco plants, i.e. reduced internode size, enhanced shoot formation, smaller flowers and compromised fertility, is reminiscent of the phenotype of tobacco plants with an impaired auxin response (Heinekamp *et al*., 2004). Thus, we investigated the auxin response in our transgenic lines. One of the typical auxin responses can be detected by epinastic leaf curvature assay, in which auxin treatment induces the curling of leaf segments as a direct effect of the hormone (Keller and Van Volkenburgh, 1997). Indeed, Fig. 5A shows that the wild-type plants responded to auxin by strong downward bending of their leaf segments. Conversely, Atu6002 plants completely lacked this response, and we did not detect any changes in leaf segment curvature before versus after the application of auxin (Fig. 5A).

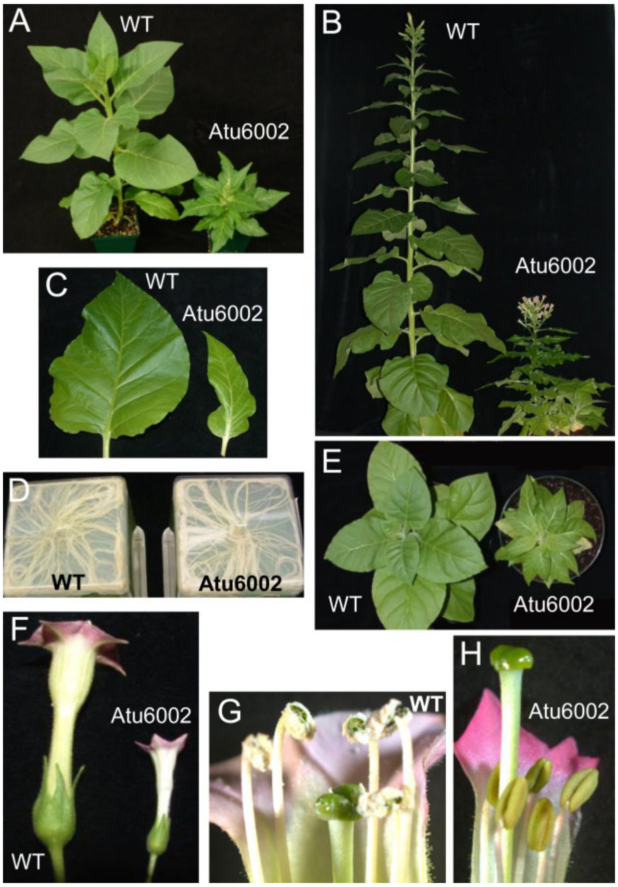

Fig. 4 Stunting and altered leaf and flower morphology of the Atu6002 transgenic line and its progeny. (A) Overall view of 3-week-old plants. (B) Overall view of 2-month-old plants. (C) Leaf shape and size of 3-week-old plants. (D) Roots of 3-week-old plants. (E) Overall view of 3-week-old plants from the progeny of a genetic cross of the Atu6002 transgenic line with a wild-type plant. (F–H) Flower morphology of 2-month-old plants. WT, wild-type plants; Atu6002, Atu6002 transgenic line 1-derived plants.

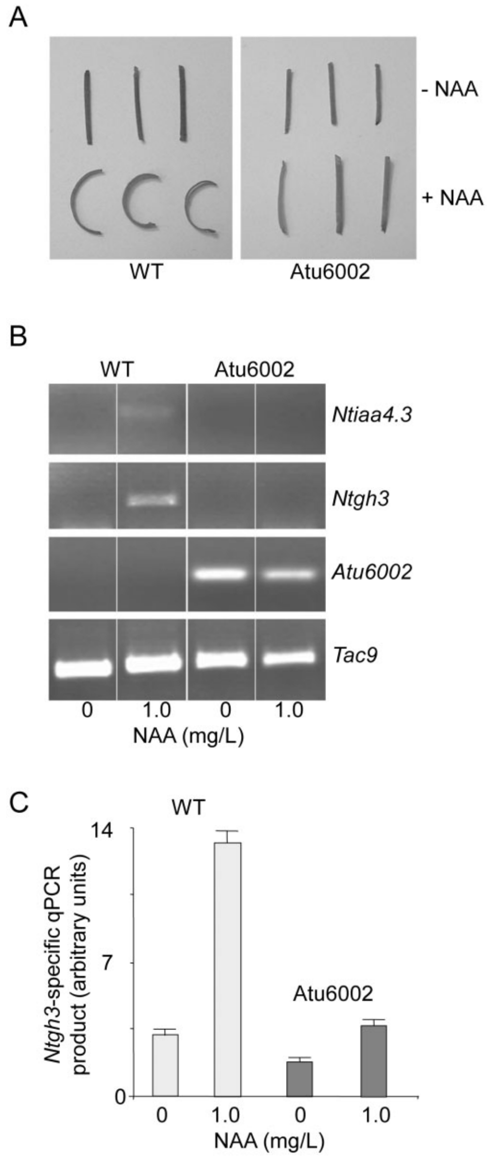

Fig. 5 Altered auxin response of Atu6002 transgenic plants. (A) Epinastic curvature of leaf segments following exposure to 1-naphthaleneacetic acid (NAA). (B) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of expression of early auxin-inducible genes *Ntgh3* and *Ntiaa4.3* in plant protoplasts treated with NAA. (C) Quantitative PCR (qPCR) analysis of *Ntgh3* gene expression in plant protoplasts treated with NAA. The data represent the average values of three independent experiments with standard deviations indicated. WT, wild-type plants; Atu6002, Atu6002 transgenic plants.

As another criterion of the auxin response, we examined the expression of two auxin early-induced genes in protoplasts derived from the wild-type and Atu6002 plants. For these experiments, we chose two well-characterized auxin-inducible genes, *Ntgh3* (Roux and Perrot-Rechenmann, 1997) and *Ntiaa4.3* (Dargeviciute *et al*., 1998), which belong to two of the three families of genes induced early after auxin treatment (Chapman and Estelle, 2009); the expression of these genes was analysed by RT-PCR. Figure 5B shows that, although protoplasts from the wild-type plants showed a typical pattern of induction of the *Ntgh3* and *Ntiaa4.3* genes after 3 h of auxin treatment, no induction of these genes was detected in protoplasts from Atu6002 plants under the same conditions. In control experiments, protoplasts from both types of plant expressed the actin gene *Tac9*, and the transgenic Atu6002 plants, but not the wild-type plants, expressed the *Atu6002* transgene (Fig. 5B). To confirm these qualitative RT-PCR data by an alternative approach, auxin-induced expression of a selected gene, *Ntgh3*, was quantified by quantitative PCR (qPCR) (Fig. 5C). As expected, the protoplasts derived from the wild-type plants exhibited substantial (*c.* four-fold) induction of *Ntgh3* transcription in response to auxin treatment. The sensitive qPCR technique also allowed us to detect very low levels of *Ntgh3* activation in protoplasts from the Atu6002 plants (Fig. 5C); thus, the auxin response in the Atu6002 plants was not completely blocked, but strongly reduced.

Atu6002 does not affect the uptake and polar transport of auxin

Does the impaired response to auxin in *Atu6002*-expressing plants result from modification in the transport of auxin into or between plant cells? To address this question, we performed tracer experiments using tritium-labelled indolylacetic acid (^{3}H-IAA). First, we measured ^{3}H-IAA uptake in mesophyll protoplasts (Delbarre *et al*., 1994) from leaves of the wild-type and Atu6002 plants. Figure 6A shows that, after 1 h, the amount of auxin in Atu6002 protoplasts was slightly higher than that in wild-type protoplasts; however, this variability appears to be in the standard deviation range. Indeed, statistical analysis detected no significant differences between the ^{3}H-IAA uptake in these systems; specifically, Student's *t*-test yielded $P > 0.05$ based on 21 885 d.p.m., SD = 504 for the wild-type protoplasts and 27 930 d.p.m., SD = 3296 for the Atu6002 protoplasts.

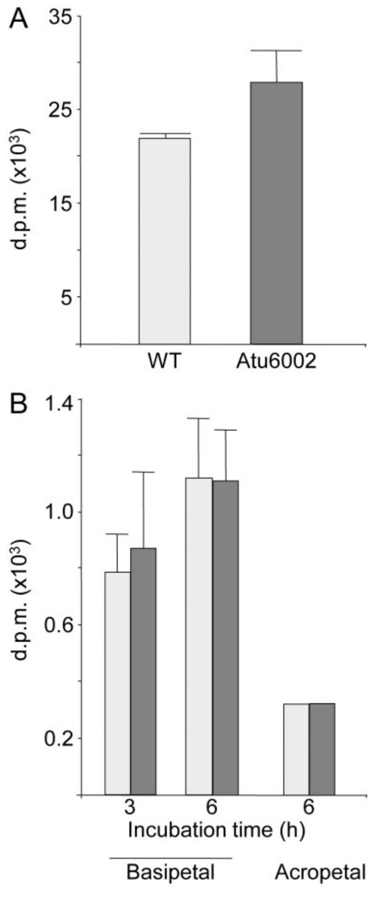

Fig. 6 Auxin uptake and transport in wild-type and Atu6002 plant tissues. (A) Uptake of tritium-labelled indolylacetic acid (^{3}H-IAA) in leaf mesophyll protoplasts after 1 h of incubation. (B) Polar transport of ^{3}H-IAA in petiole segments in basipetal and acropetal orientations at the indicated time periods after the application of ^{3}H-IAA. Measurements from the wild-type and Atu6002 plants are indicated by light and dark grey bars, respectively. All data represent average values of three independent experiments with standard deviations indicated.

Next, we assessed the polar transport of auxin by a petiole assay (Osborne and Mullins, 1969). In this classical system, the polar transport of auxin occurs efficiently in the basipetal direction, i.e. from the 'leaf' end to the 'shoot' end of the living petiole segment, whereas the opposite, acropetal movement, occurs only at very low levels (Chang and Jacobs, 1972; Osborne and Mullins, 1969). We measured the transport of ^{3}H-IAA through petioles placed vertically in a basipetal orientation (Osborne and Mullins, 1969) at different time periods, i.e. 3 and 6 h, following application of ^{3}H-IAA onto the top section of the petiole segment. Again, no significant differences between the wild-type and Atu6002 transgenic plants were observed (Fig. 6B). In control experiments, in which petioles were placed vertically in an acropetal orientation, ^{3}H-IAA movement was much less efficient (Fig. 6B). Taken together, our results suggest that *Atu6002* expression does not have a significant effect on either the uptake or transport of auxin.

DISCUSSION

Pathogenic bacteria encode a large array of effector proteins which they export into eukaryotic host cells, either directly—with the help of specialized secretion systems, such as the type III or type IV secretion system (T3SS or T4SS)—or, in the rare case of *Agrobacterium*, in the form of T-DNA genes which are then expressed in the host cell. The bacterial effector genes or proteins have evolved to acquire many eukaryotic features, allowing them to interact with the host cell machinery and to facilitate the infection process (e.g. Nagai and Roy, 2003). Functional studies of these effectors often result in the discovery of new pathways in host–pathogen interactions and contribute to a better understanding of the cellular processes of the host organism.

Unlike the effectors that are exported directly, and thus are present in the host cell only transiently, the effectors encoded by the *Agrobacterium* T-DNA reside in the transformed cell permanently, potentially having a more lasting and profound effect on its physiology. Here, we focused on one such effector, Atu6002, one of the *Agrobacterium* oncogenes with unknown biological activity. Our data show that the *Atu6002* gene is expressed only in cells that are actively dividing, such as in *Agrobacterium*-induced tumours or in calli promoted by exogenous growth regulators. This *Atu6002* expression pattern is consistent with its presumed role during the late stages of tumour development, i.e. in promoting the formation of shoots on crown gall tumours (Otten *et al*., 1999).

Subcellular localization studies combined with plasmolysis experiments suggested that Atu6002 accumulates at the plasma membrane. This is consistent with the analysis of the Atu6002 protein sequence, which predicts protein topology with six transmembrane domains and plasma membrane localization. Our data also suggested that the presence of Atu6002 in plant cells, presumably in their plasma membranes, interferes with auxin perception. When Atu6002 was constitutively expressed in transgenic plants, this activity resulted in a severe developmental phenotype, including stunting, loss of apical dominance and changes in leaf and flower morphology, characteristic of compromised auxin signalling. The lack of typical responses to exogenous auxin, such as epinastic leaf curvature and the activation of early auxin-inducible genes, suggests that auxin signalling is affected, rather than auxin biosynthesis. This targeting of the auxin response by Atu6002 may play a role in *Agrobacterium* tumorigenesis by increasing the effective cytokinin to auxin ratio and thus promoting shoot formation, one of the hallmark features of crown gall tumours. Indeed, shoot formation is more often observed in tumours induced by the C58 strain when compared with other *Agrobacterium* strains which lack the *Atu6002* gene (Hooykaas *et al*., 1982; Otten *et al*., 1999). That interference with the host auxin responses is central to tumorigenesis is supported by the observations that *Agrobacterium* has evolved to encode another T-DNA oncogene, the gene *5*, which catalyses the production of an inactive auxin analogue, thereby also reducing the cellular auxin response (Körber *et al*., 1991); however, the Atu6002 and gene 5 proteins share no sequence homology and most probably function by different mechanisms.

The strongly reduced response to auxin observed in *Atu6002*-expressing plants may result either from the inhibition of auxin uptake and/or transport or from disruption of the signalling pathway. The results of our ^{3}H-IAA tracing experiments do not support the inhibition of auxin uptake or transport scenario. Thus, Atu6002 most probably interferes with the auxin signalling pathway. For example, Atu6002, a plasma membrane protein, might impair the auxin binding protein ABP1 (Sauer and Kleine-Vehn, 2011), which functions at the plasma membrane and mediates early auxin signalling events (Sauer and Kleine-Vehn, 2011). Because the presence of the *Atu6002* gene within the *Agrobacterium* T-DNA correlates with the ability of the strain to elicit shoot formation in tumours, it is tempting to speculate that *At6002* may represent an advantage for the *Agrobacterium* strain inducing these tumours (Hooykaas *et al*., 1982; Otten *et al*., 1999). For example, the presence of shoots might extend the lifetime of the tumour and/or increase the production levels of opines used as nutrients by the bacteria.

EXPERIMENTAL PROCEDURES

Plants

Tobacco plants (*Nicotiana tabacum* var. Turk) were grown either in soil or on Murashige and Skoog (MS) medium (10 g/L sucrose, 8 g/L agar) after seed surface sterilization, and maintained *in vitro* by micro-cuttings on high-sucrose MS medium (30 g/L sucrose, 8 g/L agar). All plants were grown in an environment-controlled growth chamber under long-day (16 h light/8 h dark) conditions at 22 °C.

RT-PCR and qPCR

Total RNAs were extracted from the plant material with Trizol (Invitrogen, Grand Island, NY, USA), and the residual DNA was removed by treatment with RNase-free DNase (New England Biolabs, Ipswich, MA, USA) for 1 h at 37 °C; this extensive DNase treatment was used to ensure the removal of all traces of DNA derived from potential bacterial contamination. cDNA synthesis was performed using the Revert-Aid First Strand cDNA Synthesis kit (Fermentas, Pittsburgh, PA, USA), according to the manufacturer's instructions. PCRs were performed using ExTaq polymerase (TaKaRa, Shiga, Japan) (25 cycles for 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C) and visualized on ethidium bromide-stained agarose gels. qPCR experiments were performed using Maxima SYBR Green qPCR Master Mix (Fermentas) and an MJ MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). The absence of potential residual genomic DNA contamination was confirmed in control PCRs performed without reverse transcription.

Tumour and callus induction, and detection of *Atu6002* transcripts and GUS activity

Tobacco leaf discs (7 mm in diameter) were inoculated with *Agrobacterium* strain C58.C1 at an optical density at 600 nm (OD_{600nm}) of 0.25 for 20 min at room temperature, placed for co-cultivation on MS medium for 3 days and, finally, after a wash in water, transferred to MS medium with 100 mg/L timentin to eliminate bacterial cells. Tumours developing on these leaf explants were excised at different time periods after co-cultivation and used for RNA extraction and RT-PCR analyses (25 cycles for 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C), which employed the following primer pairs: 5'GAGGAATTCCAGCACCATCATC3'/5'CAAAGTTTGGGAAAAAGGCTCAG3' for the *Atu6002* gene, 5'TCACTGAAGCACCTCTTAACC3'/5'CAGCTTCC ATTCCAATCATTG3' for the tobacco actin *Tac9* gene, and 5'ATGCC CGATCGAGCTCAAGTTATC3'/5'TTCAGCCAGCCGTGTCTAAAAG3' for the *Agrobacterium virD2* gene, each of which amplified a corresponding 500-bp fragment.

For callus induction, leaf discs were incubated for different time periods on callus-inducing MS medium containing 1.0 mg/L NAA and 1.0 mg/L BAP. To visualize GUS activity, the leaf discs were assayed histochemically as described by Nam *et al*. (1999) and recorded under a Leica (Buffalo Grove, IL, USA) MZ FLIII stereoscope.

Plasmids

For the production of At6002pro-GUS transgenic plants, the *GUSintron* sequence from pBISN1 (Narasimhulu *et al*., 1996), amplified using the 5'GGAAGATCTATGTTACGTCCTGTAGAAACCCC3'/5'CCGGAATTCTCATTGT TTGCCTCCCTGCTGS3' primer pair, was first inserted into the *Bgl*II-*Eco*RI sites of pSAT1A (Tzfira *et al*., 2005), producing pSAT1A-GUSint. The *Atu6002* promoter sequence, corresponding to the 1038-bp intergenic region of this gene, was amplified from pTiC58 using the primer pair 5'CCGACCGGTGGCCGAAGGCTAATGGCCCTC3'/5'GGAAGATCTTGATAAC

TTGGCACAGCTTGAGGC3', and cloned into the *Age*I-*Bgl*II sites of pSAT1A-GUSint, replacing the original 35S promoter with the *Atu6002* promoter sequence. Then, the entire *Atu6002* promoter-*GUSintron* expression cassette was cloned into the *Asc*I site of pRCS2-nptII (Tzfira *et al*., 2005), resulting in pRCS2-nptII-Atu6002pro-GUSintron.

For subcellular localization experiments, *Atu6002* was amplified, using pTiC58 as template, with the primer pair 5'CCCAAGCTTCGATGGA GAACCAGAACACTCC3'/5'CGGGGTACCGAGCTCTTGAACGATCATTGAG3', and inserted into the *Hind*III-*Kpn*I sites of pSAT6A-EGFP-N1 (GenBank accession number DQ005470), forming the pSAT6A-Atu6002-GFP construct. The Atu6002-GFP expression cassette was then cloned into the I-*Ceu*I site of pPZP-RCS2 (Goderis *et al*., 2002), resulting in the pRCS2-Atu6002-GFP binary construct.

For the production of Atu6002 transgenic plants, the *Atu6002* gene was PCR amplified from pTiC58 with the primer pair 5'CCCAAGCTTATGGA GAACCAGAACACTCCGG3'/5'CGGGGTACCTTAAGCTCTTGAACGATCATTG AGG3', and inserted into the *Hind*III-*Kpn*I sites of pSAT1A. Then, the *Atu6002* expression cassette was cloned into the *Asc*I site of pRCS2-nptII, resulting in pRCS2-nptII-Atu6002.

Transient expression by agroinfiltration and microbombardment

For agroinfiltration, the binary construct pRCS2-Atu6002-GFP was introduced into the *Agrobacterium* strain EHA105, grown overnight at 28 °C and infiltrated into intact *N. benthamiana* leaves as described by Lacroix and Citovsky (2011). For biolistic delivery, 100 μg of pSAT6A-Atu6002-GFP DNA was absorbed onto 10 mg of 1-μm gold particles (Bio-Rad) and microbombarded into the epidermis of onion scales at a pressure of 90–150 psi using a portable Helios gene gun system (Model PDS-1000/He, Bio-Rad), essentially as described by Ueki *et al*. (2009). After incubation for 36–48 h at 22–24 °C, the agroinfiltrated or microbombarded tissues were viewed under a Zeiss (Oberkochen, Germany) LSM 5 Pascal confocal laser scanning microscope.

For plasmolysis, onion scales were incubated in 0.45 M mannitol as described previously (Jo *et al*., 2011; Lacroix and Citovsky, 2011) until epidermal cells were visibly plasmolysed, and examined by confocal microscopy.

Transgenic plants

Tobacco transgenic plants were produced by the classical leaf disc protocol (Horsch *et al*., 1985) using the EHA105 strain of *A. tumefaciens* carrying the pRCS2-nptII-Atu6002 or pRCS2-nptII-Atu6002pro-GUSintron binary construct. Transgenic plants were selected on MS regeneration medium (30 g/L sucrose, 8 g/L agar, 10 g/L BAP, 1.0 g/L NAA) containing 50 mg/L timentin and 50 mg/L kanamycin, and then transferred to MS rooting medium (30 g/L sucrose, 8 g/L agar). *Atu6002* transgene expression in individual transformants was assayed by RT-PCR as described for the analysis of *Atu6002* expression in tumours.

Leaf epinastic curvature

Induction of leaf epinastic curvature by auxin treatment was performed as described by Keller and Van Volkenburgh (1997). Briefly, 1×20-mm^2 segments of mature tobacco leaves were excised in parallel to secondary veins, placed in a 35×10-mm^2 tissue culture dish containing 1 mL of 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer ($MgCl_2$, 10 mM; MES, 10 mM; pH 5.6) supplemented with 1.0 mg/L NAA, and incubated in the dark at 24 °C for 20 h, before observation of the curvature of the segments.

Expression of auxin-induced genes

The transcript levels of auxin-induced genes were assayed in either leaf mesophyll protoplasts or leaf discs. Tobacco protoplasts were prepared as described previously (Lacroix and Citovsky, 2011; Yoo *et al*., 2007). Protoplast suspension (1 mL in liquid MS medium, containing 0.4 M mannitol and 0.5 g/L MES, pH 5.8), containing approximately 0.6×10^6 cells, was placed in a 35×10-mm^2 tissue culture dish, and NAA was added to the protoplast suspension to a final concentration of 1.0 mg/L. After a 3-h incubation period under light at room temperature, protoplasts were harvested, and the expression of the tested genes was analysed by RT-PCR (25 cycles for 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C) and qPCR. *Ntgh*3 gene (GenBank accession number AF123503.1) expression was detected using the primer set 5'CTCCTGCATGTGAGAAAGACGCAAAG3'/5'GTCCTT TGCCTTTGTCTAATCCGGGC3', which amplifies a 416-bp segment of the N-terminal portion of the *Ntgh3* coding sequence. *Ntiaa4.3* gene (GenBank accession number AF123506.1) expression was detected using the primer set 5'ATGGAAAGAACAGCAACATACGAG3'/5'TTAATTTGAA GGCCTATATGTCCAC3', which amplifies the entire 609-bp *Ntiaa4.3* coding sequence. For controls, transcripts of the tobacco actin *Tac9* gene and the *Atu6002* transgene were detected, as described for the analyses of expression of these genes in tumours.

In qPCR experiments, the relative abundance of the *Ntgh3*-specific product was normalized to the amount of the product specific for *Tac9*, which represented an internal control of a constitutively expressed gene. The absence of potential residual genomic DNA contamination was confirmed in control qPCRs performed without reverse transcription.

Auxin uptake and polar transport

Tritium-labelled auxin, [5-^{3}H(N)]-indolylacetic acid (^{3}H-IAA), was purchased from Perkin-Elmer (Waltham, MA, USA). Tobacco leaf mesophyll protoplast suspension (1 mL in WI liquid medium; 4 mM MES, 0.4 M mannitol, 20 mM KCl), containing approximately 0.6×10^6 cells, was placed in a 35×10-mm^2 tissue culture dish, and ^{3}H-IAA was added to a final concentration of 0.2 nM. After a 1 h incubation period under light at room temperature, the protoplasts were harvested and centrifuged at low speed (80 ***g***), and the supernatant was removed. The protoplasts were then resuspended in 1 mL of water to lyse the cells and release the intracellular content. The cell debris was pelleted by high-speed centrifugation (20 000 ***g***), and 0.9 mL of the supernatant was used to measure tritium radiation in a scintillation counter. As a control, protoplasts were harvested immediately after the addition of ^{3}H-IAA, before auxin uptake could occur, to estimate the amount of radioactive tracer adsorbed nonspecifically to the protoplast pellet. The measured tritium radiation was expressed in disintegrations per minute (d.p.m.) after quenching correction, and the value of the nonspecific ^{3}H-IAA adsorption control was subtracted from the total value of each measurement. These data were analysed by two-tailed Student's *t*-test; $P < 0.05$, corresponding to the statistical probability of greater than 95%, was considered to be statistically significant. All data were presented as average values of three independent experiments.

To estimate polar auxin transport (Osborne and Mullins, 1969), 1-cm-long petiole segments were excised and placed in a basipetal orientation into a microtube containing 0.1 mL of agarose gel in water. One microlitre of ^{3}H-IAA (0.1 μM, 0.083 MBq/mL) was placed onto the upper end of the petiole segment. After 3 or 6 h of incubation at room temperature in the dark, the petioles were discarded, the agarose gel was melted for 10 min at 60 °C in 0.4 mL of 6 M NaI and used to measure tritium radiation, which was expressed in d.p.m. As negative control, the same experiment was performed with a petiole segment placed in an acropetal orientation.

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