

Transformation of a transposon construct into tomato for functional
genomics studies

Dragana Avirovik

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Andy Pereira
Christopher B. Lawrence
Dorothea B.C. Tholl

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ABSTRACT

Tomato (*Solanum lycopersicum*) is a member of the Solanaceae family. In this research project tomato, more specifically the M82 cultivar was chosen as a model plant for *Agrobacterium*-mediated gene transfer by cotyledon inoculation. Our objective was to transform tomato with a T-DNA construct bearing a transposon from maize that can be used for mutagenesis when it transposes or moves around the genome of the tomato. The vector used is a two-component in-cis Ac-Ds system which needs a single transformation event. It was proved that it worked in *Arabidopsis* and rice according to Trijatmiko (2005). The construct consists of the BAR gene conferring resistance to herbicide Basta, hygromycin (HYG) gene conferring resistance to the antibiotic hygromycin and the green fluorescent protein (GFP) gene, which are driven by specific plant promoters. The selectable marker genes such as HYG and BAR were used to select the rare transformation events by making the transformed tomato tissue resistant to the toxic chemicals (antibiotic and herbicide) compared to the untransformed tissue in which growth was inhibited. The results described consist of developing a transformation protocol which enabled the production of transgenic tomato lines by the help of the antibiotic augmetin (amoxicillin/clavulanic acid). The transgenic lines were tested through polymerase chain reaction (PCR) and herbicide bioassays.

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Chapter 1

Introduction and Literature review

1.1 Tomato (*Solanum lycopersicum*)

In this study tomato (*Solanum lycopersicum*) was chosen as the plant for genetic transformation, using *Agrobacterium*-mediated gene transfer by cotyledon inoculation. Tomato is part of the *Solanaceae* family, which also includes other important crops such as eggplant, potato, tobacco etc. that are part of this family as well (Diez, 249). Tomato is a diploid species (having two sets of chromosomes) $2n= 24$. There are many different varieties of tomatoes available around the world with different traits such as: external and internal quality of fruits, shelf life, resistance to biotic and abiotic stresses. We chose for our research to study a tomato as our model system, more specifically the M82 cultivar. The M82 cultivar has been used for a lot of tomato breeding compared to other genotypes such as microtom, moneymaker, beefsteak, cherry and many others.

1.2 Tomato genomics and functional genomics

The tomato genome has been sequenced by the International Tomato Genome Sequencing Project that started in 2004 and was published in May 31st, 2012. To study the function of genes discovered by sequencing, methods to make mutants in tomato are required. Mutants can be loss-of-function to identify mutants with a knockout mutant phenotypes, or gain-of-function to get phenotypes of genes that are redundant and therefore no knockout phenotypes would be obtained. Activation tagging is a method of gain-of-function. It is used to generate mutations in plants or plant cells. These mutations can be dominant by a random insertion of T-DNA or transposons, which can carry different activation tag (ATag) elements that can cause transcriptional activation of plant genes. To get a good chance of activating genes of interest with ATag T-DNA insertions, a very large number of

transformed tomato plants need to be generated. The use of transposon ATag inserts needs a few transformants that can be used to generate transposon ATags by transposition in later generations. The activated chosen gene can be further analyzed in the ATag plant and can be used for functional analysis by functional genomics (Goossens, 2002).

1.3 Genetic transformation

Genetic transformation of plants since the early '80s have given plant biologists a valuable tool to study plants as well as modify them in efficient ways. However, the ability to alter the genetic makeup of living things is something that not all people accept as natural. Modern plant biotechnology can facilitate proper delivery, integration and expression of genes into plant cells, first *in vitro* to regenerated plants *in-vivo* (Ziemienowics, 2001). The development of genetically modified plants can improve crops for grain yield and fruit production, engineer tolerance to herbicides, abiotic stress, and resistance to disease. If we expect the public to understand biotechnology and its importance then we should first educate them from the basis of the simplest scientific terms in biological sciences: genes, promoters, terminators, transcription, translation, gene modification and genetic transformation.

A gene is a sequence of nucleotides in DNA on a chromosome that encodes a protein, or an RNA molecule that regulates the transcription of such a sequence. A protein encoding gene has genetic information that is passed from the gene to RNA to protein. The DNA from a specific gene is transcribed to RNA through transcription, and then the RNA is translated to a protein with a specific function. The gene has a genetic code consisting of nucleotides in series of blocks of information called codons. Each codon corresponds to an amino acid in the encoded protein. For the DNA to be transcribed to RNA there are two sites that are

important on the DNA: promoter and terminator. The promoter allows a specific protein to bind and initiate transcription, the terminator is the other site where transcription is terminated, the end product is a mRNA that will later go through translation to produce a functional protein.

Manipulating the genetic makeup in plants can be done through a few different methods such as: *Agrobacterium*- mediated gene transfer, immersion of totipotent explants and microprojectile bombardment. *Agrobacterium*- mediated gene transfer and microprojectile bombardment are the two types of methods that are most common in the research field.

Microprojectile bombardment is a method where different instruments can produce high-velocity particles that can penetrate the cell walls of the desired plant and introduce the DNA. This type of method has no bias to the kind of plant, it has enabled the injection of DNA into some of the most difficult un-orthodox plants that are very sensitive to temperature and cannot be stored (Davey et al., 2010).

Immersion of the totipotent explants or a plant tissue which is capable of growing to a mature plant is done by specific procedures such as “floral dip”. “Floral dip” is a technique in which the flowers of the plant are submerged in *Agrobacterium tumefaciens*, followed by growth of the plant until it reaches maturity, the extraction and germination of seeds and selection of transformed seedlings. There is also a related technique where if the flowers of the plant are too big for floral dip, they can be floral sprayed.

Agrobacterium- mediated gene transfer is the most widely used method for transformation. This method has been utilized to produce transgenic plants for commercial applications and for the studies of development and growth of plants.

1.4 *Agrobacterium*- mediated gene transfer

In this research we have chosen to use *Agrobacterium*- mediated gene transfer instead of the microprojectile bombardment for the following reasons: it is less expensive; our choice of plant is *Solanum lycopersicum* (tomato) a dicot and not an un-orthodox plant (monocot). Dicots or dicotyledons are flowering plants which have two embryonic leaves or cotyledons. In contrast projectile bombardment is used on monocots plants that have only one embryonic leaf. The *Agrobacterium*- mediated gene transfer is achieved by the use of a soil bacterium from the genus *Agrobacterium*. Several species from this genus can transfer a specific DNA known as T-DNA (transfer DNA) that can be genetically engineered to carry a specific DNA of interest to plant cells. Although for a successful transfer of T-DNA from a bacterium to the plants genome certain steps have to be followed. First is to choose an appropriate bacterial host, second the plant cotyledons have to be wounded before they are infected with the bacterial host. Third the bacterium must come in contact with the plant cells in a step called “inoculation”. This is when the T-DNA transfer occurs from the *Agrobacterium* inner and outer membrane and cell wall to the plant cells. Forth when the T-DNA transfer enters the plant cell it has to move into the nuclear membrane and integrate into the plant chromosome. Once the integration of the T-DNA as occurred the *Agrobacterium* is killed by the treatment of the plant cells with

antibiotics and selective agents. Selective agents are used so that it promotes the growth of only those plant cells (transgenic plant cells) that have the T-DNA integrated in the plant genome. Once the *Agrobacterium* has been completely killed, the plant tissue is moved to different media for shoot and root elongation, until they are ready to be moved to soil and exposed to the natural environment. In order to carry out *Agrobacterium* –mediated gene transfer in tomato, an appropriate vector has to be constructed.

1.5 Transfer-DNA (T-DNA) into plant cells

To mediate the transfer of T-DNA into plant cells, an appropriate bacterial host has to be chosen. *Agrobacterium tumefaciens* is the bacterium that invades plant cells by tumor formation. For a successful T-DNA transfer the plant cells have to be wounded, the bacterium has to attach to the plant cells walls, and the T-DNA has to be transferred from the bacterium to the plant. Then, the T-DNA has to be integrated into the nuclear genome of the plant; the genes from the T-DNA have to be expressed in the plant which can then lead to changes in phenotype of the new transgenic plant (Hohn, 1989). A successful invasion of the T-DNA in the plant can be visualized as a tumor/callus formed around the plant tissue that has been infected by the bacterium. Figure 1 represents the tumor formations caused by *A. tumefaciens*.

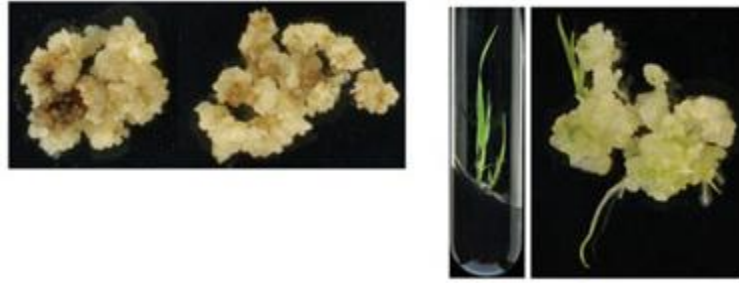


Figure 1: Tumor formation by *Agrobacterium tumefaciens*

1.6 Vector Construction

In this research the objective is to transform tomato with a T-DNA construct bearing a transposon from maize that can be used for mutagenesis when it transposes or moves around the genome. The vector construct used in this research is a two-component in-cis Ac-Ds system (Figure 2) which needs a single transformation event, and was proven to work in *Arabidopsis* and rice (Marsch-Martinez et al., 2002, Trijatmiko, 2005). The *Ac-Ds* in-cis ATag construct was shown to work in rice, reported in a thesis. The molecular analyses showed high transposition activity. The *Ac-Ds* in-cis ATag construct is comprised of Ac and Ds component. The Ac is immobile and the Ds is a mobile component that contains a selectable marker (BAR gene). Rice transformants were developed and screened and they showed active transposition of the DsAtag insert in rice (Trijatmiko, 2005).

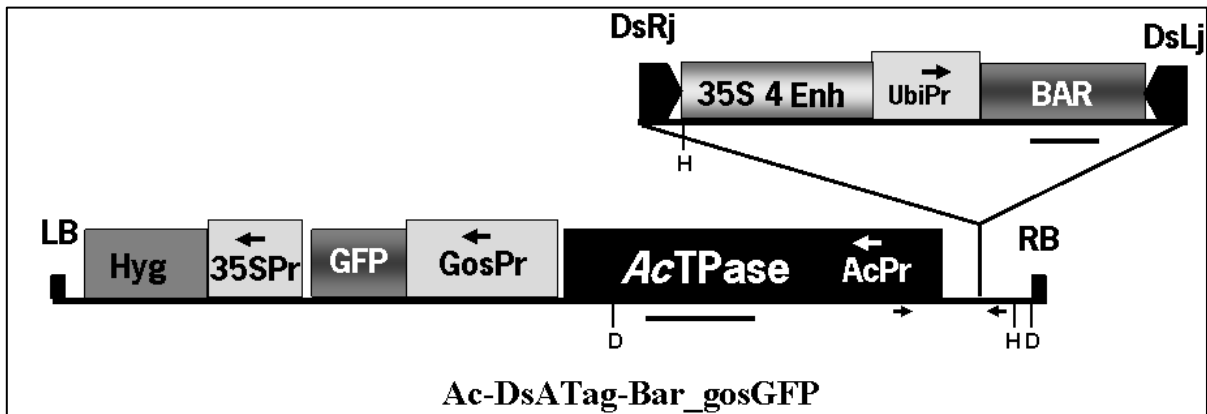


Figure 2: Activation tag constructs of the *Ac-Ds* in cis two-component system. The marker genes (dark shaded), BAR (Basta resistance), Hyg (hygromycin resistance), and GFP (green fluorescent protein) are driven by the promoters (light shaded) CaMV 35S and rice GOS with the arrows showing the direction of transcription. Abbreviations used are: RB and LB are the T-DNA right and left borders, transposon termini of *Ds* are labeled as LJ and RJ (left junction, right junction). The TPase denotes the *Ac* transposase and 35S 4Enh the tetramer of the 35S enhancer. Trijatmiko, Kurniawan R. (2005). “Comparative analysis of drought resistance genes in *Arabidopsis* and rice”. [PhD. thesis, Wageningen University, 2005], 115. <http://edepot.wur.nl/121694> (accessed Aug. 20th, 2013) Fair Use determination attached.

The marker genes (dark shaded), BAR (Basta resistance), Hyg (hygromycin resistance), and GFP (green fluorescent protein) are driven by the promoters (light shaded) CaMV 35S and rice GOS with the arrows showing the direction of transcription (Trijatmiko, 2005). The positions of primers for use in empty donor site (EDS) PCR that can reveal excision are indicated by arrows below the construct. Abbreviations used are: RB and LB are the T-DNA right and left borders, transposon termini of *Ds* are labeled as LJ and RJ (left junction, right junction). The TPase denotes the *Ac* transposase and 35S 4Enh the tetramer of the 35S enhancer (Trijatmiko, 2005). Transformation is a rare event and to select for

its occurrence the vector construct used contains selectable marker and/or reporter genes. Selectable marker genes confer resistance to toxic chemicals in transformed plants. The selection agent (antibiotic, herbicide) should fully inhibit the growth of untransformed tissue/plants, which will lead to the tissue/plant death, and in contrast do minimal damage on the transformed tissue/plant. Reporter genes in contrast to selection genes do not cause resistance to the chemical agent for the survival of plant cells. Reporter gene constructs allow the detection of products due to specific catalyzed reactions.

1.7 Green fluorescent protein (*GFP*) gene

The reporter gene used in the transposon bearing construct is the green fluorescent protein (*GFP*) gene. The expression of the *GFP* gene produces a protein *GFP* which can be used for detection under fluorescence microscope. This detection shows that the *GFP* gene is integrated in the genome of the tomato. The green fluorescent protein is responsible for the green luminescence in the *Aequorea victoria* jellyfish (Parsher, 1992). As shown in Figure 3 the modified amino acids of Ser-Tyr-Gly in the polypeptide compose the chromophore in the *GFP* protein that causes- that intense fluorescence (Parsher, 1992).

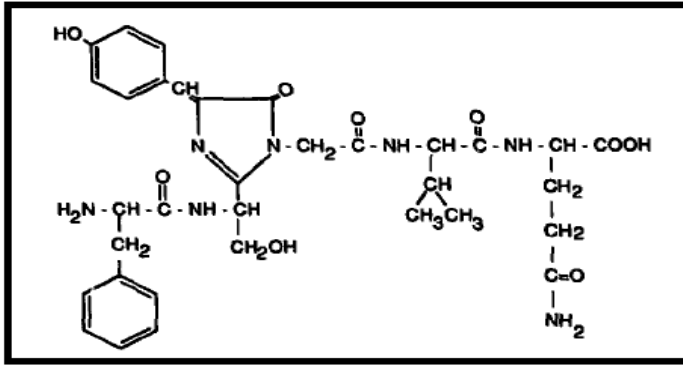
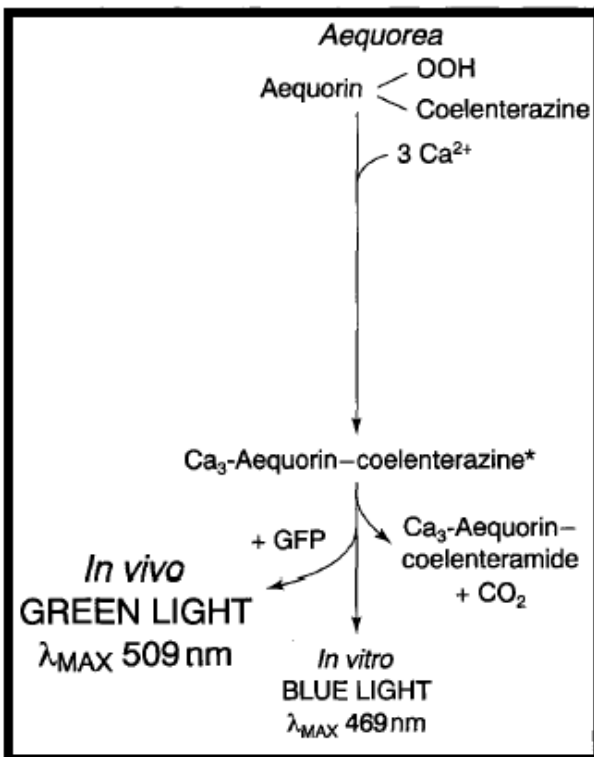


Figure 3: The chromophore in *Aequorea* green fluorescent protein (GFP). Prasher D.C., Ward, W.W., Prendergast, F.G. and Cormier, M.J. (1992). "Primary structure of the *Aequorea victoria* green-fluorescent protein." *Gene* 111: 229-233. (Accessed Aug. 20th, 2013) Fair Use determination attached.

For bioluminescence to occur Ca^{++} binds to a quorin which oxidizes coelenterazine, which is a luciferin or light emitting molecule that transfers the released energy to the green fluorescent protein, in the reaction shown in Figure 4 (Prasher, 1995). The tertiary structure of the GFP is a barrel structure. The barrel structure keeps the luciferin away from the solvents, making the GFP capable of emitting green light.



structure keeps the luciferin away from the solvents, making the GFP capable of emitting green light.

Figure 4: In *Aequorea* the aequorin protein binds calcium oxidizes coelenterazine. The oxidation of the coelenterazine releases energy and stimulates the GFP to emit green light in vivo. Prasher, D. C. (1995). "Using GFP to see the light." Elsevier Science Ltd 11(8): 320-323. (Accessed Aug. 20th, 2013) Fair Use determination attached.

1.8 BAR gene

The BAR gene was selected to be one of the two selectable markers in our vector. The BAR gene was isolated from *Streptomyces hygroscopicus* and encodes the phosphinothricin acetyltransferase enzyme. Once the BAR gene is expressed in the transgenic plant it confers resistance to phosphinothricin (PPT), which is a type of herbicide. Phosphinothricin (PPT) is an irreversible inhibitor of glutamine synthase (GS). When the plant is sprayed with this type of an herbicide sensitive tissue will undergo necrosis, whereas resistant tissue will have minimal damage. According to Lea and Ridley (1990) PPT binds to the active site on the glutamine synthase enzyme and inhibits its function. PPT is a competitive inhibitor that competes with glutamate for the enzyme's active site (Goodman & Donn, 1992). With the inhibition of glutamine synthase by PPT, the Glutamine synthase/Glutamate synthase pathway is stopped and the ammonia assimilation is disrupted. As ammonia accumulates in the plant cells because of the deficiency in glutamate photosynthesis will be inhibited and the end result is plant cell death

(Forde, 2007).

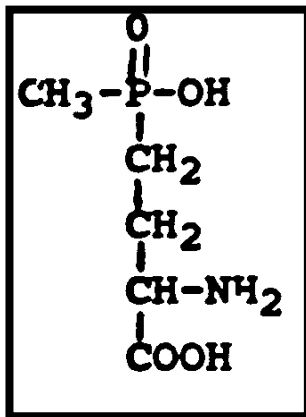


Figure 5: Chemical Structure of Phosphinothricin (PPT). Goodman, Howard M., Donn, Gunter (1992). "Plant cells resistant to herbicidal Glutamine synthetase inhibitors" United States Patent

1. <http://www.google.com/patents?hl=en&lr=&vid=USPAT5145777&id=QMgaAAAEBAJ&oi=fnd&dq=Donn,+G.,Goodman,H.+%281987%29. +%E2%80%9CPlant+cells+resistant+to+herbicidal+glutamine+synthetase+inhibitors%E2%80%9Dglutamine+synthetase+inhibitors%E2%80%9D+1987&printsec=abstract#v=onepage&q&f=false> (Accessed Aug 20th, 2013) Fair Use determination attached.

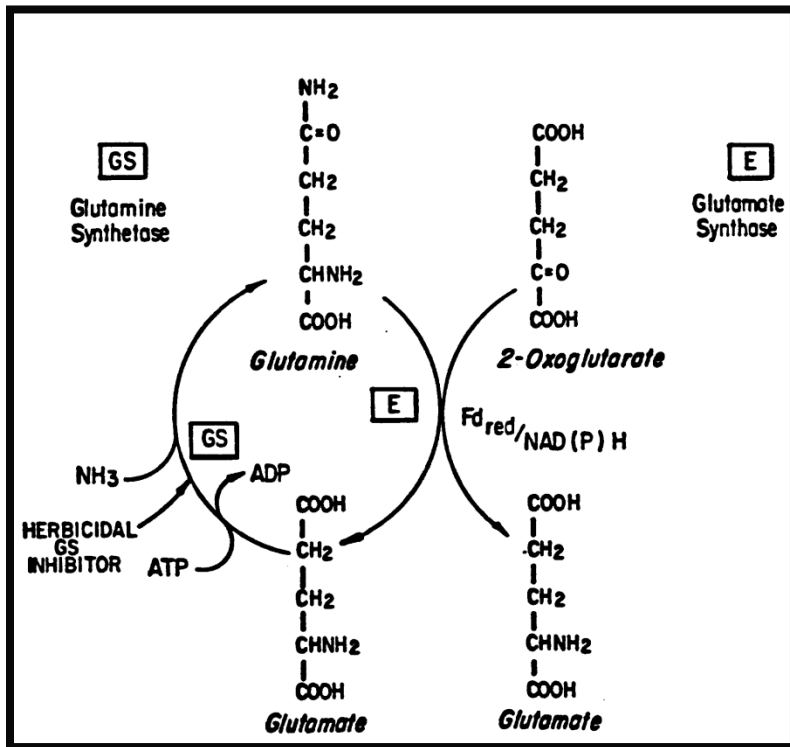


Figure 6: Glutamine Synthase/Glutamate Synthase Cycle. Goodman, Howard M., Donn, Gunter (1992). "Plant cells resistant to herbicidal Glutamine synthetase inhibitors" United States

Patent 1. <http://www.google.com/patents?hl=en&lr=&vid=USPAT5145777&id=QMgaAAA AEBAJ&oi=fnd&dq=Donn,+G.,+Goodman,+H.+%281987%29,+%E2%80%9CPlant+cells+resistant+to+herbicidal+glutamine+synthetase+inhibitors%E2%80%9Dglutamine+synthetase+inhibitors%E2%80%9D+1987&printsec=abstract#v=onepage&q&f=false> (Accessed Aug 20th, 2013) Fair Use determination attached.

1.9 Hygromycin (HYG) gene

The hygromycin gene was chosen for the vector as a selectable agent because it is widely used in producing transgenic monocot plants, for which the transposon vector was constructed. In tissue expressing the HYG gene, the activity of the hygromycin antibiotic added to the medium will be inhibited. This will in turn stop the inhibition of protein synthesis due to mistranslation of the 70S ribosome cause by hygromycin (<http://www.invivogen.com/hygromycin>). The transgenic plants which express the HYG gene will be able to resist the activity of

the hygromycin antibiotic. In contrast the plants without the HYG gene will be affected by the activity of the Hygromycin antibiotic which in turn will kill the cells and the plant would die. The figure below-shows the chemical structure of the hygromycin molecule.

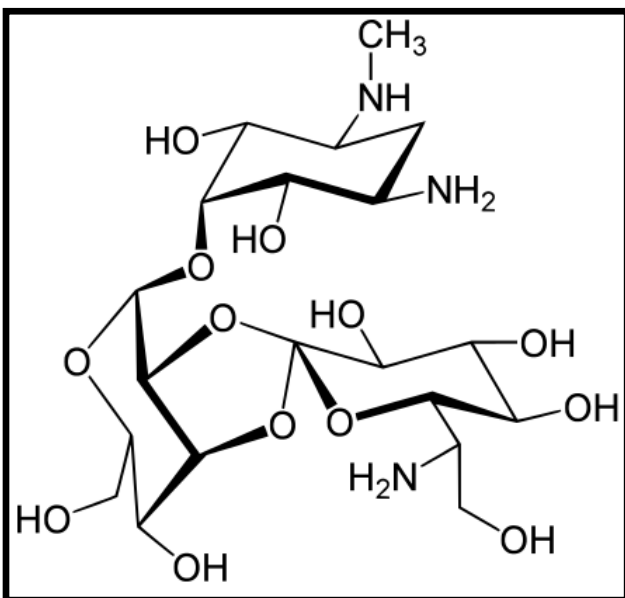


Figure 7: Chemical structure of Hygromycin. Invivogen.
<http://www.invivogen.com/hygromycin> (Accessed Aug 20th, 2013) Fair Use determination attached.

1.10 Schematic representation of the different methods used for gene transfer in plants

Figure 6 is a diagrammatic representation of the different methods used to develop transgenic plants. The description starts with the physical methods used to inject the proper vector into a chosen bacterium *Agrobacterium tumefaciens* strain. The *A. tumefaciens* strain is then used to infect the recipient plant cells. Those plant cells allow invasion of the bacterium for the T-DNA transfer are further grown on different media (with selectable and reporter agents). Once the

transgenic plant has regenerated and rooted it is moved to soil for further growth and testing (Ganeshan & Chibbar, 2010).

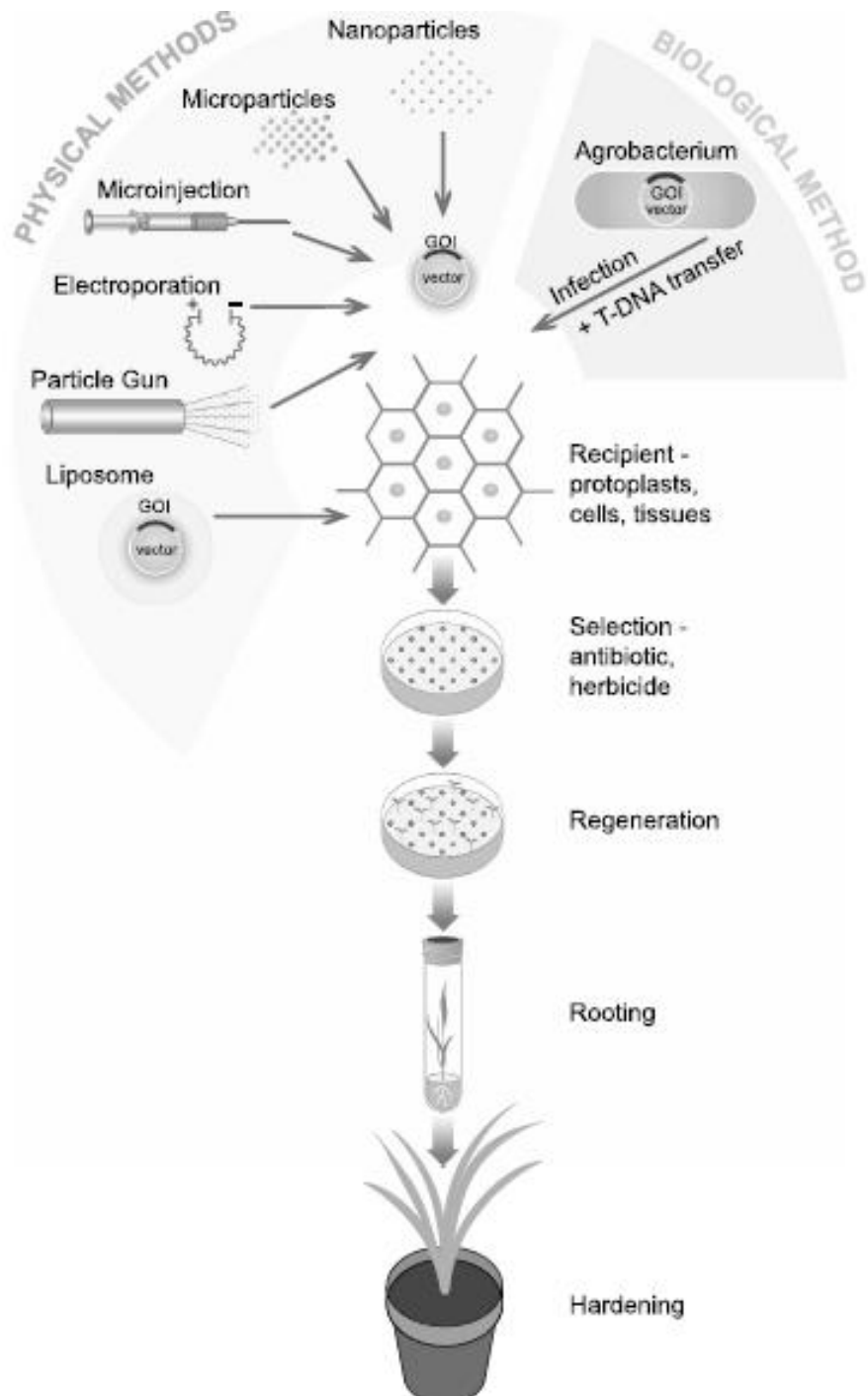


Figure 8: Scheme for developing a transgenic plant. Chibbar, S. G. (2010). "Gene Transfer Methods." Transgenic Crop Plants: 57-83. (Accessed Aug 20th, 2013) Fair Use determination attached.

Chapter 2

Materials and Methods

2.1 Research for developing the most efficient protocol

To develop tomato for functional genomics studies using transformation and insertion mutagenesis techniques, a suitable transformation methodology has to be selected. Finding an efficient transformation protocol that results in the development of sufficient number of genetically modified plants is a very long-drawn-out process. There are many published protocols available that are quite similar to each other but that differentiate themselves in some ways. Researchers chose to use different antibiotics and hormones at various concentrations (Atares, 2011), or a transformation method by fruit injection rather than floral dipping (Yasmeen, 2009). Since there are quite a number of protocols available online, we had to test a few before we found the right one to be used. The protocols found had a great deal of similarities, such as for the seed germination, co-cultivation, shoot induction and elongation, and root elongation. Most of them used Murashige and Skoog Salts (MS), which is a basal salt mixture that is used by many laboratories for cultivation of plant cells as a plant growth medium. *Myo*-Inositol is another compound used for tomato transformation because it is a good source for production of secondary messengers in eukaryotic cells, as well as a part of signal transduction in cells. The greatest difficulties were in finding the right antibiotics and hormones for our protocol. The importance of the antibiotics was when used they should sufficiently inhibit the *Agrobacterium tumefaciens* activity after the tomato transformation. The purpose of adding specific hormones was to stimulate cell elongation, cell division and cell proliferation before plant root formation.

2.2 Research on antibiotic selection

The vector construct (Figure 2) containing the marker genes (BAR-gene for tolerance to phosphinothricin (PPT), HYG-Hygromycin and GFP-green fluorescent protein) the hygromycin was chosen as one of the antibiotics used for selection of transformants in media. The other two antibiotics selected in the protocol were carbenicillin and augmentin. Carbenicillin was chosen because it is one of widely used antibiotics in plant genetic transformation having a wide range of effects on inhibition of bacterial infection, which in this case was *Agrobacterium tumefaciens* activity. At the beginning of our research we used cefatoxime instead of augmentin (amoxicillin sodium salt/potassium clavulanate), but after months of experimenting the tomato transformation was not efficient. The effects of different antibiotics were researched on the regeneration of tomato (Mamidala & Nanna, 2009), and this suggested that cefatoxime could have the largest detrimental effect on the regeneration when used at high concentration (300-400 mg/L) (Atares, 2011). Two other concentrations were used, 250mg/l as well as 50mg/l, in each medium until we doubled the carbenicillin concentration and took out the cefatoxime antibiotic in the root formation medium. Using such a high concentration of cefatoxime was highly expensive (10 g for \$1000 from Sigma), and the use of the low concentration was not as efficient on the plant regeneration. Cefatoxime is expensive and also its efficiency is low as it did not produce regeneration of transgenic lines that we required. Not only that cefatoxime had an effect over the *Agrobacterium tumefaciens* growth but it also inhibited plant regeneration. Several cotyledons that turned necrotic when treated with cefatoxime compared to the cotyledons that were treated with augmentin instead of cefatoxime. Higher efficiency of developing genetically transformed tomato was obtained by the use of augmentin as shown previously (Sun Hyeon-Jin et al, (427)), with the price more reasonable compared to cefatoxime. Kanamycin was not used in the protocol since

the vector construct did not contain the Kan gene as a selectable agent. Kanamycin was used for the growth of *A.tumefaciens* in YEP medium at 28° Celsius.

2.3 Research on hormone treatment for transformation

Selecting the right hormones was down to knowing what the functions of the different hormones available are and whether the specific hormone functions are compatible with the transformation protocol. For instance there are protocols that use NAA (1-naphthalene acetic acid), which is an auxin- that helps in plant growth and development. NAA is used in some protocols for root formation and vegetative propagation. According to the research paper by Cortina & Culianez-Macia, 2004, NAA was used in the pre-induction and co-cultivation medium which is for callus formation and not root formation, so we decided to not use NAA (1-naphthalene acetic acid) as a hormone. Another hormone called 6-Benzylaminopurine also known as BAP was used by the Cortina and Culianez-Macia labs (2004), the Harry Klee's Lab, Mike Hasegawa and Ray Bressan labs in the pre-induction and co-cultivation mediums. According to Phytotechnology Laboratories BAP is used to stimulate *in vitro* shoot development. The shoot is part of higher plants above ground; once the shoot is formed it helps to produce leaves, stems and later flowers on the plant. BAP was used on few transformations, but its presence did not improve transformation efficacy, so this hormone was abandoned. Another auxin indole-3-butyric acid (IBA) has been used as a hormone in the rooting medium (Zolman, Yoder and Bartel, 2000), helping newly transformed shoots develop roots. IBA did not show any greater efficacy in the formation of roots compared to a root regeneration media containing no IBA. In the end IAA (indole-3-acetic acid) was chosen in the protocol. Indole-3-acetic acid (IAA) is an auxin that stimulates cell elongation in combination with cytokinins such as zeatin riboside (ZR). Zeatin riboside in tissue culture stimulates cell division in the cambium and it also

promotes shoot proliferation, slows the aging process, and inhibits root formation (Sambrook and Russell, 2001).

2.4 Seed sterilization

Seed of M-82 and Micro-Tom cultivars were chosen and sterilized before they were added to sterile media that had been autoclaved. Seed sterilization is important to make sure that no bacterial contaminants are brought into the freshly autoclaved medium, and also to help make the seed ready for germination. The seeds contain two parts: the outer coat and the inner embryo that develops into the plant. The embryo in the seed is immature and can grow into a mature plant if allowed to grow under proper environmental conditions. During seed germination the seed embryo develops into a seedling and through environmental pathways will grow into a shoot. To induce seed germination as seeds are sterilized, they are submerged in water that allows water to penetrate the seed coat and cause the seed to swell, which will help the embryo grow. The seeds are sterilized with 70 % ethanol for two minutes in a 5 ml microcentrifuge tube, and by the help of a 1ml micropipette the solution is mixed that causes the seed to move in the solution. The mixing helps in making sure that the seed are cleaned thoroughly. After two minutes the 70 % ethanol is discarded and double- distilled water (ddH₂O) is added to the 5 ml microcentrifuge tube. The seeds are washed four times with ddH₂O, and further sterilized with 10 % clorox bleach (sodium hydrochlorite NaClO) for ten minutes. The microcentrifuge tube with the seeds is put on a platform shaker ten minutes. The next steps is discarding the 10 % clorox bleach from the microcentrifuge tube and add ddH₂O to it. The seed are washed further five to six more times. Once the last wash of ddH₂O is discarded the seeds are patted dry with a sterilized filter paper and moved to a germination medium.

2.5 Germination medium

Germination medium is used to plant the sterilized seeds and leave them for few days so that the seed can germinate and a seedling is formed. The plant will be kept in this medium until it has grown cotyledons, the cotyledons will be used for the transformation experiments. The germination medium consists of 1L of mixture of different chemicals. Murashige & Skoog basal salt is used (MS) at 4.43 g/L solution. Sucrose is used as source of carbon for the plant when it grows, at 30 g/L solution. Plant grade agar is a gelatinous substance, which after the medium is autoclaved and cooled, solidifies but can be melted again by heating. In our germination medium we used 8 g of plant agar for 1 L. We used 1000 μ l of 1000X vitamin solution, 10 μ l of thiamine- HCl for the 1L of medium. Thiamine (vitamin B1) HCl is used to help convert carbohydrates into fuel to produce energy for the plant. Once all of the chemicals are added to 1L of ddH₂O the pH of the solution has to be adjusted to 5.8. Finally the 1L germination medium is autoclaved at a Liquid 20, and then the liquid is cooled and poured into autoclavable PhytoCap's (jars). The seeds planted in the germination medium are left in a growth chamber at 25° C for 10-14 days.

2.6 Pre-Induction medium

Pre-induction medium is where the cotyledons are left on for a day at 25⁰ C in the growth chamber. The pre-induction medium is consisted of Basal medium (4.43 g Murashige & Skoog (MS) salt, 30 g sucrose, 8g plant agar, 1000 μ l 1000X vitamin solution, 10 μ l thiamine HCl in 1L of solution). The only difference between the germination medium and the pre-inducation medium is that in this medium we add 2, 4-Dichlorophenoxyacetic acid (2, 4-D), which is an auxin. This plant hormone is used so that it can be absorbed by the leaves of the plant and

transferred to the meristem of the plant and help in plant development. The cotyledons are cut with a surgical scalpel. These cuttings help wound the cotyledon once they are submerged in an *A. tumefaciens*. the invasion of the bacterium would be more successful.

2.7 The *A. tumefaciens* strain growth

The *A. tumefaciens* strain containing the two-component in-cis Ac-Ds system first is grown in YEP medium for 48 hours. The YEP medium contains 10 grams of yeast extract, 10 grams of bacto peptone and 5 grams of sodium chloride (NaCl) in 1L of ddH₂O. Before the YEP solution is autoclaved at Liquid 20 cycle the pH is adjusted to 7.1. Once the YEP medium is autoclaved and cooled 50mg/ml concentration of kanamycin antibiotic is added and then 500µl of freeze dried *A. tumefaciens* strain containing the two-component in-cis Ac-Ds system. The strain is grown overnight in a shaker at 28⁰ C. The next day 1 ml of the YEP medium containing the *A. tumefaciens* strain is taken and added to more YEP medium containing kanamycin. This new liquid medium is left at a shaker at 28 Celsius for 3 to 4 hours or until the OD₆₀₀=0.4-0.6. Once the OD₆₀₀ is reached the bacteria are centrifuged in a 50 ml centrifuge tube for 15 minutes. The YEP liquid medium is discarded and to the 50 ml tube Basal medium (4.43 g Murashige & Skoog (MS) salt, 30 g sucrose, 8g plant agar, 1000µl 1000X vitamin solution, 10 µl thiamine HCl in 1L of solution) is added. The *A. tumefaciens* strain pellet is dissolved in the Basal medium and then the cotyledons are soaked in the Basal medium containing the *A. tumefaciens* strain for 10 minutes.

2.8 Co-Cultivation medium

After 24 hours have passed the cotyledons from the Pre-Induction media are moved and soaked in Basal liquid medium (4.43 g Murashige & Skoog (MS) salt,

30 g sucrose, 8g plant agar, 1000µl 1000X vitamin solution, 10 µl thiamine HCl in 1L of solution) with the *A. tumefaciens* strain containing the two-component in-cis Ac-Ds system for 10 minutes. After that time period the cotyledons are taken out of the liquid medium, patted dry with a sterilized filter paper and moved to new Petri dishes containing Basal medium with 0.2 M acetosyringone. Acetosyringone is a phenolic inducer which binds to a receptor in the *A.tumefaciens*. Activation of the receptor allows virulence and higher transformation efficiency in plants. The Petri dishes are kept in dark for 3 days in a growth chamber at 25⁰ C.

2.9 Shoot and regeneration medium(S+R)

Once the cotyledons have been in the phase of infection for three days in a dark growth chamber at 25⁰ C, they are moved to a new medium – S+R (shoot and regeneration) medium – containing basal media (with the exception of using 4g of gelrite instead of 8g of plant agar in 1 L solution because only approximately half the amount of gellan gum as agar is needed to reach equivalent gel strength, and the gel is transparent). Once the medium has been autoclaved and cooled, the following hormones and antibiotics are added: 0.5 mg/ml of IAA (indole-3 acetic acid), 1.5 mg/ml ZR (zeatin riboside), 250 mg/ml carbenicillin, and 10mg/ml of hygromycin. Indole-3 acetic acid (IAA) is added as mentioned earlier it stimulates cell elongation and, in combination with cytokinins like zeatin riboside in tissue culture, stimulates cell division in the cambium. Zeatin riboside also promotes shoot proliferation, slows the aging process, and inhibits root formation (Sambrook and Russell, 2001). Hygromycin is used because the two-component in-cis Ac-Ds system construct contains a selectable gene HYG. If the cotyledons can survive the 10 mg/ml concentration of hygromycin then they have the HYG gene integrated in the plant genome. Carbenicillin is used as an antibiotic at 250 mg/ml concentration. The antibiotic will act against the further growth of *A.tumefaciens*

on the tomato cotyledons after the bacterium has formed tumor. Also this antibiotic will allow shoot regeneration of tomato to occur without having the *A.tumefaciens* invading this growth. In this medium we also used 375 mg l⁻¹ augmentin as a second antibiotic. Earlier in our project we used cefatoxime although its efficacy was not as desired so we exchanged it for augmentin. After only couple of transformation experiments we noticed that its activity not only it destroyed the *A.tumefaciens* activity but we also developed more transformants compared to when we used the cefatoxime as an antibiotic source.

2.10 Rooting medium

Once the shoot has regenerated and has elongated enough (between 5-10 cm), it is moved to a medium that only contains Basal medium (instead of 4.43 g of MS salt, 2.215 g of MS salt will be used; and instead of plant agar, gelrite will be used as mentioned earlier). No hormones are added such as the: 0.5 mg/ml of IAA (indole-3 acetic acid), 1.5 mg/ml ZR (zeatin riboside). Only carbenicillin with 250 mg/ml concentration and augmentin with 375 mg l⁻¹ concentration was added in the medium. These antibiotics were used so that no bacterial contamination occurs. The absence of ZR and IAA allowed the shoot to grow more and develop roots (hence the name Rooting medium).

This transformation protocol was modified with regard to the zeatin riboside (ZR) concentration and the use of hygromycin instead of kanamycin in comparison to the original (Qui D et al. 2007). The Table below presents a representation of the transformation protocol. The “+” sign in the Table means that the specific chemical substance is present in the solution and the “-“ means absent.

	Germination Medium	Pre-Induction Medium	Co-Cultivation	S+R	Rooting Medium	1/2 Rooting medium
Murashige Skoog (MS) salt (g/ L)	4.43	4.43	4.43	4.43	4.43	2.215
Sucrose (g/L)	30	30	30	30	30	15
Plant Agar (g/L)	8	8	8	–	–	–
Gelrite (g/L)	–	–	–	4	4	4
Thiamine HCl (mg/L)	1	1	1	1	1	1
1000X Vitamin solution (µl)	1000	1000	1000	1000	1000	1000
2, 4-Dichlorophenoxyacetic acid (2, 4-D) (1 mg/L)	–	+	–	–	–	–
Acetosyringone (0.2 M)	–	–	–	–	–	–
IAA (indole-3 acetic acid) (500 mg/L)	–	–	–	+	+	–
ZR (Zeatin Riboside) (1 mg/L)	–	–	–	+	+	–
Hygromycin(10 mg/L)	–	–	–	+	+	+
Carbenicillin (250 mg/L)	–	–	–	+	+	+
Augmentin (375 mg/L)	–	–	–	+	+	+

Table 1: Media composition for transformation of tomato

The detailed protocol above is minimized in a schematic form in Figure 9 bellow. The figure shows the steps followed in the transformation protocol tomato with an *A.tumefaciens* containing the plasmid with the genes constructed for the specific research project carried out. The protocol below shows the different concentrations of hormones and antibiotics needed for an efficient transformation protocol. . It also shows the time period of each step in the protocol for explants to stay in each specific medium. If results are observed in the proper time period, then the explants are moved to fresh medium. The reason is because the nutrients, hormones and antibiotics are depleted after specific time, if a new medium is not prepared and the cotyledons are left in an old medium for three weeks, the

antibiotic and hormonal effects will not be optimal. The time life of a specific antibiotic is limited, so if no fresh antibiotics are given after a specific time than the chance of getting contaminations increase with time.

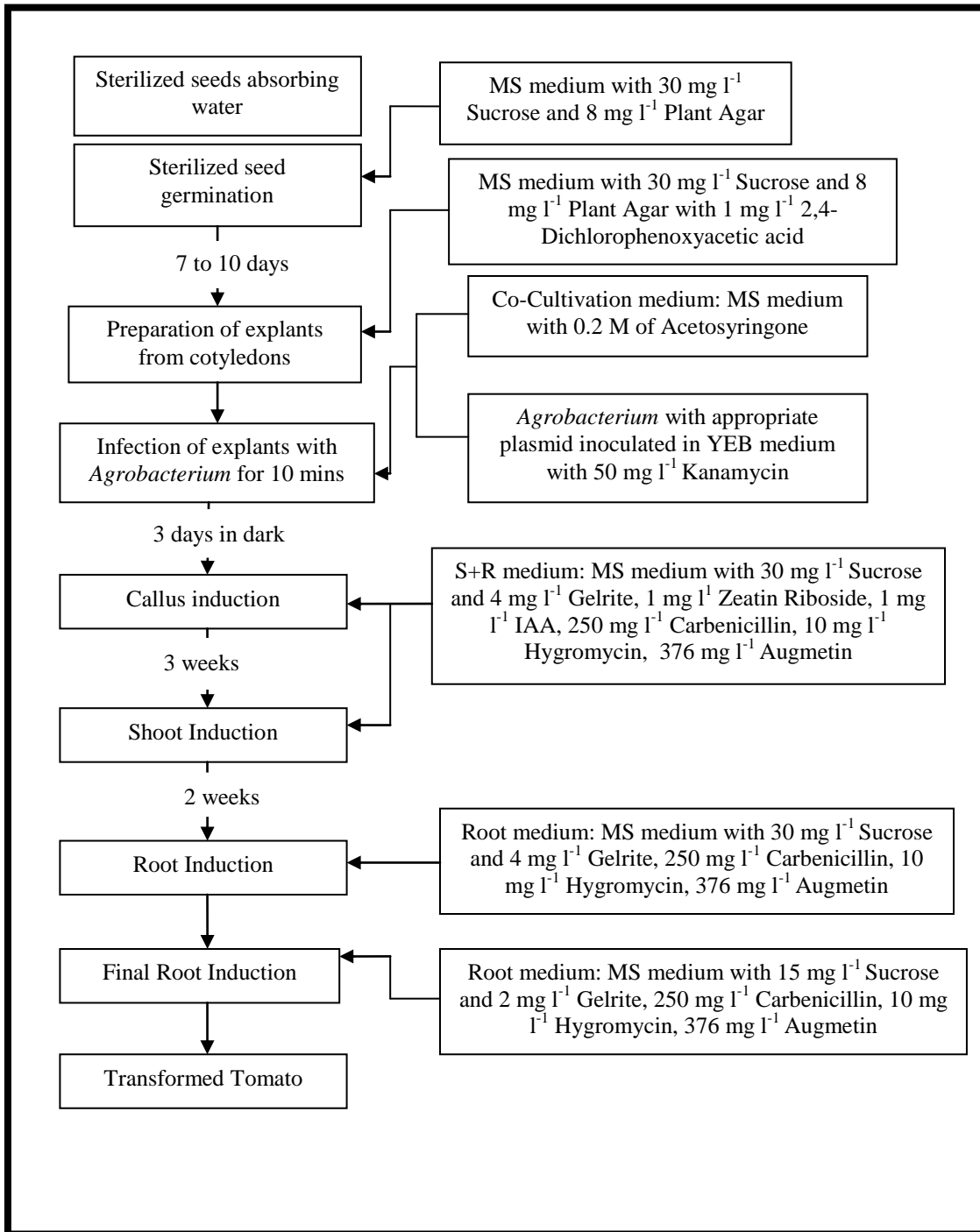


Figure 9: Schematic representation of *Agrobacterium*- mediated transformation in tomato

Once the transgenic tomato plants are selected, they have to be tested whether they contain the genes that have been integrated in the tomato genome: BAR, HYG and GFP gene. Tissue DNA has to be extracted from the transgenic lines to test whether the genes are present. The section below explains that extraction protocol

2.11 DNA extraction from plant tissue:

DNA extraction from plant tissues is important because for the primary transgenic tomatoes to be screened, a DNA needs to be isolated for different molecular analyses. The following is the protocol used during the research project:

- i. From the primary transformant, tissue is collected in a sterile 1.5 ml tube, and then frozen under liquid nitrogen.
- ii. The sample tubes are taken in the lab, and Tissue lyser from Qiagen is used to disrupt biological samples through high speed shaking for 90 seconds at 1/s Frequency 25.
- iii. Once the samples are ground into fine powder, they are left on ice and isolation buffer added at room temperature.
- iv. 400µl of 1x Isolation buffer is added to the samples: 2x Isolation buffer for 1 L (0.6 M NaCl- 35.064 g, 100 mM Tris pH=7.5- 100 ml 1M tris, 40mM EDTA- 14.8896g, 4% N-Lauroyl Sacrcosine- 40g, 1% Sodium Dodecyl Sulfate (SDS)- 100ml 10 % SDS), 10M urea (200ml 120g Urea) is added to the 2 X isolation buffer 1:1(v:v) to get the 1X isolation buffer and then the samples are vortexed.
- v. 400 µl Phenol-Chloroform with 1 % Isomylalcohol and 0.1 % hydroxyquinoline is added to the samples. The stock solution is made fresh and consists of 25ml Phenol, 25ml Chloroform, 0.1 % Hydroxyquinoline

and 500 μ l Isomylalcohol. The samples are inverted a few times, then centrifuge for 5 min at 10,000 rpm.

- vi. The supernatant is removed and transferred to new sterile tubes.
- vii. 250 μ l Isopropanol is added to the samples and mixed. The samples are left to sit at room temperature for 5 minutes, and then centrifuged for 10 minutes at 10,000 rpm.
- viii. The liquid is carefully removed without disturbing the pellet, then 200 μ l of 70 % cold EtOH (ethanol) is added to the samples, and the tubes centrifuged for 5 minutes at 10,000 rpm.
- ix. The liquid is removed again without losing the pellet and the tubes are left at room temperature for 30 minutes until the ethanol has evaporated
- x. 50 μ l of TE with RNase (50 μ l of TE and 10 μ g/ml RNase A) pH=8 is added, the concentrations of the samples are checked and, if the samples are not used immediately, they are kept at - 20 °C.(Pereira and Aarts, 1998)

SDS is used to aid in lysing cells during DNA extraction. EDTA is used to chelate divalent metal ions to inhibit DNAses and to destabilize cell membrane for lysis. N-Lauryl Sarcosine is used for solubilization and separation of membrane proteins and glycoproteins, useful in concentrated salt solutions like NaCl for cell lysis. Once phenol and chloroform is added and then centrifuged, the end result is an upper aqueous phase and lower organic phase. Nearly all of the DNA/RNA is present in the aqueous phase; and nearly all of the protein and other cell constituents partition in the interphase and organic phase. The addition of isopropanol to the aqueous DNA solution precipitates the DNA into a pellet after it is centrifuged (DNA is insoluble in salt and isopropanol). Ethanol 70% is used to purify and/or concentrate DNA from aqueous solutions. RNase A in TE is used to eliminate RNA from the DNA extracted samples (Sambrook and Russell, 2001).

This protocol is used to extract the DNA from the tissues of the primary transformants and the wild type. The wild type DNA is used as negative control for analyzing the selectable markers of the Ac-Ds in-trans system from the primary transformants through Polymerase chain reaction (PCR). A plasmid DNA for positive control can be extracted from the *A. tumefaciens* containing the Ac-Ds in-trans system. This plasmid DNA will be used in the PCR, and the extraction protocol is shown bellow.

2.12 Plasmid DNA purification

To isolate the plasmid DNA from the *A. tumefaciens* containing the Ac-Ds in-trans system, the strain is grown over-night in YEP media (10 g Bacto-yeast extract, 10g Bacto Peptone, 5 g of NaCl in 1L volume pH= 7). Bacto peptone is used as a nitrogen source for microorganisms (BD bionutrients technical manual). Bacto yeast is used to deliver water, vitamins, amino acids, peptides, and carbohydrates to the medium (Advanced bioprocessing, 3rd edition). Once the bacteria have grown, plasmid DNA purification is done using the Qiaprep spin mini prep kit from Qiagen. The plasmid DNA is isolated for use in the PCR reaction as a positive control.

2.13 Analysis of selectable markers of Ac-Ds in-trans ATag system in primary tomato transformants through PCR

Polymerase chain reaction (PCR) was used to detect the marker genes from the vector construct in the transformed tomato lines.

- i. The Initialization step.
- ii. The Denaturing step, which denatures the samples at a high temperature (94-96 °C).

- iii. The Annealing step, where the temperature is lowered to 50-65 °C, which allows the right primers (Forward and Reverse primers) to anneal to their complementary sequences.
- iv. The Elongation step, where the temperature will be raised to 72 °C, which allows the Taq polymerase to attach to each priming site and to extend a new DNA strand.

Steps ii, iii, and iv will be repeated for the number of cycles chosen. After the PCR reaction is completed, the samples are run on Ethidium bromide-stained agarose gel. When viewed under UV light, the DNA bands are visible. About 50-100ng of DNA is used for the PCR reaction from each primary transformed tomato tissue sample and positive and negative control sample.

PCR is carried out using the following primers:

- For the Hygromycin gene
HYGAP-F (5' - AAA AGT TCG ACA GCG TCT CCG ACC-3')
HYGAP-R(5' - TCT ACA CAG CCA TCG GTC CAG ACG-3')
- For the GFP gene
35S-F (5' -ATC CCA CTA TCC TTC GCA AGA CCC-3')
GFP-R(5' - GCT TGT CGG CCA TGA TAT AGA CG-3')
- For the BAR gene
BAR1-F(5' -ACC ATG AGC CCA GAA CGA CGC-3')
BAR1-R(5' - CAG GCT GAA GTC CAG CTG CCA G-3')

The PCR conditions include a denaturing step at 94 °C for 4 minutes followed by 35 cycles of 30 seconds at 94 °C; 30 seconds at 53.5 °C for Hygromycin gene, 52 °C for GFP gene and 57.5 °C for BAR gene; 1 minute at 72 °C ending with elongation step of 5 minutes at 72 °C.

2.14 Gel electrophoresis

Agarose is used for making an agarose gel for electrophoresis.. Agarose is a linear polypeptide made up of repeating units of agarobiose. Once the gel has been made a small volume of 5µl of DNA is mixed with dye and then is added to the well of the gel. The agarose gel is subjected to an electrical field for the separation of nucleic acids down the agarose matrix by their negatively charged molecules. Once the electrical field has been applied on the agarose gel at specific voltage for a specific time, the gel is moved and is viewed under UV light and the DNA can be visualized since it was bonded with Ethidium Bromide.

2.15 Screening for Basta herbicide resistance

The primary tomato transformants that test positive for all selectable markers (BAR, GFP and HYG gene) are further screened for Basta resistance. The Basta treatment is done by painting young leaves at different concentrations and following the affect of the herbicide for a time period of 0 days to 14 days. The leaves are treated at different concentrations: 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, 0.008% and 0.01%.

2.16 Extraction of seeds from fruits

Once fruits are formed from the wild type and transgenic tomatoes, the following protocol (Downie et al., 2004) explains how to extract these seeds from the fruit:

1. Slice tomato in two halves.
2. Remove seed-gel mass into beaker glass.
3. Add 0.1 M HCl to seed-gel mass and stir regularly for 1 hour.

4. Pour seed-gel mass into sieve.
5. Rinse seeds thoroughly with tap water to remove all gel tissues.
6. Spread the seeds on tissue paper and let them dry over night(Downie et al., 2004)

2.17 Screening progeny plants with Basta herbicide treatment

The seeds from the progeny of the primary transformants are grown in soil. Once they are mature, they are tested for Basta herbicide resistance the same way as the primary transformants. A polymerase chain reaction is performed to determine whether the offspring contain the genes from the two-component in-cis Ac-Ds system.

Chapter 3

Results

3.1 Transformaton of the *Ac-Ds* in cis two-component system in tomato

The goal of this project was to develop tomato transformant lines with a transposon construct for functional genomics analysis. The *Ac-Ds in cis* two-component transposon construct is meant for activation tagging, where insertions of the Ds-ATag element containing a CaMV 35S enhancer near a tomato gene would be able to enhance expression of surrounding adjacent genes and provide a gain-of-function phenotype. Transformations were done with *Agrobacterium tumefaciens* bearing a T-DNA with the *in cis Ac-Ds* ATag construct. The T-DNA contained the BAR, HYG and GFP genes as described earlier. The *Ac-Ds* vector construct containing the marker genes were used for *A. tumefaciens* mediated transformation. Seed of M-82 cultivar were germinated and grown on media and cotyledons were harvested for transformation. The *A. tumefaciens* strain containing the two-component in-cis *Ac-Ds* system was grown in YEP medium. The cotyledons were cut into three parts as shown in Figure 10 below.

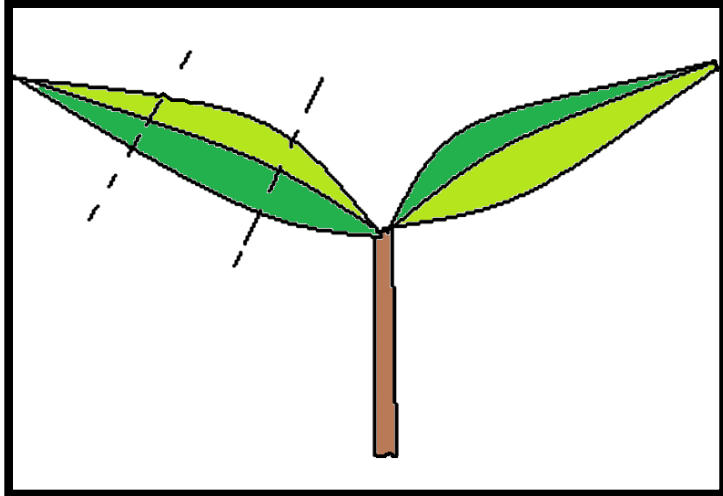


Figure 10: Cotyledon cuttings

The reason for cutting the tomato cotyledons is to cause wounds to the tomato cotyledons to allow proper attachment of the *A. tumefaciens* to the plant cell walls, which will allow transfer of T-DNA to the nuclear genome of the tomato plant. Once the T-DNA with the genes are incorporated in the chromosomal DNA of the tomato plant they will be expressed and will cause resistance to hygromycin antibiotic, detection of the GFP protein, and resistance to the herbicides. Once the infection of *A. tumefaciens* has occurred the resistance of the transformed tissue to hygromycin will allow growth of the callus. After some time a shoot will emerge from the callus with the appropriate hormones. At this point the *A. tumefaciens* growth has to be inhibited. This inhibition can be accomplished with the addition of different concentrations of antibiotics in the S+R shoot medium. These antibiotics that kill/inhibit growth of *Agrobacterium* are combinations of carbenicillin, cefatoxime and augmentin. Hygromycin antibiotic at concentration of 10mg/l Hygromycin is used to select for transformation and growth of callus and shoots. If the growth of *A. tumefaciens* is inhibited but the shoot development is not repressed, it proves that that shoot allows the resistance of the Hygromycin because of the expression of the HYG gene in the plant. With the removal of *A.*

tumefaciens the shoot will continue to grow and develop. Once the shoot is developed enough it has four to six leaves, and the plant can be moved to a rooting medium where only the specific antibiotics are added to the medium and the two hormones are removed. Indole-3-Acetic Acid (IAA) is an auxin in combination with cytokinins such as zeatin riboside it slows the aging process, it causes shoot elongation but it inhibits the root formation. That is why zeatin riboside (1 mg/l) and IAA (1 mg /l) hormones are removed from the S+R rooting medium. The transformed plant is moved to the soil for further growth and experimentation once it has grown roots and overgrown the environment where it is kept in. The figure here shows the transformed tomato in pot of soil in a controlled environment such as a growth chamber. The tomato plant is kept in the growth chamber for two more weeks for further development and then moved to the Virginia Tech's main greenhouse located on Washington Street.



Figure 11: Transformed Tomato plant in of soil

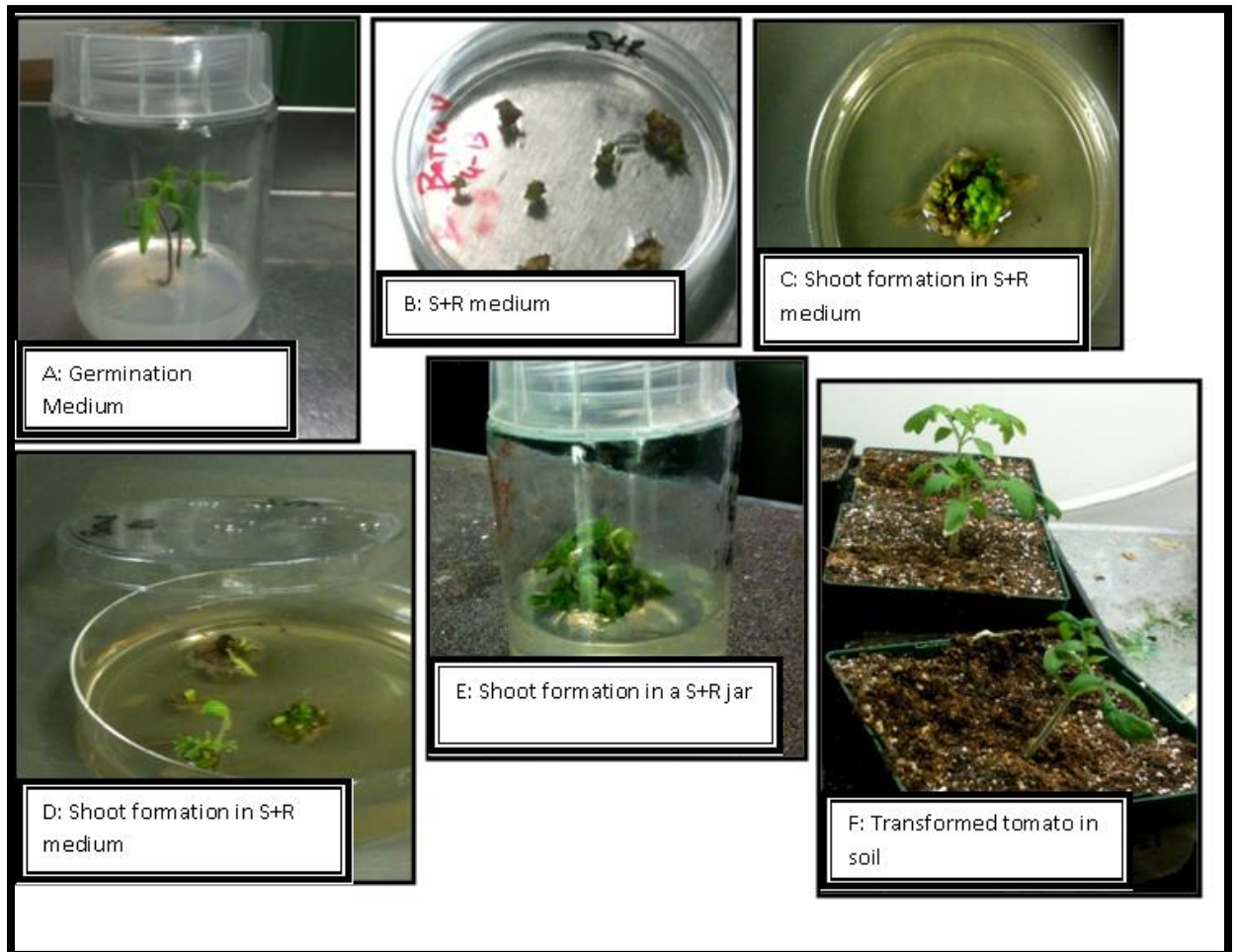


Figure 12: Pictorial representation for the generation of tomato transformants at different stages of the process. Figure 12 A represents a germinated tomato seedling with two cotyledons ready for cuttings. Figure 12B. Figures 12 C and D show the formation of transformed tomato shoots at different stages of development on S+R medium. Figure 12C is taken at the beginning of shoot formation, and Figure 12D is taken at the end of *A. tumefaciens* treatment and beginning of shoot elongation. Figure 12E above shows the S+R medium in a jar containing the transformed tomato explants with no more *A. tumefaciens* present. Figure 12F is the end product of the transformation protocol. It shows the transformed tomato plant which is moved from medium containing the necessary requirements for growth to its natural medium, soil.

3.2 Visualization expression of the GFP gene in transformed tomato lines

Once the transformed tomato lines are selected they are grown in pots of soil in growth chambers and then transferred to greenhouses, to be ready for further experiments. The first laboratory experiment that we did was detection of the GFP reporter gene. If the transformed tomato contains the two-component in-cis Ac-Ds system integrated in the tomato genome then expression of the transformed genes (BAR, GFP and HYG) can be monitored by appropriate assays. Expression of the GFP reporter gene can be detected by the intense fluorescence observed through a fluorescence microscope. Untransformed tomato tissue has background fluorescence. We tested tomato leaves and roots for GFP, but found background fluorescence. However, the newly developed young leaves showed low background fluorescence. The leaves were removed from the plants with forceps, and then immediately added to a tube containing isopropyl alcohol. This isopropanol treatment removed the chlorophyll of the plant without damaging the leaf. After removal of chlorophyll by isopropanol treatment the leaves are cleared and GFP expression can be detected by the fluorescence microscope.

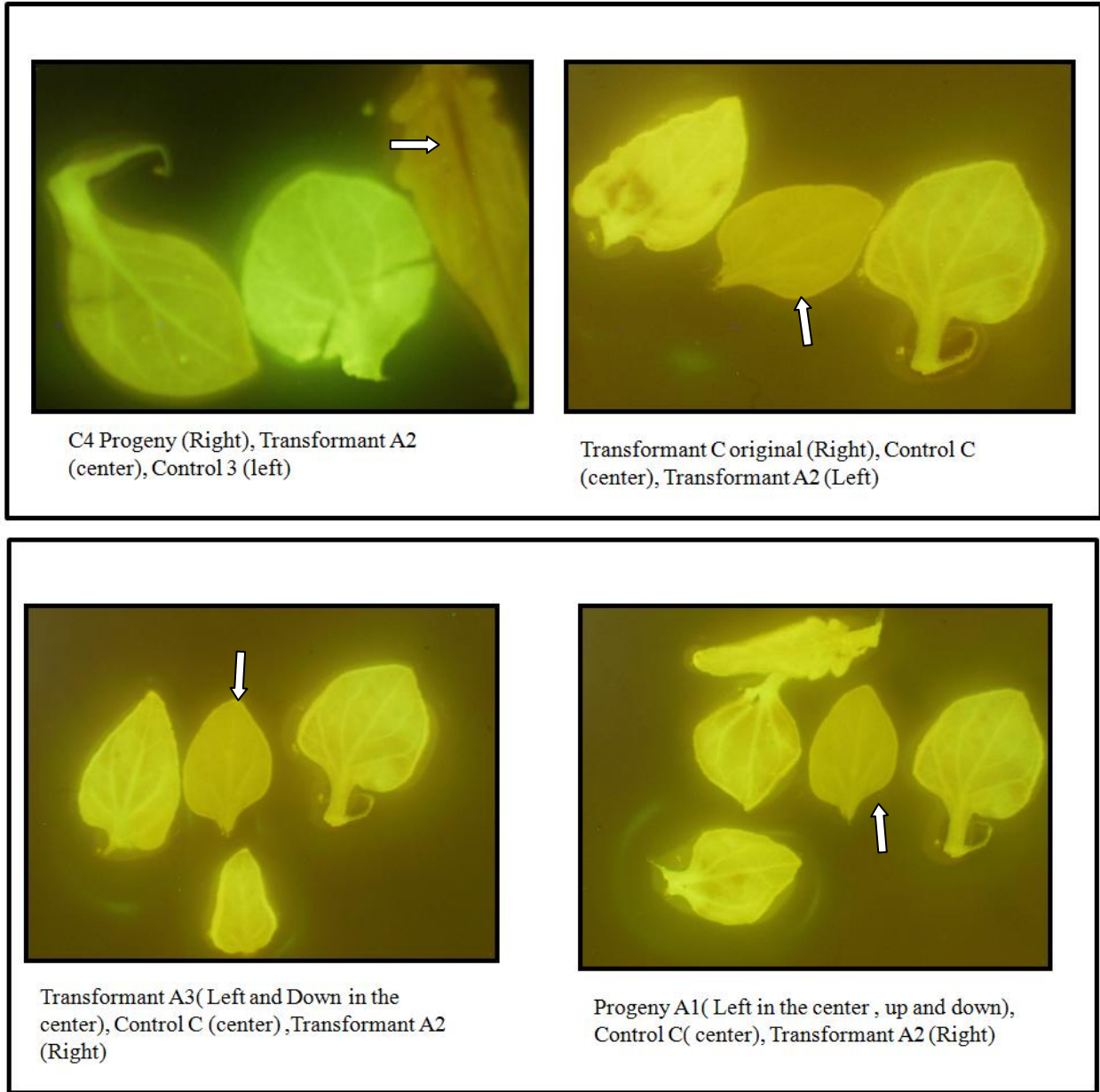


Figure 13: Images of GFP gene detection of tomato leaves by Fluorescence microscopy. The arrows in each image points toward the control tomato leaf (GFP gene not present), the rest are leaves from transformed tomatoes(containing the GFP gene). There is a distinction in fluorecence intensity between the transformed tomato leaves and the control leaves

The Figure 13 above represents a few of the fluorecence microscopy images that were taken during the process of detecting expression of the GFP gene

in the transformed tomato lines. There are distinctive differences between the transformed tomato lines and the control tomato. A control tomato refers to a tomato plant that has not been involved in any way with the transformation protocol. The control tomato also a M82 cultivar represents a group of tomatoes that can be usually found in a natural environment to which there have been no experimental techniques performed on them. The brightness seen on the leaves in the images shown in Figure 13 is due to the fluorescence of the GFP gene which is been expressed in the leaves and veins. In contrast to the leaves of the transformed lines as well as progeny lines, the control tomato leaves have low GFP fluorescence seen under the Fluorescence microscope.

3.3 Polymerase Chain Reaction (PCR) for determining the presence of the desired genes

The DNA extracted from the putative transformed tomato lines was used in further test to determine the presence of the BAR, HYG and GFP genes in the tomato lines.

Figure 15 represents a pictorial representation of the PCR process that is done by a PCR thermal cycler. As described in Chapter 3 we used six specific primers that are complementary to the maker genes: HYG, BAR and GFP genes based on our knowledge of the sequences in the construction of the *Ac-Ds* in-cis ATag construct. The sequences of six primers in the genes are shown below.

- **Hygromycin(HYG) gene:**

HYGAP-F (5' - AAA AGT TCG ACA GCG TCT CCG ACC-3')

HYGAP-R (5' - TCT ACA CAG CCA TCG GTC CAG ACG-3')

- **GFP gene :**

35S-F (5'-ATC CCA CTA TCC TTC GCA AGA CCC-3')

GFP-R (5'- GCT TGT CGG CCA TGA TAT AGA CG-3')

- **BAR gene:**

BAR1-F (5'-ACC ATG AGC CCA GAA CGA CGC-3')

BAR1-R (5'- CAG GCT GAA GTC CAG CTG CCA G-3')

The PCR conditions we used in the PCR thermal cycler were the following:

- For the denaturing step we set it up at a 94 °C temperature for 4 minutes;
- Followed by 35 cycles of 30 seconds at 94 °C temperature;
- 30 seconds at
 - 53.5 °C temperature for Hygromycin gene;
 - 52 °C temperature for GFP gene;
 - 57.5 °C temperature for BAR gene;
- And 1 minute at 72 °C temperature;
- The final elongation step was set up for 5 minutes at 72 °C temperature;
- Ending with a infinite time at 4 °C temperature;

After the PCR was done, the PCR products were loaded on an agarose gel for electrophoresis. Once the gel electrophoresis was finished the gel was visualized under UV light and photographed. Comparison of the observed bands to expected sizes was used to determine the presence of the desired genes and if the tomato lines were truly transformed. Both the GFP and HYG genes are expected to show fragments of size close to 500 base pair (bp) and the BAR gene 900 bp. Base pairs are used to measure the size of an individual DNA fragment by comparing to the standard DNA ladder marker of known size fragments. We are interested in 3 fragments indicative of the 3 marker genes of interest in the construct.

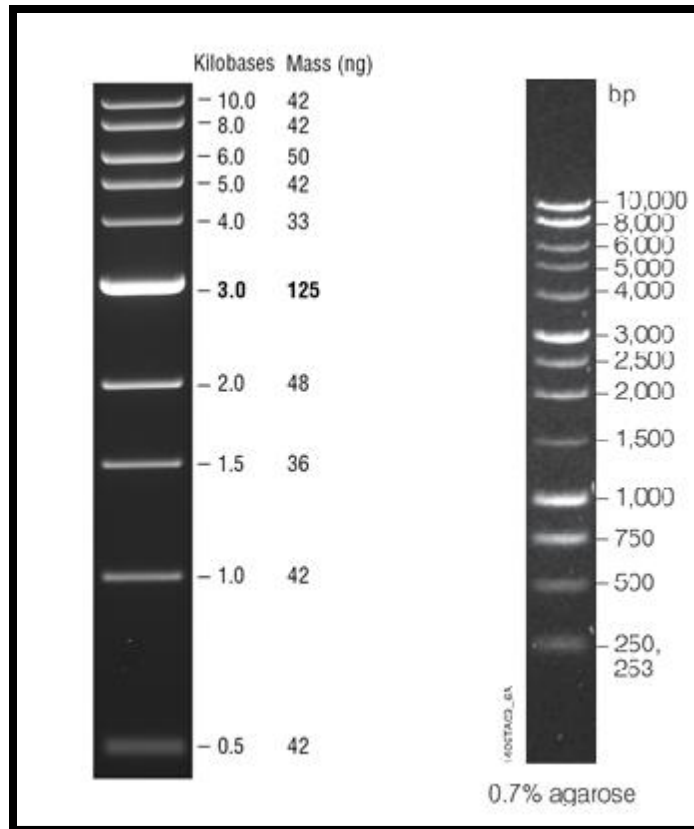


Figure 14: Pictorial representation of the 1kb DNA ladder from New England Biolabs on the left and 1kb DNA ladder from Promega on the right

The Figure 14 above represents two different 1kb DNA ladder from two different companies that were used to determine fragment sizes. By knowing the sizes for the genes being tested and comparing them to the 1kb ladder bands we are able to determine whether they match the expected size. This can further prove that the desired genes are present in the genome of the specific tomato line and that the PCR reaction yielded the results we were expecting. The Figure 15 below represents our result on determining whether the putative transformed lines growing in the green house or growth chambers were transformed as expected.

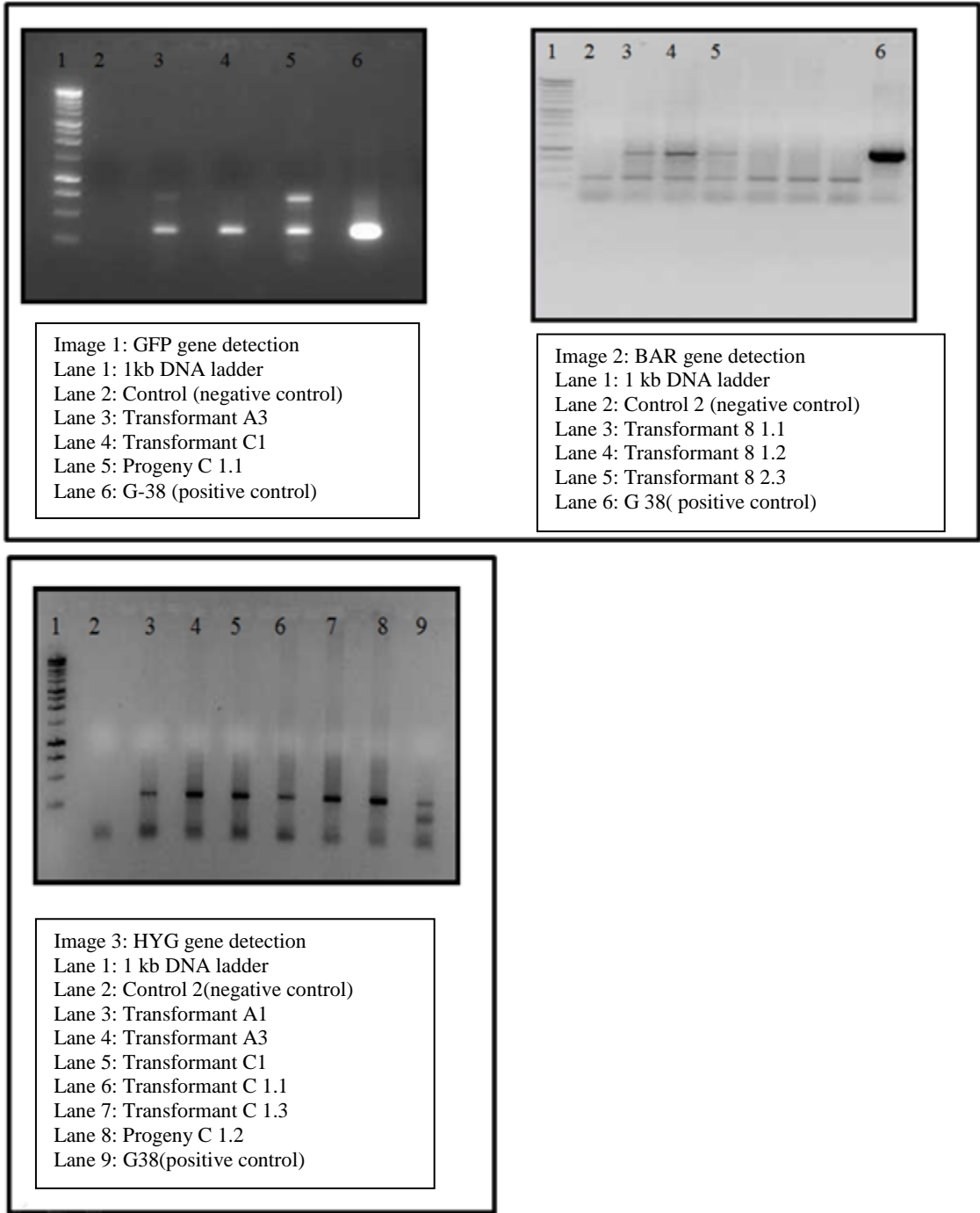


Figure 15: Images taken from Gels represents the results of three different PCR's. The Image 1 shows the results for the detection of the GFP gene by the use of 35S- F primer and GFP-R primer. In contrast Image 2 shows the results for the detection of the BAR gene by the use of BAR-F and BAR-R primers. And Image 3 represents the results for the HYG gene detection by the use of HYGAP-F and HYGAP-primers.

In Figure 15, Image 1 Lane 2 through Lane 6 represent the five different PCR samples: Control, Transformant A3, Transformant C1, progeny C1.1 and G38 respectively. As it can be seen the band representing the G-38 PCR sample shows the high fluorescence and compared to Lane 1 which is the 1 kb DNA Ladder and it has a size close to 500 bp. Comparing the rest of the bands in lines 2-5 we see that the sizes clearly match the one to the positive control (G 38). With this we can conclude that all the lines in this PCR reaction: Transformant A3, Transformant C1, progeny C1.1 contain the GFP gene because it has the same band size and presence when compared to the G38 sample our positive control. G38 sample is said to be our positive control because it contains our vector of interest: *Ac-Ds* in-cis ATag system.

Image 2 shows the image of the Agarose gel containing PCR samples for the detection of the BAR gene. Lanes 2 through 6 containing the samples of the following tomato lines: Control 2, Transformant (T) 8 1.1, T 81.2, T 8 2.3 and G38 respectively. As it can be seen the positive control sample G38 has the brightest fluorescence, which compared to the 1kb DNA Ladder shows a size of approximately 1000 bp. By looking at the other samples in Lanes 3-5 we can conclude that T 8 1.1, T81.2, T8 2.3 contain the BAR gene as well since they also have a band of size approx. 1000 bp.

Image 3 shows the image of the agarose gel on which PCR samples were run for the detection of the HYG gene. The lanes 2-9 represent the following PCR samples: Control (as negative control), TA1, TA3, T C1, TC1.1, TC1.3, Progeny C 1.2 and G38 (as positive control) respectively. The transformants A1, A3, C1.1, C1.3 and Progeny C1.2 are different from the ones above (T 8 1.1, T 81.2, T 8 2.3) is that these were developed earlier, the last band being the G 38 sample. The highest band has a size of ~500 bp, the size of fragment we expect for the HYG

gene. On comparison to the samples in lanes 3-8 it can be concluded that they also have a band of size ~500 bp. The control sample or negative control shows no band and indicates that there is no contamination or PCR carry-over in the reactions.. Based on these results we can conclude that the PCR samples for the following tomato lines: Transformant A1, Transformant A3, Transformant C1, Transformant C1.1, Transformant C1.3, Progeny C 1.2 contain the HYG gene in their genome.

3.4 Greenhouse testing for BASTA resistance in the transformed lines

Once we had proof that the genes of interest in the construct were present in the tomato genome of the transformed lines, experimentation was continued under the greenhouse conditions. From the different PCR experiments performed we determined which tomato lines had all the expected transformed gene fragments. As mentioned in earlier chapters the BAR gene is the most useful since it is present in the transposon that can move around the genome and make mutants. When this gene is present in the tomato genome we should expect certain resistance to the herbicide. The BAR gene on our *Ac-Ds* in-cis ATag system is located on the Ds transposon, which has a 35S 4Enh which is a tetramer of the 35S enhancer element, and the BAR gene. After transposition of the Ds-BAR, the insert can move around the genome to different positions. The BAR gene confers resistance to phosphinothricin (PPT), which is a competitive inhibitor that competes for the active site on the glutamine synthase enzyme and does not allow glutamate to attach to its receptor. This will cause ammonia to accumulate, inhibit photosynthesis and cause death of the plant. By expression of the BAR gene the plant can be tolerant to phosphinothricin (PPT) or in other words the plant becomes resistant to the herbicide.

To test for herbicide resistance we chose the lines of tomato that were tested positive by PCR and had the genes of interest. To develop an assay, we tested spraying, painting and treating excised leaves with the herbicide. We concluded that painting was the most appropriate as the herbicide injury would be localized. After 2 days we could determine the effect the herbicide on the plant, and did not run the risk of damaging the plants. By painting one leaf at a time with different concentrations of herbicide we could test best concentrations on the same plant. The effect of herbicide was tested on individual transformed tomato lines and compared to control plants. Control plants correspond to tomato plants from the same cultivar but which had not put under the transformation protocols and did not contain any of the desired marker genes: BAR, HYG and GFP. When the control plants were painted at certain concentration of herbicide they would not be resistant or tolerant to the effects of the BASTA the competitive inhibitor. One of the most successful BASTA herbicide screenings was using the following two concentrations: 0.001% and 0.002% for a 14-day period. Figures 17 and 18 show the tomato lines, both primary transgenic and control, on day 0. Figures 19 and 20 show the affect of the Basta herbicide by day 7. Figures 21-22 show day 14. Notice that on day 14, the leaf of the control plant treated with BASTA herbicide at 0.002% has wilted and the size has decreased by half from day 0. When compared to the control, there were no signs of effect from BASTA herbicide on

the two cuttings belonging to the primary transformed tomato from Line C during the 14-day period.

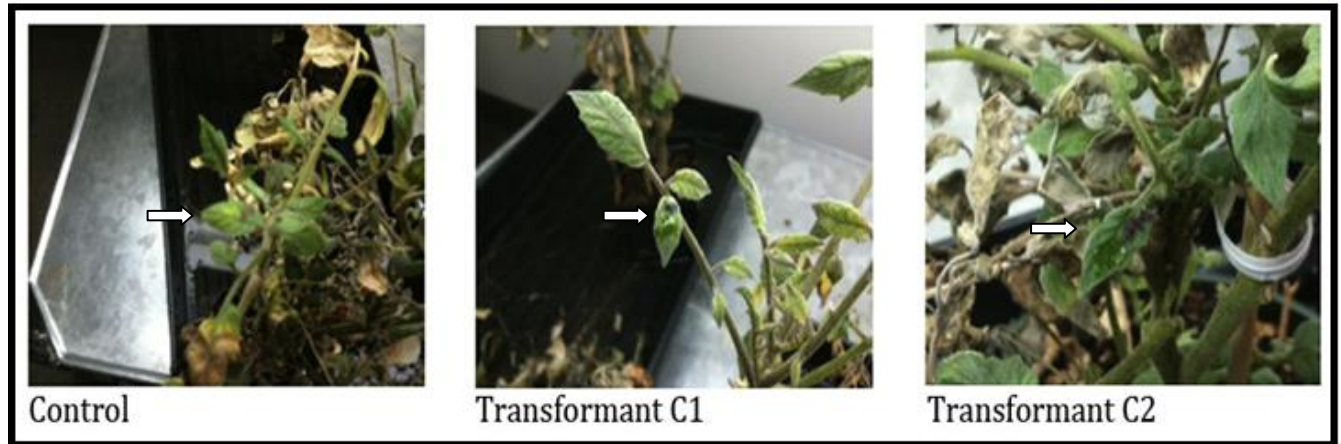


Figure 16: Treatment of control and transformed tomato with BASTA herbicide at 0.001% concentration at Day 0

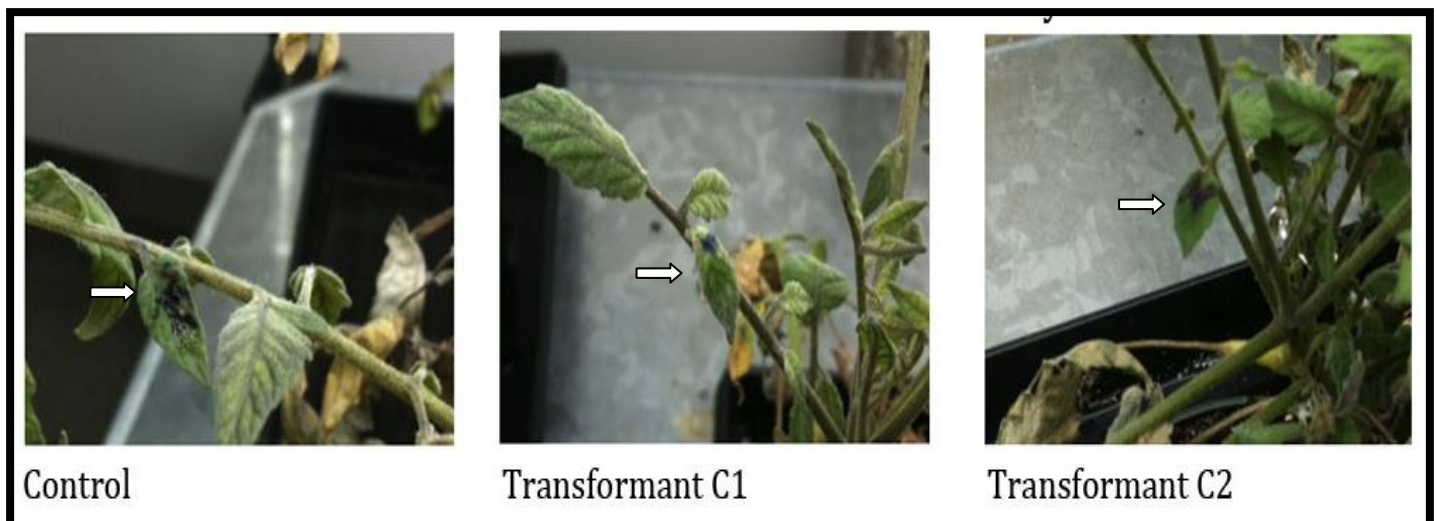


Figure 17: Treatment of control and transformed tomato with BASTA herbicides at 0.002% concentration at day 0



Figure 18: Treatment of control and transformed tomato with BASTA herbicide with 0.001% concentration at Day 5

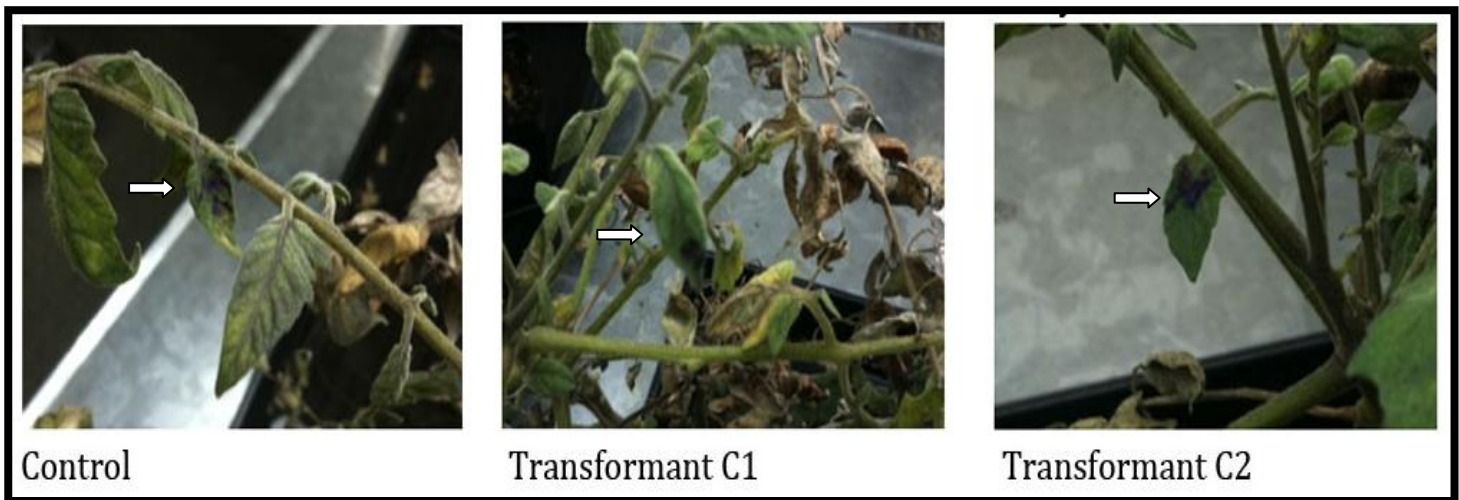


Figure 19: Treatment of control and transformed tomato with BASTA herbicide with 0.002% concentration at Day 5



Figure 20: Treatment of control and transformed tomato with BASTA herbicide with 0.001% concentration at Day 14

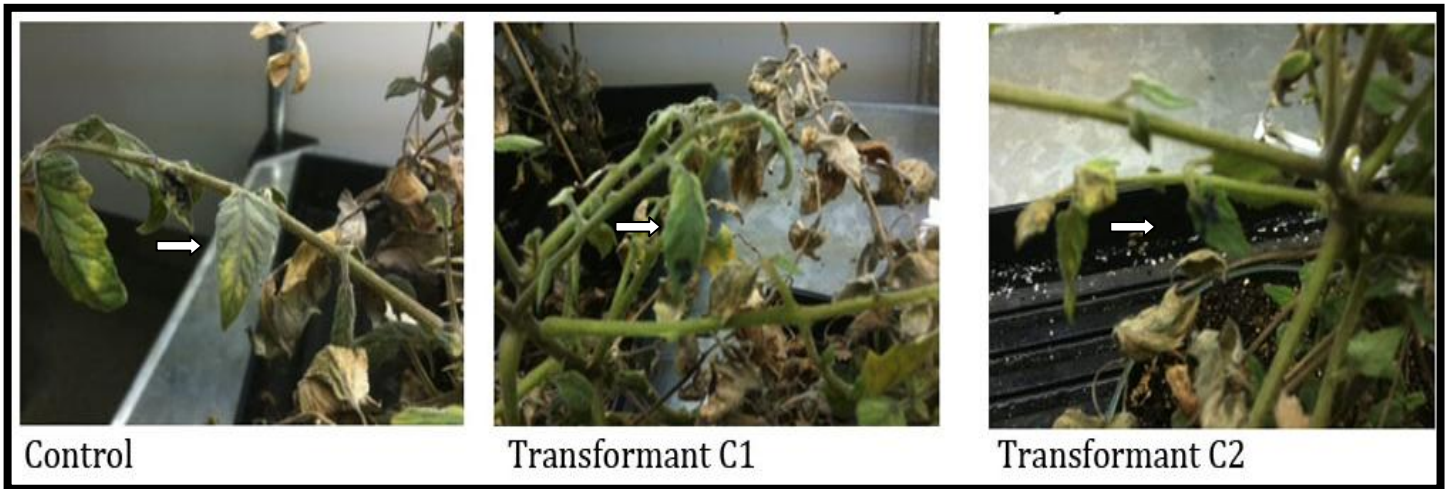


Figure 21: Treatment of control and transformed tomato with BASTA herbicide with 0.002% concentration at Day 14

3.5 Higher efficiency using Augmetin as a hormone

To determine whether the transformation protocol using augmetin as a hormone instead of cefatoxime for the development of transgenic tomato lines is more productive we used the data collected during the research project as shown in table 2, we determined that the protocol that used augmentin as a hormone increased the transformation protocol efficiency.

Trials without Augmetin	Transformed without augmetin
33	2
Trials with augmetin	Transformed with augmetin
8	3

Table 2: Data collection of trials done and how many transformed lines were developed

The result we acquired was a p-value of $8.17 * 10^{-19}$ as shown in Table 3. Since the p-value is smaller than 0.05 we can regard it as statistically different. There is a clear significant deviation between the two protocols. First when we used the protocol without the augmetin hormone there were less number of transformed tomato lines developed compared to how many trials were done. In contrast when we used a transformation protocol with augmetin we developed more transformed tomato lines in shorter time and in fewer trials.

	CHI Test
χ^2	8.17035E-19

Table 3: The p-value once the chi-square distribution was done

3.6 Conclusion

With the project coming to an end, we collected our data and determined that the transformation protocol we decided to use was successful. We were able to get transformed tomato lines in a shorter period when using 376 mg l⁻¹ augmetin. The hormone augmentin was found to be very successful in the development of transgenic tomato lines. By the use of the hormone the number of transformed tomatoes increased. Not only its efficiency was improved but also it was more cost effective. The price for purchasing augmetin was cheaper compared to the hormones we used in the past such as cefatoxime. We learned that the only way to use cefatoxime was to have it in higher concentration (around 150 to 200 mg/l). Even with the higher concentration of cefatoxime, our results were negligent; we lost more plants by cross contamination and its inability to inhibit the growth of *A.tumefaciens*.

Once the transgenic tomato lines were developed, they were further tested to detect whether the reporter gene and selectable markers genes were present. Polymerase chain reactions showed that all three GFP, HYG and BAR genes were present. We further showed that the fluorescence of the GFP gene was present in the transformed tomato lines with the help of a fluorescence microscope. Additionally, we performed BASTA herbicide resistance analysis. These analyses showed that the transgenic tomato lines had a certain degree of resistance when treated with an herbicide at 0.001% and 0.002 % concentrations. The Table bellow shows the summary of the all the transformed tomato lines that were developed. In the table it is shown which lines were grown by using cefatoxime or augmetin. The rest of the results show the detection of the marker genes (BAR, HYG and GFP).

Label	With cefatoxime	With Augmetin	GFP detection	HYG detection	BAR detection
Transformant A1	+	-		+	
Transformant A3	+	-	+	+	
Transformant C	+	-	+	+	+
Transformant C 1.1	-	+		+	
Transformant C 1.3	-	+		+	
Transformant 8 1.1	-	+			+
Transformant 8.1.2	-	+			+
Transformant 8 2.3	-	+			+
Progeny C 1.1	-	-	+		
Progeny C 1.2	-	-			
Progeny C4	-	-	+		
Progeny A1	-	-	+		

Table 4: Summary table of the results from the developed transformed tomato lines. The “+” sign means that certain chemical or gene detection is present in the transformed tomato line, the “-“ means the opposite (no presence)

The future directions for the project will consist of developing seedlings from the seeds extracted from the T₀ generations. They will be used to for detection of the marker genes (BAR, HYG and GFP) and their resistance toward the treatment by Basta. Another objective that could be researched would be determining the different transpositions of the mobile Ds component from the in-cis Ac-Ds construct compared to the immobile Ac component in the tomato genome.

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Appendix A

Annotated List of Figures

Figure 2

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Figure 3

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Figure 4

Prasher, D. C. (1995). "Using GFP to see the light." *Elsevier Science Ltd* **11**(8): 320-323. (Accessed Aug. 20th, 2013) Fair Use determination attached.

Figure 5

Goodman, Howard M., Donn, Gunter (1992). "Plant cells resistant to herbicidal Glutamine synthetase inhibitors" United States Patent **1**. <http://www.google.com/patents?hl=en&lr=&vid=USPAT5145777&id=QMgaAAA AEBAJ&oi=fnd&dq=Donn,+G.,Goodman,H.+%281987%29.+%E2%80%9CPlant+cells+resistant+to+herbicidal+glutamine+synthetase+inhibitors%E2%80%9Dglutamine+synthetase+inhibitors%E2%80%9D+1987&printsec=abstract#v=onepage&q&f=false> (Accessed Aug 20th, 2013) Fair Use determination attached.

Figure 6

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Figure 7

Invivogen. <http://www.invivogen.com/hygromycin> (Accessed Aug 20th, 2013) Fair Use determination attached.

Figure 8

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