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Modification of plant Rac/Rop GTPase signalling using bacterial toxin transgenes

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SUMMARY

Bacterial protein toxins which modify Rho GTPase are useful for the analysis of Rho signalling in animal cells, but these toxins cannot be taken up by plant cells. We demonstrate *in vitro* deamidation of Arabidopsis Rop4 by *Escherichia coli* Cytotoxic Necrotizing Factor 1 (CNF1) and glucosylation by *Clostridium difficile* toxin B. Expression of the catalytic domain of CNF1 caused modification and activation of co-expressed Arabidopsis Rop4 GTPase in tobacco leaves, resulting in hypersensitive-like cell death. By contrast, the catalytic domain of toxin B modified and inactivated co-expressed constitutively active Rop4, blocking the hypersensitive response caused by over-expression of active Rops. In transgenic Arabidopsis, both CNF1 and toxin B inhibited Rop-dependent polar morphogenesis of leaf epidermal cells. Toxin B expression also inhibited Rop-dependent morphogenesis of root hairs and trichome branching, and resulted in root meristem enlargement and dwarf growth. Our results show that CNF1 and toxin B transgenes are effective tools in Rop GTPase signalling studies.

Keywords: Rop GTPase, Cytotoxic necrotizing factor 1, toxin B, transgene, cell polarity, pathogen defence.

INTRODUCTION

Rop GTPase signalling controls polar morphogenesis of tip growing cells such as pollen tubes and root hairs (Kost, 2008; Lee and Yang, 2008; Zhang and McCormick, 2010), polar morphogenesis of leaf epidermal pavement cells (Yang, 2008) and polarizes asymmetric cell division (Humphries *et al.*, 2011). Rops also function in the development of haustoria in plant cells during biotrophic fungal growth (Hoefle *et al.*, 2011) and in lignin and secondary cell wall synthesis (Kawasaki *et al.*, 2006; Foucart *et al.*, 2009). Moreover, Rop signalling is important in plant pathogen defence as studied in rice (Kawano *et al.*, 2010a), barley (Hoefle *et al.*, 2011) and tobacco species (Schiene *et al.*, 2000; Kawano *et al.*, 2010b). The Rop GTPase molecular switch in vascular plants and mosses is regulated by a set of guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Eklund *et al.*, 2010). Known Rop effectors function in a variety of cellular processes, including actin and tubulin dynamics, production of reactive oxygen species (ROS), cell wall synthesis and polar secretion, supporting a central role for Rops as cellular signalling hubs (Yang and Fu, 2007; Berken and Wittinghofer, 2008; Nagawa *et al.*, 2010; Mucha *et al.*, 2011). Recently, several plant-specific Rop

nucleotide exchanger (PRONE) RopGEFs (Berken *et al.*, 2005) have been found to link receptor-like protein kinases (RLKs) and Rops in cell growth (Zhang and McCormick, 2007; Duan *et al.*, 2010), in pathogen associated molecular pattern (PAMP) recognition (Kawasaki *et al.*, 2009), and possibly in asymmetric cell division (Humphries *et al.*, 2011).

Functional Rop GTPase research has greatly benefited from the experimental manipulation of Rop activity levels in plant cells, using overexpression (OX) of either constitutively active or dominant negative Rop mutants, as well as antisense RNA and RNA interference (RNAi) approaches in different dicot and monocot plant species (Schiene *et al.*, 2000; Tao *et al.*, 2002; Miki *et al.*, 2005; Fu *et al.*, 2005; Hoefle *et al.,* 2011) . Additionally, GDIs, GEFs and GAPs have been over-expressed and targeted by gene knock out and RNAi methods (Kost, 2008; Lee and Yang, 2008; Zhang and McCormick, 2010).

Cytotoxic necrotizing factor 1 (CNF1) is produced by pathogenic *E. coli* strains which cause urinary tract infections and neonatal meningitis. CNF1 is a multidomain protein, consisting of a cell-binding domain, a membrane translocation domain and a C-terminal glutamine deamidase domain. It targets animal Rho GTPases by specific deamidation resulting in their constitutive activation (Flatau *et al.*, 1997; Schmidt *et al.*, 1997; Aktories, 2011).

Clostridium difficile toxin B is a pathogenicity factor associated with antibiotic-associated diarrhoea and pseudomembraneous colitis. Toxin B is a large 270-kDa multidomain protein which is constructed according to the ABCD model (Jank and Aktories, 2008), consisting of an N-terminal glycosyltransferase, an autocatalytic cysteine protease domain, a membrane translocation domain and a C-terminal cell-binding domain. Toxin B specifically glucosylates Rho GTPases (Just *et al.*, 1994), interfering with the Rho localization and activation cycles, and blocking Rho effector interactions (Belyi and Aktories, 2010). Previously, the catalytic domain encoded by the bacterial toxin B gene has been transiently expressed in pollen and shown to inhibit Rop-dependent pollen tube elongation, although *in vitro* activity on recombinant Rop protein was not documented (Kost *et al.*, 1999).

Here we show that the catalytic domains of CNF1 and toxin B are active on Rop GTPases *in vitro*. Moreover, both catalytic domains were expressed and proved to be functionally active *in planta*. Both CNF1 and toxin B inhibited Rop-dependent polar morphogenesis of Arabidopsis leaf epidermal cells. Transgenic Arabidopsis seedlings expressing CNF1 had elongated cotyledons, and developed into plants of normal size with curly elongated leaf shapes, as typical for OX of constitutively active Rop2/4 mutants. Toxin B expression inhibited cellular morphogenesis of root hairs and trichomes and plant growth generally. Our data show that these bacterial toxins with opposing effects on Rop signalling can be used effectively in plants.

RESULTS

Plant RAC/Rop GTPases as putative substrates for bacterial GTPase-modifying toxins

Amino acid sequence comparisons between Arabidopsis Rops, human Rac1 and RhoA showed that the amino acids surrounding threonine-38 (the target of glucosylation by toxin B) and glutamine-64 (target of deamidation by CNF1) of AtRop4 are well conserved, suggesting that Rop GTPases would be recognized as substrates (Figure. 1a and Fig. S2 in Supporting Information). In contrast, the asparagine-41 of RhoA, which is the target of ADP ribosylation by *Clostridium botulinum* C3 toxin (Sekine *et al.*, 1989), is followed by tyrosine in RhoA and Rac1 but by phenylalanine in AtRops. Moreover, asparagine-41 is preceded by glutamate in RhoA but by aspartate in the case of Rops and Rac1, suggesting that Rops would probably be very poor substrates for clostridial C3 toxin. Indeed, a C3 toxin assay showed that its activity towards Arabidopsis Rop4 was lower than that towards Rac1, which is already a very poor substrate in comparison to RhoA in mammalian cells (Fig. S3).

In vitro deamidation of AtRop4 GTPase by *E. coli* toxins CNF1 and CNF3 and glucosylation by *C. difficile* toxin B

Deamidation of glutamine-63 of RhoA to glutamic acid by *E. coli* toxins CNF1 and CNF3 (Schmidt *et al.*, 1997; Lerm *et al.*, 1999; Stoll *et al.*, 2009) can be observed as a shift in the mobility of the modified small GTPase on SDS-PAGE (Flatau *et al.*, 1997). A gel shift assay on reactions using CNF1, CNF3 and, as a negative control, mutated inactive CNF3(CS), showed that purified recombinant His(6×)-Rop4 was indeed a substrate for deamidation similar to RhoA GTPase control substrate. Although the shift of the deamidated plant GTPase was much smaller compared with mammalian RhoA, it was reproducibly present (Figure. 1b,c). CNF1 was active on both GDP-loaded and non-hydrolysable GTPγS-loaded AtRop4, as well as on constitutively active (CA) mutant AtRop4 G15V (Fig. S4).

To test whether the Arabidopsis Rop GTPases are a substrate for *C. difficile* toxin B, an *in vitro* glucosylation assay was performed with recombinant His(6x)-AtRop4, using native toxin B in the presence of UDP-[^{14}C]glucose. As shown in Figure. 1d, toxin B was able to glucosylate AtRop4, similarly to human Rac1 GTPase. It was reported that both GDP-bound and GTP-bound conformations of the Rho family GTPases are substrates of toxin B, with some preference for the GDP-bound conformation (Just *et al.*, 1995; Hofmann *et al.*, 1997). Both GDP-bound and GTP-bound conformations of plant Rops were also toxin B

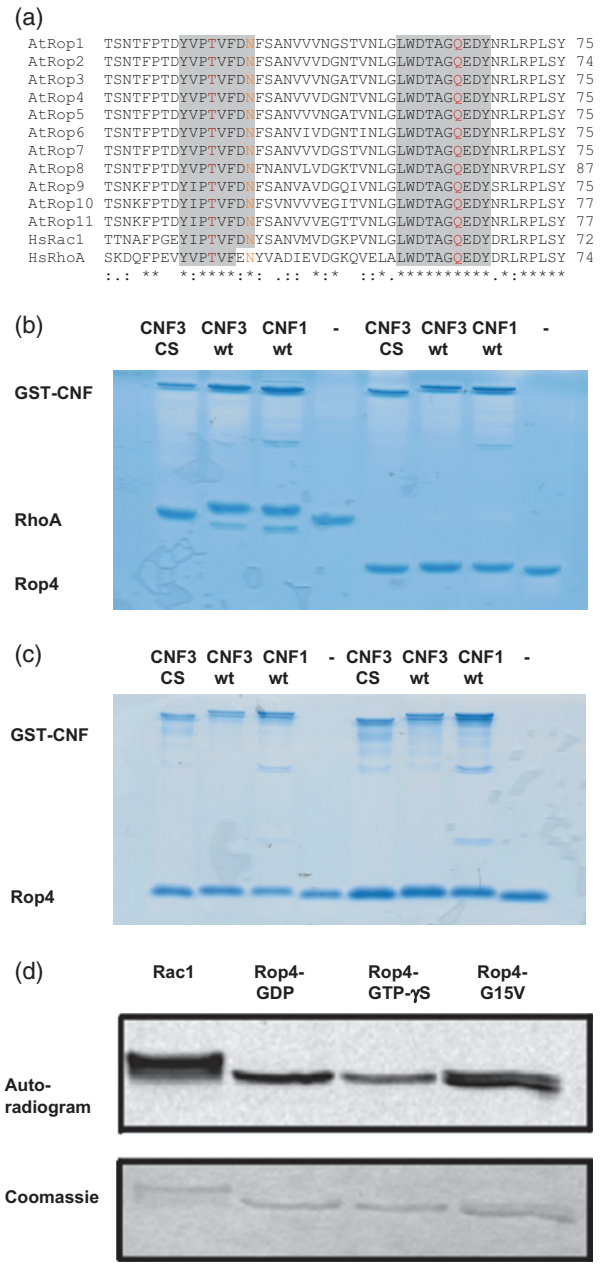

Figure 1. Rop GTPases are substrates for CNF1 and toxin B.
(a) Partial multiple sequence alignment of Arabidopsis Rop GTPases and human Rac1 and RhoA GTPases by CLUSTALW. The target amino acids T for toxin B glucosylation, N for C3 ADP-ribosylation and Q for CNF1 deamidation are shown in red. Amino acids surrounding T37 and Q63 in RhoA are conserved in Arabidopsis Rops (dark grey), but not around N41.
(b), (c) *Escherichia coli* CNF1 and CNF3 deamidate plant Rop GTPases. Coomassie stained 12.5% urea-SDS-PAGE gels of deamidation reactions showing an electrophoretic upward shift of CNF1- and CNF3-treated GST-RhoA and His(6×)-Rop4, but not in the case of inactive CNF3 CS-treated GTPases as shown *in triplo* for His (6×)-Rop4. Combinations of GTPase substrates and CNF enzymes as indicated.
(d) *Clostridium difficile* toxin B glucosylates plant Rop GTPases. Autoradiogram of SDS-PAGE gel of toxin glucosylation reactions showing the incorporation of ^{14}C glucose in human Rac1 and in Arabidopsis Rop4 protein substrates loaded with either GDP or non-hydrolysable GTPγS, and in constitutively active mutant Rop4 G15V as indicated. A corresponding Coomassie blue stained gel is shown as a loading control.

substrates, since GDP-loaded AtRop4, GTPγS-loaded AtRop4 and CA AtRop4 G15V could all be glucosylated (Figure. 1d).

CNF1 activates AtRop4 *in planta*

To express the catalytic toxin domains in plants, we transiently transformed *Nicotiana benthamiana* plants with agrobacterial strains carrying binary 35S expression vector constructs for CNF1-YFP and GFP-CNF1. Both CNF1 fusion proteins were efficiently expressed (Figure. 2a). To test the effect on substrate GFP-Rop4 by CNF1, both proteins were co-expressed and the activation status of Rops was determined, using the Rop-GTP-specific affinity reagent MBP-GBD (Molendijk *et al.*, 2008). The MBP fusion protein, containing the GTPase-binding domain (GBD) of RopGAP2, bound efficiently to GFP-Rop4-G15V, which can be considered as GTP-bound Rop4, since its GTP hydrolysis is blocked. The MBP-GBD bound weakly to GFP-Rop4 wild-type (Wt), which was probably predominantly GDP-bound due to OX, since its nucleotide exchange rate is much lower than its intrinsic hydrolysis rate (Molendijk *et al.*, 2001). The binding of MBD-GBD was marginal to GFP-Rop4-T20N, which is suggested to be in a nucleotide-free state. When co-expressed with CNF1, the amount of Rop4 Wt, which was pulled down by MBP-GBD, was much higher than when Rop4 Wt was expressed alone, indicating that this Rop protein was activated by coexpressed CNF1 *in planta* (Figure 2b).

Toxin B inactivates AtRop4 *in planta*

A synthetic gene encoding the catalytic domain of toxin B (amino acids 1–546) with increased GC content (38.5%) and optimized for expression in Arabidopsis (Figure S5) was used to replace the original bacterial toxin B catalytic coding sequence (28% GC) which was poorly expressed in transformed Arabidopsis protoplasts, and not at all in *N. benthamiana* or transgenic Arabidopsis. The codon usage-optimized GFP-toxin B and toxin B-YFP constructs could be efficiently expressed in *N. benthamiana* using *Agrobacterium*-mediated transient transformation (Figure 2c). To test for inactivation of GTP-bound Rop by toxin B, GFP-Rop4 G15V was expressed in the presence and the absence of toxin B-YFP, and the relative amounts of Rop4 G15V pulled down using MBP-GBD were determined (Figure 2d). Whereas Rop4 G15V was efficiently precipitated by MBP-GBD, co-expression with toxin B abolished recovery of Rop4 G15V in binding assays, indicating that the toxin efficiently glucosylated Rops *in planta* thereby blocking interaction with the effector.

CNF1 in tobacco does not trigger cell death

Over-expression of constitutively active Rop GTPases typically causes elevated ROS levels and a hypersensitive-like response (HR) in different plant species, including

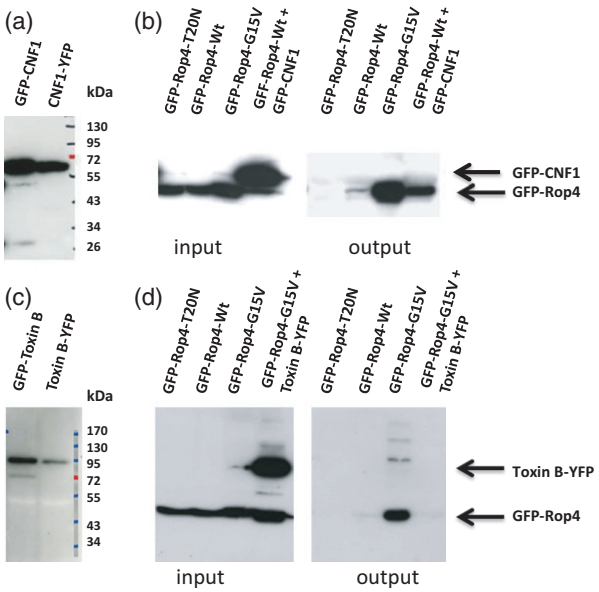

Figure 2. Modification of Rop GTPase by CNF1 and toxin B *in planta*.
(a) Immunoblot of extracts from *Nicotiana benthamiana* leaves expressing GFP-CNF1 and CNF1-YFP.
(b) CNF1 activates AtRop4 *in planta*. Pull-down assay of leaf extracts from single transformation with dominant negative (DN) GFP-Rop4 T20N, GFP-Rop wildtype (Wt), constitutively active (CA) GFP-Rop G15V constructs, and from double transformations with GFP-Rop Wt and GFP-CNF1 constructs.
(c) Immunoblot of extracts from *N. benthamiana* leaves expressing GFP-toxin B and toxin B-YFP. (d) Toxin B inactivates AtRop4 *in planta*. Pull-down assays on leaf extracts from single transformations with DN GFP-Rop4 T20N, GFP-Rop4 Wt, CA GFP-Rop4 G15V constructs, and from double transformation with CA GFP-Rop4 G15V and toxin B-YFP constructs. GFP immunoblot of 10% SDS-PAGE gel loaded with input and output samples as indicated. Molecular weight marker as shown.

N. benthamiana, in case of constitutively active rice OsRac1 (Kawano *et al.*, 2010b). Transient expression of CA-GFP-Rop4 in *N. benthamiana* also caused cell death, while dominant negative GFP-Rop4 (e.g. Rop4-T20N) and Wt GFP-Rop4 did not. Although GFP-CNF1 and CNF1-YFP were expressed efficiently, no cell death resulted unless CNF1 was expressed in combination with GFP-Rop4 Wt (Figure 3). Use of a highly efficient expression system based on amplification of constructs corresponding to pro-viral RNAs in *N. benthamiana* (Methods S1) resulted in macroscopic HR-like responses for untagged Wt Rop4, untagged constitutively active Rop4-G15V and constitutively active GFP-Rop4-G15V, but not for GFP-CNF1 transformation alone (Figure S6). The HR-like response triggered by higher expression levels of untagged Arabidopsis Rop4 Wt in this system might originate from the small subpopulation of GTP-bound molecules. Together, these results indicate that ectopic OX of constitutively active Arabidopsis Rops triggers cell death, whereas activation of endogenous tobacco Rops is not sufficient.

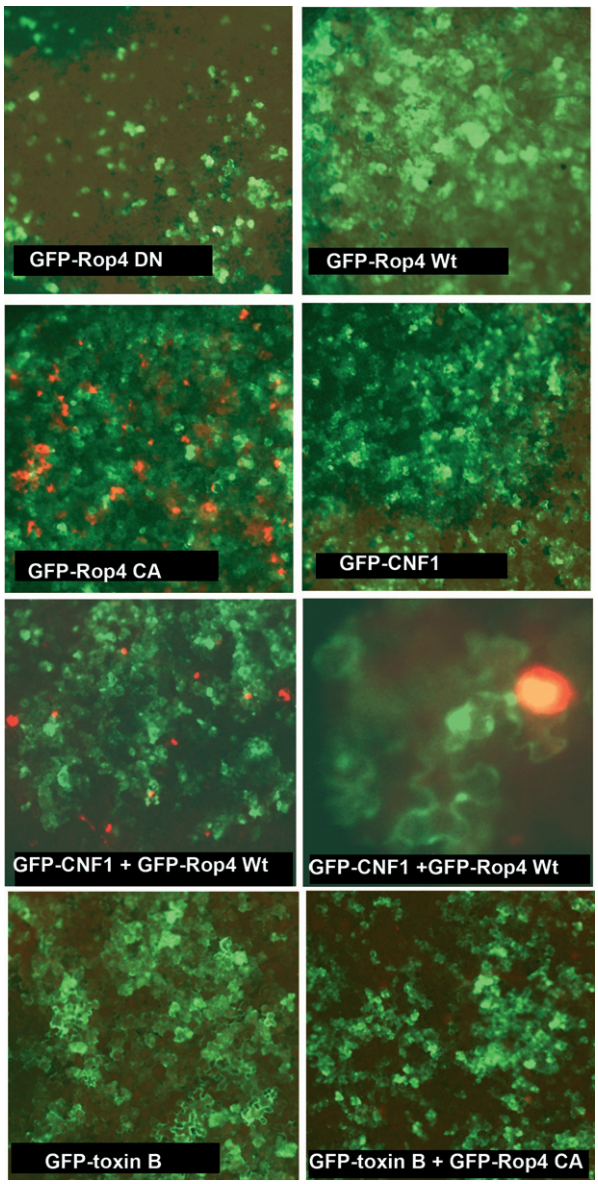

Figure 3. CNF1 activates and toxin B blocks Rop signalling-dependent cell death in *Nicotiana benthamiana* leaves.
Epifluorescence microscope images of leaf surfaces showing GFP-fusion proteins (green) and dead cell autofluorescence (red) for transformations with GFP-toxin and GFP-Rop4 constructs as indicated (DN, dominant negative mutation T20N; CA, constitutively active mutation G15V). The third row panels left and right (5× close up) show epidermal pavement cells and red dead cell in leaves co-expressing GFP-Rop4 and GFP-CNF1.

Toxin B in tobacco prevents cell death caused by over-expression of constitutively active AtRop4

To evaluate the efficacy of toxin B in blocking Rop GTPase signalling, we made use of the HR triggered by OX of constitutively active Rops in *N. benthamiana*. When either GFP-toxin B or toxin B-YFP were co-expressed with constitutively active GFP-Rop4 G15V, cell death was completely prevented (Figure 3), indicating that toxin B modified most

of the overabundant Rops, in agreement with the result from Rop-GTP-binding assays using MBP-GBD.

CNF1 and toxin B inhibit polar morphogenesis of leaf epidermal cells in transgenic Arabidopsis

Developing leaf epidermal cells undergo Rop2/4- and Rop6-dependent morphogenesis, finally resulting in cells with shapes like jigsaw puzzle pieces (Fu *et al.*, 2009; Xu *et al.*, 2010). A *Rop2*::GFP-Rop4 construct illustrates the transient nature of *Rop2* gene expression in Arabidopsis leaves, coinciding with cellular morphogenesis and early expansion of epidermal cells (Figure 4). *Rop2* was also expressed in hydathodes, stipules, root meristem, trichoblasts and root xylem, indicating a more general function for the Rop2 isoform, and pointing to additional developmental processes which might be sensitive to toxin transgene expression. Interference with Rop-dependent morphogenesis of leaf epidermal cells is a typical consequence of OX of constitutively active and dominant negative Rop GTPases (Fu *et al.*, 2002). Epidermal cell shaping thus represented a good system for testing the activity of the toxins *in planta*, using fluorescent protein fusions under control of the 35S promoter. To evaluate the specific effects of both toxins, several transgenic lines over-expressing Wt Rop, mutant Rops and the negative regulator RopGAP2 were generated in parallel. Transgenic Arabidopsis plants that expressed either GFP-CNF1 or CNF1-YFP in leaf epidermis had apolar epidermal pavement cell shapes (12 and 17 independent T_1 mature plants, respectively), as shown for cotyledons of the T_2 generation (Figure 5). Over-expression of constitutively active GFP-Rop4 also resulted in apolar pavement cells, although with slightly more residual lobes than in case of CNF1 expression. The GFP-toxin B and toxin B-YFP expressing plants produced apolar leaf epidermal cells (20 and 8 independent T_1 mature plants, respectively), as shown for the cotyledons of the T_2 generation (Figure 5). In Arabidopsis epidermal pavement cells, toxin B-YFP was targeted to motile intracellular compartments which may be related to the presence of an N-terminal anionic lipid-binding site in the cytosolic fragment of toxin B (Mesmin *et al.*, 2004). Over-expression dominant negative GFP-Rop4 (eight independent lines) developed Wt-like epidermal cells in cotyledons (Figure 5), and much less indented, more elongated cells compared with Wt in mature leaves (Figure S7). Over-expression of GFP-RopGAP2 resulted in wider necks and shorter lobes in cotyledon epidermal cells, suggesting that Rop signalling was partially blocked.

Toxin B inhibits root hair formation, trichome branching and causes lethality and dwarf growth in transgenic Arabidopsis

Root hair morphogenesis is controlled by Rop GTPases and proceeds through polar localization of Rops in membrane patches of root hair-forming cells before budding, and during budding and tip growth (Molendijk *et al.*, 2001; Jones *et al.*, 2002). Expression of constitutively active Rops may cause swelling during tip growth of root hairs (Molendijk *et al.*, 2001; Jones *et al.*, 2002; Tao *et al.*, 2002). In contrast, trichomes do not grow by tip growth but by

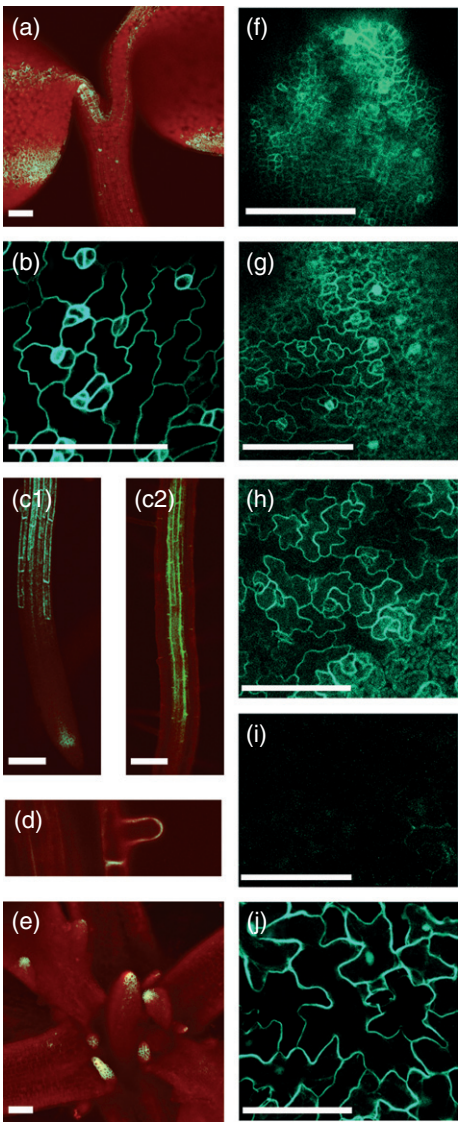

Figure 4. Expression pattern of Rop2::GFP-Rop4 promoter reporter construct in Arabidopsis.
(a)–(d) Three-day-old seedlings. (e)–(i) Twelve-day-old seedlings. Cotyledons with adaxial and abaxial expression (side view, single confocal plane) (a), adaxial epidermis of cotyledon with pavement cell and guard cells (b), root tip with positive trichoblasts in differentiation zone and positive cells around quiescent centre (c1), mature root with positive xylem (c2), growing root hair with tip-localized GFP-Rop4 (d), leaf primordia (top view, single confocal plane) with higher level expression in hydathodes, tips of leaf primordia, and stipules (e). Epidermal surface of growing leaves (successive nos 1,3,5,7) showing transient expression during epidermal morphogenesis and expansion phases (f,g,h,i). A mature leaf expressing a dexamethasone inducible GFP-Rop4 wt transgene shows finished cell shapes for comparison (j). Confocal microscope images; size bars 100 μm.

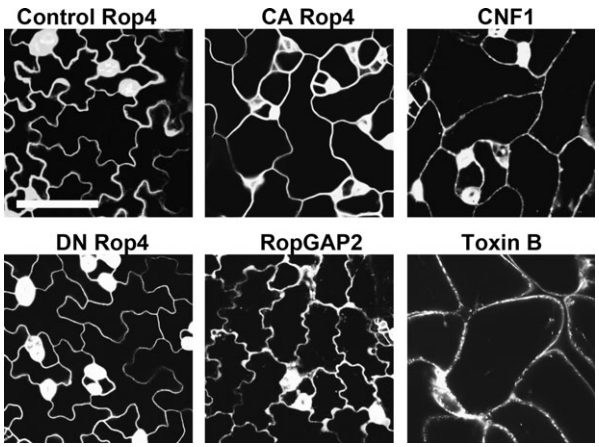

Figure 5. CNF1 and toxin B inhibit polar morphogenesis of leaf epidermal cells in Arabidopsis.
Confocal microscope images of cotyledon adaxial surfaces of 5-day-old seedlings expressing fluorescent protein fusions as indicated. Control Rop4, epidermal cells after overnight expression of a dexamethasone inducible GFP-Rop4 wt transgene showing finished cell shapes for comparison. CA Rop4, GFP-Rop4 G15V; CNF1, GFP-CNF1; DN Rop4, GFP-Rop4 T20N; RopGAP2, GFP-RopGAP2; Toxin B, toxin-B-YFP. All images at same magnification, size bar 100 μm.

diffuse growth (Schwab *et al.*, 2003), and a possible role for Rops in trichome formation has not yet been directly addressed. Therefore, the effect of toxin transgene expression was analysed for both cell types in Arabidopsis.

Most T_1 GFP-toxin B seedlings grew to Wt-sized fertile plants, while T_1 toxin B-YFP either died on soil or developed into small plants with serrated or narrow curly leaves, which were either infertile or yielded few seeds. Only 4 out of 50 T_1 toxin B-YFP plants could be recovered in the T_2 generation, and therefore T_1 primary transformants and T_2 seedlings were both analysed. Most toxin B-expressing T_1 and T_2 seedlings only showed a weak fluorescent signal, but were easily recognizable by the presence of elongated cotyledons and rounded epidermal pavement cell shapes. On average, 75% of independent toxin B-YFP (n = 77) and 35% of independent GFP-toxin B seedlings (n = 106) had very short or absent root hairs (Figure 6). Trichomes of T_1 toxin B-YFP plants (n = 7) on average had two instead of three branches, and both T_1 and T_2 seedlings (three of four lines) developed unbranched trichomes on primary leaves (Figure 6). In several GFP-toxin B lines, segregation of intermediate root hair and plant size phenotypes was observed in T_2 progeny, indicating a toxin B dosage effect. Toxin B-YFP plants generally showed more severe phenotypes than GFP-toxin B plants, suggesting that a C-terminal tag is preferable. Both GFP-CNF1 and CNF1-YFP T_2 seedlings were strongly fluorescent and had apolar pavement cells and elongated cotyledons (17 lines), similar to OX constitutively active GFP-Rop4 seedlings. T_2 seedlings that expressed CNF1 in growing root hairs (eight lines) had normal root hair shapes, while OX constitutively active GFP-Rop4 seedlings had only slightly

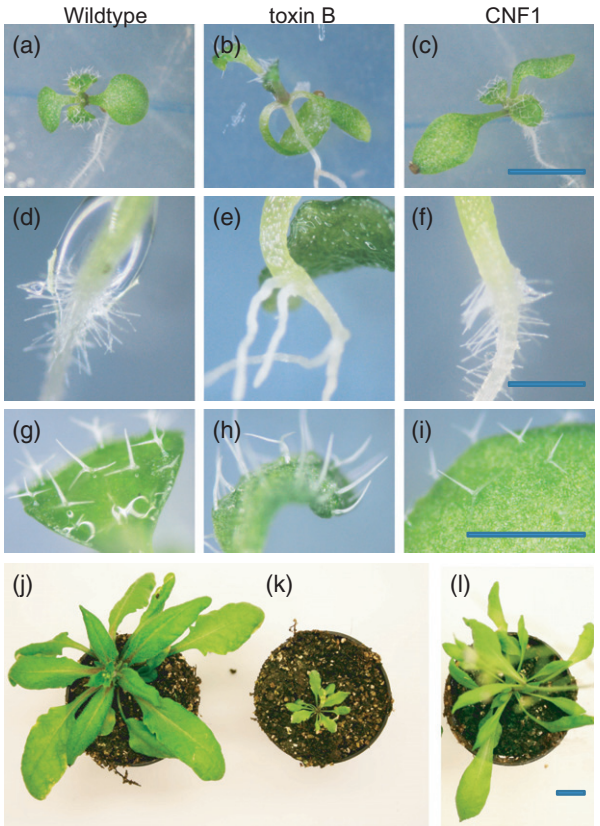

Figure 6. Phenotypes of *Arabidopsis thaliana* plants expressing toxin B and CNF1 under the 35S promoter.
Wildtype plants (a,d,g,j), toxin B-YFP plants (b,e,h,k), CNF1-YFP plants (c,f,i,l). Overview of 11-day-old seedlings, size bar 5 mm (a)–(c). Elongated cotyledons of T_1 35S toxin-B YFP (b) and T_2 35S CNF1-YFP transgenic seedlings (c). Root hairs at the hypocotyl root junction of 11-day-old seedlings, size bar 1 mm (d)–(f). Absent root hairs of T_1 35S toxin B-YFP and normal root hairs of T_2 35S CNF1-YFP transgenic seedlings. Primary leaves of 11-day-old seedlings, size bar 1 mm (g)–(i). Unbranched trichomes for T_1 35S toxin B-YFP transgenic seedling (h). Overview of plants at start of bolting (j)–(l), size bar 1 cm. Dwarf growth and serrated leaves for T_2 35S toxin B-YFP (k), and normal size and narrow curly leaves for T_2 35S CNF1-YFP transgenic plants (l).

swollen root hair tips (three lines) (Figure S8). CNF1 transformants developed into plants of normal size with curly and elongated leaf shapes (Figure 6) as characteristic for OX constitutively active Rop2 plants (Li *et al.*, 2001) and OX constitutively active GFP-Rop4 plants (three lines).

Enlarged root apical meristem in toxin B-expressing seedlings

Rop GTPases are relatively abundantly expressed in the root apical meristem (Molendijk *et al.*, 2001) and specifically so in case of Rop2 (Li *et al.*, 2005). Rops have been suggested to function in CLAVATA-like signalling, which balances differentiation and stem cell maintenance in shoot and root meristems (Trotochaud *et al.*, 1999; Miwa *et al.*, 2009). RNAi of either Rop interactor ICR1 or root quiescent centre-expressed Arabidopsis RopGEF7 results in loss of the root

stem cell population (Lavy *et al.*, 2007; Chen *et al.*, 2011). In the case of toxin B lines, we did not observe collapse of the root apical meristem (result not shown), and further examined a possible effect of toxin B expression on meristem and elongation zone sizes in roots of 5-day old T_3 seedlings. Both GFP-toxin B and toxin B-YFP expressing seedlings had longer root meristematic zones (Figure 7), while elongation zone lengths were similar to Wt with the exception of GFP-toxin B line N17. Root lengths were normal and root epidermal cells, including trichoblasts lacking root hairs, reached Wt length (not shown). These results might suggest that the absence or down-regulation of functional Rops in root meristem by using toxin B affects the balance between stem cell maintenance and cell differentiation.

DISCUSSION

Toxin CNF1 and toxin B from the bacterial pathogens *E. coli* and *C. difficile* specifically target Rho GTPases of animal host cells. Here we show that the plant Rho GTPase AtRop4 is an efficient *in vitro* substrate for the bacterial toxins CNF1, CNF3 and toxin B in both GDP- and GTP-bound conformations. Toxin B and CNF1 modify both HsRac1 and HsRhoA, with RhoA being more distantly related to Rac1 than are plant Rop GTPases. High amino acid sequence conservation exists between AtRop4, HsRac1 and HsRhoA for the switch I and II domains containing toxin B target threonine-38 and CNF1 target glutamine-64, respectively. Switch I and switch II amino acid sequences are also almost completely identical between all known Rops including those from *Physcomitrella*, strongly indicating that AtRop4 can be representative for all Rops as a functional toxin substrate.

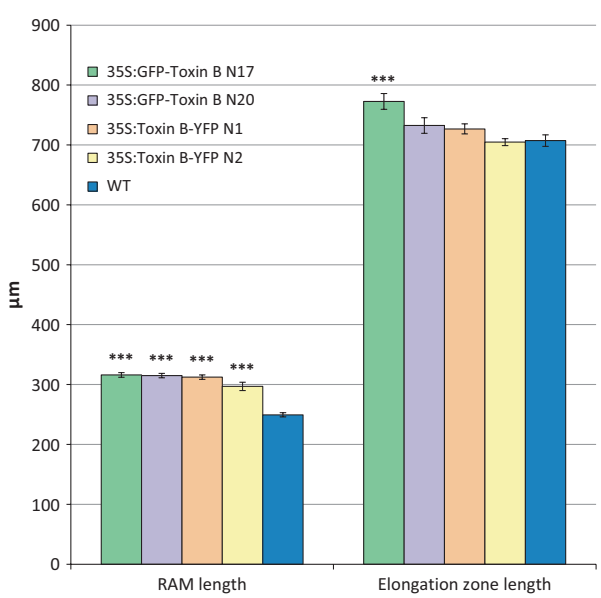

Figure 7. Expression of toxin B increases the length of the root apical meristem (RAM).
The length of the RAM and elongation zone of root tips of 5-day old T_3 seedlings from four toxin B lines as indicated. Standard errors (bars) and significant differences from wildtype (WT) according to Student's *t*-test as indicated by asterisks (***P-value < 0.001).

We made use of two Rop-dependent processes, namely the HR-like response in *N. benthamiana* upon overexpression of constitutively active Rops and epidermal cell morphogenesis in Arabidopsis, to demonstrate that the catalytic domains encoded by bacterial CNF1 and synthetic toxin B genes are active *in planta*, thereby establishing CNF1 and toxin B transgenes as novel tools to study and engineer Rop signalling. CNF1 is predicted to constitutively activate endogenous Rops irreversibly, avoiding possible side-effects associated with overexpression of constitutively active *Rop* transgenes. In animal cells, CNF1-activated Rho proteins are subsequently ubiquitinated, and almost completely depleted after 5 h of treatment with CNF1 (Doye *et al.*, 2002). In contrast, CNF1 functionally activated co-expressed Rop4 protein in tobacco over much longer periods, while transgenic Arabidopsis plants expressing CNF1 mimicked the OX CA Rop2/4 phenotype, indicating that the final outcome of the action of CNF1 in plants is an increase in Rop activity. Inactivation of Rop signalling by toxin B represents a useful alternative to RNAi and RNA antisense approaches if the aim is to target the complete Rop multigene family in different cell types or plant species. This might be especially useful in cases where expression data are incomplete, or to exclude compensatory *Rop* gene expression. Moreover RNAi silencing is not easily reversible during development, whereas regulated expression of toxin B has the potential to inactivate Rops at specific time windows. For example, expressing either toxin B or CNF1 transgenes in the *Clv1* expression domain and subsequent testing for clavata and wuschel-like phenotypes might be a promising method to delineate the role of unidentified Rop(s) in CLAVATA signalling in shoot apical meristem (Trotochaud *et al.*, 1999).

Since CA Rac1 Q61L (corresponding to Q64L in Rop4) is non-glucosylatable by toxin B (Belyi and Aktories, 2010), there would also be the possibility of introducing specific Q64L mutant *Rop* genes into a toxin B genetic background for Rop isoform-specific analysis. Another experimental option would be using either CNF1 or toxin B to target Rops only at specific cellular locations such as the nucleus or different plasma membrane domains using the appropriate localization signals.

Rop GTPases and the hypersensitive response

Overexpression of constitutively active Rop causes increased production of ROS and cell death in rice protoplasts as well as HR-like responses in rice transgenic plants and transiently transformed tobacco (Kawano *et al.*, 2010a,b). Rop GTPase activation is coupled via RopGEFs to PAMP receptor kinases (Kawasaki *et al.*, 2009), and Rops may mediate plant responses triggered by pathogen effectors (Kawano *et al.*, 2010b). Our results indicated that the

HR-like responses caused by transient OX of constitutively active Arabidopsis Rops in *N. benthamiana* leaves depended on unnaturally high levels of Rop protein, since activation of endogenous Rops by CNF1 did not result in cell death. We think that triggering of cell death in this system is primarily due to highly elevated ROS levels caused by OX constitutively active Rop, since several treatments such as high light intensity or ozone which increase ROS levels in plants are also known to cause HR-like tissue lesions. It is likely that besides the activation of Rops, other pathways are critical in induction of the HR in plant pathogen defence, as Rops also function more widely in plant growth and development. Future use of toxins CNF1 and toxin B will help to identify Rop-dependent events in effector and PAMP-triggered responses in *N. benthamiana* and Arabidopsis. For example, toxin B might be used to determine a possible functional requirement of Rops for ROS production or MAPK activation upon flg22 stimulation of FLS2 kinase signalling in transgenic Arabidopsis (Asai *et al.*, 2002; Mersmann *et al.*, 2010).

Inhibition by CNF1 and toxin B of polar morphogenesis of epidermal pavement cells

Polar morphogenesis of leaf epidermal cells depends on antagonistic Rop2/4 and Rop6 pathways. Rop2 and Rop4 promote cortical diffuse actin and lobe formation via their common effector RIC4, while Rop6 promotes cortical microtubule arrays in indentations via its effector RIC1 (Fu *et al.*, 2002, 2005, 2009). CNF1 completely inhibited formation of lobes and indentations, indicating that proper morphogenesis strictly depended on GTP/GDP cycling of the Rops, which might be necessary to establish local Rop2/4 maxima and Rop6 antagonism. The elongated cell shapes observed for OX dominant negative Rop4 were similar to knock-out Rop4 / Rop2 RNAi and OX RIC1 cells (Fu *et al.*, 2005), which might suggest that Rop2/4 antagonistic Rop6/RIC1 function (Fu *et al.*, 2009) is promoted in OX dominant negative Rop4 cells. Dominant negative Rop acts by blocking RopGEFs which generally may show specificity towards distinct Rop isoforms (Fricke and Berken, 2009). Expression of toxin B caused much more apolar cell shapes compared with dominant negative Rop4. Possibly, different isoform selective RopGEFs are involved in the shaping of epidermal cells, which might limit the effects of OX dominant negative Rop4 to isoform-specific pathways. Trichome branching, but not stalk expansion, was inhibited in toxin B-YFP expressing lines, suggesting that the shift in microtubule orientation that occurs during branching (Mathur and Chua, 2000) might require Rop action, rather than polar diffuse growth itself.

Toxin B, but not CNF1, inhibits root hair tip growth

The crinkly family receptor-like kinase Feronia activates AtRop2 via RopGEF1 and regulates Rop-mediated ROS production in root hair tip growth in Arabidopsis (Duan *et al.*, 2010). Overexpression of RopGEFs causes branching and bulging of root hairs and pollen tubes (Gu *et al.*, 2006; Zhang and McCormick, 2007; Won *et al.*, 2009). Unexpectedly, expression of CNF1 in root hair forming cells did not visibly affect root hair shapes, suggesting that the broadening and establishment of local Rop maxima underlying root hair bulging and branching phenotypes, respectively, requires a functional GTP/GDP cycle, possibly for positive feedback. Toxin B inhibited polar morphogenesis of Arabidopsis root hairs more effectively than dominant negative AtRop2 (Jones *et al.*, 2002), resulting in a complete absence of root hairs, as has previously been reported for tobacco NtRac1 RNAi and barley HvRacB RNAi plants (Tao *et al.*, 2002; Hoefle *et al.*, 2011). Toxin B severely affected root hair formation, but not trichoblast cell length (results not shown), suggesting that elongation growth is relatively independent of Rop. We anticipate that the precise information that will be obtained from studies using Rop-modifying bacterial toxins can be useful for further modelling of Rop-dependent tip growth.

Dwarf growth as a consequence of blocking Rop signalling by toxin B

Rop isoform-limited RNA silencing, using either the maize ubiquitin1 promoter in rice and barley or the 35S promoter in tobacco, results in shorter plants (Schiene *et al.*, 2000; Miki *et al.*, 2005; Hoefle *et al.*, 2011), as does expression of dominant negative AtRop2 in Arabidopsis (Li *et al.*, 2001). Using the alternative method of toxin B expression directed by the 35S promoter, we observed a more severe dwarf growth phenotype in Arabidopsis. Dwarf growth of Rop-compromised plants might be a direct consequence of a lack of Rop-dependent regulation of cytoskeletal dynamics or ROS production in cellular morphogenesis. Alternatively, less precisely known functions of Rops in transducing growth hormonal inputs to changes in gene expression, such as reported for auxin (Tao *et al.*, 2002, 2005) or in auxin–Rop crosstalk (Wu *et al.*, 2011) might have been turned off. Here, too, we think that the bacterial toxin transgenes will be important tools for increasing our understanding of auxin signalling via Rop GTPases.

EXPERIMENTAL PROCEDURES

Rop4 substrate preparation and nucleotide loading

The cDNAs corresponding to the first 179 amino acids of AtRop4 and AtRop4 G15V were cloned in-frame into pET45b and were transformed in the *E. coli* BL21 (DE3) strain for protein expression. Bacterial cells were lysed by sonication in a buffer, containing 50 mM TRIS-HCl (pH 8.4), 300 mM NaCl, 10 mM $MgCl_2$ 10 μg/ml DNAse I, 100 μM of either GDP or GTP, 1 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitors. Cell lysate, after preclearing by centrifugation at 40,000 ***g*** for 30 min at 4°C, was applied on an Immobilized Metal-Affinity Chromatography (IMAC)

column pre-charged with Ni^{2+} (His Trap® HP column, GE Healthcare, http://www.gehealthcare.com/). Column-bound His(6×)-Rop4 protein was eluted in non-denaturing conditions according to the manufacturer's instructions. A second purification step was performed using a Superdex® 200 gel filtration column (GE Healthcare) and yielded the purified protein (Figure S1).

To load the GTPases with specific nucleotide analogues, the protein sample was changed to a buffer without $MgCl_2$, supplemented with 10 mM EDTA and 400 μM nucleotide analogues and incubated for 30 min at 21°C. Subsequently, $MgCl_2$ was added to a final concentration of 20 mM, incubated for a further 15–20 min and snap-frozen in liquid N_2.

CNF1 and CNF3 assay

Recombinant purified mammalian RhoA or plant His(6×)-Rop4 were incubated with GST-CNF1 or GST-CNF3 in CNF1-reaction buffer [20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA] for 2 h at 37°C. For the negative control GTPases were incubated with a catalytically inactive form of CNF3 (GST-CNF3-C866S) or without the toxin. The molar ratio of CNF: GTPase was 1–5. Thereafter, the modification of the GTPase by CNFs was analysed by urea-SDS-PAGE, deamidated RhoA and Rop4 shift to higher molecular weight.

Toxin B assay

For the glucosylation assays, native toxin B was purified from *C. difficile* VPI 10463 as described (Just *et al.*, 1995). Approximately 10 ng of toxin and 1 μg of GST-Rac1 or His(6×)-AtRop4 proteins were added to a reaction mixture containing 10 μM UDP [^{14}C]glucose in a reaction buffer supplemented with 50 mM HEPES pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$ and 100 μg ml^{-1} bovine serum albumin (Just *et al.*, 1994, 1995). The reaction was carried out in a volume of 20 μl, for 30 min at 30°C. The enzymatic reaction was stopped by adding Laemmli loading buffer and analysed by SDS-PAGE and autoradiography.

Toxin C3 assay

The ADP ribosylation of human RhoA, human Rac1 or AtRop4 was carried out by the addition of 1.5 μg of either protein to a reaction mixture containing 50 nM C3 toxin, 50 μM [adenylate-^{32}P] NAD in ADP-ribosylation reaction buffer and incubated for 10 min at 37°C. Reactions were stopped with Laemmli loading buffer, and modified proteins were separated on 12.5% SDS-PAGE gels and analysed by autoradiography.

Transient *N. benthamiana* transformations using 35S plant expression cassette

Bacterial DNA encoding the catalytic domains of CNF and codon optimized synthetic DNA encoding the catalytic domain of toxin B (Eurofins MWG Operon) were transferred to pCK-based plasmids designed for either N-terminal GFP or C-terminal YFP tagging, and from there to binary vector pS001. Synthetic toxin B was also cloned in the GATEWAY destination vector pH7WGF2,0 (Plant Systems Biology, VIB-Ghent University, Ghent, Belgium) for N-terminal GFP tagging. Arabidopsis Rop4 Wt, Rop4 G15V and Rop4 T20N encoding cDNAs (36) were transferred to plant expression vector pCATGFP-no stop for N-terminal GFP tagging, and from there to binary vector pS001. Cultures of *Agrobacterium tumefaciens* GV3101 pMP90 carrying pS001-based constructs and *A. tumefaciens* carrying pEAQ-HT expressing the suppressor of silencing P19 were co-infiltrated into *N. benthamiana* plants using either vacuum infiltration or injection according to standard procedures. Transformed tobacco leaves at 5 days post infiltration were observed with an Axio Imager A1 epifluorescence microscope equipped with AxioCamMRc camera (Zeiss, http://www.zeiss.com/), using GFP and rhodamine filters for visualizing cells expressing GFP fusion protein and red autofluorescent dead cells, respectively. The GFP filter and red filter images were merged using Adobe Photoshop 6.0.

Assay of Rop GTPase activation status

Rop-GTP pull-down assays were performed using affinity-purified maltose-binding protein (MBP) tagged GBD of Arabidopsis RopGAP2. The cDNA encoding the GBD was cloned in pMAL-c2 (New England Biolabs, http://www.neb.com/) and MBP-GBD fusion protein purified according to standard procedures. *N. benthamiana* leaves transiently expressing GFP-Rop proteins were ground up in liquid nitrogen. Ten millilitres of leaf powder was mixed, using a cold mortar and pestle, with 20 ml of cold extraction buffer (50 mM TRIS Cl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) containing plant protease inhibitor cocktail (Sigma, http://www.sigmaaldrich.com/), 0.5 mM PMSF and 10 μg ml^{-1} leupeptin I. Extracts were passed through Miracloth (Calbiochem, http://www.merckmillipore.com), and centrifuged for 20 min at 4°C at 12 000 ***g*** rpm in a Sorvall SS34 rotor (DuPont, http://www.dupont.com/). Aliquots were taken from supernatants and 2× SDS loading buffer added to obtain input samples. To 15 ml supernatant, 200 μl of settled amylose affinity resin (New England Biolabs), containing MBP-GBD (protein concentration approximately 1 mg ml^{-1} settled resin) was added and reactions were incubated with rotation for 2 h at 4°C. After incubation, amylose affinity resin was washed once in extraction buffer with protease inhibitors, and three times in extraction buffer without protease inhibitors and without Triton X-100. Then 2× SDS-PAGE loading buffer was added to washed resin pellets and the output samples were processed for immunoblot analysis using GFP antibodies (Roche, http://www.roche.com/), and an ECL detection kit (Thermo Scientific, http://www.thermoscientific.com/).

Generation of transgenic Arabidopsis lines

Cultures of *A. tumefaciens* GV3101 pMP90 carrying 35S promoter pS001 constructs or a 35S promoter pH7WGF2,0 construct in case of GFP-toxin B, GFP-Rop4 Wt, GFP-Rop4 G15V, GFP-Rop4 T20N, GFP-RopGAP2 (At4g03100), CNF1-YFP, GFP-CNF1, toxin B-YFP and GFP-toxin B were used to transform *Arabidopsis thaliana* plants using the floral dip method. The 2.3 kb DNA region between stop codon TGA of At1g20080 and start ATG of *Rop2* (At1g20090) was obtained by genomic PCR and used as the *Rop2* promoter to construct Rop2::GFP-Rop4 Wt in binary vector pSulkan. T_1 plants were obtained using sulphonamide or hygromycin selection in case of GFP-toxin B and screened by fluorescence microscopy. Dexamethasone inducible GFP-Rop4 Wt transgenic lines have been described previously (Molendijk *et al.*, 2001). For confocal microscopy of leaf epidermal cells, a Zeiss LSM 510 with inverted Axiovert 200 microscope was used with LSM software (Zeiss).

Root apical meristem and elongation zone analysis

Wildtype Arabidopsis (Columbia 0) and four transgenic 35S toxin B lines (nos N17 and N20 in the case of GFP-toxin B and nos N1 and N2 in the case of toxin B-YFP) were used for root zone measurements. Surface-sterilized seeds were plated on agar medium

containing ½ MS salts with vitamins (Duchefa, http://www.duchefa.com/) and 0.5 mg ml^{-1} 2-(*N*-morpholine)-ethanesulphonic acid, pH 5.7. Plated seeds were incubated at 21°C for 6 h, then incubated at 4°C for 14 h, and transferred to a growth chamber at 23°C with a 16 h/8 h day/night cycle and a light intensity of 100 kcal m^{-2} sec^{-1}. Five-day old seedlings were fixed in 2% formaldehyde solution for 30 min. After clearing in methanol and chloral hydrate, seedlings were mounted on microscopic slides in 50% glycerol and observed using a Zeiss Axiovert microscope with a 20× objective. Root zone length measurements were made using AXIOVISION (Zeiss) image processing software. Positive seedlings were selected by cotyledon and root hair phenotype as well as by YFP/GFP fluorescence. The experiment was replicated twice with seedlings from T_2 and non-segregating T_3 generations of four independent toxin B lines with similar results.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Purification of Arabidopsis Rop4.

Figure S2. Sequence alignment of Rho GTPases.

Figure S3. C3 assay on Rho GTPases.

Figure S4. CNF1 assay on GDP and GTP Rops.

Figure S5. Synthetic DNA sequence of catalytic domain of toxin B.

Figure S6. CNF1 and the hypersensitive response in tobacco.

Figure S7. Epidermal cell shapes of DN-Rop4 and toxin B plants.

Figure S8. Root hairs of CA-Rop4 and CNF1 plants.

Methods S1. Tobacco transformation using proviral vectors.

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