

# Tracking of plasmodesmata localization with the use of fluorescently labelled VAP27 protein

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**Abstract.** Plasmodesmata play a crucial role in plant development processes, biotic and abiotic stress responses. The thick space inside of a plasmodesma between the plasma membrane and desmotubule is tethered by spoke-like elements. VAP27 is a transmembrane protein localized in plasmodesmata that acts as their tether. The aim of the study was to investigate the integration of fluorescently labelled VAP27 protein into plasma membrane in *Nicotiana benthamiana* plasmodesmata which would enable the tracking of *N. benthamiana* plasmodesmata localization *in vivo*. The research was done by infiltration of *N. benthamiana* with the *Agrobacterium tumefaciens* cells. *A. tumefaciens* were transformed with the GreenGate assembled plasmid containing the gene coding for N-terminally labelled VAP27 with EGFP. The EGFP fluorescence was detected by confocal imaging of *N. benthamiana* leaves. Prior to confocal imaging, the leaves were infiltrated with aniline blue, which acted as plasmodesmata marker. The findings revealed the colocalization of positions of VAP27-EGFP expression and plasmodesmata. The conclusion was made that the integration of VAP27-EGFP into the plasma membrane in plasmodesmata was successful.

## 1 Introduction

Plasmodesmata are series of pores between plant cells that enable the intracellular transport of metabolites vital for the normal cell functioning of the whole plant organism [1]. They play a crucial role in plant development processes, biotic and abiotic stress responses.

A plasmodesma can be described as a tubule along which in its inner side there is an endoplasmic reticulum (ER) strand, called desmotubule, which is tightly bound to the plasma membrane (PM) of the inner surface of the tubule. The thick space (around 10 nm) between the PM and desmotubule, which is tethered by spoke-like elements, acts as an intracellular conduit for a wide range of metabolites [2].

VAP27 is an evolutionarily conserved transmembrane protein which is known to localize in the plasmodesmata and takes part in cell-to-cell signaling [3].

The aim of the study is to develop a marker for *N. benthamiana* plasmodesmata based on N-terminally fluorescently labelled VAP27 protein with EGFP [4] that would allow plasmodesmata localization tracking *in vivo*. The introduction of the VAP27-EGFP into *N.*

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*benthamiana* was done by infiltration of the *N. benthamiana* leaves with *A. tumefaciens* [5] that was previously transformed with the GreenGate assembled plasmid [6] containing the gene for the VAP27 protein with N-terminally fused EGFP. The GreenGate assembled plasmid was previously amplified in *Escherichia coli* TOP10 cells.

The study of VAP27-EGFP expression in plasmodesmata was done by confocal imaging.

## 2 Materials and methods

### 2.1 In-Fusion cloning

The pGGC plasmid, carrying origins of replication of both *E. coli* and *A. tumefaciens*, was used as a cloning vector. The gene for VAP27-EGFP was cloned to the vector by the In-Fusion cloning method. The VAP27 protein UniProt identifier is Q9SC83. The EGFP protein UniProt identifier is A0A348GST9. The forward primer for pGGC linearisation is AAGTGAAGCTTGGTCTCAGGCTCTTACCAAAGAT. The reverse primer for pGGC linearisation is AGGGCGAGAATTCGGTCTCACTGAGGTTTTTTTCAG. For the VAP27-EGFP PCR amplification, the forward primer AGTTCTTCGCCCTTAGAAACCAT was used. The reverse primer used was CTGGGTTACCTGCTGAAAAAACC. The primers were automatically generated with the programme Takarabio (powered by Teselagen).

The linearization and amplification of pGGC were done according to the Phusion Plus Green PCR Master Mix protocol. The amplification of VAP27-EGFP was done according to the PrimeSTAR GXL protocol. The 10 µM concentration of the primers stock solutions was used. The preparative 1% agarose gel electrophoresis was conducted. By using the UV light, gel fragments containing the target amplicons were cut and placed into 2 ml tubes for the DNA extraction according to the NucleoSpin Gel and PCR Clean-up protocol. After the extraction, the concentrations of VAP27-EGFP and linearized pGGC were measured by NanoDrop. For each cloning reaction, 160 ng of the linearized pGGC and 40 ng of VAP27-EGFP were mixed. Additionally, 2 µl of 5X In-Fusion HD Enzyme Premix was added and nuclease-free H<sub>2</sub>O was added up to 10 µl. Each sample was incubated for 15 min at 50°C, then placed on ice for further use.

### 2.2 Agarose gel electrophoresis

In order to check the efficiency of PCR reactions, the 1% agarose gel electrophoresis was done. As an intercalating agent, the peqGREEN DNA/RNA Dye was used (4 µl / 100 ml of agarose solution). As a running buffer, 1X TAE (40 mM Tris base, 20 mM AcOH, 1 mM EDTA pH 8.0) was used. 5 µl of ladder solutions were injected into one gel lane. The gels ran by constant 120 V for 25-40 min.

### 2.3 Transformation and seeding of *E. coli*

Competent *E. coli* TOP10 cells were used for transformation. 2.5 µl of the In-Fusion reaction mixture were added to 50 µl of bacterial cells.

For making LB-Agar, the 10 g of the LB-medium and 10 g of the agar were dissolved in 500 ml of ddH<sub>2</sub>O and then autoclaved. After slight cooling down of the agar solution, carbenicillin was added to the LB-agar until its concentration was 100 µg/ml. Then, the agar was poured onto Petri dishes.

The seeding of the transformed *E. coli* cells was done as follows:

- Out of 550  $\mu$ l, 25  $\mu$ l were taken and seeded on the half of a plate;
- Centrifugation at 11000 g for 1 min was done;
- 450  $\mu$ l of supernatant was discarded;
- The pellet was resuspended in the remaining volume and was seeded onto LB-agar in another plate.

The transformed plated *E. coli* cells were stored at 37°C for 24 h.

## 2.4 Colony PCR

For the sake of validation of transformation efficiency, the colony PCR was done for each bacterial colony according to Colony PCR with the Sapphire Amp Fast PCR Master Mix protocol. The forward primer, complementary to the backbone SP6 promoter (ATTTAGGTGACTATAGAAG), and the reverse primer, reverse complementary to VAP27 (TTACCTGCTGAAAAAACC), were used for this purpose. The criteria of choosing the primers were universal for constructs with different insertion sites, yielding the amplified fragments no longer than 3000 bp, the melting temperatures differing no more than 3°C and the lengths of the primers (16-28 bp). Subsequently, the 1% agarose gel electrophoresis was done in order to monitor yielded amplicons.

## 2.5 Miniprep

The extraction of plasmids from *E. coli* TOP10 was conducted according to the protocol of the Isolation of high-copy plasmid DNA from *E. coli*. The concentration of the extracted constructs was measured with NanoDrop.

## 2.6 Sequencing

The sequencing sample was sent to the company MicrosynthSeqlab LLC. For each sequencing reaction, 48 ng/ $\mu$ l of the plasmid and 4  $\mu$ M of the primer were used in the final volume of 15  $\mu$ l. The two primers were used: ATTTAGGTGACTATAGAAG (forward) and TTACCTGCTGAAAAAACC (reverse).

## 2.7 GreenGate assembly and *E. coli* transformation and plating

For *E. coli* TOP10 transformation, 15  $\mu$ l of GreenGate reaction was used. The transformed cells were seeded onto Petri dishes with LB-agar with 100  $\mu$ g/ml of spectinomycin. The plating was done as written above.

## 2.8 Restriction digest

In order to verify whether the GreenGate cloning was done correctly, the restriction digest was performed with the endonucleases EcoRV (restriction site GAT|ATC) and BglII (restriction site A|GATCT) according to the rCutSmart Time-Saver protocol. After that the 1% agarose gel electrophoresis was done in order to monitor yielded restricted fragments.

## 2.9 Transformation, plating and inoculation of *A. tumefaciens*

Competent *Agrobacterium* (CV3101, Ti pMP90 pSOUP) cells were thrown on ice. Then, 20  $\mu$ l of *Agrobacterium* was put on a new 1.5 ml Eppendorf, 2  $\mu$ l of DNA was added and the tubes were gently flicked to mix. The mixture was kept on ice for 45 minutes. A heat shock

was performed at 37°C for 5 min. Afterwards, the samples were incubated on ice for 30 min. 300 µl of the LB-medium was added, and the sample was put in a shaker at 28°C for 2-3 hours. 100 µl of cells was plated on LB containing spectinomycin (50 µg/ml), rifampicin (50 µg/ml), and tetracycline (2.5 µg/ml). The cells were incubated for 2 days at 26-28°C in order for colonies to form.

Also, another set of transformation was done with the p19 plasmid which carries the gene for RNA silencing suppressor p19 that disrupts the RNA silencing antiviral defence system of the host plant [7]. The cells which were transformed with the p19 plasmid were seeded onto the LB-agar containing kanamycin (50 µg/ml) and rifampicin (50 µg/ml). The transformed cells were then incubated at 28°C for 60 h.

For inoculation of the *A. tumefaciens* cells, many colonies were picked and placed into one tube. The cells were incubated in a shaker at 220 rpm and 28°C overnight.

## 2.10 Infiltration of *N. benthamiana* leaves with *A. tumefaciens*

After inoculation, the desired OD<sub>600</sub> value of *A. tumefaciens* was between 0.8 and 1.2. Prior to centrifugation for re-buffering of *A. tumefaciens* in the infiltration solution, the OD<sub>600</sub> values of the cells containing the construct with VAP27-EGFP and the *A. tumefaciens* sample with p19 plasmid were 0.83 and 0.82, respectively. Afterwards, 500 µl of the *A. tumefaciens* liquid culture in the LB-medium were mixed with 500 µl of 50% (v/v) glycerol and stored at -80°C for use in the future. The remaining *A. tumefaciens* were centrifuged at 4000 g for 20 min and the pellet was resuspended in the infiltrate solution containing 1 mM of MES, 0.1 mM of MgCl<sub>2</sub> and 150 µM of acetosyringone. The volume of the infiltrate solution was calculated, so that the OD<sub>600</sub> would be 0.6. Then, *A. tumefaciens* were kept in darkness for 1 h. Prior to infiltration, the *A. tumefaciens* cells transformed with VAP27-EGFP and p19 plasmid were mixed together in the final volume of 5 ml in the infiltration solution, so that the final OD<sub>600</sub> of the VAP27-EGFP-transformed cells was 0.3 and that of p19-transformed cells was 0.1. Next, the infiltration of the *N. benthamiana* leaves with the obtained mix took place.

For the infiltration, the chosen leaves were not too young or too old. A very small puncture on the abaxial side of each selected leaf was made. Leaf veins were avoided. With a 1 ml syringe, the *A. tumefaciens* mix was applied against the leaf at the abaxial side to the puncture.

## 2.11 Induction of VAP27-EGFP expression in *N. benthamiana* leaves

Prior to confocal imaging, the VAP27-EGFP expression was induced for around 16-18 h by spraying the 20 µM of β-estradiol with 0.1% (v/v) Tween-20.

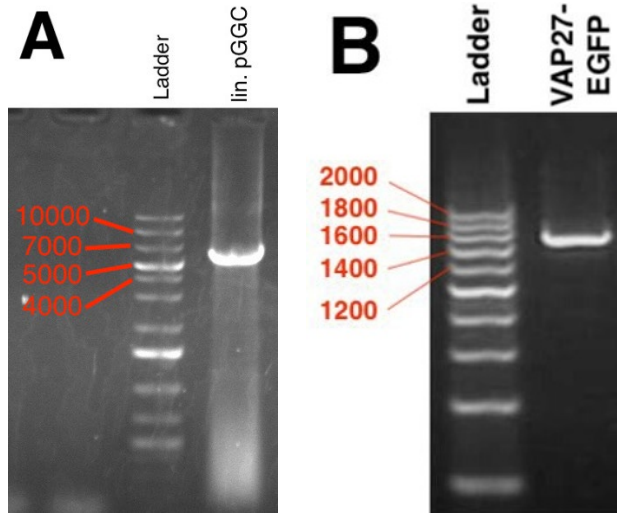
## 2.12 Confocal imaging of *N. benthamiana* leaves

The confocal imaging of the *N. benthamiana* leaves was done from the abaxial side. Prior to imaging, the leaves were infiltrated with the 0.1% (w/v) aniline blue solution, which acted as a plasmodesmata marker. The infiltration position was close to the position where previously the infiltration with *A. tumefaciens* had taken place. The microscope used was C-Apochromat 40x/1.20 W Korr FCS. For the EGFP fluorescence, the EGFP-T1 channel was used (excitation and emission wavelengths were 488 nm and 525 nm, respectively); for plasmodesmata staining – DAPI-T2 channel (excitation and emission wavelengths were 405 nm and 456 nm, respectively). The fluorescence intensities were optimized with the help of the OMERO programme.

### 3 Results

#### 3.1 In-Fusion cloning

The amplified fragments of the linearized pGGC and VAP27-EGFP are shown on the agarose gels (Fig. 1).



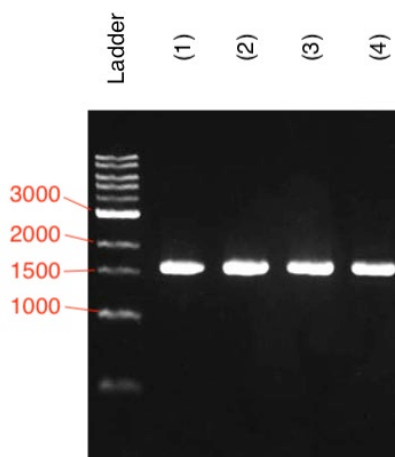
**Fig. 1.** 1% agarose gel electrophoresis of linearized pGGC (A) and VAP27-EGFP (B). The ladder bands of 1 kb Plus DNA Ladder – (A) and 200 bp DNA Even Ladder – (B) are marked in red.

The linearized pGGC is ~5200 bp long, its band can be clearly seen in the position between 5000 and 7000 bp ladder bands close to the 5000 bp band (Fig. 1, A). The brightness indicates a big amount of amplified fragments. The same can be said about VAP27-EGFP amplicon (Fig. 1, B), which is ~1500 bp long. It is located between the ladder bands of 1400 and 1600 bp. It is to conclude that both PCR reactions were performed successfully.

Concentrations of the linearized pGGC and the VAP27-EGFP fragment measured by Nanodrop were 22.4 ng/μl and 49.7 ng/μl, respectively. Since the concentrations of target DNA samples are sufficient, the In-Fusion cloning reaction was done with the subsequent *E. coli* TOP10 transformation. The transformed cells were seeded onto the 20 g/l of LB-agar with 100 μg/ml of carbenicillin on Petri dishes and incubated at 37°C for approximately 24 h. After the 24 h incubation, relatively big and well separated colonies could be observed for each construct.

#### 3.2 Colony PCR

In order to verify the efficiency of the transformation, the colony PCR of four *E. coli* colonies was carried out (Fig. 2).



**Fig. 2.** Colony PCR of 4 transformed *E. coli* TOP10 colonies. (1), (2), (3) and (4) stand for different colonies. The bands of the ladder (1 kb Plus DNA Ladder) are marked in red.

It can be clearly seen that the positions on the gel of all 4 samples are close to 1500 bp, which indicates the correct In-Fusion assembly of the construct.

### 3.3 Miniprep

The transformed *E. coli* cells were inoculated and colony 1 was further used for the plasmid extraction. The yielded concentration of the construct after extraction is 1245 ng/ $\mu$ l.

### 3.4 Sequencing

The sequencing results of the extracted pGGC-VAP27-EGFP construct revealed its correct assembly. No point mutations were to be found.

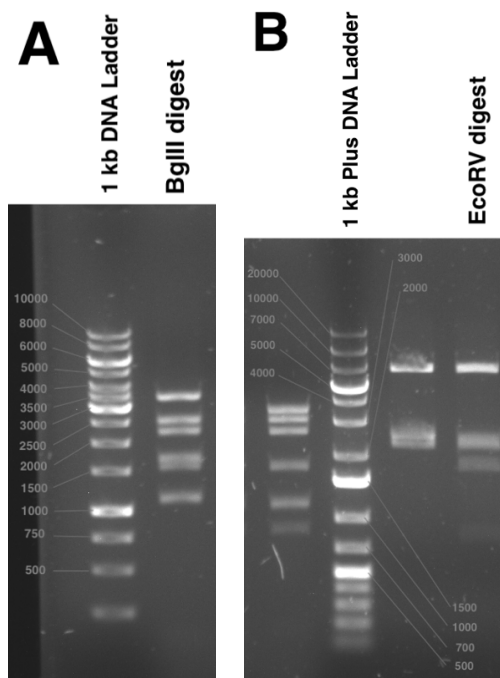
### 3.5 GreenGate cloning, *E. coli* transformation and cultivation, and plasmid extraction

After the GreenGate cloning, the subsequent extra-ligation, *E. coli* TOP10 transformation and its overnight incubation at 37°C on LB-agar containing 100  $\mu$ g/ml of spectinomycin, the cells grew well and formed relatively big and well separated colonies. The negative control did not show any untransformed cell growth, which indicates a good quality of the spectinomycin stock solution used.

The GreenGate assembled construct was extracted from the *E. coli* cells, the concentration of the construct was 420 ng/ $\mu$ l. Since the concentration was sufficient for the *A. tumefaciens* transformation, it was decided to perform a restriction digest of the sample with the extracted plasmid rather than perform plasmid extraction from the 3 remaining inoculation samples.

### 3.6 Restriction digest

In order to verify whether the GreenGate assembly was done correctly, the restriction digest was carried out and the restricted fragments were monitored by the agarose gel electrophoresis (Fig. 3).



**Fig. 3.** Restriction digest of the GreenGate construct by BglII (A) and EcoRV (B) endonucleases. 1 kb DNA Ladder (A) and 1 kb Plus DNA Ladder (B) were used as references.

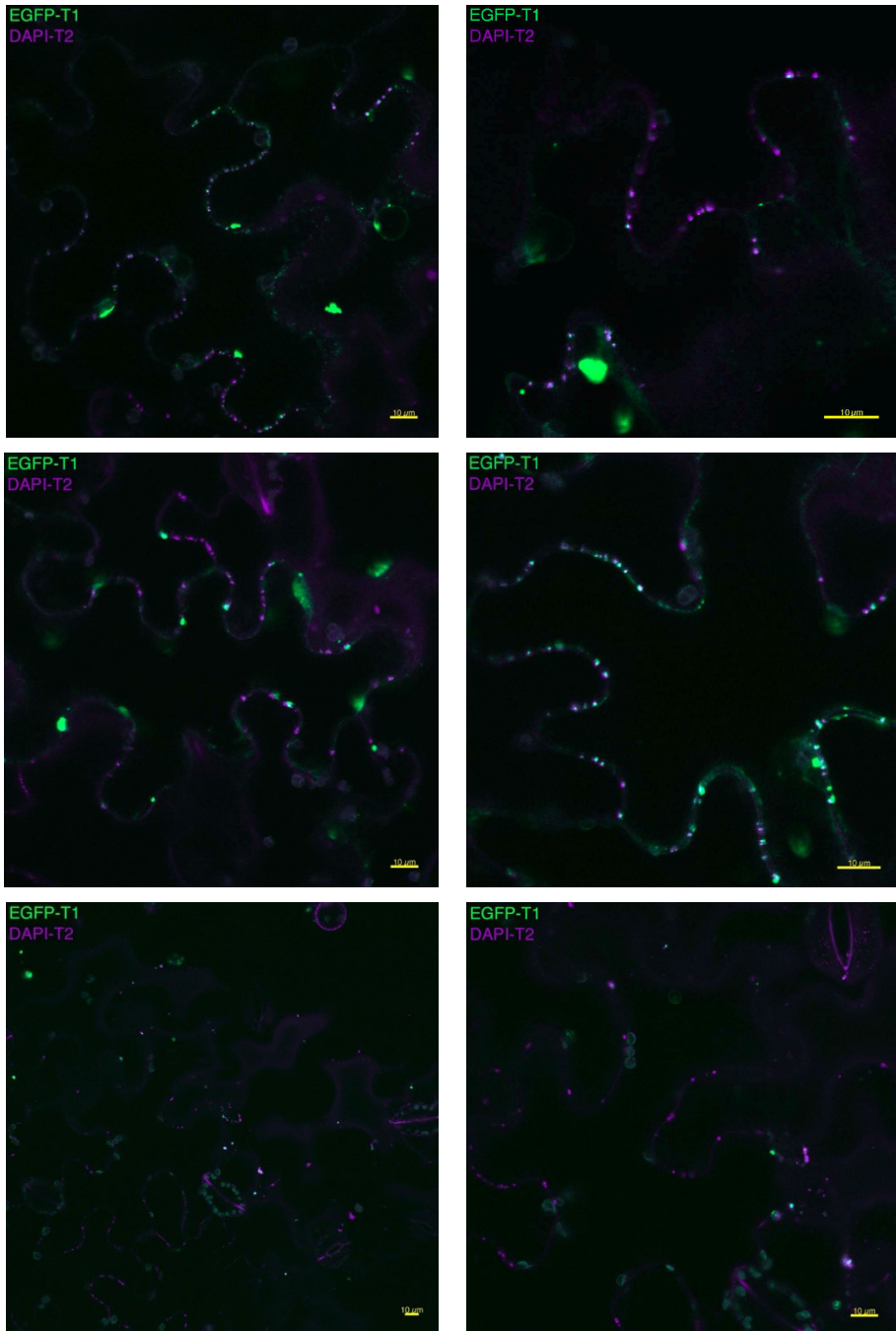
The restriction digest was also done *in silico* with the use of Geneious programme. The theoretical lengths of the fragments after digestion by BglII are 1036, 1090, 1501, 1648, 2177, 2464 and 3294 bp. The theoretical lengths of the fragments after digestion by EcoRV are 758, 1728, 2175, 2272 and 6396 bp.

The fragments observed on the agarose gel (Fig. 3) coincide with those of the *in silico* restriction digest. The GreenGate construct was further used for the *A. tumefaciens* transformation.

### 3.7 A. *tumefaciens* transformation and growth

After the 2-day incubation at 26-28°C of the transformed *A. tumefaciens* cells, many relatively small, but well separated colonies were formed. Many colonies were picked at once and inoculated into one tube containing the LB-medium having the same concentration of the corresponding antibiotics. The cells were incubated in a shaker overnight.

### 3.8 Confocal imaging



**Fig. 4.** Confocal microscopy of *N. benthamiana* leaves with expression of VAP27-EGFP. Plasmodesmata staining with aniline blue is shown in purple, VAP27-EGFP fluorescence is shown in green. For monitoring aniline blue fluorescence, excitation and emission wavelengths were 405 nm and 456 nm, respectively. For monitoring EGFP fluorescence, excitation and emission wavelengths were 488 nm and 525 nm, respectively.



The plasmodesmata localization is recognizable (Fig. 4, purple). The green fluorescence of EGFP is also perfectly visible (Fig. 4, green). It is concentrated in small bright dots which cannot be the chloroplasts autofluorescence. The colocalization of positions with the biosensor expression and plasmodesmata is visible (Fig. 4, white), which indicates the successful integration of VAP27-EGFP into the plasma membrane in plasmodesmata.

## 4 Discussion

### 4.1 pGGC and VAP27-EGFP amplification for In-Fusion cloning

The linearization and amplification of pGGC and amplification of VAP27-EGFP for the later In-Fusion cloning were done successfully (Fig. 1); the choice of primers (generated by Takarabio) was appropriate for all the PCR reactions.

### 4.2 Colony PCR and Sequencing for evaluation of In-Fusion constructs

Both the sequencing and the colony PCR with the subsequent agarose gel electrophoresis (Fig. 2) show correct In-Fusion cloning.

### 4.3 Restriction digest of the GreenGate assembled construct

The restriction digest revealed correct GreenGate assembly (Fig. 3). However, it remains debatable whether EcoRV was one of the most fitting endonucleases for the restriction digest since only 3 fragments were yielded, the lengths of which were close to each other (Fig. 3, B); thus, the fragments resolution was relatively low. In contrast to EcoRV, the restriction digest by BglII yielded 7 fragments (Fig. 3, A), which allowed a high fragments resolution. The only drawback of this endonuclease is that the 2 gel bands around 1 kb long could be badly resolved.

### 4.4 A. *tumefaciens* transformation and growth

It is to be assumed that the transformation of *A. tumefaciens* was done successfully due to its growth on the medium with antibiotics, which allowed the selection of effectively transformed cells. On the other hand, in order to verify the quality of the GreenGate constructs after the *A. tumefaciens* transformation, their extraction from the cells with the subsequent evaluation by colony PCR or the restriction digest could have been performed. For that, one would probably require another miniprep protocol suitable for lysis of *A. tumefaciens* [8].

### 4.5 Confocal imaging of *N. benthamiana* leaves

The results presented above indicate successful expression and integration of the VAP27 and EGFP fusion into the plasma membrane in plasmodesmata of *N. benthamiana* cells (Fig. 4). It is to expect that EGFP, which was covalently fused to VAP27, did not hinder the correct folding of the latter although the two proteins have the same molecular mass (around 27 kDa). Moreover, the correct folding of EGFP was not hindered, either. If that was the case, no EGFP fluorescence would be observable as the formation of the p-hydroxybenzylidene-imidazolidinone fluorophore by T65, Y66 and G67 EGFP amino acids

is required. The formation of the EGFP  $\beta$ -barrel domain that stabilizes the fluorophore [9] occurred successfully as well.

Moreover, transformation of *N. benthamiana* is a complicated, multiple-step process including attachment of an agrobacterium cell to a plant cell with the subsequent transition of the target DNA fragment to the latter and its integration into the plant genome [7]. Confocal imaging confirmed successful occurrence of these biological steps.

The p19 plasmid expression in the *A. tumefaciens* cells after the *N. benthamiana* infiltration was sufficient for silencing the defence system of the latter [10].

## 5 Conclusion

The study proves the suitability of the N-terminally fluorescently labelled VAP27 with EGFP for tracking the localization of plasmodesmata in *N. benthamiana in vivo*. The GreenGate plasmid containing the gene for VAP27 with N-terminally fused EGFP was correctly assembled, which was confirmed by the restriction digest. Confocal imaging of *N. benthamiana* leaves with induced VAP27-EGFP expression demonstrated the colocalization of the VAP27-EGFP fusion and plasmodesmata, indicating the successful integration of the fusion protein into the plasma membrane. This study demonstrates the potential of this methodology to explore plasmodesmata dynamics and cell-to-cell signalling in plants. Further investigations with different plasmodesmata proteins and other plant species could shed more light on the intricate mechanisms that govern intercellular communication in plants. This knowledge may have significant implications for agriculture and biotechnology offering opportunities to improve plant growth, stress tolerance, and crop yields.

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