

Imaging the intracellular targeting of vacuolar membrane proteins in living cells

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Summary

Following development of a genetic construct for Red Fluorescent Protein (RFP) HDEL which remains in the lumen of the Endoplasmic Reticulum (ER) after translation, it was co-expressed with a construct for Photo Activatable Green Fluorescent Protein (PA-GFP) bound to Alpha-TIP (Tonoplast Intrinsic Protein). These were co-infiltrated into tobacco plants (*Nicotiana rustica*) which were then viewed under the confocal microscope. By activating the PA-GFP in the ER lumen, the path of the tonoplast protein through the cell was observed over a period of time. The results of this study are discussed, and their relevance to future studies are assessed.

Keywords: RFP-HDEL, Photo Activatable GFP, Alpha-TIP, vacuolar membrane proteins, vacuole, confocal microscopy, *Nicotiana rustica*.

Introduction

Fluorescent proteins used in confocal microscopy are fundamental to the process of cellular imaging. The original fluorescent protein, GFP, originates from the jellyfish *Aequorea victoria*¹, emitting light in the green part of the spectrum. This has since been adapted to excite and emit light at other wavelengths, including RFP, which emits light in the red part of the spectrum. Even GFP itself has been modified, through cloning, to absorb light of a frequency closer to the emission spectrum. The GFP used in these studies is photo activatable, meaning a very high intensity UV light is required to activate the protein to emit light like normal GFP. When activated it absorbs light at a frequency of 488nm and has a peak of emission at 507nm. The RFP used absorbs light at a frequency of 558nm and has a peak of emission at 583nm.

With access to the recently installed Leica TCS SP5 confocal microscope, it was possible to obtain high resolution, high speed images of samples expressing fluorescent proteins. This includes monitoring of the sample over a period of time and even doing this as a Z-stack, consisting of many images which together make up a 3D view of the sample.

One use of PA-GFP is to monitor the flow rate of proteins within a system and this is how it was used by Runions *et al.* when they provided proof that the

golgi bodies of the ER are in fact attached to the ER instead of moving along the surface, and that the lumen of the ER moves far faster than could be expected by diffusion physics alone.² However, in this study, the reason for using PA-GFP instead of normal GFP was a matter of wanting to only illuminate the protein in a specific part of the cell and then follow its progress through the cell.

The intention of this study was to produce a construct for RFP-HDEL which would be retained within the lumen of the ER where it could act as a marker under the microscope. RFP-HDEL could then be co-infiltrated with already prepared Alpha-TIP PA-GFP. Very little is known about the trafficking pathway of tonoplast bound proteins within the cell as the signalling mechanisms of these proteins appear to be very complicated.³ By getting the plant to co-express the two proteins, some of the cells within the plant could be viewed under the confocal microscope over a time period to find out the route taken by Alpha-TIP after translation.

The initial hurdle in this process was in producing the RFP-HDEL marker. By using only a template of the RFP protein, and by designing primers for each end of the sequence, RFP-HDEL was easily assembled. The forward primer for the sequence was already available, as it was used in a

previous study, but the reverse primer containing the HDEL sequence had to be designed. Several criteria had to be fulfilled by the design: the primer should be stable both thermally and chemically; it should be written in reverse order and complementary to the leading strand; it should begin with a restriction site after a tail of junk DNA to make binding to the restriction site more favourable. With these criteria in mind, and with the help of some computer software, a primer was designed and ordered, consisting of a tail of A and T (8 bases long), the *SacI* restriction site (6 bases), a stop codon (3 bases), the codons for L E D and H (12 bases) and the reverse order complementary sequence for RFP (17 bases).

This primer, when used in a PCR reaction with the forward primer and a template of the RFP, produced a large quantity of DNA material which were run on a gel to identify the correctly sized product which was present in large quantities as can be seen in figure 1.

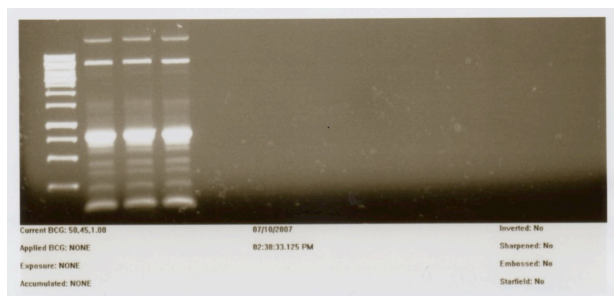


Figure 1 - PCR Product

After purifying the product, digestion of the restriction sites and inserting into the 35s vector containing ampicillin resistance, the ligation product was ready to transform *E. coli* cells to clone the vector overnight. These cells were grown on a nutrient rich medium containing ampicillin, which would selectively only allow *E. coli* cells which had been transformed by the vector to grow.

After an overnight incubation at 37°C, colonies had grown on the plate, and these were screened for the insert by using a PCR reaction to amplify the vector from the genome of each colony. The PCR products were run on a gel to identify the size of the vectors. As can be seen in figure 2, all thirteen colonies tested contained the insert, as they were all around 1.2 kbp in size.

All thirteen colonies were cultured overnight at 37°C in a nutrient rich medium. Since all thirteen colonies grew successfully, but a microcentrifuge would only accommodate twelve tubes, one colony was disposed of. The vectors were extracted from the remaining colonies using a miniprep kit, the product was digested with *EcoRV* which divided the backbone and the 35s cassette. Running the product of this on a gel separated the backbone from the 35s cassette as can be seen in figure 3.

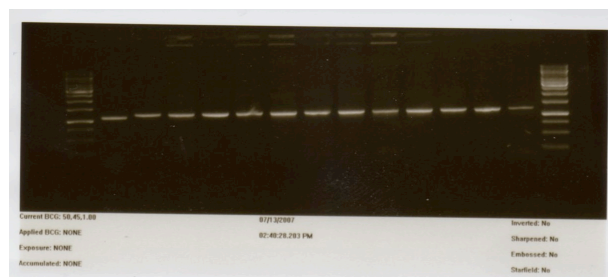


Figure 2 - Amplified vectors from transformed *E. coli* colonies

After the cassette was cut from the gel and purified using the miniprep kit, it was then ligated with pGreen; another vector, this time containing resistance to kanamycin. The whole process was followed again, cloning the vector using *E. coli* once more.

Once colonies of the *E. coli* had been identified as containing the full insert in the pGreen vector, the vector was used to transform *Agrobacterium* which would be used to infiltrate the tobacco plants. To screen the *Agrobacterium* for presence of all the components that make up the construct, it was incubated on a nutrient rich medium with tetracyclin, kanamycin and rifamycin at 28°C overnight. Colonies that grew on the plate were then cultured overnight in nutrient rich medium containing kanamycin. Next day the cells were separated from the medium by centrifugation and stored in glycerol at -80°C.

Previously, this process has also been done by Paul Hunter to generate *Agrobacterium* which contained the DNA for the PA-GFP tonoplast membrane protein to be observed in this study. *Agrobacterium* is the genus name of a gram-negative bacteria which causes tumours in plants. They inject their DNA into the plant to genetically modify the plant to do as the bacteria requires. By placing the DNA for the fluorescent proteins into the *Agrobacterium*, they can be used to infiltrate the plant with genes for the proteins. If, by chance, the same cell is infiltrated with both fluorescent proteins, then that cell will become viable for analysis under the microscope.

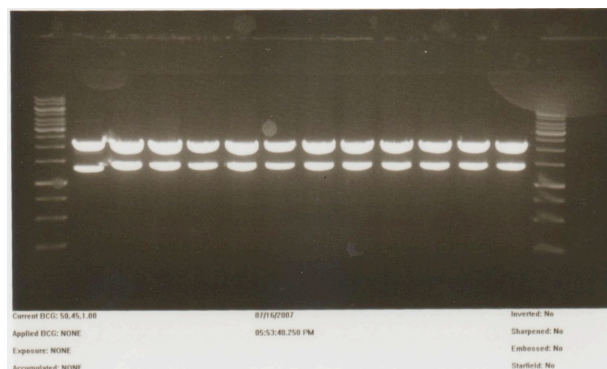


Figure 3 - The 35s cassette separated from the backbone

To infiltrate the tobacco plants, cultures were produced overnight at 28°C by taking a small piece of the glycerol stock and placing it in a nutrient rich medium with kanamycin. The cultures were then washed down by centrifugation and resuspending in infiltration medium. A spectrophotometer was used to standardise the concentration between different cultures before combining the solutions to create a co-expression solution. This was injected into the underside of the leaf to be infiltrated using a standard syringe without a needle. After a few days, the leaf was ready to be viewed under the microscope.

Preparation for the confocal microscope involved placing a piece of double sided tape on a standard slide before cutting a small square of leaf from the infiltrated area and placing it top down on the tape. Sterile water was then placed on the piece of leaf and a cover slip used to limit water evaporation and to present a flat surface for the high powered microscope.

Objective oil was then placed onto the coverslip surface and the slide was placed with the coverslip downwards over the objectives of the microscope. Having brought the Z position up to view the surface cells, the lasers were used to fluoresce the proteins within the cell. The RFP outlines the ER very well, and shows visible signs of it moving rapidly around the cell. The GFP was invisible until the UV laser was used in pulses to activate it, at which point it would also illuminate the ER as this was the area of the cell currently in focus.

Results

The first task, having reached the stage of viewing the sample, was to ensure that the cells were being viewed at the correct time in their development after infiltration. This period of time was to be just after the proteins were beginning to be expressed, but not late enough so that the Alpha-TIP protein had moved to the tonoplast. This was decided by infiltrating one plant, then viewing and activating the PA-GFP of samples taken from it under the microscope at 24 hourly intervals until it could be seen to be fluorescing. After 4 days, hints of fluorescence could be seen, 5 days gave strong fluorescence and 6 days presented fuzzy hazes of green fluorescence where the Alpha-TIP had moved to the tonoplast, instead of remaining co-localised to the RFP-HDEL. At all stages, the red of the RFP-HDEL was overlayed and where the two proteins were co-localised, the image would appear orange, whereas red on its own or green on its own indicated that the proteins were no longer co-localised.

Looking at figure 4, it can be seen that the 4 day sample is pale, but that all the activated PA-GFP is

in the same location as the RFP-HDEL; within the lumen of the ER. However, activated PA-GFP after 6 days is no longer retained within the ER lumen and can be seen as a green haze across the entire cell.

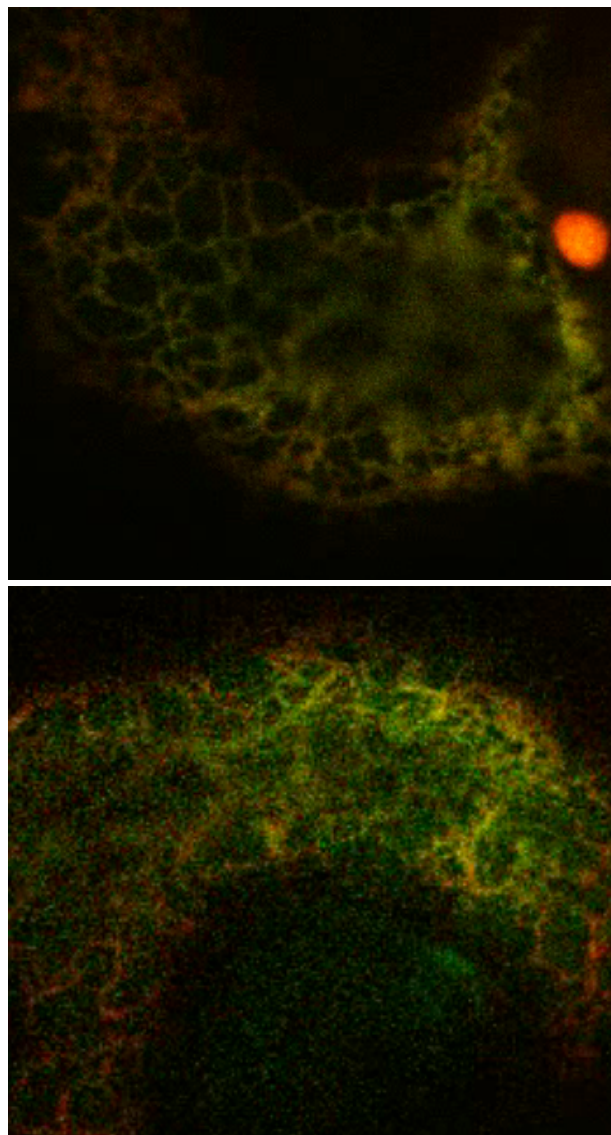


Figure 4 - The appearance of activated PA-GFP overlaid with RFP-HDEL at 4 day (top) and 6 days (bottom) after infiltration

After realising that the best time to view the sample after infiltration was approximately 5 days, a second issue had to be overcome in which very few cells were expressing both proteins within the plant, making it very difficult to perform the study since it takes a long time to prepare the microscope over a single cell and prepare to accurately activate PA-GFP in exactly the right areas of the cell. This is partly due to the fact that activation often would only occur if the zoom level was approximately 200x or greater. At these levels, the UV laser would be very concentrated, allowing activation. Below this zoom level, the laser strength was too dispersed and would not activate the protein cor-

rectly. By the time a cell was identified as having both proteins expressed, it was already partially activated and quite often partially photo bleached due to the time it took to find it. For this reason, infiltrations with a greater concentration of Alpha-TIP versus RFP-HDEL were used. Initially, 1:2 and 1:3 ratios were used. It was found that 1:3 ratio left too few cells expressing RFP in the lumen of the ER, but at a ratio of 1:2, enough cells were found to be expressing both proteins to be able to find one which is co-expressing more easily.

As previously mentioned, the UV laser was unable to activate PA-GFP from a distance, but it would still cause the lumen of the ER in cells which were expressing PA-GFP to glow whilst the UV laser was active; the fluorescence would not persist once the laser was turned off though. This worked to the favour of the study when trying to find a cell that was expressing both proteins. By turning the UV laser on at 63x zoom (the lowest zoom level of the oil objective) cells expressing Alpha-TIP would fluoresce bright green without activating the PA-GFP significantly.

One question proposed by this research was how fast can the entire lumen of the ER be photo activated to a sufficient level to study. The answer to this is very obviously subjective, but in general, by activating a very small patch of the cell's ER (less than a tenth of the entire cell surface), the lumen would flow so fast that the entire cell would be fluorescing within approximately 2 minutes. In order to establish a strong fluorescence that would persist for a longer period of time, it would be advisable to pulse the UV laser for periods of no more than around 10 seconds at a time, and repeat after a further 10 seconds. This would allow fresh inactivated PA-GFP to enter the field of view on the cell and activate that as well. In either case, the cell would have a sufficient quantity of activated GFP within 5 minutes at the very most.

It was noted, during the preliminary tests, as mentioned, that the samples, if left for any period of time, would start to tire. This appeared to be partly due to photo bleaching of the proteins, but also because the sample was starting to dry out, since the heat from the lasers was evaporating the water from under the cover slip. This was not a problem during single sessions of looking at the cells, but the intention was to run a 16 hour overnight time course under the microscope, and for this the sample had to be maintained as much as possible. In order to prevent the water evaporating on these longer time courses, nail varnish was used to seal the edges of the coverslip. Longer coverslips were trialled so that more water could be trapped against the sample, but these presented bad optical reflections under the microscope, so the original, smaller, coverslips were used instead.

The second issue, as already mentioned was that the samples were photo bleaching when exposed to the laser too much. The only way to overcome this was to set the microscope to only expose the sample when absolutely necessary by reducing the frequency of images taken during a time course, but this presented little problem when it was established that the protein's trafficking through the cell was very slow.

One final consideration, before setting up an overnight time course was that the sample would be likely to move during the time period of observation. This is mainly due to the cells changing size whilst submerged in water. This caused the cell to drift in all three dimensions during the time course. To balance this, the microscope's field of view was set to give some allowance around the whole cell in case it moved.

The first overnight time course was technically a huge success. The microscope was set up to take a Z-stack 3D image of a section of leaf which was infiltrated 5 days before. The Z-stack was repeated every half hour, using line averaging to reduce noise in the image. Unfortunately, the problem came from the sample deteriorating rapidly. Having been removed from the leaf, the sample was beginning to senesce and the proteins were being adversely affected by this. The image became more and more fuzzy in both the RFP-HDEL and the PA-GFP as the time continued, and no loss of co-localisation was observed during the first 3 hours, before the images lost too much definition.

At this stage, it became obvious that the approach of removing a section of leaf and observing it over a period of time was never going to show what was being looked for. For this reason, an alternative approach was trialled, in which the PA-GFP would be activated on a UV transilluminator by placing the leaf, still attached to the plant, on top of the transilluminator and allowing it to activate the PA-GFP throughout the entire leaf without causing the leaf to senesce. Then, over a course of time, samples could be removed from the leaf and observed under the microscope to see what progress was being made by Alpha-TIP. The problem, as previously anticipated, was that the transilluminator was nowhere near strong enough to activate PA-GFP and so when a sample was removed and viewed under the microscope, no GFP was visible. By using the UV laser, some of the cells were photo activatable, proving that the transilluminator had not been strong enough. Further time on the transilluminator did not change the situation.

As a final resort, a different RFP construct was used. ST-RFP localises to the golgi bodies of the cells and would act as a marker to find out if the Alpha-TIP passes through the golgi bodies at any stage. If they did, the golgi bodies would become orange instead of the red caused by ST-RFP.

Photo activation of PA-GFP was performed in the same way as before, but this time, the time course was set to run for only 6 hours and a Z-stack of the cell was made every 15 minutes. After 6 hours, 21 of the 25 Z-stacks showed a clear image, representing the first 5 hours after activation. After this time, the image disappears completely; perhaps due to the oil losing contact between the objective and the slide. The Z-stacks were each combined into a single image which shows approximately one third of the cell as a 2D image.

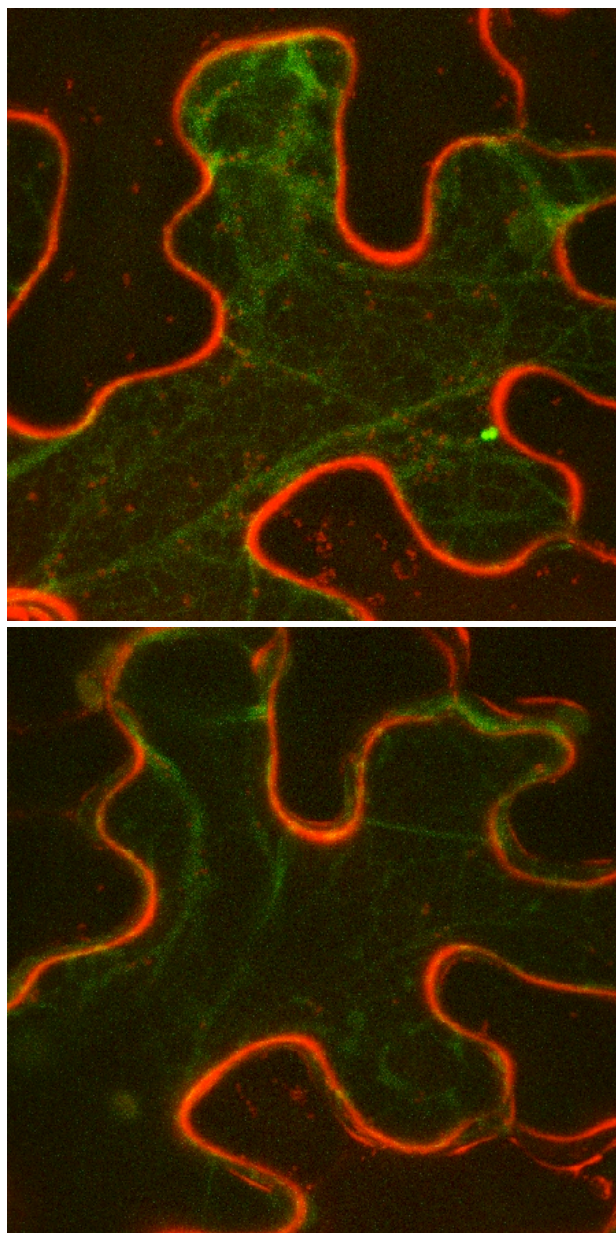


Figure 5 - Maximum projection images of Z-stacks taken in the time course for ST-RFP and PA-GFP at time zero (top) and after 5 hours (bottom)

The 21 slides show very clearly that over the 5 hour period, no co-localisation occurs in the golgi bodies. Figure 5 shows the first and last time point in this series. As can be seen, there is a clear dis-

tingtion between the red golgi bodies and the green of the Alpha-TIP at both time points. I would seem that the Alpha-TIP has begun to localise to the tonoplast (the large dark area of the cell) after 5 hours, but at no point goes via the golgi bodies.

Discussion

Although the original aim of this study, to find the path taken by Alpha-TIP between the ER and the tonoplast, was not achieved as was intended, a number of facts have been brought to light which were previously unknown and form a solid foundation for future studies of this kind.

The first discovery is that RFP-HDEL is much more easily infiltrated into plant cells than Alpha-TIP combined with PA-GFP. It might be that the transcription of RFP-HDEL is promoted more within the plant cell than Alpha-TIP, but if this were true, it would be expected that at least a small amount would be present in all cells, but this isn't the case. It seems much more likely that the *Agrobacterium* find it easier to infiltrate the RFP-HDEL than the Alpha-TIP PA-GFP. Given this information it is now known that when co-infiltrating these two genes, the ratio of RFP-HDEL to Alpha-TIP PA-GFP should be around 1 to 2.

It is also known that the best time to view the sample after infiltration is more than 4 days but less than 6. This time varies from leaf to leaf and weather conditions, but in general, the best time to view the leaf was after 5 days. Any longer than this and the Alpha-TIP had already begun to progress into the tonoplast membrane.

It has also been established that the entire lumen of the cell could be activated in no longer than 5 minutes using short pulses of UV laser light on small areas of the ER. Once activated, the fluorescence of the GFP would remain for many hours, even under intermittent observation by the microscope; only bleaching when excessive exposure to the lasers was used.

During a 3 hour time course, the co-localised nature of the RFP-HDEL and Alpha-TIP was not lost, indicating that Alpha-TIP takes longer than 3 hours to begin trafficking within the cell. This gives an indication as to the time period in which future studies should be looking to observe changes in the localisation of Alpha-TIP as this presents huge technical problems in terms of keeping the microscope looking at the right part of the cell in question and maintaining the sample whilst it is under the microscope. The issue of desiccation of the sample appears to be easily resolved by simply sealing the coverslip, but this doesn't stop the leaf from senescing and disrupting the proteins being observed.

Whilst activating the PA-GFP on a transilluminator failed on this occasion, it may still be possible to take this approach so that the PA-GFP can be activated on a live leaf sample by using a more powerful UV source. Another alternative would be to use non photo activatable GFP on the Alpha-TIP protein and ensure that the sample was monitored from the first signs of GFP presence. This would mean that the UV laser would be unnecessary, but would make the results more complicated to interpret since the GFP would never leave the lumen of the ER but would show in other locations. If those locations happened to be areas that are in close proximity to the ER such as the golgi bodies, then it may be hard to identify the presence of GFP in these new locations.

The final experiment involving the use of ST-RFP to highlight the golgi bodies was a huge success in comparison to previous attempts to obtain results. It is quite obvious from this time course that the Alpha-TIP does not, at any stage, co-localise with the golgi bodies but yet it does reach the tonoplast, indicating it is taking a route that does not involve the golgi bodies. Paul Hunter is currently working on a theory that the protein has a tag called a C-terminal vacuolar sorting signal (ctVSS)⁴ that indicates the protein should not use the golgi bodies as a transport mechanism and instead go straight to the tonoplast. He is attempting to successfully run a similar co-infiltration between ST-RFP and Alpha-TIP with PA-GFP and a C-terminal deletion to see if this mutant protein will pass through the golgi bodies. Unfortunately, this has so far lead to unexpressed copies of the mutant protein, presumably because the plant doesn't see the new protein as a viable gene. In any case, this is a very obvious progression on the work performed in this study.

In conclusion, the study has been a great success in uncovering some of the mysteries behind the trafficking of Alpha-TIP in *Nicotiana rustica*, even if the whole story isn't yet written. However, with a little more time and patience, it may be possible to get even more useful information out of some of the techniques presented here.

Experimental Procedures

Primer Design

Whilst taking into consideration all the factors mentioned, the sequence of the reverse primer is shown in figure 6. Where more than one codon is

present for a particular amino acid, the one used preferentially in the RFP sequence was selected.

PCR Specifications

When performing PCR reactions, a generic formulation was used to produce the reaction solution, as shown.

H ₂ O	39.25µl
Buffer	5.00µl
Template DNA	1.00µl
Sense (forward) primer	1.25µl
Reverse primer	1.25µl
dNTPs	1.25µl
pwo polymerase enzyme	0.30µl
	~ 50.00µl

Digestion

In order to digest the restriction sites, a generic formulation was used, as shown.

H ₂ O	11.0µl
DNA	5.0µl
Restriction Enzyme 1	1.0µl
Restriction Enzyme 2	1.0µl
React™ buffer	2.0µl
	20.0µl

This was then allowed to incubate at 37°C for 90 minutes.

ATATATAT GAGCTC TTA CAG CTC GTC GTG GCGCCGGTGGTGGAGTGGC

Figure 6 - The sequence of the reverse primer

Ligation

During ligation, a standard formulation was used. Both positive solutions (containing the insert) and negative solutions (without the insert) were produced. The formulation is as shown.

	+ve	-ve
H ₂ O	2.5µl	6.5µl
Insert DNA	4.0µl	---
Vector DNA	0.5µl	0.5µl
Ligase Buffer	2.0µl	2.0µl
Ligase	1.0µl	1.0µl
	10.0µl	10.0µl

Transformation of *E. coli*

In order to transform *E. coli* cells with the DNA plasmid required, a specific protocol was followed as shown:

1. 90µl of TCM were combined with 10µl of the product from the ligation process.
2. The *E. coli* cells were allowed to thaw on ice after which the above is added to them.
3. The cells are allowed to continue on ice for 30 minutes.
4. The cells are then thawed completely at room temperature for 10 minutes.
5. 800µl of Luria-Bertani (LB) medium were added to the cells and this was allowed to incubate in a shaking 37°C incubator for 1 hour.

Transformation of *Agrobacterium*

In order to transform *Agrobacterium* with the DNA plasmid required, a specific protocol was followed as shown:

1. 1µl of the product from the ligation process were added to the frozen *Agrobacterium* cells.
2. The cells were thawed at 37°C for 5 minutes.
3. Liquid nitrogen was used to flash freeze the cells again.
4. The cells were then thawed again at 37°C for 5 minutes.
5. 1 ml of LB was added to the cells and they were left at 28°C for 2-4 hours.
6. The culture was then centrifuged at 3,000 RPM for 5 minutes to compact the cells and 800µl of the supernatant was disposed of, leaving the remaining 200µl to resuspend and plate the cells.

Acknowledgements

For their assistance in this work, I would like to thank Nicholas Tolley, Christian Craddock and Paul Hunter of the Frigerio laboratory at the University of Warwick. Without their assistance and advice, this work would not have been possible.

I would also like to thank Dr. Lorenzo Frigerio of the Biological Sciences Department of the University of Warwick, whose expertise on successful usage of the confocal microscope were invaluable whilst attempting to obtain useful results from this study.

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