Expression, Purification and *in vitro* Enzyme Activity Assay of Plant Derived GTPase

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[Abstract] Based on gene expression data after biotic stress, the GTPase *RabA4c* has been suggested to regulate pathogen-induced callose biosynthesis in the model organism *Arabidopsis thaliana*. We studied the function of *RabA4c* in its native and dominant negative (dn) isoform. *In planta*, *RabA4c* overexpression prevented penetration of the virulent powdery mildew *Golovinomyces cichoracearum* into epidermal leaf cells. This penetration resistance was caused by enhanced callose deposition at sites of attempted fungal penetration at early time points of infection. By contrast, *RabA4c (dn)* overexpression did not increase callose deposition or penetration resistance.

In this protocol, we describe the expression, purification and activity assay of the heterologously expressed GTPase *RabA4c* from *A. thaliana* based on the publication Ellinger *et al.* (2014). We fused *RabA4c* to the fluorophore mCitrine and expressed this protein in the yeast strain *Pichia pastoris GS115*. For purification of *RabA4c*, we used the *GFP-Trap_A* kit (Chromo Tek) which specifically binds to GFP derivatives like mCitrine. The enzyme activity assay was done by using the *GTPase Assay Kit* from Innova Biosciences. In general, we followed the instructions made by the manufactures.

Materials and Reagents

A. Protein expression
   1. pGAPZ A, B, & C *Pichia pastoris* Expression Vectors (Life Technologies, catalog number: V200-20)
      Note: Currently, it is "Thermo Fisher Scientific, Invitrogen™, catalog number: V200-20".
   2. Zeocin™ (InvivoGen, catalog number: ant-zn-5b) for selection
   3. 1 % yeast extract (Carl Roth GmbH + Co., catalog number: 2363.3)
   4. 2 % peptone (BD, catalog number: 211677)
   5. D(+)-Glucose (Carl Roth GmbH + Co., catalog number: X997.3)
   6. Yeast Peptone Dextrose (YPD) media (see Recipes)

B. Protein purification
   1. Acid washed glass beads (Sigma-Aldrich, catalog number: G 8772)

2. GFP-Trap® (ChromoTek GmbH, catalog number: gta-20)
3. NanoOrange Protein Quantification Kit (Invitrogen, catalog number: N-6666)
   Note: Currently, it is "Thermo Fisher Scientific, Molecular Probes™, catalog number: N-6666".
4. 10 mM Tris (pH 7.5)
5. 50 mM NaCl
6. 0.5 mM EDTA (pH 8.0)
7. 100 mM Glycine (pH 2.5)
8. 1 M Tris (pH 10.4)
9. Dilution buffer (see Recipes)
10. Wash buffer I (see Recipes)
11. Wash buffer II (see Recipes)
12. Elution buffer (see Recipes)

C. GTPase activity assay
   1. GTPase Assay Kit (Innova Biosciences Ltd., catalog number: 602-0121)
   2. Negative control: expressed and purified GFP using *P. pastoris* as expression system (plasmid pGAPZ::eGFP)

Equipment

1. 300 ml flasks
2. Shaker (160 rpm)
3. Centrifuge for 50 ml Falcon tubes (3,220 *x g*)
4. Vortex
5. Tube rotator at 4 °C and room temperature
6. Plate Reader (BioTek Instruments, model: Synergy HTX Multi-Mode Reader)
7. Tabletop centrifuge (cooling)

Procedure

A. Protein expression
   1. Strain: *Pichia pastoris GS115*
   2. Expression vector: pGAPZ
   3. Pre-culture: incubate one colony from a YPD (+ zeocin) plate in 4 ml liquid YPD (+ zeocin) media at 28 °C and 160 rpm for one day.
   4. Main-culture: Incubated 1 ml of the pre-culture in 100 ml fresh YPD (+ zeocin) media at 28 °C and 160 rpm for 3 days.
   5. Strains: *pGAPZ::RabA4c-mCit[NA]/ [DN]*, control *pGAPZ:e:GFP* [NA= native form, DN= dominant negative, please refer to Ellinger *et al.* (2014) for strain generation].

6. Yeast cells were transferred in 50 ml Falcon tubes and harvested by centrifugation (3,220 *x g*, 10 min, 4 °C).
7. Discard the supernatant and wash the cell pellet with sterile ddH_2O.
8. Mechanical lysis: Add an identical volume of sterile acid washed glass beads to the cell pellet and vortex the beads-cell suspension for 30 sec at maximum speed followed by chilling on ice for 30 sec, repeat these steps A8-10 times.
9. Spin down the glass beads at 453 *x g* at 4 °C for 2 min.
10. Transfer the supernatant in a new 2 ml tube, wash the glass beads with 1 ml dilution buffer (see above), centrifuge again and transfer the supernatant also to the 2 ml tube.
11. Separate the cell debris by centrifugation at max speed at 4 °C for 30 min.
12. Transfer the clear supernatant in a new tube and store at 4 °C until starting with protein purification. Store the pellet at -20 °C for SDS-PAGE analysis.

B. Protein purification
1. Equilibrate 25 µl (for one sample) *GFP-Trap_A* agarose beads in dilution buffer (use a 2 ml tube): Wash 3 times with 500 µl ice cold dilution buffer, between the wash steps spin beads down at 719 *x g* at 4 °C for 2 min and discard the supernatant.
2. Add the crude extract to the equilibrate beads and incubate the sample for 4 h at 4 °C under constant mixing (*e.g.* tube rotator).
3. Spin down sample at 719 *x g* at 4 °C for 2 min and carefully remove the supernatant by pipetting. Store an aliquot for “non-bound proteins” analysis at 4 °C.
4. Wash beads 3 times with 1 ml ice cold wash buffer I and II through centrifugation at 719 *x g* at 4 °C for 2 min; discard the supernatant.
5. Beads fused to the protein of interest are ready-to-use for enzyme activity assay.

C. GTPase activity assay (for more information, please refer to the GTPase Assay Kit Manual)

GTPases [also known as G proteins (guanine nucleotide-binding proteins)] function as molecular switches that cycle between an active and inactive state. The cycle is linked to the binding and hydrolysis of GTP. In the active state, these enzymes interact with GTP to perform diverse cellular functions. Inactivation occurs through the association of GAP (GTPase-activating protein). This protein hydrolyzes GTP to GDP and free phosphate. To return to the active state, the enzyme GEF (guanine nucleotide exchange factor) is required to catalyze the exchange of GDP for GTP (Figure 1).

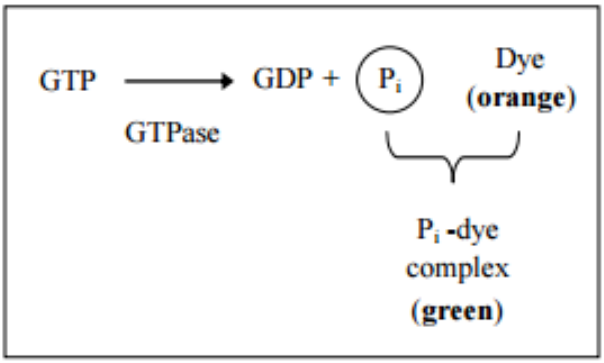

Figure 1. Principle of the GTPase assay kit (Innova Bioscience)

1. Prepare the substrate buffer mix (SB-Mix) and samples for a standard curve as described in the GTPase Assay kit Manual.
2. Add 350 µl SB-Mix buffer to the purified beads-protein solution.
3. Incubate samples at room temperature for 30 min under constant mixing.
4. Spin down beads at 1,073 *x g* for 5 min using a tabletop centrifuge.
5. Put 100 µl of reaction buffer in one well of a 96-well plate (triplicates) and add 100 µl pure H_2O; prepare samples for the standard curve in the same way.
6. Prepare the Gold-Mix buffer (see GTPase Assay kit Manual) and add 50 µl to each well to stop the reaction.
7. After 2 min add 20 µl stabilizer.
8. Incubate plate at room temperature for 30 min in the dark.
9. Read plate at a wavelength of 650 nm (590- 660 nm, see GTPase Assay kit Manual).
10. For calculation of enzyme activity, follow Appendix 1 of the GTPase Assay kit Manual (Figure 2).

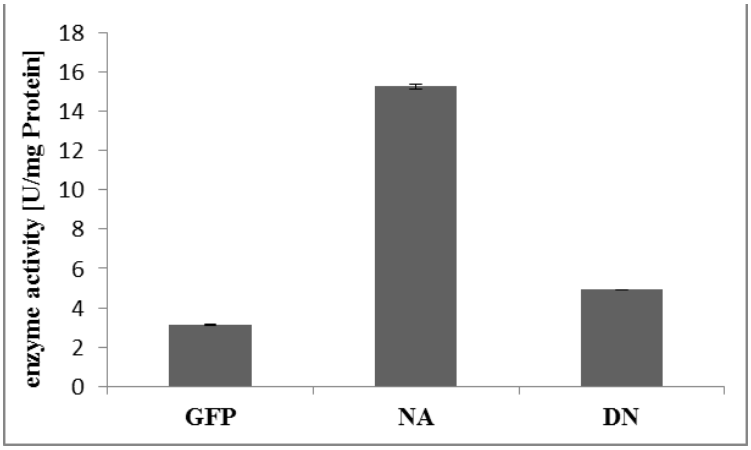

Figure 2. Specific enzyme activity of *RabA4c*-mCit [NA]/ [DN] and GFP as control. The GTPase assay was performed as described above (Procedure). Error bars represent standard deviations.

D. SDS-PAGE and protein quantification
   1. After GTPase Assay: Elute the target protein from the *GFP-Trap_A* beads by adding 200 µl 100 mM glycine (pH 2.5), mix shortly and incubate at room temperature for 2 min.
   2. Add 20 µl 1 M TRIS base (pH 10.4) for neutralization.
   3. Spin beads down at 1,073 *x g* at room temperature for 2 min, transfer supernatant to a new tube.
   4. Protein quantification follows the instructions of the manufactures (NanoOrange Protein Quantification Kit, see above), the protein amount is necessary to determine the enzyme activity.

Recipes

1. Yeast Peptone Dextrose (YPD) media
   1% yeast extract
   2% peptone
   D(+)-Glucose
2. Dilution buffer
   10 mM Tris (pH 7.5)
   150 mM NaCl
   0.5 mM EDTA (pH 8.0)
3. Wash buffer I
   10 mM Tris (pH 7.5)
   300 mM NaCl
   0.5 mM EDTA (pH 8.0)
4. Wash buffer II
   10 mM Tris (pH 7.5)
   500 mM NaCl
   0.5 mM EDTA (pH 8.0)
5. Elution buffer
   100 mM glycine (pH 2.5)
   1 M Tris (pH 10.4)

Acknowledgments

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References

1. Ellinger, D., Glöckner, A., Koch, J., Naumann, M., Stürtz, V., Schütt, K., Manisseri, C., Somerville, S. C. and Voigt, C. A. (2014). Interaction of the *Arabidopsis* GTPase *RabA4c* with its effector PMR4 results in complete penetration resistance to powdery mildew. *Plant Cell* 26(7): 3185-3200.