Bacterial toxins: a novel tool to study plant RAC GTPase signaling

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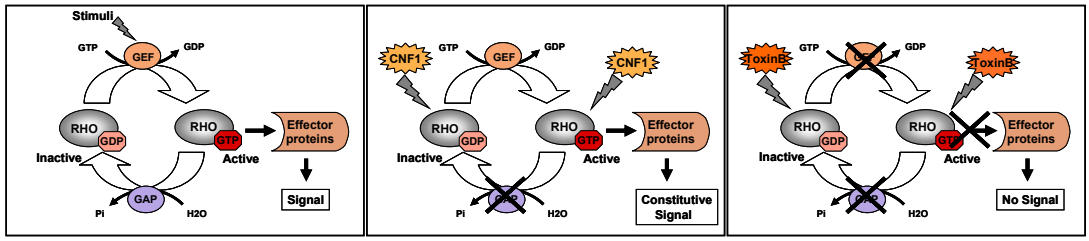
Introduction

Cycling between an active GTP-bound state and an inactive GDP-bound state, RHO family of monomeric GTPases play a very important role in signal transduction. The proper functioning of these GTPases are tightly regulated by Guanine nucleotide exchange factors (GEF) and GTPase activating proteins (GAP). One class of bacterial toxins can modify RHO family GTPases to render them either constitutively active or constitutively inactive, and thereby manipulate the function of the intoxicated cells without directly killing them. The CNF1 toxin from pathogenic strains of *Escherichia coli* deamidates a glutamine amino acid in the active site of RHO GTPases. The deamidation causes loss of the ability to hydrolyze bound GTP, resulting in constitutive activation of GTPase. The toxin A/B from *Clostridium difficile* glucosylate a threonine residue in the effector loop region of RHO GTPases. The glucosylated GTPase functions as constitutively inactive, due to inability to interact with regulators or effectors.

The property of *Escherichia coli* toxin CNF1 to constitutive activate and of *Clostridium difficile* toxin A/B to constitutively inactivate the RHO family members has been widely used to study the RHO signalling in animal cells. Here we report the ability of CNF1 and toxin A/B to enzymatically modify the Arabidopsis RAC GTPase and expression of their catalytic domains, which are sufficient for enzymatic activity, in the plant cell.



Protein Sequence analysis suggests Arabidopsis RAC GTPase modification by toxins A/B and CNF1

Amino acid sequence comparisons between Arabidopsis RACs, human Rac1 and RhoA suggested that the AtRAC proteins would function as substrate for Clostridial Toxins A and B and E.coli toxin CNF1, as the amino acids surrounding the T38 and Q64 residues of AtRAC5, corresponding to T37 and Q63 of Rho A, are well conserved.

Target of Toxin A/B ▼ Target of CNF1 ▼

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AtRAC8   ---NKFPTDYIPTVFDNFSVNVVVEGITVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC10  ---NKFPTDYIPTVFDNFSANVVVEGTTVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC7   ---NKFPTDYIPTVFDNFSANVAVDGQIVNLGLWDTAGQEDYSRLRPLSYRGA----
AtRAC1   ---NTFPTDYVPTVFDNFSANVVVNGATVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC6   ---NTFPTDYVPTVFDNFSANVVVNGATVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC11  ---NTFPTDYVPTVFDNFSANVVVNGSTVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC3   ---NTFPTDYVPTVFDNFSANVIVDGNTINLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC4   ---NTFPTDYVPTVFDNFSANVVVDGNTVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC5   ---NTFPTDYVPTVFDNFSANVVVDGNTVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC2   ---NTFPTDYVPTVFDNFSANVVVDGSTVNLGLWDTAGQEDYNRLRPLSYRGA----
AtRAC9   ---NTFPTDYVPTVFDNFNANVLVDGKTVNLGLWDTAGQEDYNRVRPLSYRGA----
HsRac1   ---NAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPLSYPQT----
HsRhoA   ---DQFPEVYVPTVFENYVADIEVDGKQVELALWDTAGQEDYDRLRPLSYPDT----
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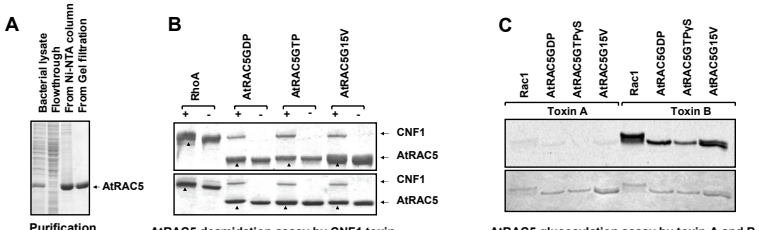
Biochemical assays to check Arabidopsis RAC GTPases modification by toxins A/B and CNF1

To check the activity of toxins A,B and CNF1, Arabidopsis RAC5 GTPase was expressed in *E. coli*, purified to homogeneity (A) and was used as a substrate for biochemical assays.

Deamidation of RHO GTPases by *E. coli* CNF1 can be observed by a shift in the mobility of the modified protein on SDS-PAGE [1]. The shift in electrophoretic mobility of CNF1 treated AtRAC5 proteins indicates that CNF1 can deamidate the AtRAC5 *in vitro.*

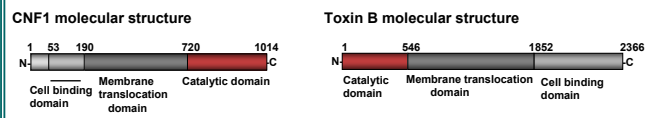
Toxins A and B from *C. difficile*, were able to glucosylate the Arabidopsis RAC5 GTPase, as detected by incorporation of [^{14}C]glucose .



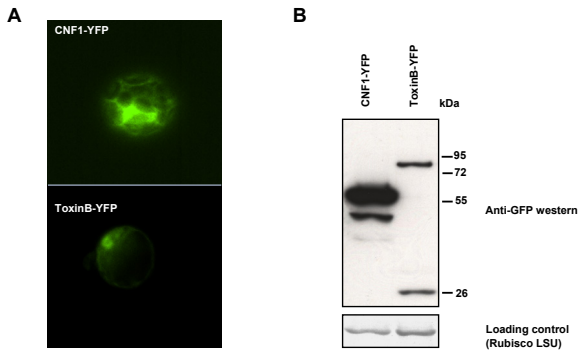
AtRAC5 deamidation assay by CNF1 toxin
(Upper panel and lower panel: Images of two Urea-SDS-PAGE gels (15%) showing a difference in the electrophoretic mobility of CNF1 treated (+) and non-treated (-) RhoA, AtRAC5 proteins. Arrow head indicates the upward shift)

AtRAC5 glucosylation assay by toxin A and B
(upper panel:autoradiograph, lower panel:coomassie stained gel as loading control)

Expression of catalytic domains of toxin B and CNF1 in Arabidopsis Protoplasts



Catalytic domains of both CNF1 and toxin B, which are sufficient for enzymatic reaction both *in vitro* and *in vivo* [2, 3], could be efficiently expressed in Arabidopsis protoplasts as detected by fluorescence microscopy (A) and Western blot analysis (B).



Expression of catalytic domains of toxin B and CNF1 with a C-terminus YFP tag in Arabidopsis protoplasts.
A: Images taken by an epifluorescence microscope. B: Western blot of protoplasts expressing CNF1-YFP and toxin B using anti GFP antibodies.

Summary and Outlook

Using biochemical assays, we have demonstrated the ability of *E. coli* CNF1 and *Clostridium difficile* toxin A/B to enzymatically modify Arabidopsis RAC5 GTPases. Our results also show the efficient expression of the catalytic domains of both toxins in the plant cell. Although, the functionality of toxins expressed in planta remains to be shown, the toxins described here are simple protein molecules, and no modification is required for their functionality, indicating that there is very high possibility for them to be functional in plants.

We plan to use these bacterial toxins as a novel tool to manipulate the activity of endogenous levels of plant RAC GTPases. Therefore, transgenic Arabidopsis plants expressing catalytic domains of the toxins, under the control of an inducible promoter, will be generated and used to study the role of AtRAC family GTPases in key cellular processes.

References

[1]. Flatau, G., Lemichez, E., Gauthier, M., Chardin, P. Paris, S., Florentini, C. and Boquet, P. (1997) Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. Nature 387:729–733.

[2]. Lemichez, E., Flatau, G., Bruzzone, M., Boquet, P.and Gauthier, M. (1997) Molecular localisation of the *Escherichia coli* cytotoxic necrotizing factor CNF1 cell-binding and catalytic domains. Mol. Microbiol. 24:1061–11070.

[3]. Hofmann, F., Busch, C., Prepens, U., Just, I. and Aktories, K. (1997) Localization of the Glucosyltransferase Activity of *Clostridium difficile* Toxin B to the N-terminal Part of the Holotoxin. J. Biol. Chem. 272:11074-11078.