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Transformation of tobacco with the BA5 cement protein gene from Balanus amphitrite

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Abstract

Barnacles are sessile, marine organisms that adhere to myriad surfaces and compete with each other via fast growth and swamping techniques. Little is currently known about their novel adhesion mechanism whereby a protein matrix displaces water at the substrate, creating a strong bond under wet conditions. Commercial availability of biodegradable, wet-surface glue could be useful in biomedical fields, e.g. joining bones. Understanding the properties of barnacle cement could also aid the shipping industry in preventing barnacle attachment to ship hulls – biofouling, or the adhesion of barnacles to a man-made surface en masse, is known to increase ship drag and fuel costs as well as degrade alloys over time. In an attempt to produce the barnacle glue in plants, one of the major cement protein genes (BA5) has been cloned from the barnacle B*alanus amphitrite*. The cloned gene has been sequenced, recombined with plant-specific promoters and transferred into *Agrobacterium tumefaciens*, from where it was transferred into tobacco cells. Once sufficiently grown, the transformed tobacco plants will be maintained and tested for the expression of the BA5 gene. Parallel attempts are being made to clone genes for other glue component proteins so that they can be tested in different combinations to produce the actual barnacle glue in vitro. In addition to testing for the presence of the BA5 gene within the genetically engineered tobacco, two antibiotic treatments, cefotaxime and carbenicillin, were tested for efficacy on hindering the growth of *A. tumefaciens* post-transformation. Observation of tissue on these selective media showed carbenicillin to be superior in preventing *A. tumefaciens* growth.

Introduction & Background

Barnacles (*Balanus spp.*) are sessile, invertebrate suspension feeders with calcified plates that make up their carapaces (Walters, 2004). These attributes lend to their classification as arthropods and members of the infraclass Cirripedia in the subphylum Crustacea. They are exclusively marine organisms and their habitats generally include substrates located in shallow waters or the intertidal zone (Walters, 2004). What is particularly interesting about these species is their ability to survive and thrive in the face of predation, competition with limpets and mussels and an ever-changing marine environment (Doyle, 1996). Their calcite plates, which can be grown onto substrate along with a stalk for additional adhesion, protect adults from whelk predation and desiccation when not submerged – two calcite plates can close over the feeding aperture.

Additionally, many *Balanus* species effectively compete for space using fast growth and swamping strategies. Rapid growth enables barnacles to resist displacement and reach higher up in a water column for better feeding while swamping involves a massive concentration of barnacles adhering to a large substrate surface to ensure that at least some barnacles survive (Doyle, 1996). The barnacle's journey from birth to adulthood involves multiple stages and steps. The first larval stage is the nauplius which can be described as a one-eyed larva with only a head and a telson. The barnacle grows in this form until reaching the cyprid stage where it then begins its search for a suitable substrate to adhere to. Once adhesion takes place, the barnacle can then begin filter feeding on plankton with use of its cirri to continue growth.

Balanus spp. is considered by some to be a scourge of the ocean related industries due to the fouling they cause underwater pipelines and ship hulls – extensive growth of organisms on ship hulls greatly reduces propulsion efficiency and may lend to metal corrosion (Marine, 1952).

The problem lies within their ability to strongly adhere to a substrate using specialized cement – a network of proteins with attributes ranging from high hydrophobicity to a strong charge and high cysteine content (Kamino 2000; 2001). This cement is able to efficiently solve several problems associated with underwater adhesion: it is able to avoid random aggregation in the barnacle's secretory glands during transport, maintain solubility during movement to the attachment site and can displace water at the attachment site for ensure effective binding to the substrate (Kamino. 2001). The source of these proteins in the adult barnacle is believed to be a cement organ and corresponding excretion duct that lie within the basal portion, near the ovariole (Kamino, 2001). Research on the proteins involved in barnacle adhesion has been difficult due to the fact that the cement is highly insoluble (Wiegemann, 2005). At this time, barnacle cement has only been partially dissolved in a lab setting. The resulting analysis of this partial dissolution is that the substance is approximately 90% protein and 4% inorganic ash (comprised of calcium and other minerals) with the remainder of the cement's makeup being carbohydrates and lipids (Dickinson, 2009). Specifically, it is believed that, based on preliminary research with individual barnacle species, the cement is made up of up to ten major proteins (Dickinson, 2009). However, only a fraction of these have been successfully isolated and studied for composition and cement makeup may vary with species.

Before delving deeper into the conjectured mechanism of adhesion of a cyprid to a substratum, it must be made clear that the quality of the adhesion taking place is directly linked to the spreading of the adhesive's liquid phase and the wettability of the substratum's surface (Wiegemann, 2005). Additionally, liquids tend to spread more freely on hydrophilic surfaces than on hydrophobic ones. However, despite the perceived favoritism, fouling organisms such as the barnacle can adhere to a surface based on a range of substrate attributes that possibly involve either surface charge (Wiegemann, 2005). Attachment begins by a small amount of liquid cement being excreted onto the surface by the cement duct; the attachment organs of the antennules embed themselves in this liquid cement (Wiegemann, 2005). It is important to stress that barnacles are able to prevent aggregation of the proteins that make up the cement within the body but rather mix the proteins just as they are each leaving their respective cement ducts to prevent internal hardening. After initial attachment, the cyprid undergoes metamorphosis into a juvenile barnacle. Additional cement excretion fills gaps between the base plate and the surface via capillary action that excludes water from the surface (Wiegemann, 2005).

Research has already been done on a few particular barnacle species, while others have had little exposure. However, it is important to highlight what is currently known about major species of barnacle as there may be an overlap between cement composure and functionality. For example, there are currently five known cement proteins that have been identified from *Megabalanus rosa*; these are designated as M. rosa cement protein (Mrcp): Mrcp-100k (accession # AB033942.1), Mrcp-52k, Mrcp-68k, Mrcp-20k (accession # AB035415.1) and Mrcp-19k (accession # AB242294.1) (Urushida, 2007). Each of these proteins is considered novel and demonstrate differences between them. Mrcp-100k (N-terminal amino acid sequence of HRPSFERR*XX*G*X*LRSPVAADLDDDEIGM with X most likely being Cys) and Mrcp-52k were both attributed to the insolubility and hydrophobicity of the barnacle cement for stability in seawater and may act as bulk proteins in the cement complex (Kamino, 2000; 2001; Urushida, 2007). Mrcp-68k is defined as having 57% of its total amino acid residues consisting of Ser (16%), Thr (14%), Gly (15%) and Ala (12%); this composition is unusual compared to other cement proteins (Kamino, 2001). Mrcp-20k has an experimentally determined molecular mass of 23689 Da when all Cys residues were alkylated and exhibits six repeats of a Cys-rich sequence

which lends to its charged nature. The N-terminal amino acid sequence of Mrcp-20k is HEEDGVCNSNAPCYHCDANGENXSXNXELFDXEAKKP with X most likely being Cys (Kamino, 2001). A fifth cement protein, Mrcp-19k, had a mature N-terminal sequence of VPPPXDLGIASKVKQKGVTGGGASVSTT where X is most likely Cys (Urushida, 2007). The mature N-terminus amino acid sequences were not found for Mrcp-52k (accession # AB623048.1) and Mrcp-68k. Conversely, the proteins isolated from other species such as the BA5 cement protein gene of *Balanus Amphitrite* (accession # E30232) or the BA6 gene from *Semibalanus balanoides* are relatively unstudied in terms of function and amino acid composition but may yield similar conclusions upon more in-depth study.

Expressing barnacle cement proteins such as the BA5 or BA6 cement proteins individually within plant vectors would greatly contribute to the study of barnacle cement as a whole since it is notoriously difficult to dissolve in the lab setting (Kamino, 2001). Not only would individual isolation and analysis of the proteins that make up the glue matrix aid in understanding how they effectively work together, such analysis may lead to the developments in the shipping industry and biomedical field. Understanding the protein structure and producing these proteins in bulk may open up avenues for the development of ship hull and pipeline coatings that affect the wettability of the surface in question to hinder successful barnacle attachment and thus reduce drag and corrosion to decrease fuel and maintenance costs. Additionally, a derivative of the cement proteins may find use in the biomedical field as a way to join bone to bone or tendon to bone after an injury due to the cement's ability to displace water (or bodily fluids). This potential for scientific advancement can be made possible by using plant vectors such as tobacco for *in vitro* studies; advantages of such a system would be the ability to control environmental conditions, develop cost-effective transformation and protein recovery mechanisms and regulate protein synthesis (Doran, 2000). All of these factors would greatly enhance commercial viability of protein production via plant vectors. This research may also allow for further advancement in the use of plant systems for the mass production of animal-derived proteins since the biomass production can be large-scale and low-cost through agriculture and there is generally a low risk of mammal-borne viruses, blood-borne pathogens, oncogenes and bacterial toxins (Doran, 2000).

Two methods of gene transfer to plants involve the use of *Agrobacteria* in bacteria-plant horizontal gene transfer or a gene gun. Found normally in the soil, species within the genus *Agrobacterium* are gram-negative bacteria that are capable of inter-kingdom gene transfer; this is a unique characteristic (Pitzschke, 2010). Evidence of gene transfer to plant species by this bacterium is found in the formation of plant tumors with tumor proliferation occurring with or without the presence of the bacterium. This bacterium-absent proliferation is the result of plant cell transformation on a genetic level (Pitzschke, 2010). Genetic manipulation for bioengineering purposes utilizes the species *A. tumefaciens.* DNA sequences that are to be injected into plant cells are first incorporated into the tumor DNA (T-DNA) region that has its tumor-inducing (Ti) plasmid 'disarmed.' This plasmid is then introduced into the bacterium which is then transferred into plants via the aforementioned horizontal gene transfer method (Pitzschke, 2010). Since the genes that govern tumor growth in plants have been removed, the plant cells transformed by DNA exchange and incorporation can successfully pass the engineered DNA to their progeny (Pitzschke, 2010). Current work with the BA5 cement protein gene from *B. amphitrite* involved the cloning of the gene and subsequent transfer into first E. coli vectors and then agrobacterium vectors.

Gene guns are just that: guns that are capable of directly delivering foreign DNA into regenerative cells to achieve genotype-independent transformation in plants (Christou, 1992). One specific way gene guns work is through microparticle bombardment: plasmid DNA is complexed onto dense metal particles such as gold or tungsten, although tungsten may result in cellular death due to toxicity with some plant organisms (Furth, 1997). This method of gene injection is typically used for plants due to the low tissue layer penetration that affects cells within 500 µm of the injection site (Furth, 1997). This technique is also generally more expensive than other gene transfer techniques.

The objectives of this research are to successfully transform tobacco leaf tissue with *A. tumefaciens* containing the pMDC83/BA5 plasmid and test for the presence of the BA5 gene within the transformed tissue as well as gauge the efficacy of carbenicillin and cefotaxime in preventing *A. tumefaciens* growth post-transformation.

Experimental Design & Methods

Basic experimental approach leading to the transformation of tobacco leaf tissue with A. tumefaciens – Accomplished prior to current research on the BA5 gene, *Balanus Amphitrite* specimens were collected from the coastal areas of New Hampshire and mRNA was isolated from their glue glands using the Zymo Research RNA purification kit. This mRNA was then reverse transcribed into cDNA using RT-PCR, at which time the BA5 gene was amplified from the newly acquired cDNA with the use of specific PCR primers. The PCR products were then confirmed as correct using gel electrophoresis. The BA5 gene was then cloned into a pCR8 GW TOPO gateway compatible entry vector via a TOPO cloning reaction, after which the BA5 gene was cloned again, this time into both a pMDC83 vector for plant expression and a pDEST17 vector for bacterial expression – the bacterial component of past research was simply to confirm that the BA5 gene had been successfully transferred to a bacterial vector, similar to current work on transforming a plant vector with its respective plasmid (figure 1) – this was done via an LR-clonase reaction and then then confirmed using dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Primer design and sequencing of the BA5 gene – Sequencing reactions were prepared by first designing the appropriate forward and reverse primers for the BA5 gene. This design was carried out by obtaining the accession number for the BA5 gene (E30232) through the NCBI website and then pasting the FASTA sequence into BioEdit Ver. 7.1.3.0. The NCBI page for the BA5 gene was then referenced for the correct start and stop codons in the appropriate reading frame so that the largest portion of the gene was selected. For the forward primer, the first twenty nucleotides were selected beginning at the start codon (ATG). A reverse compliment was then performed on the sequence using BioEdit commands and once again the first twenty base pairs were selected to obtain the reverse primer. Each primer was then selected and analyzed using Oligoanalyzer 3.1 to ensure that selected primers had a low enough GC content and did not form self-dimers. Additionally, the melting temperatures of both primers were checked to be sure that they were within 5°C of each other for proper PCR amplification. The final primer designs are as follows: Forward (F1) BA5 primer 5'-(ATG-CTG-CGG-CTG-ATC-CTG-TT)-3' and Reverse (R1) BA5 primer 5'-(GCA-CCC-GTA-GCT-CTT-GCA-G)-3'.

The second step in the BA5 gene sequencing process involved obtaining and resuspending 100x concentrations of BA5 gene primers in double distilled water (ddH_2O); water

was added using the formula 1000 multiplied by the weight of the primer in milligrams (i.e. 190 µl of ddH_2O was added to 0.19 mg of primer). After each primer had resuspended thoroughly, 10x dilutions were made by adding 5 µl of the 100x suspension to 45µl ddH_2O. The sequencing solutions were then made by adding 1µl of the forward or reverse primer (10x dilution) to 2µl of the pMDC83/BA5 plasmid DNA stock from the -80°C freezer and 3µl ddH_2O. These solutions were sent to the Hubbard Center for Genomic Studies for processing and analysis of the BA5 cds sequence.

Restriction digestion – BamHI and BstEII were used in the restriction digestion of the pMDC83/BA5 plasmid; the former was implemented to confirm that the BA5 gene was in the correct orientation within the plasmid backbone and the latter to confirm the presence of the BA5 gene. Table 1 shows the components of each prepared digestion. The Master mixes were briefly centrifuged down and then both were incubated for 2.5 hours; the BamHI mix was incubated in at 37°C bath while the BstEII mix was incubated in a thermomixer at 60°C. The resulting digestions were then run on a 1% SeaKem LE agarose gel in 1x TAE buffer at 80 volts for 40 minutes. The gel was then soaked in 50 µg/ml ethidium bromide for 15 minutes, after which the gel was transferred to a ddH_2O bath for 10 minutes. The washed gel was then taken to the UV chamber where a UV photo was taken of the gel using PCimage.

Table 1

	BamHI	**BstEII**
Buffer	1.0µl BamHI buffer (10x)	1.0µl Buffer 3
10x BSA	1.0µl	1.0µl
Enzyme	0.3µl	0.3µl
pMDC83/BA5 plasmid DNA	2.0µl	2.0µl
ddH_2O	5.7µl	5.7µl

Transformation of tobacco leaf tissue – In preparation for the tobacco tissue transformation, basal medium was prepared to the specifications in table 2. The pH of all prepared media was adjusted to a value between 5.50 and 5.70 from an initial pH of around 3.8. The medium was autoclaved on the liquids cycle for approximately 20 minutes (121°C) and then the plates were poured (approximately 30 ml of medium per plate). At the same time, a subculture of electrocompetent *A. tumefaciens* containing the pMDC83/BA5 plasmid had been incubating in liquid Luria Broth (LB) medium for 16 hours after an initial liquid culture of LB medium for 16 hours – both the culture and subculture were incubated in a 28°C shaker. On the day of the transformation, acetosyringone (15 µl of a 50 mM stock concentration) was added to the liquid subculture followed by a 4 hour incubation time, after which the subculture was spun down in a large centrifuge (10,000 rpm) for 5 minutes. The supernatant was then discarded and the pellet was resuspended in 30 ml of 0.9% (w/v) NaCl solution; the resulting solution was further diluted with the same NaCl solution until the OD_{600} of the solution was between 0.6 and 0.7 – this resulting solution is the Agro wash.

Within a laminar flow hood, the Agro wash was poured into a Petri dish. Tobacco leaves were then extracted from magenta boxes from the growth room and cut into roughly 1 cm^2 pieces – six pieces per plate. These freshly cut pieces were submerged in the Agro wash and then stabbed repeatedly with a scalpel. The leaf pieces were then left to soak for 15 minutes, after

which they were dried on sterile paper towels and placed onto non-selective Basel Medium with the greener leaf side facing upward and as much of the leaf as possible touching the medium. The plate was then labeled and wrapped with micropore tape to minimize the potential for contamination. The leaf pieces were then allowed to sit in the growth room for 24 hours, during which time selective medium was prepared.

Table 2

Concentration	Component	300 ml solutions (10 plates)
30 g/L	Sucrose (sigma)	9 g
4.3 g/L	MS salt	1.299 g
2 ml/L	500x Vitamin Solution (Lin 3/27/12)	300 µl
1.0 mg/L	BAP (Lin 9/19/2011)	300 µl
0.1 mg/L	NAA (Lin 1/24/12)	300 µl
2.4 g/300 ml	Plant tissue agar – Type A	2.4 g

Antibiotic treatment options – Two types of selective media were used, one containing hygromycin (50 mg/L) and carbenicillin (300 mg/L) and one containing hygromycin (50 mg/L) and cefotaxime (3 g/L). Two sets of 10 plates were tested with carbenicillin while one set of 10 plates was tested with cefotaxime, both of which being transferred to the selection medium from the non-selective media after soaking each leaf piece in a ddH_2O bath containing their respective antibiotics with the same concentrations as the plates.

Results

Sequencing results showed that only the forward primer designed for the BA5 gene matched up to the actual BA5 cds sequence taken from the NCBI website (Appendix A). The reverse primer did not link up properly with the BA5 cds. Conversely, the pairwise alignment BLAST of the forward and reverse primers against the BA5 cds on the NCBI site showed that both of the designed primers matched up with the gene sequence (figure 4).

Digestion of the pMDC83/BA5 plasmid with BsteII yielded faint banding in the vicinity of 402 base pairs, a cut of the plasmid used to determine orientation within the plasmid of the BA5 gene (figure 3). However, the digestion of the plasmid with BamHI, the restriction enzyme used to test for the presence of the BA5 gene, yielded no banding at all (figure 3).

The transformation of the tobacco leaf pieces with the transformed *A. tumefaciens* appeared to be successful – the development of callus and shoots is being closely monitored. One thing to mention specifically is the efficacy of the two chosen antibiotic treatments, carbenicillin and cefotaxime, on the hindrance of *A. tumefaciens* on the exterior of the plant tissue and growth media. The selective media containing hygromycin and carbenicillin showed normal tobacco tissue cell death (preceding the callus stage) with no *A. tumefaciens* growth (figure 5). Conversely, the selective media containing hygromycin and cefotaxime showed extensive *A. tumefaciens* growth and thus, contamination of the media (figure 6).

Discussion & Conclusions

The importance of confirming that the BA5 gene had been successfully transferred into *A. tumefaciens* from the bacterial expression vector preceding it was paramount for the eventual transformation of the tobacco leaf tissue (figures 1 and 2). Since the orientation of the plasmid fragment cut out of the pMDC83/BA5 vector was demonstrated via the gel picture but not the BamHI cut of where the actual BA5gene was supposed to be, sequencing was needed to further ensure its presence. However, only the forward primer annealed to the BA5 cds sequence. A pairwise alignment BLAST of the forward and reverse primers against the BA5 cds was required to confirm that the primers had been designed correctly, leading to the belief that there had been some error in the actual sequencing process, leading to the reverse primer failing to anneal (figure 4). At this time, the tobacco leaf cultures are being monitored for signs of contamination while they grow on selective media (hygromycin and carbenicillin plates are the only ones that have, thus far, remained uncontaminated).

While individual cement proteins are being cloned and transferred into plant tissue for amplification, work is already being done on providing non-toxic coating solutions for the shipping industry; previous means of biofouling prevention such as the use of biocidal antifouling paints containing copper and tributyltin (TBT) have come under scrutiny since safe interaction with the marine ecosystem is unknown for these compounds (Pettitt, 2004). Work is currently being done to develop silicone-based alternative treatments that are known to be non-toxic and exhibit low energy, low modulus properties (Pettitt, 2004). However, such research is still in experimental stages, so a better understanding of barnacle cement's properties as a whole would go a long way in aiding preventative measures. A more comprehensive knowledge of the cement's properties may also allow for other solutions such as the use of bacteria to prevent adhesion. It is already known that there are physical and chemical stimuli involved with barnacle larvae and their preference for a substrate (Maki, 1988). It has been shown that substrates coated with certain kinds of bacteria can positively or negatively affect the rate of successful adhesion for other sessile marine organisms (Maki, 1988). If cement protein research explored more avenues in biological adhesion deterrents, an effective solution may come to light that limits the amount of potentially harmful chemicals released into marine ecosystems (i.e. a ship hull or pipeline coating that may degrade over long periods of time).

Despite developments in other aspects of barnacle cement not pertaining to its genetic makeup, it is safe to say that understanding how cement works at the molecular level will benefit all areas of research on this topic. Future work would involve confirming that the BA5 gene successfully transferred to the tobacco and continuing to grow and maintain surviving tobacco leaf tissue that has been transformed (figure 7). Transforming tobacco and other plants (such as Arabidopsis) with the BA6 cement protein gene from *Semibalanus balanoides* will also be under consideration. Such work would enable the streamlining of the plant transformation process as well as allow for mass accumulation of said genes of interest for individual study. The testing of antibiotics on the selection media is especially important as the correct type and its usage will enable a higher success rate for plant tissue transformations.

References Cited

Christou P & D. McCabe (1992) Particle gun transformation of crop plants using electric discharge (ACCELL™ Technology). Probe 2 (2)

Dickinson G, I. E. Vega, K. J. Wahl, B. Orihuela, V. Beyley, E. N. Rodriguez, R. K. Everett, J. Bonaventura & D. Rittschof (2009) Barnacle cement: a polymerization model based on evolutionary concepts. J Exp Biol (212): 3499-3510.

Doran P. M. (2000) Foreign protein production in plant tissue cultures. Current Opinion in Biotechnology (11): 199-204.

Doyle P, A. E. Mathers, M. R. Bennett & M. A. Bussell (1996) Miocene barnacle assemblages from southern Spain and their palaeoenvironmental significance. Lethaia (29): 267–274**.**

Furth P.A. (1997) Gene transfer by biolistic process. Molecular Biotechnology (7): 139-143.

Kamino K (2001) Novel barnacle underwater adhesive protein is charged amino acid-rich protein contituted by Cys-rich repetitive sequences. Biochem J 356: 503-507.

Kamino K, K. Inoue, T. Maruyama, N. Takamatsu, S. Harayama & Y. Shizuri (2000) Barnacle cement proteins: Importance of disulfide bonds in their insolubility. J Biol Chem 275 (35): 27360-27365.

Maki J, D. Rittschof, J. D. Costlow & R. Mitchell (1988) Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films. Marine Biology (97): 199-206.

Marine Fouling and Its Prevention, U.S. Naval Institute, Annapolis, Maryland, 1952.

Pettitt M, S. L. Henry, M. E. Callow, J. A. Callow & A. S Clare. (2004): Activity of Commercial Enzymes on settlement and Adhesion of Cypris Larvae of the Barnacle *Balanus amphitrite*, spores of the Green Alga *Ulva linza*, and the diatom *Navicula perminuta*. Biofouling (20): 6, 299-311

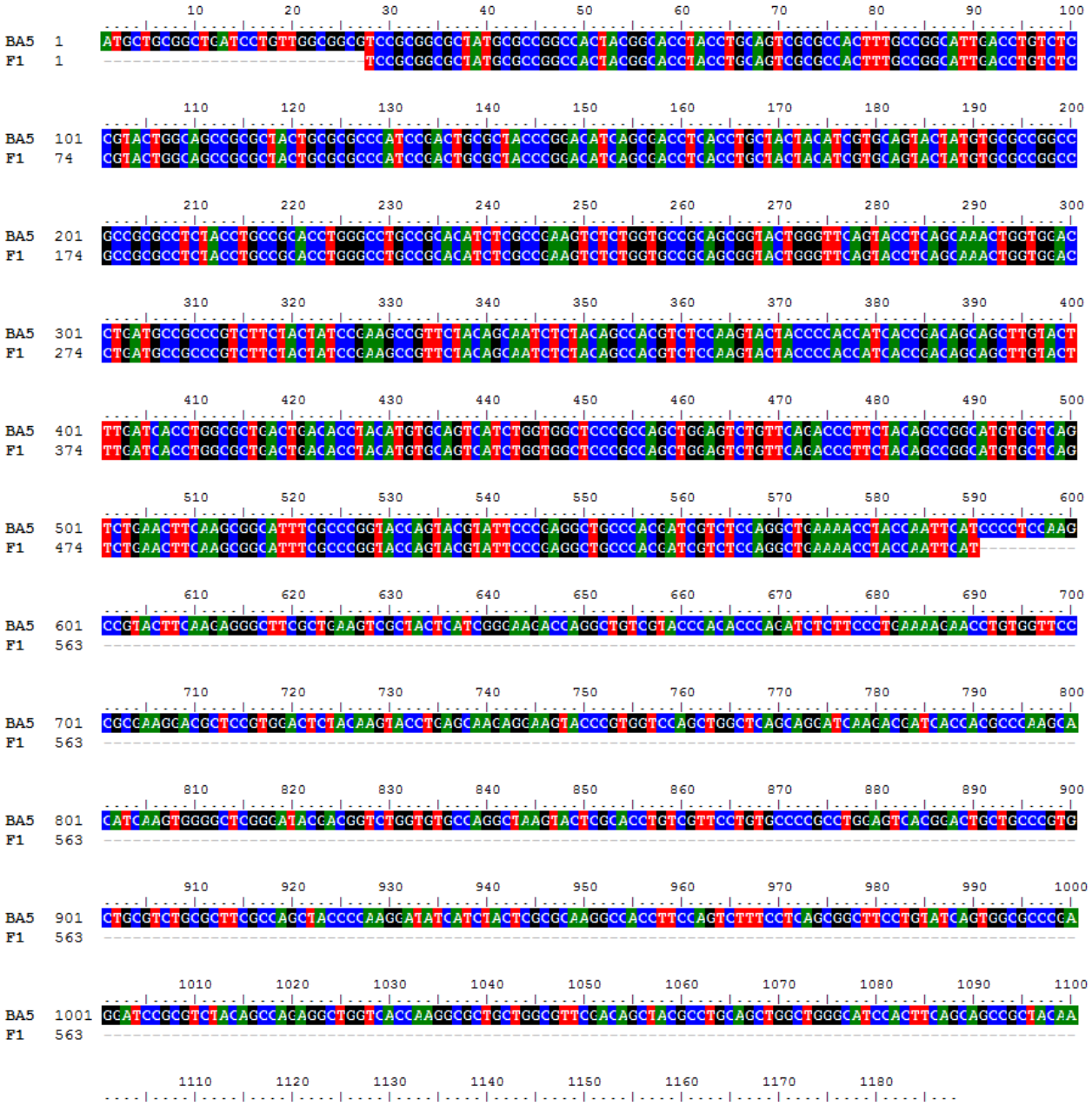
Pitzschke A & H. Hirt (2010) New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. EMBO J. 2010 (29): 1021-1032.

Urushida Y, M. Nakano, S. Matsuda, N. Inoue, S. Kanai, N. Kitamura, T. Nishino & K. Kamino (2007) Identification and functional characterization of a novel barnacle cement protein. FEBS Journal 274 (2007): 4336-4346.

Walters, Martin, and Jinny Johnson. *The World of Animals*. Bath [England: Parragon, 2004. Print.

Wiegemann M (2005) Adhesion in blue mussels (*Mytilus edulis*) and barnacles (genus *Balanus*): Mechanisms and technical applications. Aquatic Sciences 67 (2): 166-176.

Appendix A



Sequencing results of the BA5 barnacle cement protein gene cds within BioEdit Ver. 7.1.3.0. This cds sequence was matched up and compared to the created sequences based on the forward and reverse primers sent into the Hubbard Center for Genomic Studies. It should be noted that only the forward primer (F1) designed for the BA5 cds sequence aligned accordingly while the reverse primer (R1) did not – R1 is not compared to the BA5 cds in BioEdit as a result.

Figures

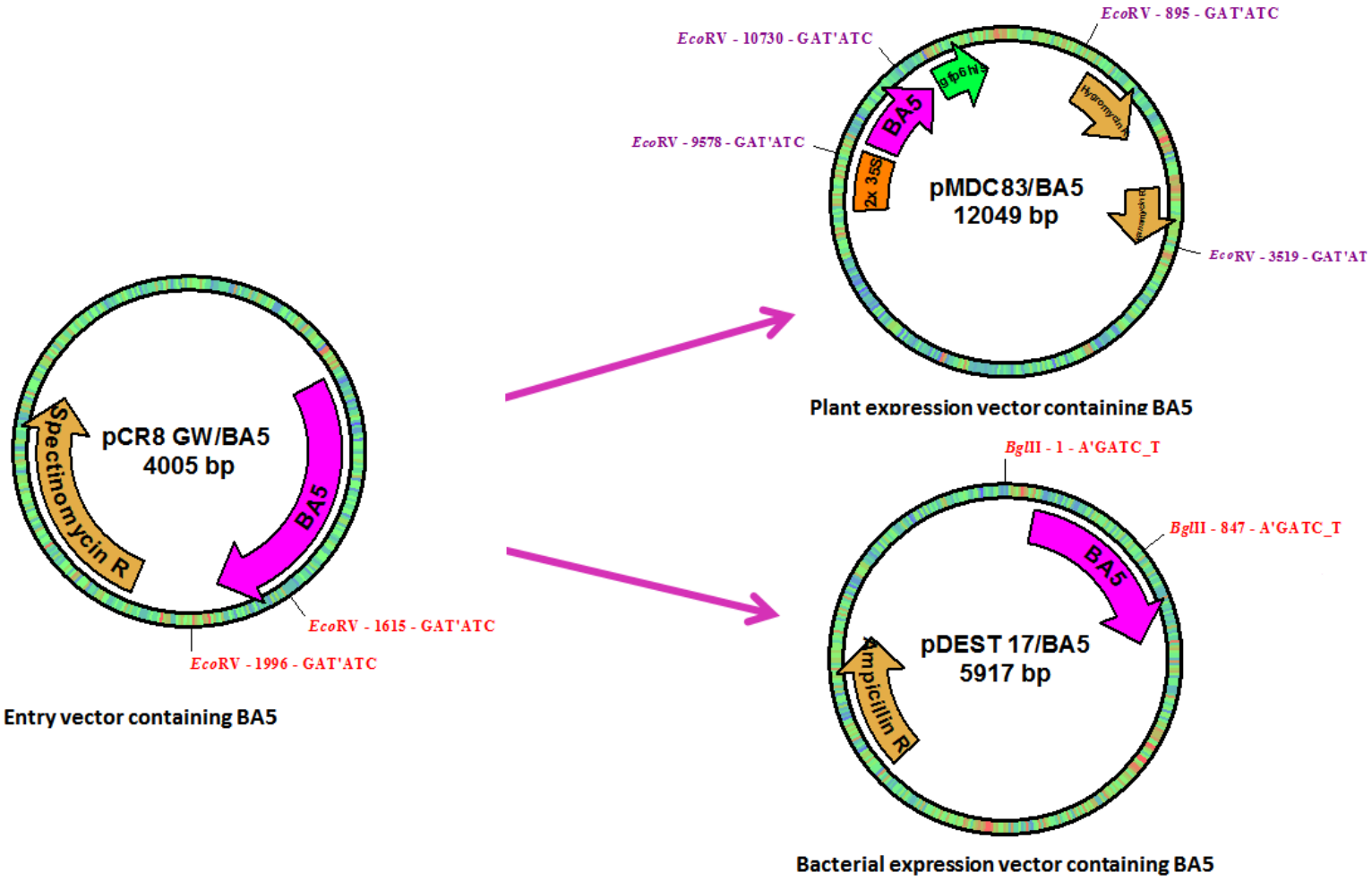


Figure 1 – The overall path of the BA5 gene from entry vector to the bacterial expression vector and finally the plant expression vector. Only the pMDC83/BA5 vector was used for the purpose of plant tissue transformation as previous work had already been done with the pDEST17/BA5 plasmid.

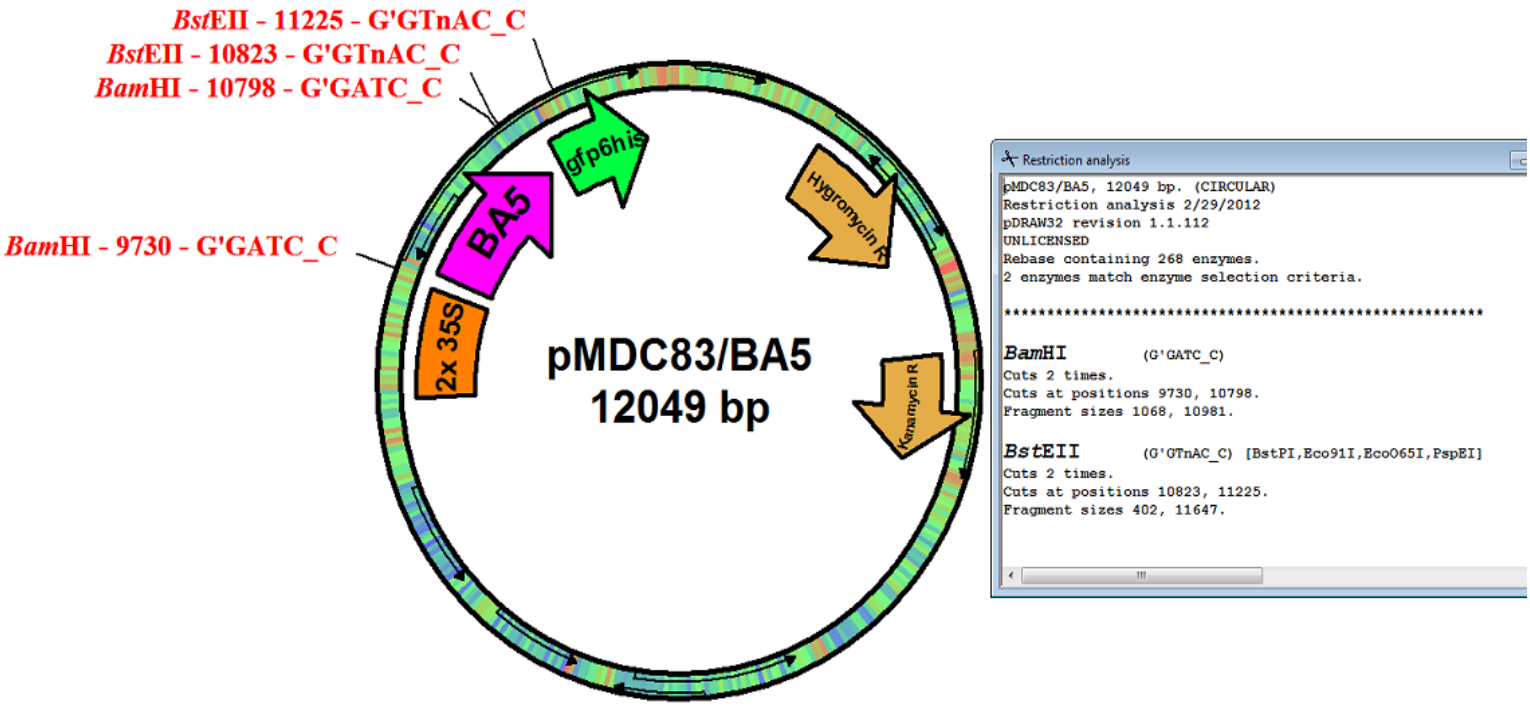


Figure 2 – The pMDC83/BA5 plasmid contained within a transformed *A. tumefaciens* bacterial cell, ready for transfer into a tobacco cell. Also shown are the two restriction enzymes used in the restriction digest to confirm the presence and orientation of the BA5 gene. The pMDC83/BA5 plasmid should include the BA5 gene attached to a gfp6his tag and a 2x35s promoter sequence. Additionally, the plasmid should have hygromycin resistance for when the tissue is being transformed.

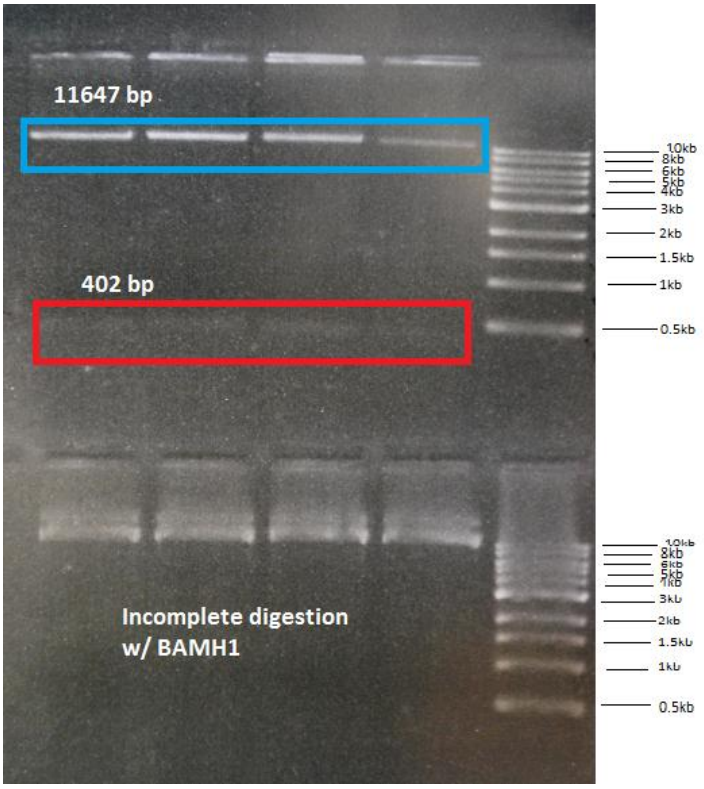
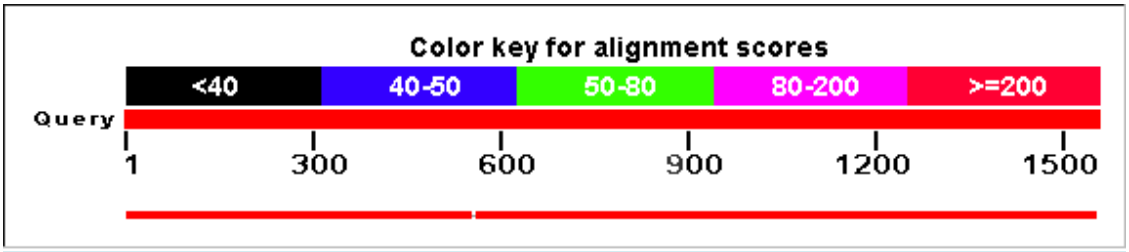


Figure 3 – Gel electrophoresis results for the restriction digestion of the pMDC83/BA5 plasmid using BamHI and BstEII. The BstEII restriction enzyme was able to successfully cut the plasmid at its designated sites to show orientation of the BA5 gene within the plasmid backbone but bands showing the BamHI restriction enzyme's cut were absent.



Accession	Description
E30232.1	Fifth adhesive protein gene of barnacles

Max score	Total score	Query coverage	E value	Max ident
1784	2779	99%	0.0	99%

Figure 4 - Pairwise alignment BLAST of the forward/reverse primer and BA5 gene. Sequences created via the forward (F1) and reverse (R1) primers sent in for processing at the Hubbard Center for Genomic Studies were put together and compared to the BA5 cds via the NCBI website(http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=blast2seq). The resulting alignment scores showed a max identity value of 99%, confirming that the sequences created based on the designed primers were not the reason behind the failure for the R1 primer to anneal to the BA5 sequence.

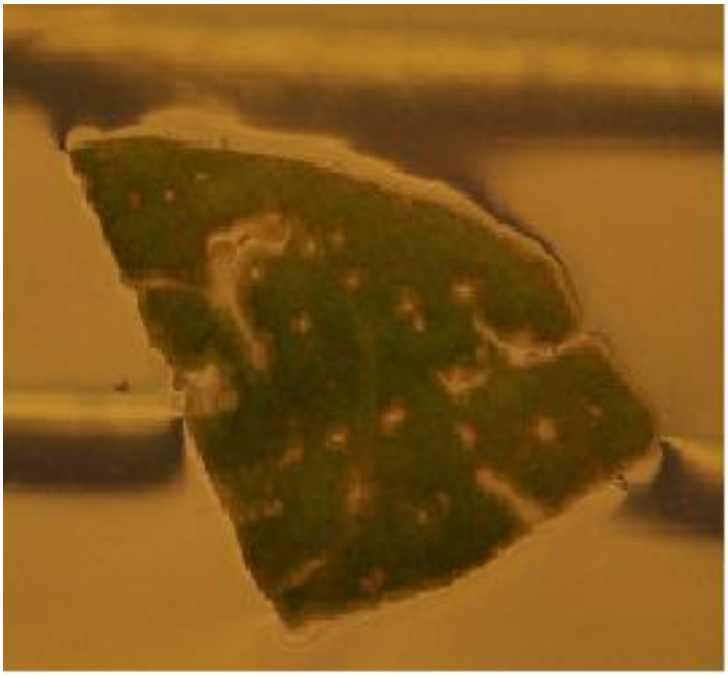


Figure 5 – Transformed tobacco leaf tissue on selective media containing hygromycin and carbenicillin. Photographed three days after plating. The lack of contamination by *A. tumefaciens* indicates the effectiveness of carbenicillin in selective media post-transformation.

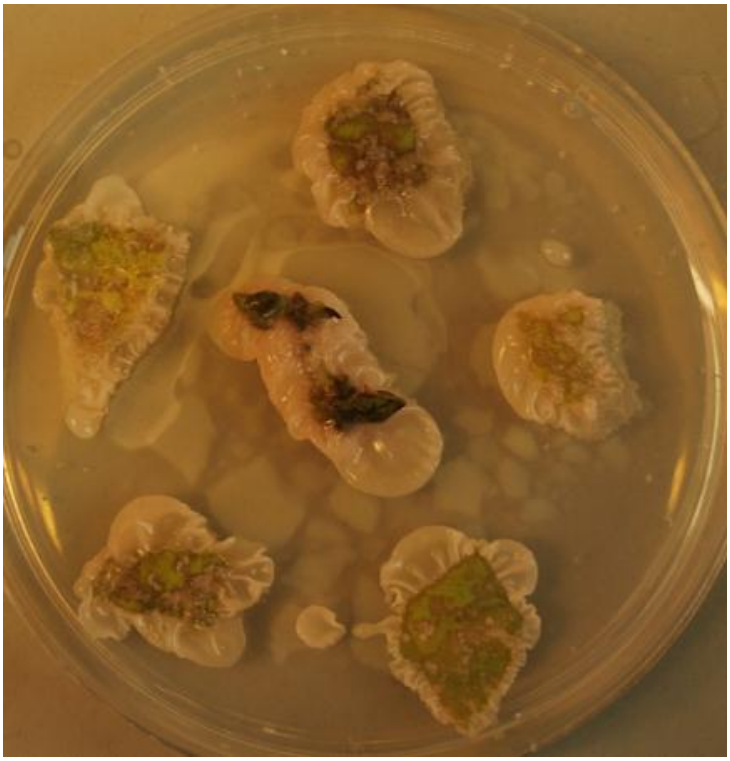


Figure 6 - Transformed tobacco leaf tissue on selective media containing hygromycin and cefotaxime. Photographed three days after plating. The resulting contamination of the tobacco tissue pieces demonstrates how ineffective cefotaxime is in hindering *A. tumefaciens* growth relative to carbenicillin.

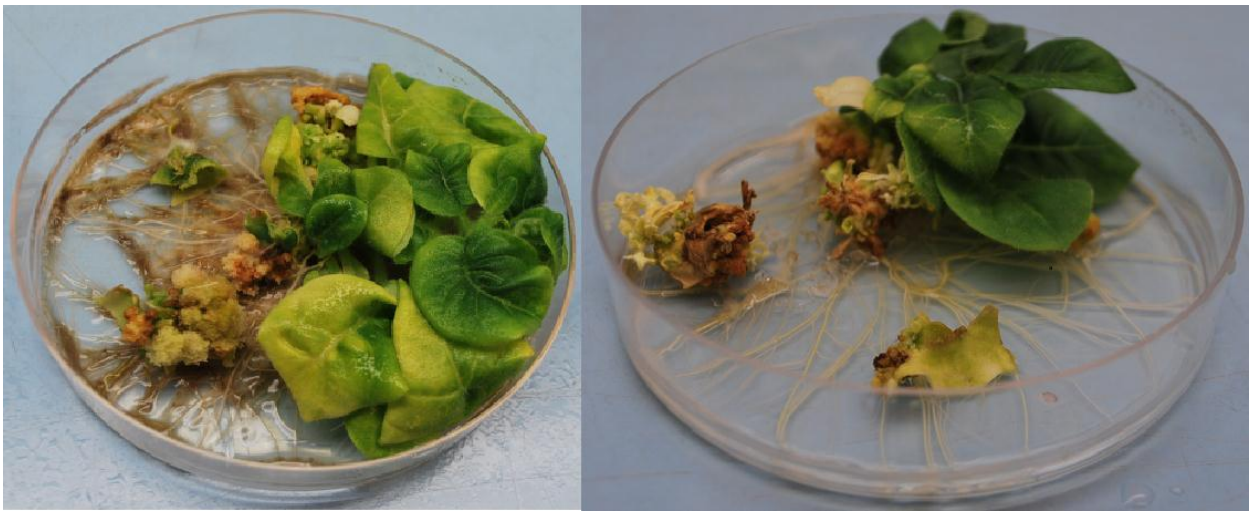


Figure 7 – Expected growth of the transformed tobacco leaf cultures as they grow callus and shoots and then sprout into fully fledged tobacco plants containing the BA5 gene at which point they will be tested for the presence of said gene.