Chapter 34

Transient Expression of P-type ATPases in Tobacco Epidermal Cells

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Abstract

Transient expression in tobacco cells is a convenient method for several purposes such as analysis of protein-protein interactions and the subcellular localization of plant proteins. A suspension of *Agrobacterium tumefaciens* cells carrying the plasmid of interest is injected into the intracellular space between leaf epidermal cells, which results in DNA transfer from the bacteria to the plant and expression of the corresponding proteins. By injecting mixes of *Agrobacterium* strains, this system offers the possibility to co-express a number of target proteins simultaneously, thus allowing for example protein–protein interaction studies. In this chapter, we describe the procedure to transiently express P-type ATPases in tobacco epidermal cells, with focus on subcellular localization of the protein complexes formed by P4-ATPases and their β-subunits.

Key words *Nicotiana benthamiana*, *Agrobacterium tumefaciens*, Epidermal cells, Fluorescent protein, Co-expression, Infiltration

1 Introduction

Transient expression in tobacco epidermal cells has advantageously been applied for the study of membrane proteins. The technique can be used to express fluorescently tagged proteins and thereby determine the subcellular localization of proteins of interest [1, 2]. In combination with the split-GFP system, the method is also commonly used as an in vivo system to detect protein-protein interactions [3].

For introduction of foreign DNA into tobacco, advantage is taken of the natural lifecycle of *Agrobacterium tumefaciens*. *Agrobacterium* is a soil bacterium, which naturally infects plants and causes the tumor-related disease crown gall [4]. For the tumor to develop, a fragment of the bacterial DNA (the so-called transferred DNA; T-DNA) is directly transferred from the bacteria and integrated into the plant genome. The machinery responsible for this process is encoded by a tumor-inducing (Ti) plasmid including the T-DNA and virulence genes (*vir*-genes) important for the

Maike Bublitz (ed.), *P-Type ATPases: Methods and Protocols*, Methods in Molecular Biology, vol. 1377,
DOI 10.1007/978-1-4939-3179-8_34,

transfer of T-DNA. With only relatively minor modifications of this natural plant transformation process, genes of interest can be introduced and expressed in plants [5, 6]. Thus, keeping as little as 25 bp from each side of the T-DNA from the original Ti-plasmid (left and right border (LB, RB), Fig. 1) is enough for integration of the T-DNA into the plant genome, meaning that almost all of the T-DNA region can be exchanged with the gene of interest, in this way removing genes involved in tumor formation. In addition, the Ti-plasmid, which originally consists of approximately 200,000 bp, can be split into two plasmids such that the *vir*-genes and the T-DNA become separated in space while both gene-fragments are

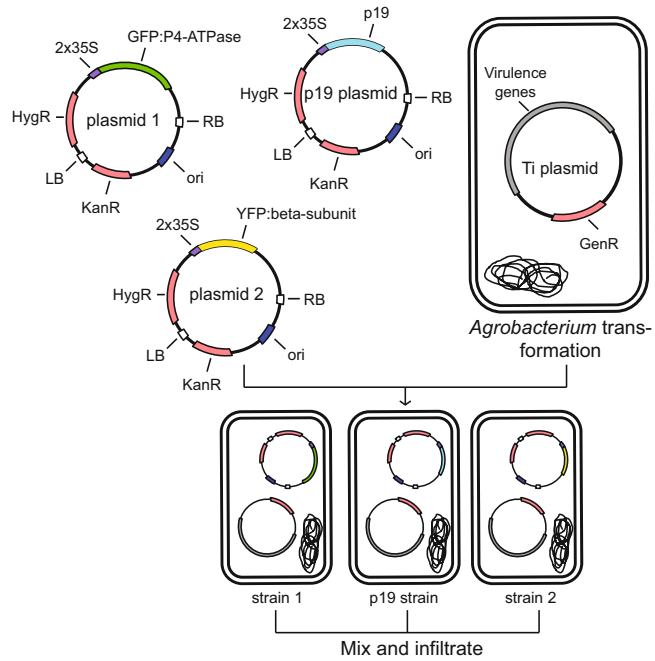

Fig. 1 Using *Agrobacterium tumefaciens* for transient expression. Genes of interest are cloned under the control of the desired promoter into so-called binary plasmids in between DNA sequences denominated left border (LB) and right border (RB) that mark the DNA region, which will be transferred to the plant. Although this is not required for transient expression, most binary plasmids also carry a selection marker for plants between LB and RB. The plasmids also contain origins of replication for *E. coli* and *Agrobacterium* (ori) and a selection marker for bacteria. Laboratory *Agrobacterium* strains carry helper plasmids that contain the virulence genes necessary for transfer of DNA to the plant cell. Plasmids are individually transformed into *Agrobacterium* and strains are mixed prior to infiltration to achieve the desired combination of target proteins. We routinely mix strains carrying the genes of interest with a freshly transformed strain containing the silencing suppressor p19 gene. Other abbreviations: *KanR* kanamycin resistance, *BaR* Basta resistance, *HygR* Hygromycin resistance

fully functional and still work in concert [7, 8]. In such a binary plasmid system employing two vectors the *Agrobacterium* host line is harboring the plasmid containing the *vir*-genes after removal of tumor-inducing genes (Fig. 1), while the small T-DNA fragments (LB and RB) are placed on a smaller plasmid (vector) which can be amplified in both *Escherichia coli* and *Agrobacterium* (Fig. 1) [7, 8]. The gene of interest is then cloned between LB and RB under the control of the desired gene promoter (*see* Subheading 1.3).

1.1 Choice of Agrobacterium Strain and Application System

There are several *Agrobacterium* strains available which can be used for transient expression [7]. They all share the common feature of carrying a modified Ti plasmid (helper plasmid) for transfer of genes of interest into plants. However, each of the strains has different capabilities, which should be taken into account when making a choice. For instance, the widely used *Agrobacterium* strain, GV3101, has been shown to generate higher expression levels compared to another well-known strain named C58C1 (approximately 50 % of GV3101) [9]. In this protocol, strain C5851 will be used, as in our hands this strain provides more consistent results when co-expressing two membrane proteins at the same time.

For gene transfer between *Agrobacterium* and plants, the bacteria need to be infiltrated into the plant tissue. This process can be executed in several ways. The two least invasive methods known to date are vacuum infiltration and direct injection. To vacuum-infiltrate plant tissue, the desired plant area is submerged in an *Agrobacterium* solution, after which the whole plant or leaf disk is exposed to short vacuum treatment. During this treatment the *Agrobacterium* solution is forced into the plant tissue via the stomatal pores. This method can be used for both stable and transient expression [10]. For transient expression in leaves, direct injection can be advantageous. In this case, only the area which has been chosen for expression is being exposed to the *Agrobacterium* solution, which is directly injected into the leaf tissue through stomata without affecting the rest of the plant [11].

1.2 Choice of Tobacco Species

Several tobacco species are commonly used for transient expression, but as for the *Agrobacterium* strains, each species shows specific abilities. As an example, compared to other *Nicotiana* species, *Nicotiana benthamiana* is often used for transient expression and is known to produce a high degree of recombinant protein within a short timeframe [12]. However, due to the small biomass yield from this species, large scale production of recombinant proteins may be facilitated by using species that are more sizeable such as *N. excelsior* [12]. The hybrid species *N. excelsiana* (*N. benthamiana* × *N. excelsior*), which recently has become available, has a higher biomass production than *N. benthamiana* and is easier to infiltrate than *N. excelsior* [9]. The commonly used *N. benthamiana* will be employed as an expression host in this protocol.

1.3 Choice of Promoter

Although in many cases, the native gene promoter can be used for expression in tobacco epidermal cells, it is usual practice to utilize strong constitutive promoters corresponding to other genes of plant (e.g., ubiquitin) [13] or viral origin (e.g., 35S from cauliflower mosaic virus) [14]. This circumvents the limitations derived from low expression or from the presence of tissue-specific or growth condition-specific regulatory sequences in the native promoters. However, high expression derived from constitutive promoters, especially from the viral 35S sequences, can result in posttranslational gene silencing [15]. Co-expression of viral silencing repressors, such as the p19 protein [16], is widely used to overcome this problem. In this protocol, overexpression of membrane proteins will be achieved under the control of a strong double 35S promoter combined with co-expression of a p19 silencing suppressor.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C). Keep all *Agrobacterium* growth media at 4 °C for long-term storage.

2.1 Tobacco Plants

1. *N. benthamiana* seeds.
2. Small round plastic pots 5 cm in diameter.
3. Standard soil.
4. A green house or growth chamber under the following conditions: 16 h light at around 140 μmol photons m^{-2} s^{-1} light intensity followed by 8 h darkness, humidity around 70 %, temperatures ranging between 24 °C during the day and 17 °C during the night.

2.2 Plasmids

1. cDNA fragments (*see* **Note 1**) corresponding to P4-ATPases fused to the coding sequence for green fluorescent protein (GFP) generated in binary plasmids pMDC43 and pMDC84 [17] containing a double 35S promoter (*see* **Note 2**).
2. C-terminally Yellow fluorescent protein (YFP)-tagged versions of the corresponding P4-ATPase β-subunits generated in binary plasmid pEarleyGate101 [18], bearing a single 35S promoter.
3. A plasmid containing the p19 gene encoding the viral silencing suppressor p19 protein [16], to facilitate high expression of recombinant proteins.

2.3 Agrobacterium Preparation

1. YEP plates [20]: 1 % yeast extract, 1 % peptone, 0.5 % NaCl, 1.5 % agar. Sterilize by autoclaving at 120 °C for 20 min. Let the media cool down to around 60–65 °C and add 50 μg/mL

kanamycin and 25 μg/mL gentamicin (*see* **Note 3**), before pouring into round petri dishes. Antibiotic stocks are 5 mg/mL gentamicin and 50 mg/mL kanamycin in water. Filter-sterilize and keep at –20 °C.

2. *Agrobacterium tumefaciens* strain C58C1 [19] (*see* **Note 4**) transformed with each of the plasmids of interest individually and selected on YEP plates.
3. LB liquid medium [21]: 1 % Tryptone, 0.5 % Yeast Extract, 1 % NaCl. Sterilize by autoclaving at 120 °C for 20 min. Let the medium cool down to around 60–65 °C before adding 50 μg/mL kanamycin and 25 μg/mL gentamicin from stock solutions as above.
4. 14-mL round-bottom culture tubes.
5. An orbital shaker and a static incubator at 28 °C.
6. A table centrifuge suitable for 2 mL Eppendorf tubes.
7. A spectrophotometer for measuring absorbance at 600 nm and standard plastic cuvettes of 1.5 mL volume.

2.4 Other Media

1. Infiltration solution: 10 mM $MgCl_2$ and 100 μM 4′-Hydroxy-3′,5′-dimethoxyacetophenone (acetosyringone) in water. Prepare a stock solution of acetosyringone at 10 mM in 50 % ethanol and store at -20 °C (*see* **Note 5**). Keep a 1 M $MgCl_2$ stock solution in water at room temperature. Right before starting your experiment, take equal volumes of acetosyringone and $MgCl_2$ stocks to a 50-mL plastic tube and make a 1:100 dilution in water (i.e., for 50 mL final volume of infiltration solution, you will need to mix 500 μL of each reagent). Keep at room temperature.

2.5 Visualization

1. Leica SP2 UV MP or SP5 II spectral confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) with a 63×/1.2 NA water immersion objective and equipped with laser lines at 488 and 514 nm.
2. Standard microscopy slides and cover glasses.

3 Methods

Due to the variability inherent to working with living organisms, each transient expression experiment should be repeated at least three independent times, with a new batch of tobacco plants and fresh *Agrobacterium* transformations, to ensure reproducibility of the results.

3.1 Preparation of Tobacco Plants

1. Plant *N. benthamiana* seeds in individual small pots containing standard soil and grow them in a greenhouse or growth chamber.

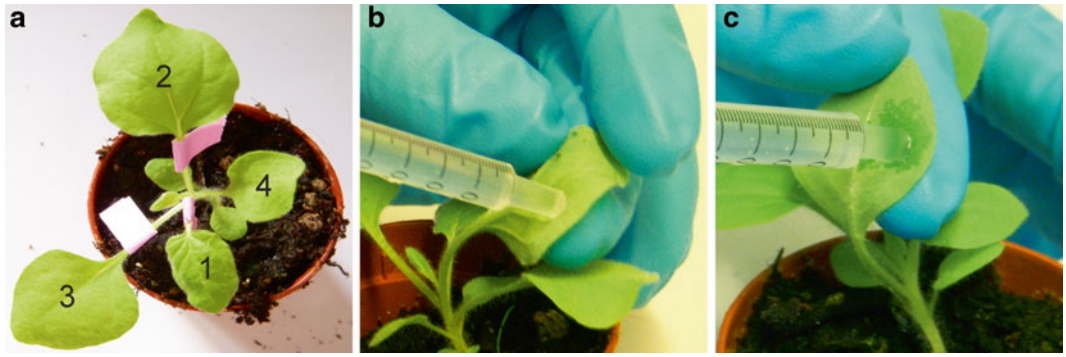

Fig. 2 Infiltration procedure. (**a**) 3-week-old tobacco plant suitable for infiltration. Leaves are numbered starting from the uppermost leave with a reasonable size. Leaves numbered 1, 2, and 3 would be infiltrated in such a plant; (**b**) A syringe without needle is used to force an *Agrobacterium*-containing solution into the intracellular space of tobacco epidermal cells. The lower side of the leave is used for infiltration. The tip of the syringe is pressed against the leave and a counter-pressure is applied from the other side by using a finger; (**c**) The *Agrobacterium* solution is released by applying pressure on the syringe piston. A *darker green* area appears where the solution has entered the leaf

2. Remove 3-week old *N. benthamiana* plants at the 4-5 leave stage from the growth chamber (*see* **Note 6**) and allow them to acclimate to ambient laboratory conditions for at least 1 h before infiltration (*see* **Note 7**).
3. Select and label leaves to be infiltrated (*see* **Note 8**) by hanging a piece of tape from the leaf attachment to the stem (Fig. 2a).

3.2 Preparation of Agrobacterium

1. For each DNA construct freshly transformed in *Agrobacterium* (*see* **Note 9**), transfer one colony to a 14-mL round bottom culture tube containing 2 mL of antibiotic-containing LB medium. For the p19-containing *Agrobacterium*, inoculate 3–4 colonies, as this strain presents a reduced growth due to toxicity of the p19 protein.
2. Incubate cultures at 28 °C for 14–16 h in an orbital shaker with 160–180 rpm agitation.
3. Transfer the cells to 2-mL Eppendorf tubes.
4. Centrifuge at 2500 × *g* for 4 min at room temperature.
5. Discard supernatant and resuspend in 1 mL infiltration solution by pipetting very gently up and down (*see* **Note 10**).
6. Repeat **steps 4** and **5** once more.
7. Incubate without shaking at 28 °C for 1–2 h (*see* **Note 11**).
8. Measure OD_{600} for each *Agrobacterium* suspension using a 1:10 dilution in water.
9. For each plant to be infiltrated, prepare 1–2 mL (*see* **Note 12**) of infiltration mix by diluting the desired combination of *Agrobacterium* strains to a final $OD_{600} = 0.3$ each in infiltration solution (*see* **Note 13**).

3.3 Infiltration of Epidermal Cells

1. Transfer the cell suspension to a 1- or 2-mL syringe without needle (*see* **Note 14**).
2. Avoiding the veins, place the syringe tip against the lower side of the leaf and apply a gentle pressure against it from the other side of the leaf (*see* **Note 15**) (Fig. 2c).
3. Push slowly the syringe piston to force the cell suspension into the leaf. As the apoplastic space gets filled, the infiltrated area will turn a darker shade of green (*see* **Note 16**) (Fig. 2d). Infiltrate a leaf area of about 0.5–1 cm around the infiltration point.
4. Water the plants and return them to the growth chamber or the greenhouse. Avoid watering during the last 24–30 h prior to visualization (*see* **Note 17**).

3.4 Visualization

1. Cut a piece of about 3 × 3 mm from the desired infiltrated leaf, starting by the area closest to the wound inflicted by the syringe tip (*see* **Note 18**).
2. Place the leaf fragment onto a microscopic slide with the infiltrated side up, add a drop of water on top and cover with a cover slide (*see* **Note 19**).
3. Mount the sample in the confocal microscope and excite GFP at 476 nm (100 % laser intensity) and record its emission spectra between 492 and 500 nm. Excite YFP at 514 nm (40 % laser intensity) and record emission between 545 and 560 nm. Use sequential scanning between lines to follow both fluorescent proteins at once (*see* **Note 20**). Images are scanned with a line average of 2 (*see* **Note 21**).

For each localization experiment, visualize at least 3 independent samples per plant, each corresponding to a single infiltrated leave (*see* **Note 22**).

4 Notes

1. In some cases, expression levels might improve when using genomic DNA fragments.
2. As the role of the protein termini is still relatively unknown for P4-ATPases, we routinely generate and test constructs tagged at both termini.
3. Gentamicin will select for the helper plasmid, while kanamycin will select for the plasmid bearing the gene of interest.
4. Other *Agrobacterium* strains, such as GV3101, have been used with identical positive results.
5. We have used acetosyringone stock solutions up to 1-year old with positive results.

6. It is very important to use healthy unstressed young plants.
7. This acclimation period facilitates subsequent infiltration.
8. Younger leaves tend to express at higher levels than older leaves, as the cells are actively dividing. However, young leaves are also more difficult to infiltrate and to visualize microscopically due to a more evident vascular tissue. Therefore, we infiltrate three leaves per plant, starting by the uppermost leave with a reasonable size. We routinely infiltrate several plants with the same DNA construct(s) either in the same or in independent experiments, to account for variability of the biological replicates.
9. Transformed *Agrobacterium* cells can also be kept at −80 °C and directly inoculated from the glycerol stock, although final expression levels in the infiltrated tobacco leaves tend to be lower.
10. Vortexing dramatically diminishes the efficiency of the infiltration procedure.
11. Shaking at this step reduces the efficiency of the infiltration procedure.
12. The amount of infiltration solution required will depend on personal skills and plant conditions.
13. Although we have assayed different final OD_{600} for the different *Agrobacterium* strains, an equal concentration of each provides the best results in our hands. However, this should be assessed in a case-to-case basis. Up to four different strains can be successfully combined.
14. Use a syringe with a narrow tip to minimize the wounded area caused during contact with the leave surface.
15. As the leaf will get wet with *Agrobacterium* suspension on both sides, use always gloves when infiltrating.
16. Although it is possible in some cases to infiltrate the whole leaf using one infiltration point, best results are obtained by infiltrating at several different positions within the same leaf. In other cases, especially for very young leaves, the infiltrated areas will be small due to the difficulty to force the cell suspension into the apoplastic space. At this point, it is possible to mark the extent of the infiltrated area by using a black marker pen, if desired. Watch out for squirting *Agrobacterium* suspension.
17. The time required to properly express the P-type ATPase will depend on the *Agrobacterium* strain, the tobacco species, the promoter used and the protein of interest. Typically, overproduction of the desired protein during the first stages of transient expression saturates the cell machinery, causing retention in the endoplasmic reticulum. Progressively, the first wave of high expression subdues and trafficking of the nascent protein

to its target membrane becomes more effective. Times as short as 48 h can be sufficient for proper localization in some cases. For P4-ATPases and their β subunits, the optimal is around 3–5 days in our hands, although longer times might be required for some specific proteins.

18. Do not prepare all your samples at once. Each sample should be cut from the leaf right before visualization.
19. As the leaves are quite thick, it is sometimes useful to use double-sided tape to adhere the cover glass to the slide. That will generate a separation between the two glass pieces that will better accommodate the leaf fragment.
20. In our hands, co-infiltration of a GFP-tagged protein with another YFP-tagged protein, always results in a population of cells expressing either of the two proteins individually. Therefore, it is always possible to find cells that show GFP signal but no YFP signal and *vice versa*. We use these cells to establish the visualization parameters (laser intensity, pinhole aperture, photomultiplier sensitivity) where we cannot detect bleed-through of the fluorescent signals (Fig. 3). These parameters are then used for all the samples in a batch. If any parameter change is necessary, bleed-through should be reassessed.
21. If the signal is too low, it might be necessary to use a higher line average to reduce to background noise. This, however, increases the working time and the concurrent bleaching of the fluorophores, which occurs each time the sample is excited. Therefore, cells with high expression levels are chosen for

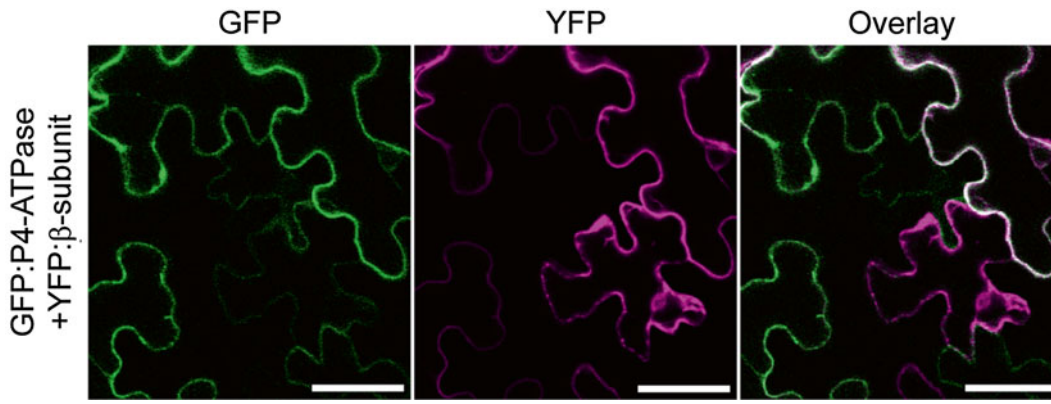

Fig. 3 Bleed-through assessment. Microscopic visualization of tobacco epidermal cells infiltrated with a GFP-tagged P4-ATPase and an YFP-tagged β-subunit. The image shows an example where cells only showing GFP fluorescence (overlay image, *green* signal), only showing YFP fluorescence (overlay image, *magenta* signal) or showing both signals simultaneously (overlay image, *white* signal) are present. Cells only showing the fluorescent signal for one of the proteins are used to optimize acquisition parameters such as photomultiplier sensitivity, pinhole aperture and laser intensity. Once defined, the parameters are kept unchanged throughout visualization of a whole plant set. Scale bar, 50 μm

visualization in applications that require long acquisition times. Bleaching of GFP is faster than YFP. For this reason, it is advisable to use YFP when only one protein of interest is expressed.

22. The expression levels for the desired protein(s) will vary with leaf age, plant health, and even seed batch. Too high expression will result in endoplasmic reticulum retention, while low expression will make the fluorescent signal difficult to distinguish from the background noise. For co-localization experiments, different cells may be expressing each tagged protein at a different level. Therefore, it is important to take a large number of images that represent the biological variability of the sample when analyzing results. This is especially relevant in subcellular localization experiments. In tobacco epidermal leaves, the vacuole occupies most of the cell volume, pressing all other membrane structures against the cell wall. Some organelles, like Golgi or prevacuolar compartments, will appear as easily distinguishable vesicular bodies constantly moving around the cell. The endoplasmic reticulum can be easily spotted as a patchy signal around the nucleus and as membrane threads with a high mobility that cross the cytoplasm (a feature named cytoplasmic streaming). The tonoplast and the plasma membrane are, in contrast, difficult to discern, as both appear as a sharply defined signal along the contour of the cell. One way to distinguish them is to zoom in on the cell nucleus, since the plasma membrane encloses all cellular organelles, while the tonoplast surrounds them. However, this visualization may become an arduous task for an untrained eye and the digital zooming required might compromise image resolution for publication. An easier method consists in infiltration of the leaf with a 1 M solution of mannitol in water 2–5 min before visualization, using the same protocol as for *Agrobacterium* infiltration described in this chapter. The high osmotic pressure of the mannitol solution causes the vacuole to lose water and shrink, in a process termed cell plasmolysis. In our standard procedure, we first take one single sample of a leaf that has been infiltrated at several positions and check that our protein(s) of interest are expressed. If the levels of expression are adequate for visualization, we choose another infiltrated area on the same leaf and inject mannitol as close as possible to the first infiltration wound. Then we cut out a sample from the wet area generated by the mannitol solution entering the apoplast and proceed to visualization. In this case, a drop of mannitol solution is placed between the sample and the cover glass instead of water. After plasmolysis, the plasma membrane remains attached to the cell wall by strips of membrane called Hechtian strands, while the vacuole fragments and appears as large round intracellular membrane structures.

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