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Study of Protein phosphatase subunit B'ϕ - impact on *Arabidopsis thaliana* and plant-microbe interaction.	
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ABSTRACT.

The protein phosphatase 2A (PP2A) complex accounts for a considerable part of the protein dephosphorylation. PP2A is made up of three types of subunits, a catalytic(C), scaffolding (A) and regulatory (B) subunit. A certain subgroup of B' called B′ϕ (B'phi) clade appears to be essential for the interactions between plants and microorganism (especially mycorrhiza) and is the focus in this thesis. The B'phi gene is not present in the model plant *A. thaliana*. The B'phi gene from Heinz tomato was isolated in our lab, cloned into a plasmid vector and transferred to *A. thaliana*. These plants are now to be further investigated in the master project.

The main objectives of this project are to check the expression of B'phi (RNA isolation with subsequent RT-PCR). Selection on Basta to check if the plants are homozygous for the insert. Effects of microbes like *Pseudomonas* will be tested. Stress conditions, salt, microbe-associated molecular patterns (chitin) and others are to be tested. Three mutants (B'phi 13, 16 and 20) used in this experiment were homozygous for the insert.

Flowering between Mutant and Wild type plants in two different sets of experiment only B'phi 13 was used showed faster flowering in Mutant plants than in the wild type plants.

Gene expression analysis was also carried out and the result of this experiment showed that the gene of insert B'phi is expressed. Gene sequencing showed a 100% match confirming that the gene B'phi is what has been used for all the analysis in this thesis.

Co-cultivation with Pseudomonas showed increase in chlorophyll in the Mutant plants (B'phi 13 and 16). For Salt stress, the Mutant plants (B'phi 13 and 16) had better survival response than the Wild type plants and had higher chlorophyll content than the Wild type plants except in B'phi 13 (1) and 16 (2) experiments where their chlorophyll content was low at concentrations 50 mM and 0 mM respectively. This might have occurred because of transplanting of poorly selected 1-week old seedlings to the salt medium. Chlorophyll is an important pigment for photosynthesis which is known to help in the growth and development of plants. Therefore, B'phi can bring about improvement in Agriculture especially because of its effect seen in this work, thereby reducing the challenge of food shortage.

ABBREVIATIONS.

A. Thaliana	Arabidopsis Thaliana.
bp	Base pairs.
CaMV	Cauliflower mosaic virus.
cDNA	Complementary DNA.
LB	Luria-Bertani.
NCBI	National Centre for Biotechnology Information.
MCS	Multiple cloning site.
MS medium	Murashige and Skoog medium.
PCR	Polymerase chain reaction.
PP2A	Protein phosphatase 2A.
PPP	Phosphoserine/phosphothreonine. specific protein phosphatase.
PPM	Metal ion dependent protein phosphatase.
PTP	Phosphotyrosine phosphatase.
PTS	Peroxisomal targeting signal.
qPCR	Quantitative Real Time Polymerase. Chain Reaction.
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction.
T1	First generation transgenic.
T2	Second generation transgenic.
T-DNA	Transfer-DNA.
Ti-plasmid	Tumor inducing plasmid.
WT	Wild Type.

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1. INTRODUCTION

1.1 PROTEIN PHOSPHATSES AND KINASES.

Protein Phosphatases are enzymes involved in the flow of information in the cell (Signal Transduction), they are a variant class of proteins grouped by their specificity for their substrates, which are phosphorylated tyrosine, serine, threonine residues. These enzymes are responsible for dephosphorylation of proteins in the cell (Barford, Das et al. 1998).

Experimentally, just a small portion of the several thousands of protein phosphatases and kinases have been characterized. However, from the results of these characterized enzymes it is observed that they are very important in the growth and development of plants. It is necessary to have an equilibrium between kinases and phosphatases for the best or most favorable regulatory function in a cell. Protein kinases and phosphatases responsible for reversible phosphorylation encrypts a great portion of the sequenced gene order of Arabidopsis. Protein phosphatases are grouped into three different families, first, PPP and PPM families include the Ser/Threonine phosphatases and the PTP family consisting of tyrosine specific and dual specificity phosphatases (Luan 2003).

Structurally, there are four different groups of phosphatases, they are: Number one, The PPP group (Serine/Threonine protein phosphatases) are composed of PP1, PP2A, and PP2B (these enzymes are controlled by calcium/calmodulin regulated, PP2B, also called calcineurin). The second structural class has PP2C, which is a Serine/Threonine phosphatase with a different arrangement from the PPP family. Number three, in this phosphatase structural family is the protein tyrosine phosphatases, (PTPs), they have substrate specificity for phosphotyrosine (pTyr) with proteins and dual -specificity phosphatases, and then dephosphorylation of the three phosphorous residues. The fourth class of enzymes are called low molecular weight PTPs catalyze the dephosphorylation esters and phosphotyrosine proteins making a distinct structural class from the PTPs.

In 1989, the foremost plant protein kinase sequences were announced then in the middle of 1998, about 549 more of these sequences including 175 in Arabidopsis Thaliana (*A. Thaliana)* alone

were publicized. Depending on their sequence relationships *A. Thaliana* can be classified into about twelve major groups. The Protein Serine/Threonine kinases network in plant cells kind of act as a central processing unit, receiving input information from receptors that are sensitive to environmental factors, phytohormones and other conditions from the environment translating it into suitable outputs like gene expression, cell growth and division, metabolism (Hardie 1999).

Protein kinases catalyzes protein phosphorylation reactions in contrast with the activity of protein phosphatases, the functions of these enzymes are closely regulated in vivo. Studies have shown that phosphatases can align with kinases or can counter their reactions. Also, amongst a structural class, their phosphatase area integrity is maintained, thereby making the special characteristics within the group of the protein phosphatase mainly by the inherent features of the non-catalytic regulatory and targeting domains or associated domains (Barford, Das et al. 1998).

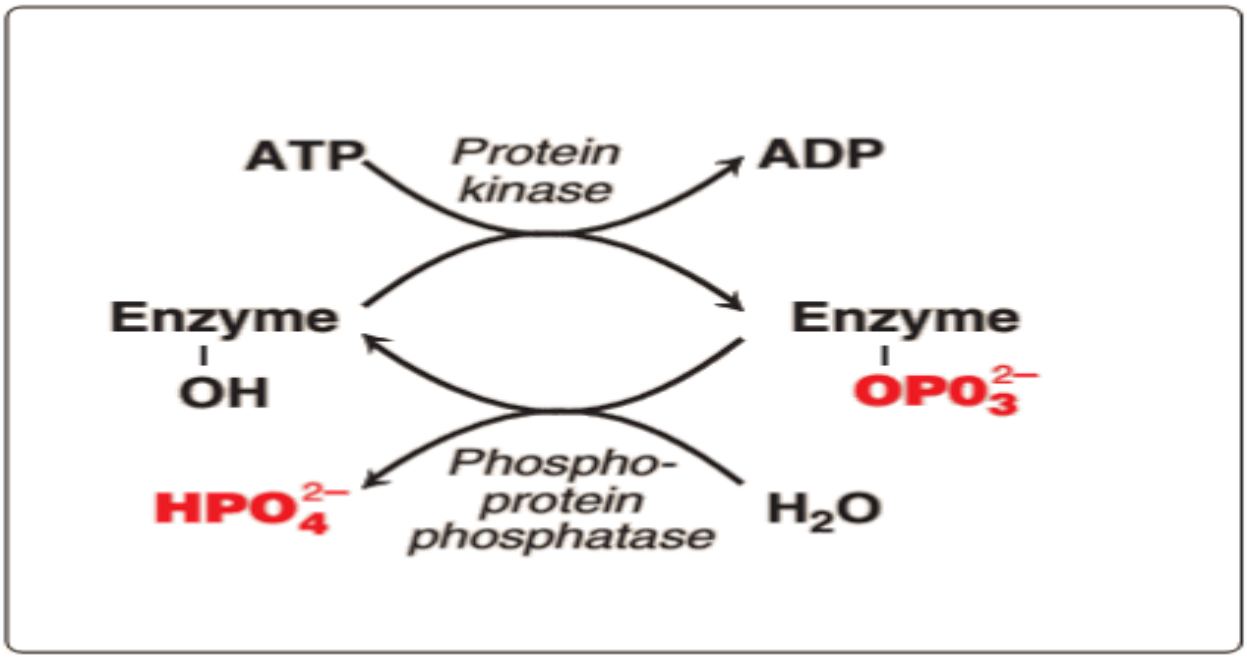


FIGURE 1: Enzymes involved in phosphorylation and dephosphorylation.

https://tuitiontube.com/enzymes-phosphorylation-dephosphorylation/

1.2 PROTEIN PHOSPHORYLATION AND DEPHOSPHOSPHORYLATION.

Controlling cell activities in reaction to outside stimuli is crucial to all living organisms. The key feature for the several and diverse processes unfolded to interface or pass these stimuli across cell membranes thereby bringing about changes in reactions and roles of proteins in the cell is the process of reversible phosphorylation. Protein phosphorylation controls the functions of enzymes by straight up obstructing or serving as a plug to enzyme catalytic sites, or by liaising compliant allosteric changes (Barford, Das et al. 1998).

Protein phosphorylation is the covalent addition of a phosphate group to a protein by Protein kinase. The removal of a phosphate group from a protein by a protein phosphatase is called dephosphorylation, these reactions are the start and off control in cell regulatory functions. The enzymes involved in phosphorylation and dephosphorylation are classified as Serine/Threonine and Tyrosine phosphatases and kinases. The performance and arrangement characteristics of the proteins are affected by the presence or absence of a phosphate group. The vast range of cell functions is greatly dependent on intracellular proteins that can be reversibly phosphorylated, and the quantity of protein kinases and phosphatases present to accelerate the reaction. Some functions of phosphorylation include activating some proteins by phosphorylating them to get their designated place in the cell where they perform their role or roles, also reaction between proteins that partner together to form complexes for proper activity can be controlled by phosphorylation and so many other functions. Some proteins need to be phosphorylated in order to target the destination site in the cell where they function. Therefore, nearly all aspects of cell function involve reversible phosphorylation. These include metabolism, cell cycle progression, ion transport, developmental control, and stress responses. This diverse spectrum of cellular functions is reflected by the large number of intracellular proteins that are subject to reversible phosphorylation and the number of protein kinases and phosphatases that catalyze the reactions. In plants newer studies reveals that, protein phosphorylation is observed in nearly all signaling pathways. Phosphorylation on Serine/Threonine is the most common alteration of proteins found in plants, while the role of tyrosine phosphorylation has just been lately identified in plants (Luan 2003).

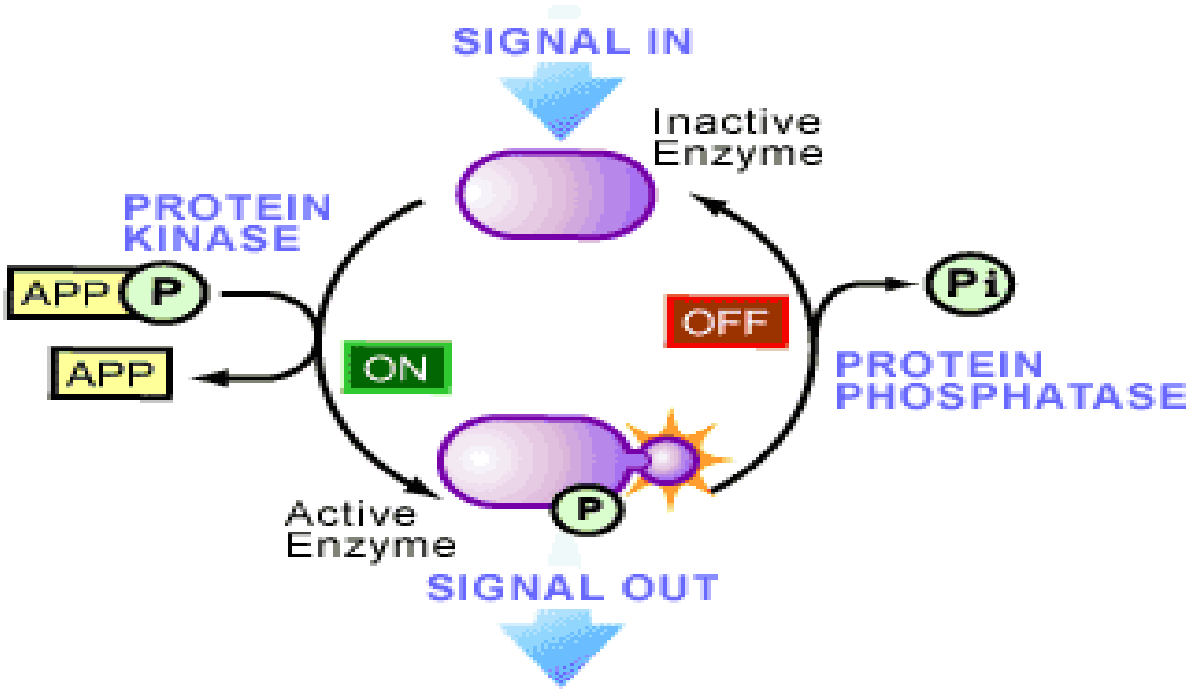


FIGURE 2: Protein Phosphorylation: A global regulator of cellular activity by Davidsecko. (Secko 2003)

1.3 PROTEIN PHOSPHATASE 2A COMPLEX. (A, B AND C SUBUNITS).

PP2A is part of a family of some phosphatases that have common heterotrimeric structure. Its core enzyme is composed of a scaffolding subunit A or PR65 with molecular weight of ~65-kDa and a catalytic C subunit of ~36-kDa molecular weight (Cho and Xu 2007).

This complex is a vital serine/threonine phosphatase, a holoenzyme composed of a heterodimeric core enzyme (Xu, Xing et al. 2006). Processes involved in comprehension of the role and construction of the PP2A holoenzyme complex has not been forthcoming regardless of recent developments. The architectural information about any of the regulatory subunits is still uncertain as to how the regulatory subunit identifies the scaffolding or the catalytic subunits. The formation of a holoenzyme occurs as a result of the methylation of the carboxyterminal of the catalytic subunit (Xu, Xing et al. 2006).

Various signal transduction and developmental pathways as well as, nitrate reductase activation, synthesis of isoprenoid, apical-basal auxin transport, defense reactions, regulation of flowering time, brassinosteroid signaling pathway, peroxisomal β-oxidation.(Kataya, Heidari et al. 2015)

PP2 can be classified by their reliance on divalent cations into PP2A, 2B, 2C. Ca^{2+} are controlled by PP2B while Mg^{2+} regulates PP2C, divalent ions are not needed for the proper functioning of PP2A. Another way to characterize PP1 and PP2 enzymes is by inhibiting effect of some drugs like calyculin A, okadaic acid and cantharidin (inhibits only PP2A), which affects the activity of PP1 and PP2A but does not inhibit PP2B and PP2C.

A more organized method for grouping these enzymes have been proposed when a vast number of genes encrypting these phosphatases were characterized from eukaryotic organisms.

PP1, PP2A and PP2B are the most predominant eukaryotic Serine/Threonine phosphatases called PPP family because they were more comparable after the sequence and structure of these gene products were studied, although the PPP family group of enzymes have the same catalytic region of 280 amino acids (residues), when analyzing (Luan 2003). Their N- and C- terminal non-catalytic domains they are distinct and can be differentiated by their corresponding regulatory subunit (Barford, Das et al. 1998).

PP2C, pyruvate dehydrogenase phosphates and many different Mg^{2+} Serine/Threonine reliant phosphatases are more comparable and related to each other and are thereby called PPM family (Luan 2003).

To properly comprehend the complete steps of phosphorylation of proteins condition, always in the cell is mechanism that is influenced by protein kinases and phosphatases interactions with their specific substrates. Therefore, to figure out firstly, the steps involved in cellular proteins phosphorylation, we need to have an insight of the mode of operation of how these enzymes (protein kinases and phosphatases) identify their substrates. Secondly, their catalyzing technique, thirdly, how some subcellular locations are picked out, or aimed by these enzymes. Lastly, how are these three mechanisms controlled (Barford, Das et al. 1998).

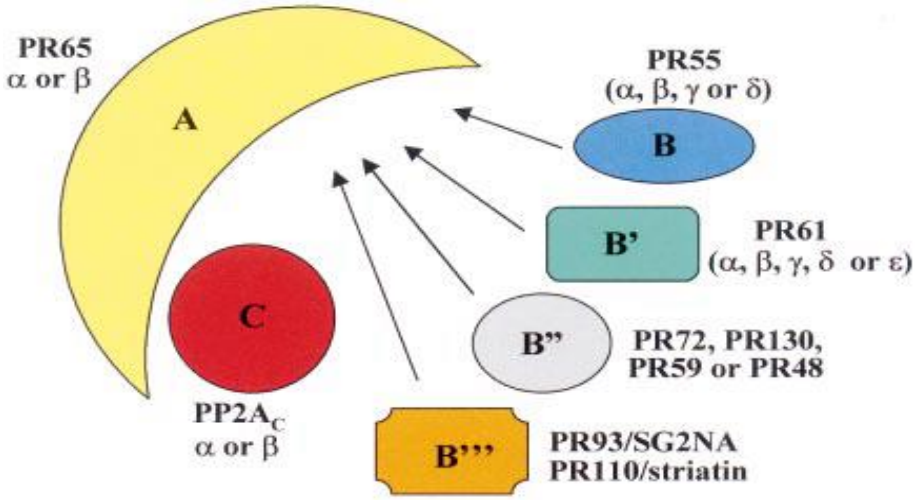


FIGURE 3: A is the second constant regulatory or structural subunit, and the third variable subunits B/B'/B''/B''' are the architecturally non-related subunits, and also catalytic subunit C. (Janssens and Goris 2001).

1.4 B REGULATORY SUBUNIT.

B regulatory subunit is one of the three subunits of the enzyme PP2A heterotrimeric protein complex. This subunit has several isoforms that has been isolated from various organisms and further classified into 3 categories. The feature of B regulatory subunit is responsible for PP2A's effectiveness to act on a wide range of substrates. Also, there is an assumption that the variable B regulatory subunit, regulates the specificity of an enzyme for a substrate and, its mode of action due to the structural differences between the three categories of B-regulatory subunit B, B', B'' (Zhong, Jiang et al. 2014).

Mechanisms of the PP2A complex are greatly regulated by the binding of at least one of the 18 regulatory B subunits to the AC core complex, which is responsible for the governing function of PP2A's specificity for its substrate, its function as an enzyme and its location in the cell. Studies have shown that carboxylate group methylation of the C-terminal residue Leu 309 encourages the binding of the B, B', B'' subunits to the AC core dimer. When the C-terminal tail of C subunit is

removed, it stops the methylation of the carboxyl C-terminal and the recruitment of the B, B', B'' subunits to the AC core enzyme (Xu, Xing et al. 2006).

The B family, (also known as PR55) consists of four genes, Bα, Bβ, Bγ, and Bδ, resulting in proteins having molecular masses of about 54-57 kDa.

For the B' class, also known as B56 or PR61, is made up of not less than seven isoforms encrypted by five genes B′α, B′β, B′γ, B′δ, and B′ε, with molecular masses in the range of 54 and 74 kDa (Strack, Ruediger et al. 2002).

Interestingly, like the A-scaffolding subunit the B56/PR61 subunit has a huntingtin-elongation-A subunit-TOR-like (HEAT-like) recurrent structure. Concurrently, the regulatory B'/B56/PR61 subunit interrelates with the catalytic subunit and with the preserved ridge of the scaffolding subunit. At the meeting point between the B'/B56/PR61 subunit and the scaffolding subunit, the carboxyterminal of the catalytic subunit identifies a surface groove (Xu, Xing et al. 2006).

B'' family are labelled in order of their molecular masses as PR48, PR59 and PR72/130 (Strack, Ruediger et al. 2002).

The major B family is the B56 of the B' family with a minimum of eight members in the group. They are all a part of a common preserved core domain with 80% sequence identity, having profound functions in cell cycle, cell proliferation and Wnt signaling, this happens by dephosphorylation of a few crucial controllers of cell mechanisms plus APC, Akt, Erk, Mdm2, paxillin, cyclin G and p53 (Xu, Xing et al. 2006).

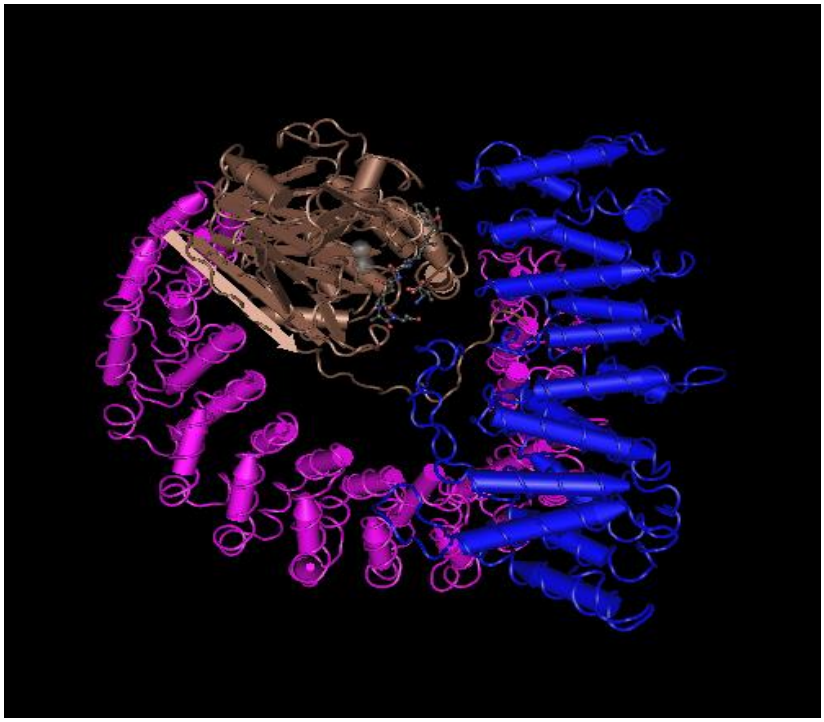


FIGURE4:ProteinPhosphatase2A-B'phi.

https://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?Dopt=s&uid=84468

1.5 *ARABIDOPSIS THALIANA* AS A MODEL ORGANISM.

A. thaliana (mouse-ear cress) is an angiosperm or flowering plants, it is a tiny dicotyledonous breed/genre, it is also a part of the mustard or the Brassicaceae family group. Looking at its agronomic importance, *Arabidopsis* is not a useful plant like its affiliated counterparts that are economically significant like broccoli, canola, cabbage, turnip, radish (Meinke, Cherry et al. 1998).

A. thaliana is the most researched amongst other flowering plants, this conclusion is as a result of the number of publications for its study. 3500 *Arabidopsis* publications were added to the PubMed in the year 2008 as opposed to 65 altogether before the year 1979 and 7 publications of *A. thaliana* in 1979. Friedrich Laibach is the first researcher to conduct experiments with *Arabidopsis* (McCourt and Benning 2010).

Several characteristics of *Arabidopsis* makes it a great choice for biochemical, physiological and genetic researches for more than 40 years. It is a photosynthetic plant that for the accomplishment of its life cycle, need just air, a few minerals, light and water.

Here are some advantages of this plant as a model organism: A fast life cycle, from seed to seed (8 weeks) or from germination to mature seed (6 weeks). *Arabidopsis* is very productive with lots of seeds from one plant, cross fertilization is not necessary because *Arabidopsis* can fertilize itself therefore seeds can be separated from a plant also, and easily grown in an isolated place. It was sequenced in year 2000 and has a small genome with a total of 125 Mb, the plant is transformation of the plant is effectively carried out using *Agrobacterium tumefaciens* (Meinke, Cherry et al. 1998).

The B subunits of *Arabidopsis* which is the regulatory subunits are grouped as unconnected families as follows B/B55 (a and b), B' (a(α), b(β), g(ɣ), d(ẟ), e(ε), z, h, u(θ), and k), and B'' (a, b, g, d, e, and TON2). The B, family is further classified into 3 smaller groups a, h, and k, the h sub-group is composed of similar analogues B'h, B'g, B'u, and B'z with 83% sequence arrangement similarity. B'g and B'z sequence arrangement similarity is 88% and that of B'h and B'u pair is 81%. The localization of the preserved sequence similarity is mainly located in the core region and is made up of the distinct domains' accountable interrelation with the AC heterodimer (Kataya, Heidari et al. 2015).

In *Arabidopsis*, three genes code for A-subunit, five genes code for C-subunits and 17 genes code for B-subunits. The catalytic subunits of Arabidopsis are five in number and are grouped into two. The B subunits of *Arabidopsis* and the catalytic subunit are joined by a hook-like structure of the isoforms of *Arabidopsis* PP2A subunits A. Two PP2A 55 kDa B-subunit isoforms that has five degenerate WD-40 (has 43-48% resemblance with the B subunit of animals and yeast) but not much has been discovered about the activities of this 55 kDa B-subunits. Nine PP2A B' subunits are found in *Arabidopsis*, AtbB'α and AtB'β alone have recognized sequences for nuclear targeting (Kataya, Heidari et al. 2015).

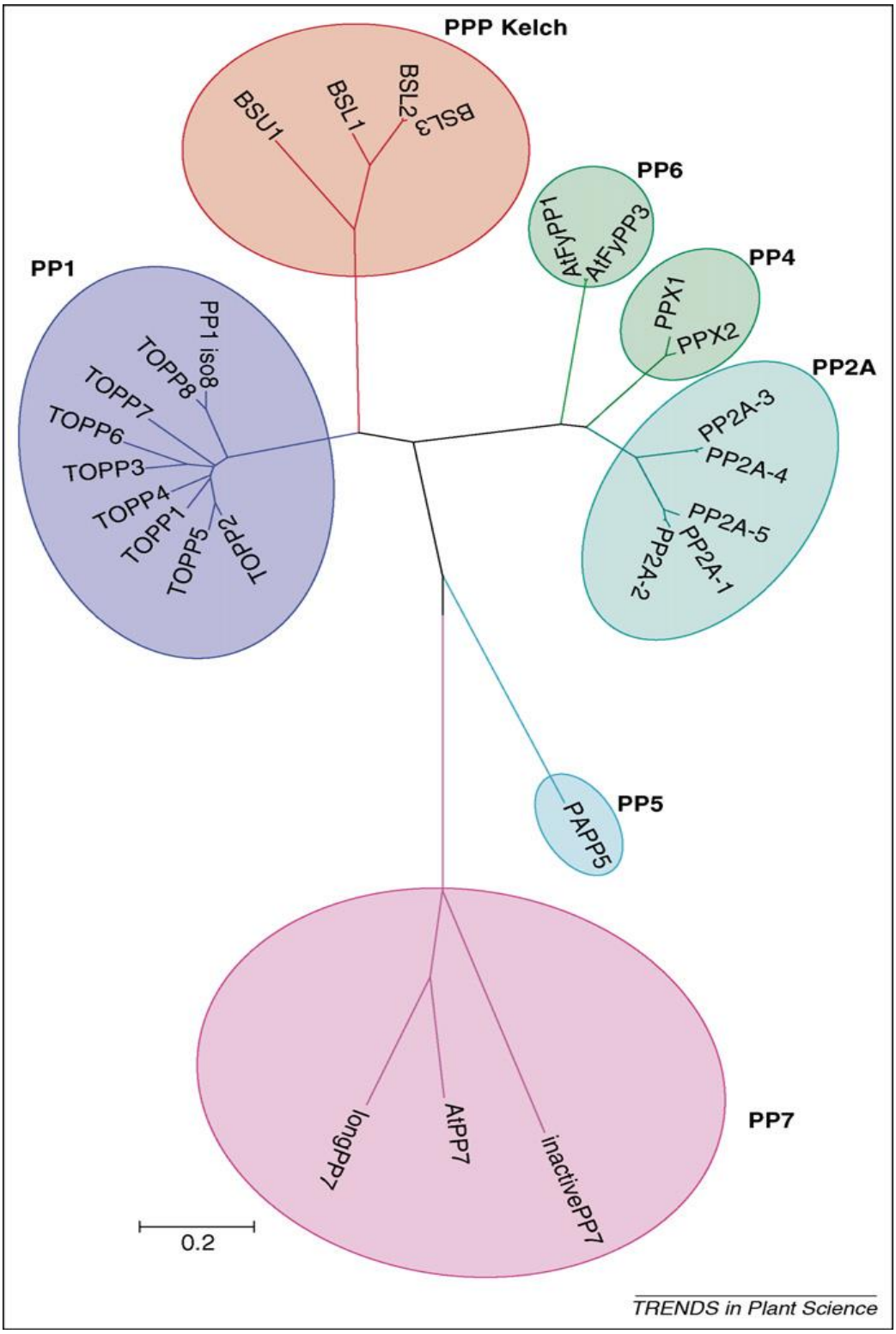


FIGURE 5: Schematic diagram for Phosphoprotein phosphatase (PPP) of *A. Thaliana's* catalytic subunits. (Farkas, Dombradi et al. 2007).

1.6 MAIN OBJECTIVES OF THIS PROJECT.

Protein phosphatase 2A-B'phi is a regulatory subunit of Protein phosphatase 2A. this subunit appears to be important for interactions with microorganisms in some plants (potatoes and tomatoes) especially mycorrhiza (Charpentier, Sun et al. 2014).

Mycorrhiza is a symbiotic association of certain fungi and plant roots which facilitates the uptake of mineral nutrients by roots, the host plants in return supplies associated mycorrhizal fungi with carbohydrates, helping each other to survive. Arbuscular mycorrhizae and ectomycorrhiza are the two main types of mycorrhizal symbiosis found in about 90% of terrestrial plants. for example, in potato and tomato plants (Molina and Trappe 1984).

B'phi has not been much studied and may also be important for regulation of other processes. B'phi gene is not present in our model plant *A. thaliana.*

The B'phi gene from Heinz tomato was isolated in our lab (I. Averkina, E. Asare), cloned into a plasmid vector (pBA002) containing the 35 CaMV promoter for constitutive expression of the gene, and transferred to *A. thaliana* -together with the gene giving Basta resistance as a selective marker. Then, these plants are now further investigated in the master project.

- Selection on Basta to check if the plants are homozygous for the insert.
- To check the expression of B'phi (RNA isolation with subsequent RT-PCR).
- Effects of Microbe *(Pseudomonas*)
- Stress conditions, (salt).

Chlorophyll assay of plants infected with Pseudomonas WCS 417 and the salt stressed plants.

Analysis

1) Seeds (about 50) will be sown from each plant to check if progeny is Basta resistant, e.g. test if the seedlings are homozygous for Basta resistance (and then also likely the B'phi gene). Seed batches with homozygous seeds are used in further experiments.

2) The B'phi gene was fused with the 35S CaMV promoter for constitutive expression, but

expression needs to be checked. First, about ten plants should be tested for B'phi expression.

3) Growth and development of plants expressing B'phi will be recorded and compared with wild type (WT) plants. Parameters to be tested are (a) germination, hypocotyl length and root morphology, (b) time to flowering, growth, size and other visible traits.

4) PP2A activity will be tested

5) Effects of plant growth promoting bacteria *(Pseudomonas*) will be tested.

6) Test of stress conditions, especially salt stress.

2 .MATERIALS AND METHODS.

2.1 MATERIALS.

Biochemicals used in this study were purchased from Bio-Rad (Hercules, CA, U.S.A), Invitrogen (Carlsbad, CA, U.S.A), Merck (Damstadt, Germany) and Sigma- Aldrich/Fluka (St. Louis, MO, U.S.A).

2.1.1 Plants used for this study.

A. thaliana ecotype Columbia was used in this study. The T-DNA plants *A. thaliana* used for this study were transformed with an empty vector PB002 (a binary vector from *Agrobacterium tumefaciens*) using "the floral dipping method" (I. Averkina, E. Asare).

2.1.2 Bacteria used in this Study.

Pseudomonas simiae (formerly *Pseudomonas fluorescens*) *WCS 417* in glycerol stock.

Table 1: List of kits used in this study

Kits	**Function**	**Supplier**	**City, Country**
RNeasy Plant Mini Kit	Used to extract plants for PCR	QIAGEN	Hilden, Germany
Phire® Plant Direct PCR Kit	Genotyping of plants	Thermo Fisher Scientific Baltics UAB	Vinus, Lithuania
SuperScript IV VILO Master Mix	For cDNA syntheses	Invitrogen Thermo Fisher Scientific	Vinus, Lithuania
SuperScript® III Reverse Transcriptase		Invitrogen	Carlsbad, CA, USA.

GFX™ PCR DNA and Gel Band Purification Kit	For agarose gel band purification before sequencing	GE Healthcare	Buckinghamshire, UK

Table 2: List of Biochemicals.

Reagent	Classification	Supplier	City, Country
Taq Polymerase	DNA Polymerase Enzyme	Invitrogen	Carlsbad, CA, USA
PCR buffer, 10X PCR reaction buffer	Buffer	Invitrogen	Carlsbad, CA, USA
T4 DNA Ligase 10X buffer	Buffer	Promega	Madison, MI, USA
Molecular Biology Grade Water	Chemical	Thomas Scientific	Swedesboro, NJ, USA
dNTP's, 2.5 mM of each NTP, total 10 mM	Chemical	Bioline	Luckenwalde, Germany
Agarose	Chemical	Sigma Aldrich	St Louis, MO, USA
Agar-Agar	Chemical	Merck	Darmstadt, Germany
LB Agar	Chemical	Merck	Darmstadt, Germany
Hyper Ladder™ 1kb	Marker gel electrophoresis	Bioline	Luckenwalde, Germany
$MgSO_4$		Merck	Darmstadt, Germany
P-jord	Planting Soil	Tjerbo	Rakkestad, Norway
Agra-vermiculite	Phyllosilicate group of minerals	RHP	Rhenen, The Netherland
Glycerol	Chemical (alcohol)	Merck	Darmstadt, Germany

Rifampicin	Antibiotic		
Bacteriological Agar	For preparing Agar media	VWR Chemicals	United Kingdom
Sucrose	For preparation of Agar.	ThermoFisher	Kandel, Germany

Table 3: Oligonucleotide Primers for Gene expression

Primers	**Sequence**	**Annealing Temperature**	**Extension time**	**Amplicon Size**
Short forward and Short reverse Primers	B'phi_short_For: AAGGCACCCTGGAATCGGAG B'phi_short_cDNA_Rev: CGCGTTCAGCAACCTGCGAG	59 °C	45 s	400 nucleotides
Short Forward and Complete reverse Primers.	B'phi_short_For: AAGGCACCCTGGAATCGGAG B'phi_complete_Rev: TCACATTGCTGCATTTTCAATTTTTTCCC	55 °C	1 min	670-Cdna 944-gDNA

2.2 METHODS.

2.2.1 Basta Selection of B' phi Mutants.

Seed Sterilization and Sowing of *A. thaliana* Seeds.

First, 25 ml 1% (w/v) Ca-hypochlorite + 1 drop of Tween was made the mixture was Shook for homogenization and then, left to settle.

Then, 1 ml of the solution (supernatant) was pipetted into 9 ml 96% ethanol (to be used the same

day).

The seeds were placed in Eppendorf tubes (0.1 ml or less) and Add 1 ml of the ethanol/hypochlorite solution was added. The seeds and solution mixture was shaken and allowed to stand for 4min (if the seeds are left longer than 4 mins the solution will kill the seeds)., The supernatant was pipetted off, 1 ml of 95% ethanol was added, pipetted off, and this washing twice was repeated twice making sure that the ethanol is well removed.

The seeds were left to dry over-night in the sterile hood.

The next day. Petri dishes with half strength MS medium (with 1% sucrose) were placed in the

hood (with N and without N). The spatula was deepened in 70% ethanol, and then burn it in the flame before spreading the seeds.

Approximately 40 seeds were sown in each Petri dish giving enough space for each seed in the agar. Petri dishes were closed with parafilm. And left in a dark place for 2days. Two Petri dishes were made for each treatment of Wild Type, Wild Type with Basta, Basta B' phi 13, 16 and 20 (making a total of 10 dishes).

Two days later, the dishes were placed vertically in continuous light and observation was made after three weeks. Describing the progeny seedlings and determining if they are resistant to the pesticide BASTA (homozygous for BASTA resistance).

The batch of seeds with homozygous seeds are used for further experiments.

2.2.2 FLOWERING STATISTICS FOR *ARABIDOPSIS THALIANA* WILD TYPE AND TREATED TYPE PLANTS.

Flowering Observation was done twice for B'phi 13 and Wild type plants, for comparison.

2.2.2.1 SOWING OF SEEDS ON THE 20TH OF SEPTEMBER 2018. (1st Flowering Experiment).

Arabidopsis grows well in soil mixtures that include ample peat moss, vermiculite or perlite for aeration. Vermiculite and soil in the ratio 1:3 respectively were used to sow *Arabidopsis* seeds in pots with perforations was placed in tubs for sub irrigation, which is a better method.

Water was poured into the tub or tray and left for thirty minutes to make the soil wet and moist before sowing seeds. A maximum of two seeds were sown in each pot. The seeds were dropped on the soil and not covered with soil because they need light for germination. Fifty pots of *A. thaliana* wild type (25 pots in each tray) and fifty pots of Mutant *A. thaliana* (25 pots in each tray) were sowed on the 20th of September 2018.

After sowing the, seeds were covered with a plastic dome to maintain high humidity until the seedlings are one week old. The seeds were kept in a cold room for 3 days at a temperature of 4°C to imbibe them. Seeds are typically dormant, this means that they do not germinate immediately, but this can be overcome by imbibing the seeds at 2-4°C hence the reason they were kept at 4°C.

After imbibing for a period of 3 days, the seedlings were moved to a growth room chamber with photo period of 16hrs./8hrs. room with a temperature of 16°C. The plastic dome was not removed until the seedlings started to germinate. The seedlings were put under cool-white fluorescent bulbs. Watering was done regularly, to keep the soil damp and not oversaturated with water. 1x Hoagland nutrient solution was added on the same day once every week. 1x Confidor was used to spray the plants to protect them from insects. Confidor is a chemical used to control aphids, mealy bugs, scale thrips, whitefly and other sucking insects on ornamentals, roses and vegetables. It belongs to a new chemical group; therefore, it is excellent to spray with other insecticides, its advantage over older formulations is that it controls pests resistant to older formulations). Its toxicity is low, it is a water-based formulation, it is absorbed through the foliage and moves throughout the plant to control pests- it works from the inside out. Confidor's active ingredient is 0.125g/L IMIDACLOPRID. was used to spray plants to protect them from insects.

These plants were monitored and maintained for a period of 71 days. The 32nd day after sowing, was the first day of recording flowering statistics (that is when the first white buds started to appear), but this was not the actual day that flowering was first noticed.

Hoagland solution is a hydroponic nutrient solution recipe (formula) named after the researcher who developed it. Hydroponics provides means to grow in areas where they would not otherwise be viable, but plants still need the right nutritional support for healthy growth. It is a universal nutrient solution for plants grown in a hydroponic environment. Hoagland was developed in 1938 by researchers Hoagland and Arnon. Anon revised the solution in 1950 to include chelated. It contains a lot of Nitrogen and Potassium.

Hoagland Solution.

Table 4: Solution Composition (Hoagland and Arnon 1950).

Chemicals	Concentration
KH_2PO_4	1 mM
KNO_3	5 mM
$Ca(NO_3)_{2:4}H_2O$	5 mM
$MgSO_4:7H_2O$	2 mM
Fe-EDTA	1 μM
H_3BO_3	46.23 μM
$MnCl_2:4H_2O$	9.2 μM
$CuSO4:5H_2O$	0.36 μM
$ZnSO4:7H_2O$	0.77 μM
$Na_2MoO_4: H_2O$	0.12 μM

2.2.3 Sample preparation for Polymerase Chain Reaction.

Finnzymes' Phire® Plant Direct PCR Kit is designed to perform PCR directly from plant leaves and seeds without prior DNA purification. Fresh plants, plant material stored at +4°C or frozen are all suitable templates for this kit, as well as plant material stored on commercially available cards such as Whatman 903® and FTA® Cards. A list of plants tested with this kit is available at www.finnzymes.com/directpcr. The kit employs Phire® Hot Start II DNA Polymerase, especially engineered enzyme with a DNA-binding domain that enhances the processivity of the polymerase. Phire Hot Start II DNA Polymerase also exhibits extremely high resistance to many PCR inhibitors found in plants. The Phire Plant Direct PCR Kit contains reagents and tools for two alternative methods: direct and dilution protocols. A 0.5 mm Harris Uni-Core™ puncher and a compatible Harris Cutting Mat™ are included for convenient sample handling. Dilution Buffer is included for optional sample treatment before PCR (see 'Dilution protocol' in Section 4). It can be used to treat larger/more difficult samples (e.g. more fibrous or latex containing samples), or when multiple PCR reactions are performed from a single sample. It is also a useful choice when longer DNA fragments (> 1 kb) are amplified. The kit includes control primers for amplification of a highly conserved region of chloroplast DNA.1 The kit is recommended for end-point PCR

A piece of a presumed transformant (e.g. a punch approximately 2 mm in diameter) was dropped in 20 µl of Dilution Buffer provided in the Phire® Plant Direct PCR kit. The plant tissue was crushed with a 100 µl pipette tip by pressing it briefly against the tube wall until the solution was greenish in color. The supernatant was used as a template for a 10 µl PCR reaction. The composition of the reaction mix shown in Table 1.

This experiment was carried out twice because there was contamination in one of the samples WT34. Hence the reason for tables showing experiment 1 and experiment 2.

A piece of a presumed transformant (e.g. a punch approximately 2 mm in diameter) was dropped in 20 µl of Dilution Buffer provided in the Phire® Plant Direct PCR kit. The plant tissue was crushed with a 100 µl pipette tip by pressing it briefly against the tube wall until the solution was greenish in color. The supernatant was used as a template for a 10 µl PCR reaction. The composition of the reaction mix shown in Table 10. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013358_Phire_Plant_Direct_PCR_UG.pdf

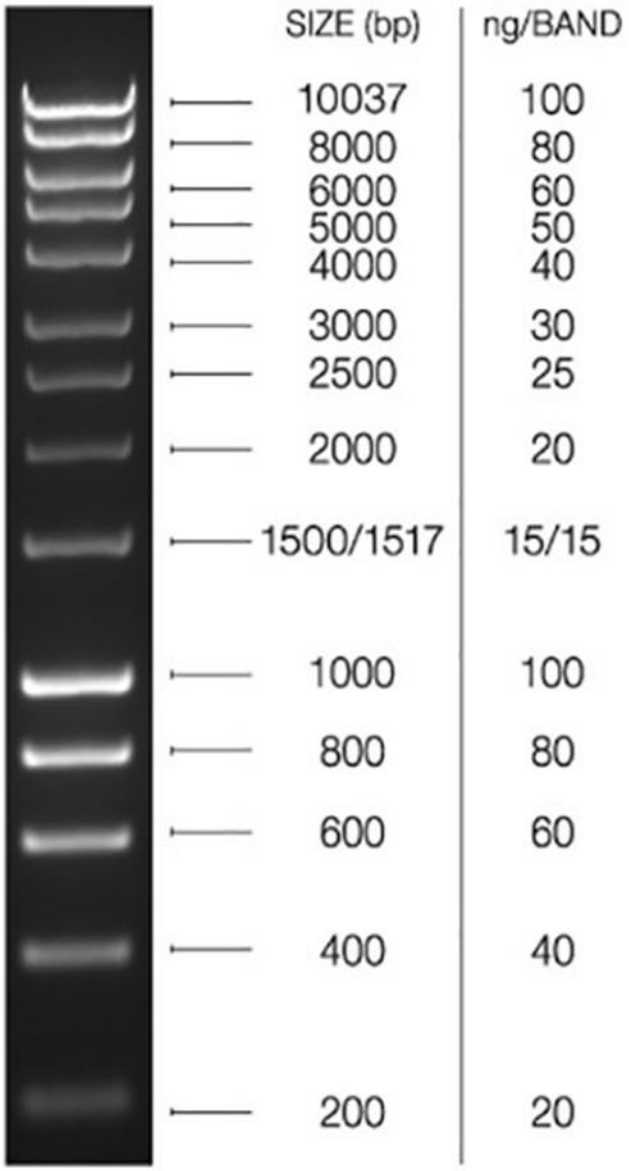


FIGURE 6: HyperLadderTM 1kb Ready to use molecular weight marker for easy orientation and size determination of DNA up to 10kb.' https://www.bioline.com/us/hyperladder-1kb.html

Table 5: The composition of PCR mix. The composition of PCR Mix for experiment 1 PCR Test

Component	10 µl reaction	Final concentration	Mastermix for 19 reactions
dH2O	3.4 µl		64.6
2xPCR buffer	5 µl		95
Forward primer	0.5 µl	0.5 µM	9.5
Reverse primer	0.5 µl	0.5 µM	9.5
DNA Polymerase	0.2 µl		3.8
Plant extract	0.4 µl		0.4

9.6 µl of the master mix was pipetted into each PCR tube prior to adding 0.4 µl of plant extract. The PCR program was designed considering the manufacturer's recommendations (Thermo Fisher Scientific (2010)).

Master mix was prepared, for the PCR, using the Phire Plant Direct PCR kit.

Table 6: The composition of PCR Mix for experiment 2 (WT 34) PCR Test:

Component	10 µl reaction	Final concentration	Master mix for 6 reactions
dH2O	3.4 µl		20.4
2xPCR buffer	5 µl		30
Forward primer	0.5 µl	0.5 µM	3
Reverse primer	0.5 µl	0.5 µM	3
DNA Polymerase	0.2 µl		1.2
Plant extract	0.4 µl		0.4

For this experiment, 9.6 µl of the master mix was pipetted into each PCR tube prior to adding 0.4 µl of plant extract. Master mix was prepared, for the PCR, using the Phire Plant Direct PCR kit. The PCR program was designed considering the manufacturer's recommendations (Thermo Fisher Scientific (2010)).

Table 7: PCR program for experiments 1 and 2.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	98 °C	5 min	1
Denaturation Annealing Extension	98 °C 54 °C 72 °C	5 s 5 s 45 s	40
Final extension	72 °C 4 °C	1 min Hold	1

The annealing temperatures for primer pairs were calculated using New England Biolab calculator (https://tmcalculator.neb.com). Extension time depend on fragment length for Expand High Fidelity Hot Start II DNA Polymerase For extension, use 20 s for amplicons < 1kb or 20s/kb for amplicons >1kb.

Table 8: Expected PCR products

Sample name	NCBI Reference Sequence:	Full length gDNA, bp	Expected PCR amplicon with vector sp. pr., bp	Expected PCR amplicon with gene sp. pr., bp
Tomato Heinz, MM, AC	XM_010317091.2	1486	-	1486
Plants transformed with the pBA002-T-DNA vector	XM_010317091.2	1486	1762	1486

Two master mix were prepared: with vector-specific primers and with gene-specific primers.
For the PCR, the Phire Plant Direct PCR kit will be used.

Table 9: Master Mix for Loading Buffer for PCR experiment 1.

Component	Volume	Master Mix for 18 reactions
Loading Buffer	2 µl	36 µl
Gel Red	1 µl	18 µl

For Hyper ladder, a mixture of 5 µl of hyper ladder to 1 µl of gel red was loaded on the gel.
3 µl of the loading buffer master mix to each sample of PCR mixture (10 µl) before loading on the gel.

Table 10: Master Mix for Loading Buffer

Component	Volume	Master Mix for 6 reactions
Loading Buffer	2 µl	12 µl
Gel Red	1 µl	6 µl

Table 11: Hyper-ladder mixture for PCR experiment 1 and 2 (WT 34).

Component	Volume	Total volume for loading
Hyper-ladder	5 µl	10 µl
GelRed	1 µl	2 µl

Samples separated by electrophoresis which carried out at 90 for 45 min in a Power Pac Basic electrophoresis chamber (Bio-Rad) using 1x TAE buffer as running buffer. Agarose gels were visualized with UV-light using ChemiDoc™ Imaging Systems (Bio-Rad) (Hercules, CA, USA)

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used for determination size and concentration of PCR products, restriction digest and for purification of digested vectors. Agarose gel comprise of 0.5% (w/v) agarose melted in 1x TAE buffer. Before loading the sample on agarose gel, Crystal 5x DNA Loading Buffer Blue (Bioline) was added. GelRedTM (Biotium) fluorescent nucleic acid dye was add previously to loading buffer to make DNA bands visible under UV-light exposure.

2.2.5 Phenotypical Response and Chlorophyll Content of A. thaliana Inoculated with Pseudomonas WCS 417

The main purpose of this experiment is to obtain a more pronounced phenotypical response of the *A. thaliana* to inoculation with Pseudomonas fluorescens WCS 417.

pH value and Sugar concentration may inhibit the positive effect of bacteria growth on plant or the growth may not be evident, if the pH value or Sugar concentration is not optimized. For *Pseudomonas-A. thaliana* interaction the optimized values for pH and Sugar concentration are 5.8 and 0.5% respectively.

Pseudomonas fluorescens WCS 417 (Pieterse et al. 1996) provided by Corne Pieterse at University of Utrecht, The Netherlands.

2.2.6 Plant Growth Conditions.

Surface sterilized seeds of Arabidopsis WT were sown on solidified ½ MS-medium (Murashige and Skoog 1962) with 1% sucrose, pH 5.8, and 0.7% agar. The plates were placed in the dark at 4°C for two days for stratification prior to cultivation with a 16 h photo period for 5days. To study the plant bacteria interaction, the seven-day old seedlings were then transferred to new plates (5 plants per plate) with bacteria inoculated with ½ MS medium composed specifically for the bacteria.

2.2.7 Plant growth medium for *Pseudomonas-A. thaliana* co-cultivation.

Half-strength Murashige and Skoog (1/2 MS) was prepared by mixing appropriate volumes of stock solutions with distilled water and pH was adjusted to 5.8. The medium was added 0.5% (w/v) sucrose. The solution was mixed well with 0.7% (w/v) agar with subsequent autoclaving and cooling to approximately 45-50°C. The medium was poured into square petri dishes (12 mm x 12 mm) with 40 ml in each and left to solidify for at least 30 min.

2.2.8 Inoculating of agar plates with *Pseudomonas.*

The bacteria culture of *Pseudomonas* from the glycerol stock was streaked on a petri dish with LB agar supplemented with 50 µg/ml rifampicin and incubated overnight at 28°C. The grown bacteria culture was loosened in 10 ml of 10 mM MgSO4 (Pieterse et al. 1996). The bacteria solution was collected in a 15 ml Falcon tube and centrifuged at 5000 rpm for 5 min, the supernatant was removed, and the cells were re-suspended in new 10 ml of 10 mM MgSO4 with subsequent centrifugation at 5000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in another 10 ml of 10 mM MgSO4, the OD600

was measured and the suspension was diluted with 10 mM MgSO4 to obtain OD600 = 0.005 (Verhagen et al. 2010). The bacterial suspension, 500 µl, was spread evenly with a flamed and cooled spreader on each 12x12 square Petri dish prior to the plant transplantation.

2.2.9 Chlorophyll Assay for Bacteria inoculation.

Shoots were extracted in a mortar with 2 ml 95% ethanol. Filtered (or centrifuge away particles at max speed). Diluted with ethanol as follows: took 0.30 ml of the extract plus 1.20 ml of ethanol. Determined absorbance at 654 nm. Calculated the chlorophyll content given in µg/ml of extract

according to the formula: Chlorophyll content = Absorbance654 x 25.1 x 10 (µg/ml) (if diluted with another factor, change 10 with the other factor).

2.2.10 SALT STRESS.

2.2.10.1 Plant Growth Conditions.

Surface sterilized seeds of *Arabidopsis* WT were sown on solidified ½ MS-medium (Murashige and Skoog 1962) with 1% sucrose, pH 5.8, and 0.7% agar. The plates were placed in the dark at 4°C for two days for stratification prior to cultivation with a 16 h photo period for 5days. To study the plant bacteria interaction, the seven-day old seedlings were then transferred to new plates (3 plants per plate) with ½ MS medium and different salt concentration.

Plant growth medium for different salt concentrations-*Arabidopsis* cultivation

Half-strength Murashige and Skoog (1/2 MS) was prepared by mixing appropriate volumes of stock solutions with distilled water and pH was adjusted to 5.8. The medium was added 0.5% (w/v) sucrose. The solution was mixed well with 0.7% (w/v) agar with subsequent autoclaving and cooling to approximately 45-50°C.

Spreading different salt concentrations on agar plates.

For this experiment 5M of NaCl was autoclaved and used. Different concentrations of 5M NaCl, 0 mM, 50mM, 100mM, 150mM, 200mM (3 petri dishes for each concentration). Different volumes of the 5M NaCl was added to 120 ml of the Half-strength Murashige and Skoog (1/2 MS) then, 40 ml of the mixture was divided into three different petri dishes and was spread evenly with a flamed and cooled spreader on each 12x12 square Petri dish prior to the plant transplantation.

This step was done for the five different concentrations so in the end 15 petri dishes were prepared.

2.2.10.2 Chlorophyll Assay for Salt Stress.

Shoots were extracted in a mortar with 2 ml 95% ethanol. Filtered (or centrifuge away particles at max speed). Diluted with ethanol as follows: took 0.30 ml of the extract plus 1.20 ml of ethanol. Determined absorbance at 654 nm. Calculated the chlorophyll content given in µg/ml of extract according to the formula: Chlorophyll content = $Absorbance_{654}$ x 25.1 x 10 (µg/ml) (if diluted with another factor, change 10 with the other factor).

2.2.11 GENE EXPRESSION ANALYSIS.

2.2.11.1 Harvesting of Plant Samples.

Mortar, pestles, scalpels, twizers, spatulas were autoclaved and stored in liquid nitrogen before use. Liquid Nitrogen was Pumped for use in this experiment. Foil was cut out and labelled with name of each plant sample and stored in liquid nitrogen, the foil is to keep the harvested plant sample in and store in the liquid Nitrogen.

Autoclaved cryotubes were labelled with each plant name. Time of harvesting was recorded prior to harvesting. A table was made for recording weight of tubes without cover, expected weight minimum of 50 mg and maximum of 100mg of crushed plant sample plus tubes because that is the recommended quantity for the Qiagen kit used for this experiment, in this experiment 50 mg was used. Real weight was measured after weighing the crushed sample in the tube.

Autoclaved twizers and razors were used to harvest plants (rosette) the fresh ones (new ones), then wrapped them in the labelled foil (labelled before harvesting) and put in liquid nitrogen until time to store in -80°C freezer.

Liquid nitrogen was poured into the mortar and pestle to cool them down to avoid thawing of the plants before crushing is over. Cryotubes were weighed without cover, the crushed sample was scooped into the cryotubes and weighed, work was done quickly to avoid thawing of the crushed plant samples.

Spatula kept in liquid nitrogen was used to put the crushed sample in the tube quickly without thawing the sample and quickly put the tubes in the liquid Nitrogen.

Samples were taken out of the liquid nitrogen and stored in a box and frozen at -80°C until further treatment.

RNAeasy Qiagen Kit.

The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer,

which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to a RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl water. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for mRNA since most RNAs

RNA stabilization using RNA later technology RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes need to be avoided for all reliable quantitative gene expression analyses, such as microarray analyses, quantitative RT-PCR, such as TaqMan® and LightCycler ® technology, and other nucleic acid-based technologies.

Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi Up to 100 mg of sample is first ground in liquid nitrogen and then lysed under highly denaturing conditions. The RNeasy Plant Mini Kit provides a choice of lysis buffers: Buffer RLT and Buffer RLC, which contain guanidine thiocyanate and guanidine hydrochloride, respectively. The higher cell disruption and denaturing properties of Buffer RLT frequently make it the buffer of choice. However, some tissues, such as milky endosperm of maize or mycelia of filamentous fungi, solidify in Buffer RLT, making the extraction of RNA impossible. In these cases, Buffer RLC should be used instead. After lysis with either buffer, samples are centrifuged through a QIAshredder homogenizer. This simultaneously removes insoluble material and reduces the viscosity of the lysates by disrupting gelatinous material often formed in plant and fungal lysates. Ethanol is added to the cleared lysate, creating conditions which promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water. RNA Cleanup This protocol can be used to purify RNA from enzymatic reactions (e.g., DNase digestion, RNA labeling) or to desalt RNA samples (up to 100 µg RNA). Buffer RLT and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column.

Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.

2.2.11.2 RNA extraction (Qiagen RNeasy).

Crushed, weighed and stored samples in the -80°C freezer for later use from the previous step is kept in liquid Nitrogen before preparing the buffers RTL and RDD.

- RLT buffer with **ß-**Mercaptoethanol was prepared (in the fume hood, **ß-**ME is toxic)
  Add 10 µl **ß-**ME per 1 ml RTL.
- RDD buffer was prepared by:
  Adding 10 µl DNase 1 stock to 70 µl RDD buffer (fridge +4°C)
  The solution was mixed gently by inverting the tube and centrifuged briefly to collect residual liquid.

Procedure:

RTL of 450 µl buffer (added **ß-**ME) was added in the hood to a max of 100 mg tissue powder

Vortexed vigorously for 30 sec to 1 min.

Then transferred to a lilac QIA shredder spin column which was assembled in a 2 ml collection tube (in the hood).

It was spanned for 2 mins, 14.00 x g.

The supernatant of the flow-through was carefully transferred to a new 2 ml Eppendorf tube without disturbing the pellet. Only this supernatant was used in subsequent steps.

Ethanol (96-100%) with a volume of 0.5 was added. Here this was about 225 µl.

It was mixed by pipetting.

The sample (around 650 µl) was transferred including any precipitate to a RNeasy pink spin column assembled in a 2 ml collection tube.

Spinning was done for 15 secs, 13.000 x g.

The flow through was discarded.

Optional steps, to remove genomic DNA.

350 µl RW1 buffer was added to the RNeasy pink spin column, then closed gently.

Spinning was done for 15 secs at 13.000 x g. Flow through was discarded.

Also, 80 µl DNase 1 incubation mix solution was added to the center of the membrane.

Incubation was done at room temperature (20-30°C) for 15 mins.

Then 350 µl RW1 buffer was added to the RNeasy spin pink column.

It was spanned for 15 secs at 13.000 x g.

Flow-through was discarded.

Continue protocol step 8.

RPE buffer of 500 µl was added to the RNeasy spin column, the lid was closed gently.

Then spin for15 secs 13.000 x g.

Flow-through was discarded.

Also add 500 µl RPE buffer.

Again, spin for 2 mins 13.000 x g.

After centrifugation, the column was removed carefully to prevent flow-through carryover.

The column was placed onto a new centrifugation tube.

Then spin for 1.5 mins 14.000 x g to remove ethanol and RPE.

Column was placed in a new 1.5 ml collection tube (supplied).

Also add 30 µl RNase free water.

It was spanned for 1min at 13.000 x g to eluate RNA.

The eluate was kept. If needed the last step can be repeated but not necessary.

The column was placed onto a new collection tube, and 30 µl RNase free water was added and spinning was done for 1min, 13.000 x g.

The eluate was kept, and the concentration was measured by nanodrop then cDNA synthesis experiment was the next.

Table 12: RNA Quality and Concentration by Nanodrop.

Samples	ng/ml	A260/280	A260/230
WT4	925.9	2.17	2.00
WT 17	605.1	2.15	1.85
B'phi 44	290.9	2.13	0.81
B'phi 47	379.3	2.16	0.62
B'phi 50	299.4	2.13	2.28

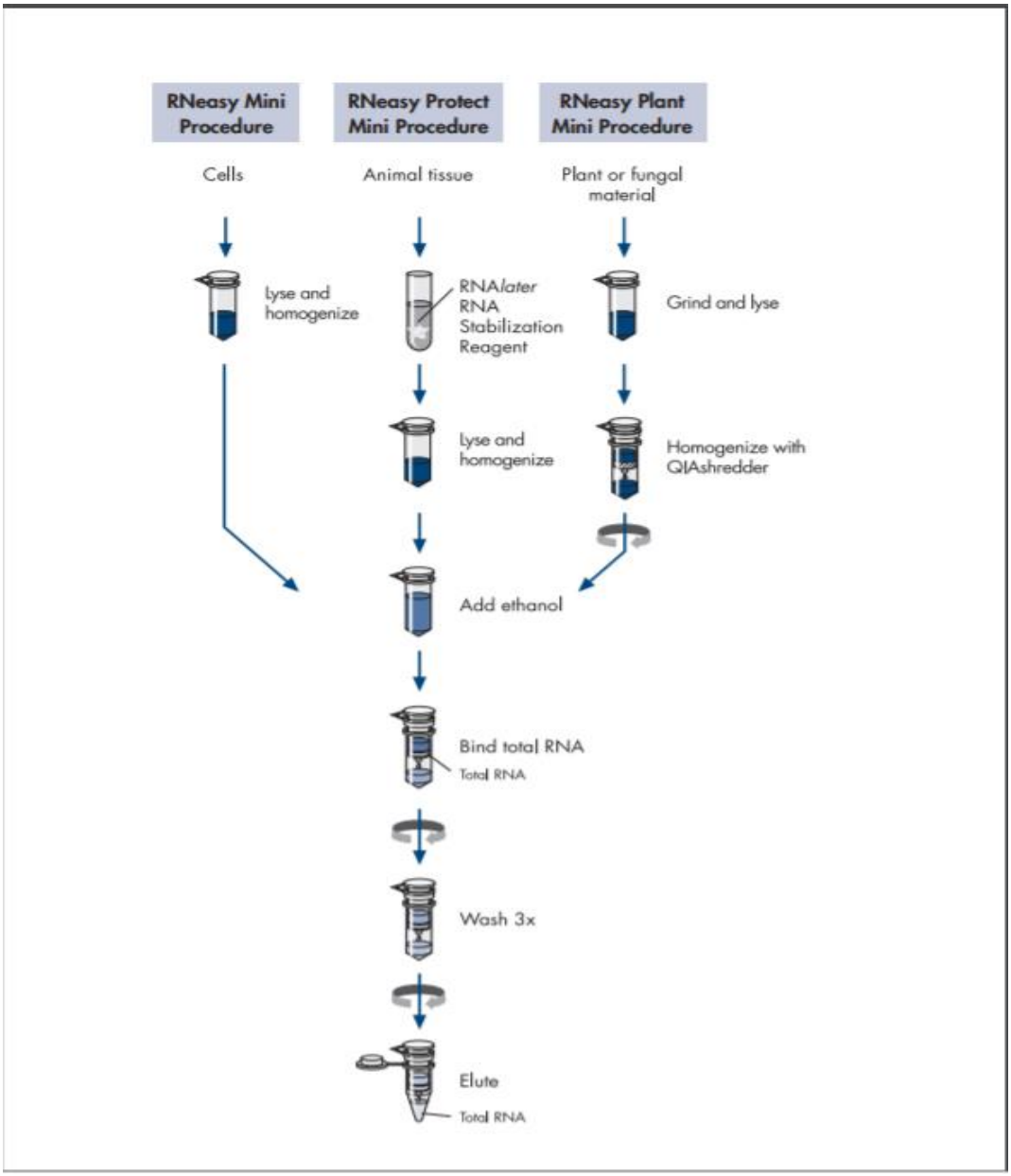


FIGURE 7: Illustration of RNeasy Plant Mini PRoceedure Experment.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013358_Phire_Plant_Direct_PCR_UG.pdf

cDNA SYNTHESIS.

SuperScript IV VILO Master Mix (Highest cDNA yields in the shortest time).

Invitrogen™ SuperScript™ IV VILO™ Master Mix is a cDNA reaction master mix designed for two-step quantitative PCR (RT-qPCR) applications. The master mix format elevates the trusted Invitrogen™ VILO™ technology (Variable Input, Linear Output) to the next level by combining optimized buffer conditions with Invitrogen™ SuperScript™ IV Reverse Transcriptase (RT), known for its high processivity and thermostability.

This new formulation allows the cDNA reaction to occur at higher temperatures with shorter reaction times, resulting in greater cDNA yields and sensitivity even with samples of suboptimal purity and low template levels.

The Invitrogen™ SuperScript™ IV VILO™ Master Mix with ezDNase enzyme is an optimized solution for first strand cDNA synthesis in two-step RT-PCR applications.

The 5x master mix includes SuperScript™ IV Reverse Transcriptase, a propriety recombinant RNase inhibitor, helper proteins, stabilizer proteins, oligo (dT)18, random hexamer primers, $Mgcl_2$ and dNTPs.

The SuperScript™ IV VILO™ No RT control contains all the components of the SuperScript™ IV VILO™ Master Mix except the reverse transcriptase enzyme. The No RT Control is used in a RT minus reaction to verify the absence of genomic DNA (gDNA) contamination in the RNA sample.

ezDNase enzyme (Cat. No. 11766051) is a novel double-strand specific thermolabile DNase that is used to remove gDNA contamination from template RNA prior to the Rt reaction. The enzyme is available as part of the kit or as a standalone product (but I am not using this in my cDNA synthesis experiment because I am doing the Reverse transcription experiment for SuperScript™ IV VILO™ Master Mix without ezDNase enzyme treatment).

2.2.11.3 Reverse transcription protocol

SuperScript™ IV VILO™ Master Mix (without ezDNase enzyme treatment).

1.Prepared RT and No RT Control reaction mixes (on ice).

Table 13: components added to an empty RNase-free tube on ice.

Component	RT reaction	No RT Control reaction
SuperScript™ IV VILO™ Master Mix	2 µl	-
SuperScript™ IV VILO™ No RT Control	-	4 µl
Template RNA (1 pg to 2.5 µg total RNA)	varies	varies
Nuclease-free Water	To 10 µl	To 10 µl

2. To anneal primers, the mixture was gently mixed and incubated at 25°C for 10 minutes.
3. Then reverse transcribe RNA was done by Incubating mixture at 50°C for 10 minutes.
4. To Inactivate enzyme, mixture was Incubated at 85°C for 5 minutes.

5. qPCR amplification

Use the diluted or undiluted cDNA for qPCR or store at –20°C for up to one week, or –70°C for long term storage.

Table 14: Reverse Transcriptase Reaction.

Sample	Concentration	Master Mix	RT Control	Total RNA	Nuclease free water
WT 4	925.9	2	-	1.1	6.9
WT 17	605.1	2	-	1.7	6.3
B'phi 44	290.9	2	-	3.4	4.6
B'phi 47	379.3	2	-	2.6	5.4
B'phi 50	299.4	2	-	3.3	4.7

Table 15: No Reverse Transcriptase Reaction.

Sample	Concentration	Master Mix	RT Control	Total RNA	Nuclease free water
WT 4	925.9	-	2	1.1	6.9
WT 17	605.1	-	2	1.7	6.3
B'phi 44	290.9	-	2	3.4	4.6
B'phi 47	379.3	-	2	2.6	5.4
B'phi 50	299.4	-	2	3.3	4.7

Table 16: Primers used in experiment their Annealing temperature, Extension Time and Amplicon Size.

Name of Primers	Sequence of Primers	Annealing Temperature (°C) T_a	Extension Time.	Amplicon Size- bp.
Short forward and Short reverse Primers	B'phi_short_For: AAGGCACCCTGGAATCGGAG B'phi_short_cDNA_Rev: CGCGTTCAGCAACCTGCGAG	59 °C	45 s	400 nucleotides
Short Forward and Complete reverse Primers.	B'phi_short_For: AAGGCACCCTGGAATCGGAG B'phi_complete_Rev: TCACATTGCTGCATTTTCAATTTTTTCCC	55 °C	1 min	670-Cdna 944-gDNA

Table 17: PCR Set up for the Experiments.

Cycle step	Temperature	Time	Number of cycles	Number of cycles
Initial denaturation	98 °C	5 min	1	1
Denaturation Annealing Extension	98 °C Varies 72 °C	5 s 5 s Varies	35 B'phi_short_For B'phi_short_cDNA_Rev	40 B'phi_short_For B'phi_complete_Rev

2.2.12 SEQUENCING.

Illustra GFX PCR DNA and Gel Band Purification Kits.

This kit is a product of GE Healthcare-Life Sciences.

Designed for the rapid purification and concentration of PCR products or DNA fragments ranging in size from 50 bp to 10 kb. This kit can be used to purify DNA from reaction volumes up to 100 µl or agarose gel slices up to 900 mg.

This kit combines a versatile chaotropic buffer with a glass fiber matrix supported in a spin column for the purification of DNA from both solution and agarose gel. Typical recoveries range from 60% to 80% for DNA fragments from agarose gel to as high as 95% for PCR products from solution. DNA purity is exceptional; 99.5% of contaminants are removed.

Illustra GFX PCR DNA and Gel Band Purification Kit contains the following components in sufficient quantities: GFX columns, collection tubes, color-coded bottles of capture buffer, wash buffer, and two elution buffers (Tris-HCL and sterile water), and an instruction booklet.

The Basic Principle.

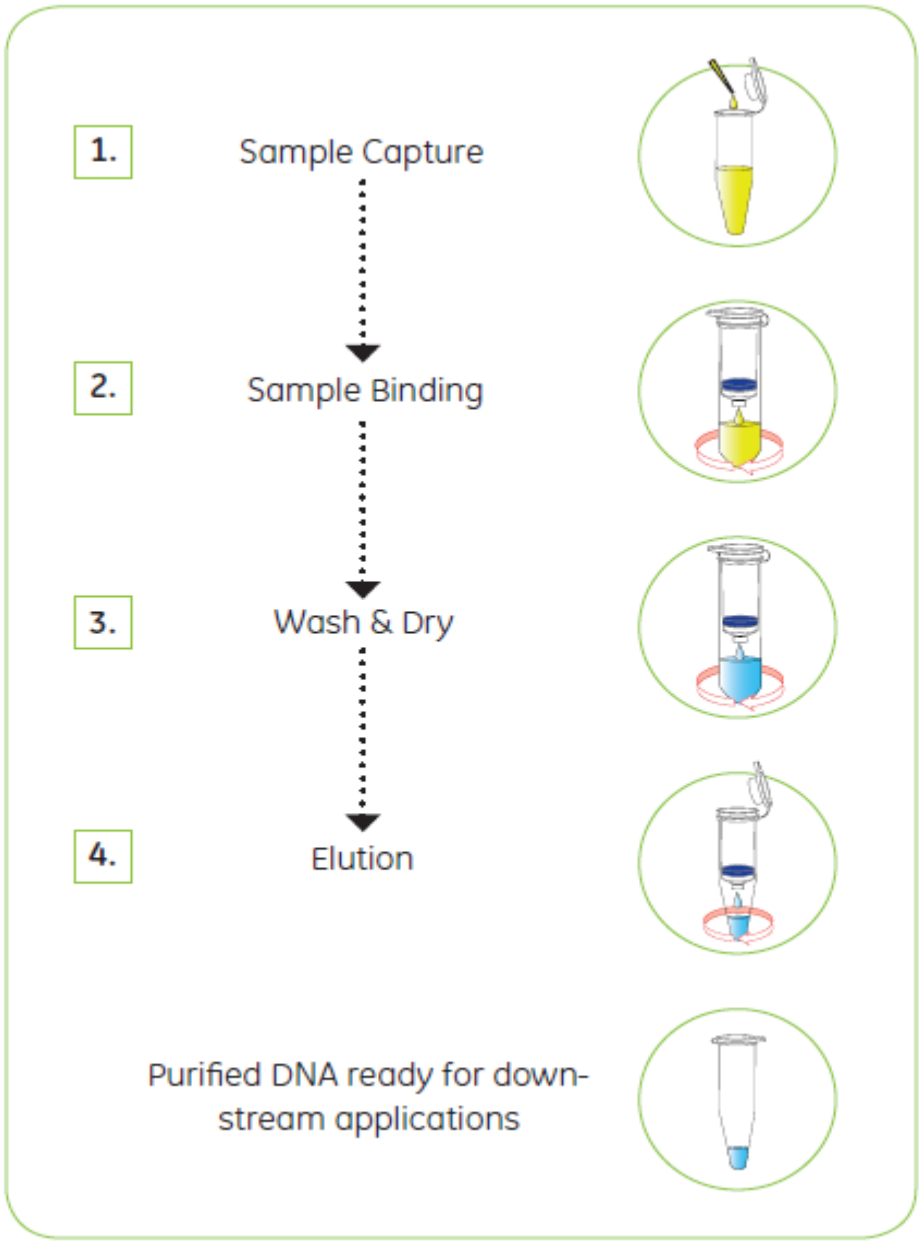


FIGURE 8: Use of the Illustra GFX PCR DNA and Gel Band Purification Kit involves the following steps. http://www.blossombio.com/pdf/products/UG_28951562AA.pdf

Materials to be supplied by user (these are not included in the kit)

Disposables:

1.5 ml DNase-free microcentrifuge tubes (2 per purification from agarose gels, 1 per purification from PCR mixtures and enzyme reactions)

Chemicals:

Absolute ethanol

Equipment needed

Microcentrifuge that accommodates 1.5 ml microcentrifuge tubes Vortex mixer

For purification of DNA from agarose gels only: Clean scalpel or razor blade Water bath or heat-block for 60°C incubation.

The illustra GFX PCR DNA and Gel Band Purification Kit is designed for the purification and concentration of DNA from PCR mixtures, restriction enzyme digestions, solutions and agarose gel bands. DNA ranging in size from 50 bp up to 10 kbp can be purified from solution volumes of up to 100 µl and from gel slices of up to 900 mg. No modifications are required for purification of DNA from gels run in borate-based buffers (e.g. TBE). The Capture buffer type 3 contains a pH indicator that changes color at various pH levels to visually indicate whether the Capture buffer plus sample mix is at the optimal pH for DNA to bind to the silica membrane. For efficient binding of DNA to the silica membrane the Capture buffer-sample mix requires a pH $\leq$ 7.5. The pH indicator will appear a yellow or pale orange color in this range. If the pH is > 7.5 (which can occur if the agarose gel electrophoresis buffer is not refreshed, is incorrectly prepared, or if the pH of the sample exceeds the buffering capacity of Capture buffer) DNA adsorption will be inefficient and yield may be reduced. The pH indicator will appear a dark pink or red color in this range. If the pH of the binding mixture is > 7.5 it can be adjusted by addition of a small volume of 3 M sodium acetate pH 5.0 before loading onto the GFX Microspin column. The indicator dye does not interfere with DNA binding and is completely removed during the wash step. In addition, using a

colored as opposed to a clear binding mixture allows easy visualization of any insolubilized agarose. Complete solubilization is necessary to obtain maximum yields.

If pH indicator is a yellow of pale orange color, Capture buffer sample mix is at optimal pH for efficient DNA binding to the silica membrane:

Figure 9: optimal pH for efficient DNA binding to the silica membrane

Optimal pH

If the pH indicator is a dark pink or red color, the pH of the Capture buffer-sample mix is too high to achieve efficient DNA adsorption to the silica membrane: pH too high

Figure 10: pH too high for DNA binding to the silica membrane.

Typical reactions from which DNA can be isolated include: • Sequential restriction enzyme digests, where the enzymes involved have differing buffer requirements; perform the first digest, purify the sample using the illustra GFX PCR DNA and Gel Band Purification Kit, and proceed to the second enzyme reaction. • DNA modified by an enzymatic reaction, including phosphatase reactions with CIP or SAP, filling-in or removal of overhangs to form blunt ends e.g. with DNA (Klenow) polymerase I, large fragment or T4 DNA polymerase or proof-reading polymerases, and nuclease reactions e.g. S1 nuclease or mung bean nuclease. To concentrate your DNA sample, use an elution volume that is less than the starting volume of the sample being purified. For optimal recovery, use 50 µl elution volume. Table 1 opposite shows example percentage yields obtained when purifying a 910 bp fragment.

Percentage yield obtained with illustra GFX PCR DNA and Gel Band Purification Kit

Table 18: Sample sources and Expected yield.

Sample Source	Elution volume (µl)	Yield (%) *
PCR	10 50	65 82
300 mg agarose	10 50	57 91
900 mg agarose	50	55

*910 bp PCR fragment at 8.4 ng/µl was purified from the PCR mixture or from the weight of agarose indicated. Percentage yield was determined by A260 readings. Fragments ranging in size from 48 kbp to 50 bp can be purified.

Table 19: Product Specifications.

Sample Type:	PCR mixtures, enzyme reactions, DNA solutions, agarose gel slices
Sample size range	48 kbp-50 bp
Input volume	100 µl solution or up to 900 mg agarose
Elution volume	10-50 µl (into one of two elution buffers provided)
Major subsequent applications	Further PCR amplification, sequencing, labelling, restriction enzyme digestion, ligation, cloning.
Yield obtained when purifying a 910 bp fragment at a starting concentration of 8.4 ng/µl	PCR 10 µl elution volume-65% PCR 50 µl elution volume-82% 300 mg agarose 10 µl elution volume-57% 300 mg agarose 50 µl elution volume-91%

When purifying a PCR fragment less than 50 bp but greater than 10 bp in length, use an Illustra Microspin G-25 column. This kit cannot be used for the purification of RNA.

Preparation of working solutions.

Capture buffer type 3

Capture buffer type 3 contains a pH indicator that changes color at various pH levels to visually indicate whether the Capture buffer plus sample mix is at the optimal pH for DNA to bind to the silica membrane. Refer to section 4.1 for more information.

Wash buffer type 1

Prior to first use, add absolute ethanol to the bottle containing Wash buffer type 1. Add 100 ml of absolute ethanol to the Wash buffer type 1 in kit 28-9034-70 or add 250 ml ethanol to the Wash buffer type 1 in kit 28-9034-71. For the sample pack size, add 10 ml absolute ethanol to the Wash buffer type 1. Mix by inversion. Indicate on the label that this step has been completed. Store upright in an airtight container.

Elution buffers type 4 and 6

Two elution buffers are provided with this kit to enable optimal performance of the purified sample in as wide a range of downstream applications as possible.

Elution buffer type 4

(10 mM Tris-HCI, pH 8.0) -the sample should be eluted into this buffer for use in a range of downstream applications and for long term storage of the sample.

Elution buffer type 6

(sterile nuclease free water) -the sample should be eluted into this buffer for the best results with sequencing applications, especially when using a salt-sensitive capillary loading analyzer.

In this experiment **Elution buffer type 6** was used because this buffer is best for sequencing applications, which is the experiment carried out here.

Sample verification

When purifying DNA from either PCR mixtures or restriction enzyme digestions, we recommend running an analytical gel prior to purification to check for a single band representing the DNA

species of interest. If multiple bands are present, we recommend performing a preparative gel and excising the band of interest and following 5.4 Protocol for purification of DNA from TAE and TBE agarose gels.

Protocol for purification of DNA from TAE and TBE agarose gels

1. Sample Capture

a. Weigh a DNase-free 1.5 ml microcentrifuge tube and record the weight.

b. Using a clean scalpel, long wavelength (365 nm) ultraviolet light and minimal exposure time, cut out an agarose band containing the sample of interest. Place agarose gel band into a DNase-free 1.5 ml microcentrifuge tube (user supplied).

c. Weigh the microcentrifuge tube plus agarose band and calculate the weight of the agarose slice.

Note: The sample may now be stored at -20°C for up to 1 week.

d. Add 10 µl Capture buffer type 3 for each 10 mg of gel slice, for example, add 300 µl Capture buffer type 3 to each 300 mg gel slice.

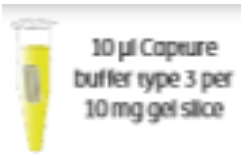


FIGURE 11: Capture buffer sample mix.

Note: If the gel slice weighs less than 300 mg, add 300 µl Capture buffer type 3. DO NOT add less than 300 µl Capture buffer type 3 per sample.

Note: To save time when purifying multiple samples of gel bands (each weighing less than 500 mg), add 500 µl Capture buffer type 3 to each gel slice. DNA recovery will be unaffected providing the volume of Capture buffer type 3 is in excess of the weight of each gel slice.

e. Mix by inversion and incubate at 60°C for 15–30 minutes until the agarose is completely dissolved. Mix by inversion every 3 minutes.

FIGURE 12: Incubation.

Note: If sample contains DNA greater than 5 kb, do not vortex, as this may cause shearing of the DNA.

f. Once the agarose has completely dissolved check that the Capture buffer type 3-sample mix is yellow or pale orange in color.

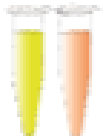


FIGURE 13: Expected color of sample mix after incubation.

Note: If the color of the binding mixture is dark pink or red, add a small volume (~ 10 µl) of 3 M sodium acetate pH 5.0 and mix. Ensure that the binding mixture turns a yellow or pale orange color before loading onto the GFX Microspin column.

FIGURE 14: Dark pink requires further experiment.

3. RESULTS.

The B'phi gene from Heinz tomato was isolated in Lillo Lab, cloned into a plasmid vector and transferred to *A. thaliana* (together with the gene giving Basta resistance as a selective marker). Basta is a Pesticide.

In this experiment, Basta was used for selection of transformed plants, to check if the seedlings sown from each generation of transgenic *A. thaliana* plant are homozygous for Basta before use in further experiments.

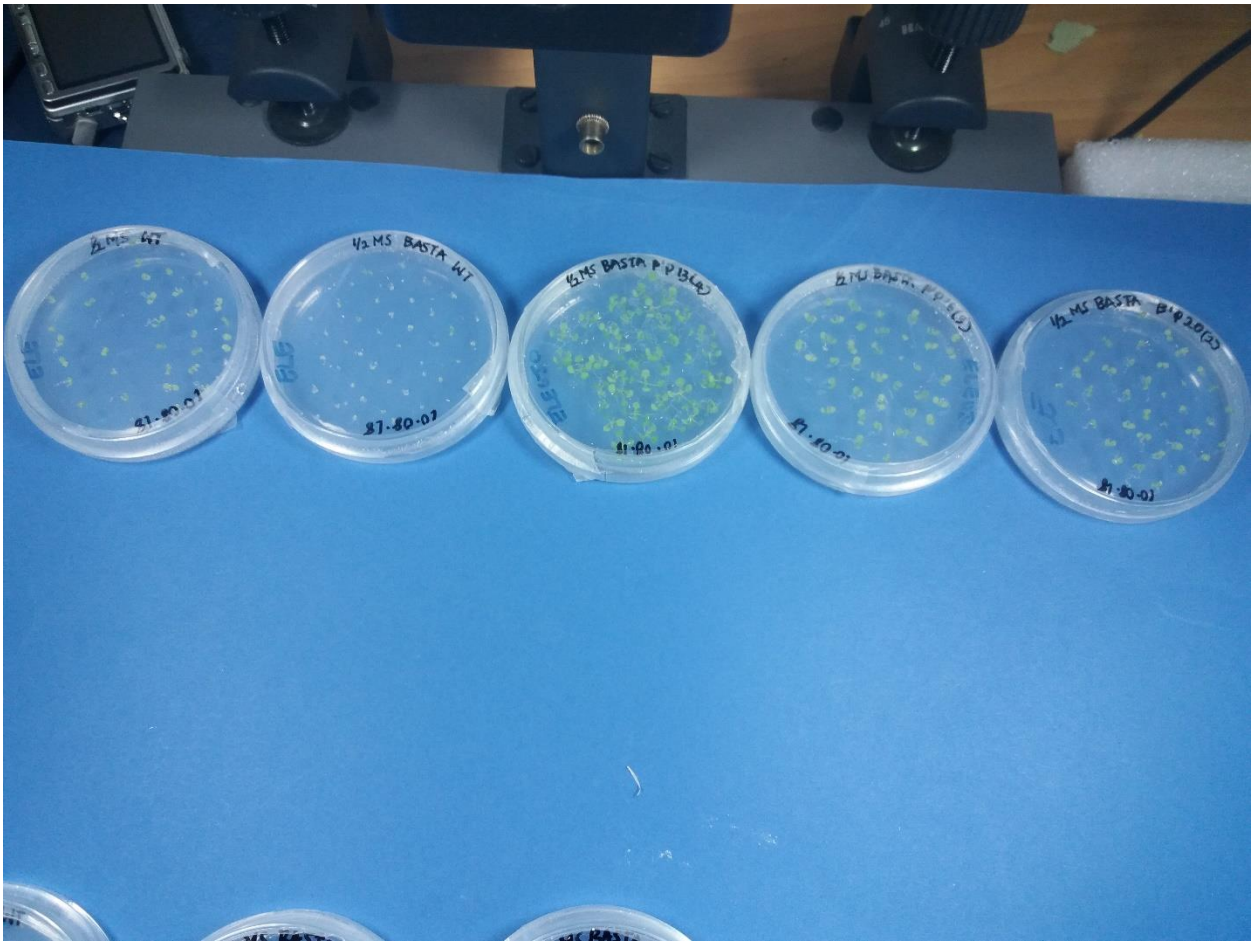


FIGURE 15: Plates of sown Wild type on MS media and Wild type and B'phi *A. thaliana* on ½ MS Media plus Basta.

TABLE 20: The growth statistics for seeds sown for Basta experiment.

SEEDS SOWN	Good Growth Plants	Poor Growth Plants or no Growth	Percentage Resistance to Basta (%)
½ MS WT	34	6	85.0
½ MS WT	38	2	95.0
½ MS WT BASTA	4	36	10 (POOR)
½ MS WT BASTA	5	35	12.5(POOR)
½ MS BASTA B'PHI13(4)	40	0	100
½ MS BASTA B'PHI13(4)	39	1	97.5
½ MS BASTA B'PHI16(3)	38	2	95.0
½ MS BASTA B'PHI16(3)	40	0	100
½ MS BASTA B'PHI20(2)	39	1	97.5
½ MS BASTA B'PHI20(2)	37	3	92.5

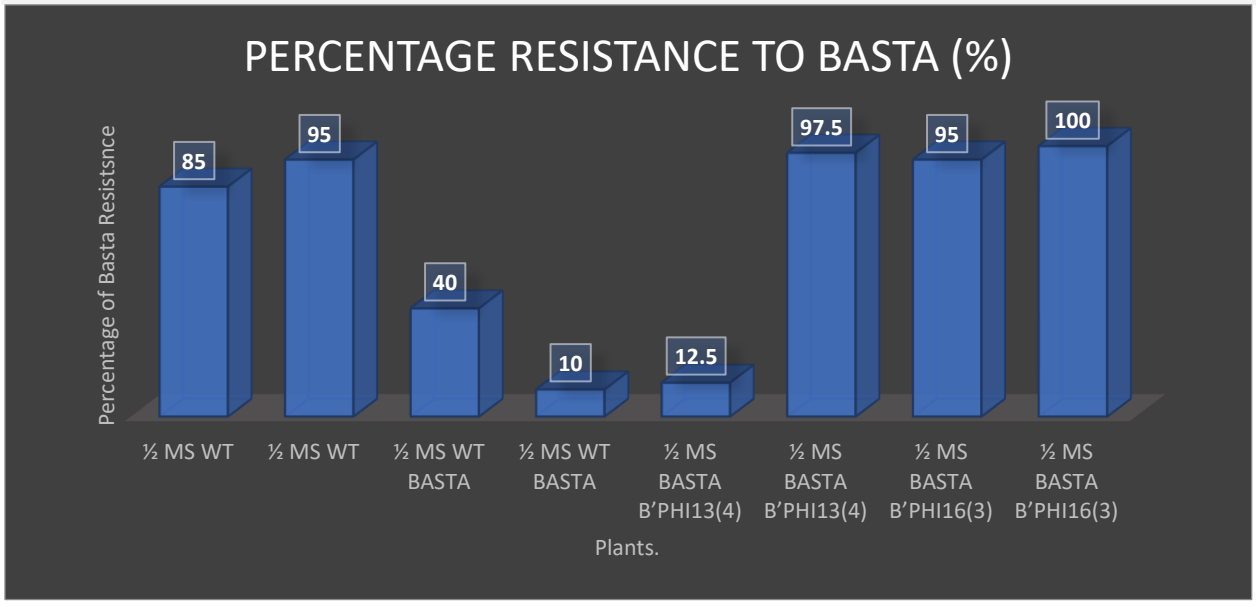


FIGURE 16: Percentage of Resistance to Basta (%).

Basta (Phosphinothricin or Glufosinate) is one of the most widely used broad spectrum herbicide used to control a wide range of weeds in agricultural and industrial areas (Kang, Song et al. 2014). Glufosinate (trade name Basta) contains an active compound called phosphinothricin that inhibits the action of glutamine synthase (Leason, Cunliffe et al. 1982) ,disturbs the gas exchange and chlorophyll fluorescence emission and consequently disrupts photosynthesis that could lead to death of plant cells (Nada 2016).

To check for homozygosity, three generation of transformed *A. thaliana* plants B'phi 13, 16 and 20 (all have been infused with a gene resistant to Basta) were checked and Wild type *A. thaliana* with and without Basta were checked, that is a total of 5 sets of plants. Petri dish plates with ½ MS Media and 1% sucrose were prepared, and two petri dishes were prepared for each treatment of the five sets of plants, making a total of 10 Petri dishes. In eight Petri dishes except for a set of treatment (Wild type *A. thaliana* without Basta) a concentration of Basta of 0.01 mg/ml was added to the growth agar in which the seeds were sown.

After stratification for 3 days and the petri dishes have been in continuous light (16hrs./8hrs.) for three (3 weeks). It was observed after the experiment, during statistics and after coalition of results that: The Wild type plants with Basta had the poorest survival rate with a percentage resistance to Basta of 10.0 and 12.5 for each set of its treatment, compared to the Wild type without Basta with a percentage resistance to Basta of 85 and 95 and all the transgenic *A. thaliana*, B'phi 13, 16 and 20 with Basta in the growth agar on which they have been sown, having a percentage resistance to Basta of 100 and 97.5% for the set of B'phi 13, 95 and 100 % resistance to Basta for the set of B'phi 16 and finally for B'phi 20 a percentage resistance to Basta of 97.5 and 92.5.

3.1 FLOWERING EXPERIMENT.

When to flower is a crucial developmental decision in the life cycle of a plant that is directly associated with its reproductive success. Subsequently the timing of flowering is regulated both by the developmental status of the plant as well as external cues. The major environmental factors that modulate flowering include the quantity and quality of light, vernalization (exposure to winter conditions) and growth temperature. *A. thaliana* is a facultative long day plant in which flowering is accelerated by long days (16 hrs. light). The molecular genetic basis of the photoperiodic

induction of flowering has been deciphered using mutants that are late flowering specifically in long days (Balasubramanian and Weigel 2006).

Flowering in *A. thaliana* is modulated by several environmental factors (Bernier et al, 1993). It is therefore important to perform flowering-time measurements under a controlled environmental condition, including light quality and intensity, day length, temperature, watering, and spacing between plants. Because of the large influence of environmental effects, it is imperative that only plants grown at the same time and same place must be compared (This rule was observed in this thesis work). Flowering is also modulated by endogenous factors and can thus be affected by genes that are not primarily involved in the control of flowering time. Also, the variation of flowering time between ecotypes (laboratory strains) is generally large. Therefore, only mutants that are induced in the same background can be compared (The same B' phi 13 seeds were sown and compared in the two-flowering experiment in this work). Flowering time can be defined in terms of different criteria. Upon the transition from vegetative to the reproductive phase in *A. thaliana*, leaves cease to form, and flowers emerge instead. The onset of flowering can be defined as the day on which flower buds are first seen at the apex with or without the aid of magnifying glass; when the first flower opens; when the stem has bolted 1cm etc. As long as flower opening, stem elongation rate is not affected in the mutant line, any of the above criteria are valid for comparison with the wild type. In wild type plants a good correlation exists between the number of days to flower and the number of leaves produced under certain conditions, so flowering can be defining in terms of leaf number (Koornneef, Hanhart et al. 1991).The most meaningful value related to flowering time is total number of leaves (i.e., rosette and cauline leaves) (Page and Grossniklaus 2002)

Early flowering ecotypes, such as Columbia (used in this thesis), Landsberg erecta, and WS, have mutations in the FRI gene (Johanson, West et al. 2000).Three genetic pathways promote flowering of *A. thaliana* under long photoperiods. These pathways are represented by the genes *CO*, *FCA*, and *GA1*, which act in the long-day, autonomous, and gibberellin pathways, respectively (Reeves and Coupland 2001). The plants were kept in 16hr light (long day) in this experiment, so *CO* is the representative of the genetic pathway that promotes flowering in Arabidopsis for in this thesis work), Many mutations that delay flowering of *A. thaliana* have been isolated, but none of them prevent flowering under all conditions. (Reeves and Coupland 2000) Mutations affecting the long-

day pathway delay flowering under long but not short days, whereas mutations affecting the autonomous pathway delay flowering irrespective of photoperiod (Koornneef, Hanhart et al. 1991).

A. thaliana is a facultative long day plant in which flowering is accelerated by long days (16 hrs. light), which was the same light condition used in this thesis work. The molecular genetic basis of the photoperiodic induction of flowering has been deciphered using mutants that are late flowering specifically in long days. The nuclear protein CONSTANS plays a major role in integrating the effects of long days. The expression of *CO* as well as the activity of the CO protein itself is under light regulation. Analysis of naturally occurring flowering time variation resulted in the identification of the vernalization pathway that promotes flowering in response to winter conditions. Vernalization is a mechanism that plants appear to have evolved in order to avoid flowering in peak winter. Previous studies have shown that in long days there is a delay in flowering under lower temperatures (16°C LD) and the analysis of flowering time mutants revealed that Cryptochrome Circadian Regulator 2 (*cry2)* photoreceptor mutants show an extensive delay in this response. It is also known that the early flowering behavior of another photoreceptor mutant Phytochrome B (*phyB)* is temperature sensitive and this early flowering effect was abolished at lower temperatures. Since previous studies have indicated that flowering is not induced by a simple heat shock, in this experiment by (Balasubramanian and Weigel 2006). They tested plants by growing at different temperatures that will not induce a typical heat shock response. They realized that growing plants at 25°C or 27°C, results in early flowering compared to the normally used 23°C in short days (Balasubramanian and Weigel 2006). In the flowering experiment for this thesis work, the temperature of the growth chamber was 22°C I long day 16 hrs. light.

A. thaliana flowers rapidly in long-day photoperiodic conditions of 16 hrs. or continuous light. However, under short-day conditions of 8 to 10 hrs. of light, the plants display a much more extensive period of vegetative growth before flowering. Genes that control this daylength response were identified originally via mutations that cause late flowering under long days but that do not alter flowering time in short-day conditions. Examples of photoperiod pathway genes include *CONSTANS, GIGANTEA, FE, FD,* and *FHA.* A second group of genes, which includes *LUMINIDEPENDENS, FCA, FVE, FY,* and *FPA,* form an autonomous pathway that

monitors the developmental state of the plant and is active under all photoperiodic conditions. Mutants for this second class of genes flower later than wild-type controls irrespective of daylength (Koornneef, Hanhart et al. 1991).

Two sets of flowering experiments were carried out using the B'phi 13 transgenic plants. It was done twice to check for reproducible or comparable. Hence the 1st and 2nd flowering experiments.

3.2 FIRST FLOWERING OBSERVATION EXPERIMENT FOR B'PHI 13 AND WILD TYPE *ARABIDOPSIS THALIANA* PLANTS.

In the first experiment, Rosette leaves and Cauline leaves were counted for each plant on each day of observation. Observation for the first experiment was done between 22nd of October till the 30th of October.

Please refer to appendix for the tables for this first flowering experiment.

1st Flowering Experiment.

WILD TYPE:

On the 22nd of October:

Only 2 Wild Type Plants had flowered. Percentage for this date = 2/50 * 100 % = 4 %

On the 24th of October:

A total of 17 Wild Type Plants had flowered. Percentage for this date = 19/50 * 100 % = 38 %

On the 26th of October:

A total of 27 Wild Type Plants had flowered. Percentage for this date = 46/50 * 100 % = 92%

On the 30th of October:

A total of 4 Wild Type Plants had flowered. Percentage for this date = 50/50 * 100 % = 100%

MUTANT TYPE:

On the 22nd of October:

Only 7 Mutant Plants had flowered. Percentage for this date = 7/50 * 100 % = 14 %

On the 24th of October:

A total of 28 Mutant Plants had flowered. Percentage for this date = 28/50 * 100 % = 56 %

On the 26th of October:

A total of 47 Mutant Plants had flowered. Percentage for this date = 47/50 * 100 % = 94%

On the 30th of October:

A total of 50 Mutant Plants had flowered. Percentage for this date = 50/50 * 100 % = 100%

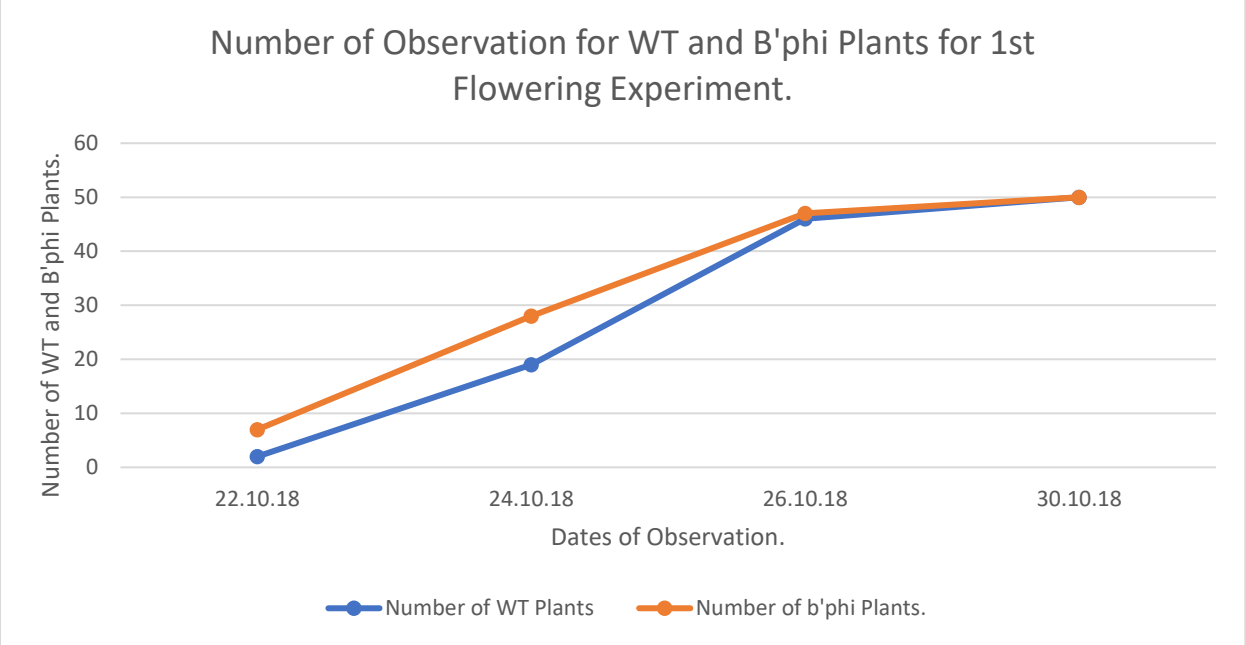


FIGURE 17: Flowering Observation for 50 Wild and 50 Mutant Type B'phi *A. thaliana* plants (Numbers).

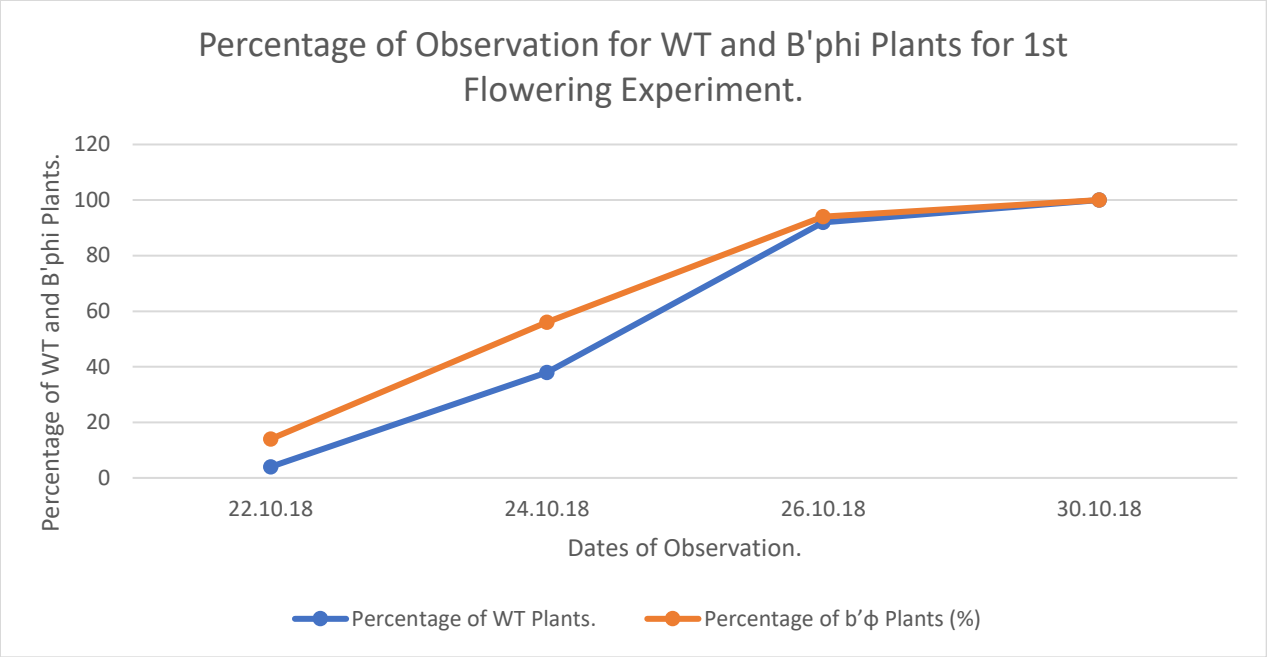


FIGURE 18: Percentage of Flowering Observation for 50 Wild and 50 Mutant Type B'phi *A. thaliana* Plants.

From the statistics, the flowering in Treated plants was faster than the Wild Type Plants. From the first day of observation, 14 % of the Mutant plants had flowered and just 4% of the Wild Type Plants had flowered. On this day, it was observed that the rate of flowering in Mutant was higher than the Wild Type Plants.

By the second time of observation, there was still higher flowering rate in the Mutant Plants than the wild type plants on this day, still favoring the Mutant Type Plants, over the Wild Type Plants.

The third time of observation the percentage rate of flowering for the Wild Type Plants was 92 % and the Mutant was 94 % of the Wild Type, with flowering in the Mutant still faster than the wild type

After 4 days of the third observation, another observation showed that both Wild Type Plants and Mutant Type Plants had flowered 100 %.

All 50 plants, in both treated and Mutant had flowered by the last day of observation.

Pictures of Wild Type and B'phi 13 *A. Thaliana* plants for 1st flowering Experiment.

Please refer to appendix section for more pictures of first flowering experiment.

FIGURE 19: Second Picture of Wild Type and Treated Type Plants numbers (1-50) respectively, taken on the 22.10.2018.

3.3 SECOND FLOWERING OBSERVATION EXPERIMENT FOR B'PHI 13 AND WILD TYPE *ARABIDOPSIS THALIANA* PLANTS.

2nd Flowering Experiment.

In total, 100 *A. thaliana* plants were observed. 50 Wild types and 50 B'phi. In the course of this flowering experiment, the plants were infested by aphids therefore, nine Wild type plants and two B'phi plants didn't survive due to poor survival response were either dead or were too little to be observed at the time of observation and statistics. Hence, bringing the total of Wild type plants 49

and 48 for B’phi plants. In this second flowering experiment, cauline and rosette leaves were not counted.

Statistics was not taken for Nine Wild Type plants and two B’phi plants because the plants were eaten by insects, both the wild type and the B’phi plants. It was observed that the response of the wild type plants after being eaten by insects (aphids) was poor compared to the B’phi plants hence leading to loss and poor growth of nine Wild type plants and two B’phi plants. Unlike the first experiment, Cauline and Rosette leaves were not counted.

It must be noted that the first time of flowering observation and statistics was not the first time that flowering was noticed.

Please refer to appendix for the tables for this second flowering experiment.

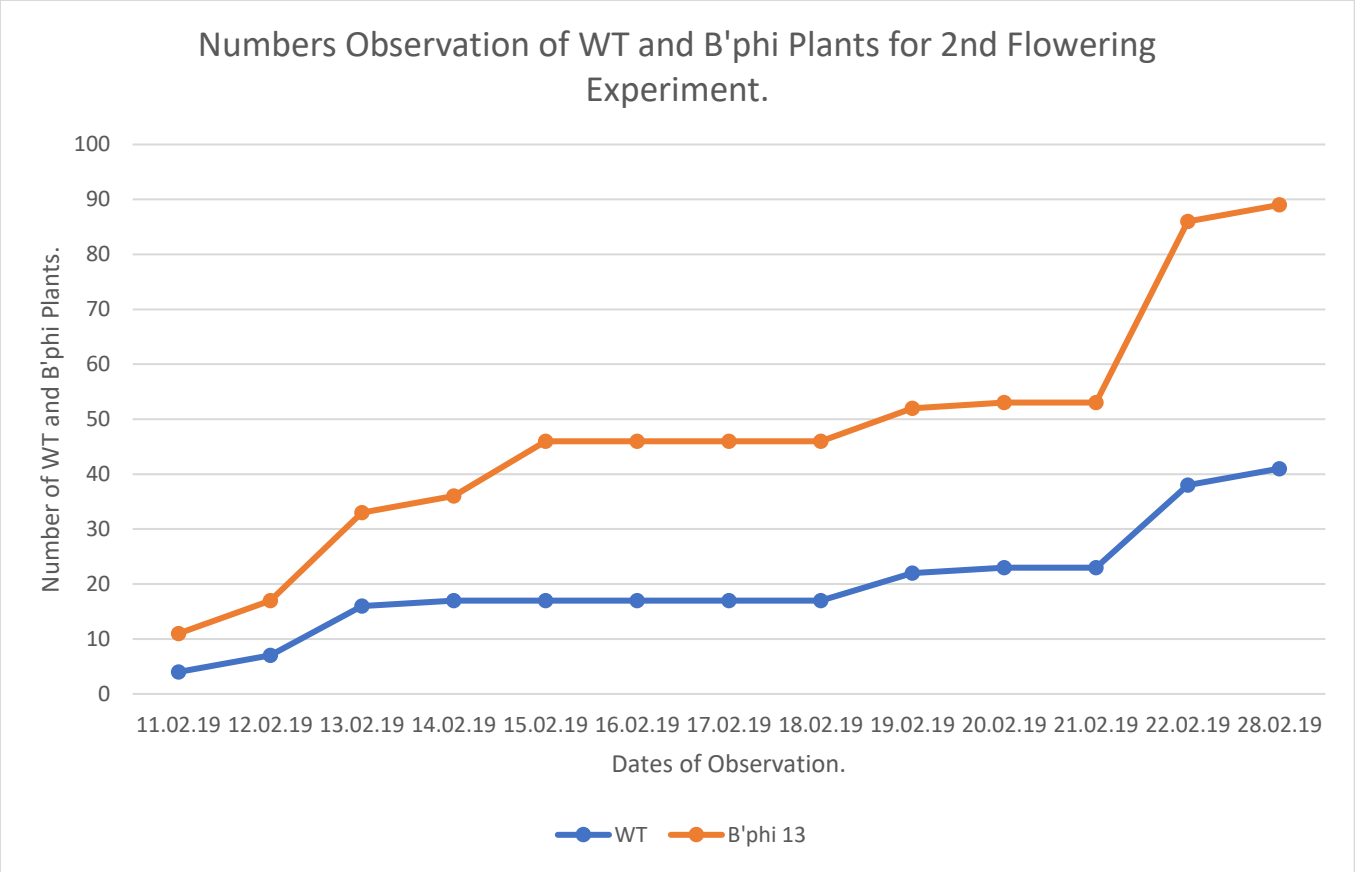


FIGURE 20: Flowering observation for 41 Wild type and 48 Mutant B'phi 13 *A. thaliana* plants.

On the first day of flowering observation, 4 of the Wild type plants out of 41 plants had flowered and 7 of the Mutant plants out of the 48 plants had flowered, the following day, statistics was taken and an increase in the number of plants that had flowered was the same, 3 plants each had flowered for both Wild and Mutant plants. The 2nd day another observation was done showing that 9 plants in Wild type flowered and 7 in Mutant plant flowered, with the Mutant plant still higher in flowering by just 1 plant.

On the 4th day of observation, 1 wild type plant and 2 Mutant plants had flowered. Observation on the 5th day of flowering showed no increase in flowering in the Wild type plants and it remained constant for the next 3 days showing only 17 plants flowered at this time. While on the 5th day of flowering observation for Mutant plants, 10 more Mutant plants had flowered making a total of 29

by this day, this number also remained constant for the next 3 days just like the Wild type plants with the Mutant plants still having the highest number of plants that had flowered. On the 9th day of observation, there was an increase in flowering again in both Wild Type (5 more) and Mutant Plants (1 more) after remaining constant for 3 days, having a total of 22 and 30 respectively.

On the 10th day of statistics for flowering, 1 Wild type plant had flowered and for the Mutant plants flowering remained constant at 30 plants for the next 2 days. By the 11th day, 15 more Wild type plants flowered, and 18 more Mutant plants had flowered, and flowering was complete for all plants.

Pictures of Wild Type and B'phi 13 *A. thaliana* plants for 2nd flowering Experiment.

Please refer to appendix section for more pictures of first flowering experiment.

FIGURE 21: B'phi 13 plants (Numbers 25-50).

FIGURE 22: Wild Type plants (Numbers 1-25).

3.4 BACTERIA INNOCULATION AND CHLOROPHYLL ASSAY.

Plant roots are colonized by an immense number of microbes, referred to as the root microbiome. Selected strains of beneficial soil-borne bacteria can protect against abiotic stress and prime the plant immune system against a broad range of pathogens. *Pseudomonas* spp. rhizobacteria represent one of the most abundant genera of the root microbiome (Zamioudis, Mastranesti et al. 2013).

Selected strains of soil-borne beneficial microbes, collectively referred to as plant growth-promoting bacteria (PGPR) and plant growth-promoting fungi (PGPF), have long been demonstrated to promote plant growth, improve host nutrition, and protect plants from various forms of abiotic stress and soil-borne diseases (Schwachtje, Karojet et al. 2011) .Similar to the immunostimulatory properties of human probiotics, root colonization by selected PGPR and PGPF strains primes the whole-plant body to efficiently defend itself against a broad range of pathogens and even insects. This form of systemic resistance is called induced systemic resistance (ISR) and widely occurs in monocotyledonous and dicotyledonous plant species (Zamioudis and Pieterse 2012). Colonization of the roots by ISR-inducing rhizobacteria and fungi does not directly activate the plant immune system but primes the aboveground plant parts for an accelerated defense response upon pathogen or insect attack, thus providing a cost-effective protection against plant diseases (Conrath, Beckers et al. 2006).Both *WCS 417* and *WCS358* are capable of triggering ISR in *Arabidopsis* (Pieterse, Van Wees et al. 1998).

In the experiment of Unraveling Root Developmental Programs Initiated by Beneficial *Pseudomonas spp*. Bacteria *(WCS417* Inhibits Primary Root Elongation. (Zamioudis, Mastranesti et al. 2013)

To analyze in detail the molecular mechanisms underpinning *Pseudomonas spp.*-stimulated effects on root development, they focused their analysis on developmental responses to the model strain *WCS417*. After 8 days of cocultivation, the primary root length of seedlings exposed to *WCS417* bacteria was reduced by approximately 40% compared with mock-treated roots, suggesting a suppressive effect of *WCS417* on primary root elongation. To test the possibility that the reduction of root length in *WCS 417*-treated roots is due to effects on cell elongation, the length

of root epidermal cells in the elongation and differentiation zones was assessed after 8 days of cocultivation with *WCS 417*. This number was found to be reduced by approximately 40% in *WCS 417*-treated roots compared with mock-treated roots. Collectively, their data indicates that reduced primary root elongation in response to WCS417 is due to inhibitory effects on cell expansion rather than on the organization and function of the root meristem (Zamioudis, Mastranesti et al. 2013).

The main purpose of this experiment is to obtain a more pronounced phenotypical response of the *A. thaliana* to inoculation with *Pseudomonas fluorescens WCS 417*.

pH value and Sugar concentration may inhibit the positive effect of bacteria growth on plant or the growth may not be evident, if the pH value or Sugar concentration is not optimized. For *Pseudomonas-A. thaliana* interaction the optimized values for pH and Sugar concentration are 5.8 and 0.5% respectively.

Total number of petri dishes used to sow plants in each set of this experiment for the transfer of seedlings that have been stratified and kept in continuous light 16 hrs./8 hrs. for 1 week were 12. Three petri dishes for Wild type control plants (5 plants in each plate) and 3 petri dishes for Wild type with *Pseudomonas WCS 417* (5 plants in each plate). Another 3 petri dishes for B'phi control plants (5 plants in each plate) and 3 Petri dishes for B'phi with *Pseudomonas WCS 417* (5 plants in each plate). After seedlings were transferred the plants were kept in continuous light 16 hrs./8 hrs. for 2 weeks before analysis of the Shoot weight, Root weight, Root length and Chlorophyll assay.

Five sets of these experiments were done three B'phi 13 (1,2,3) and two B'phi 16 (1,2) so that they can all be compared to see if they are reproducible.

Only two pictures and tables of the experiments of *A. thaliana-Pseudomonas* interaction are represented her one from the B'phi 13 group and the other from the B'phi 16 group. Please refer to the appendix for the pictures and tables of the other three experiments.

Chlorophyll Assay Pictures for B'phi13 and 16 Wild Type *A. thaliana* Plants.

FIGURE 23: Second B'PHI 13 Bacteria/ chlorophyll experiment.

Table 21: Data for Harvested Treated and Untreated Wild Type and Mutants of *A. thaliana* inoculated with *Pseudomonas WCS 417 on* LB agar medium. (2nd B'Phi 13 experiment).

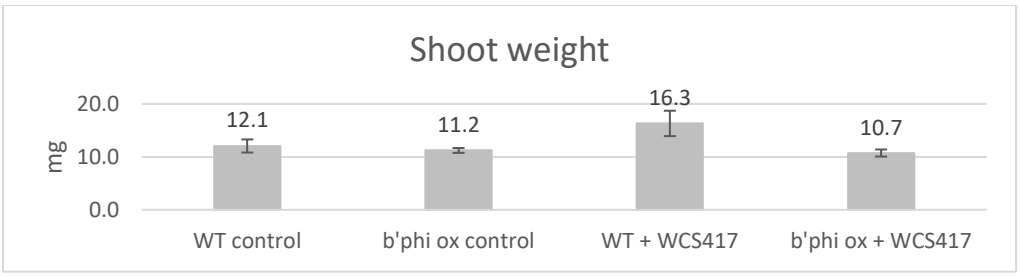
Sample ID	Shoot Weight	Absorbance	Root Weight	Root Length				
				Plant1	Plant2	Plant3	Plant4	Plant5
WT C1	52.5	0.168	11.5	100.831	102.772	99.71	65.143	119.051
WT C2	72.5	0.406	24.3	119.124	118.653	105.319	117.455	99.909
WT C3	55.8	0.18	15.4	95.452	117.618	113.71	108.201	63.514
B'phi C1	59.4	0.228	14.7	108.55	89.497	134.591	113.516	137.777
B'phi C2	51.7	0.163	13.5	95.688	110.457	106.895	103.407	98.565
B'phi C3	57.1	0.197	16.5	110.263	113.357	107.607	117.894	105.717
WT T1	103.1	0.349	23.2	36.273	35.155	25.207	35.443	39.03
WT T2	61.7	0.35	20.6	35.513	33.951	35.066	32.852	31.683
WT T3	79.9	0.368	19.6	33.298	37.18	32.451	29.859	30.144
B'phi T1	48.1	0.238	20.3	28.893	31.347	30.794	33.63	34.445
B'phi T2	53	0.233	21.4	29.426	28.72	30.894	27	35.949
B'phi T3	59.7	0.316	19.3	40.409	34.246	27.267	32.806	37.767

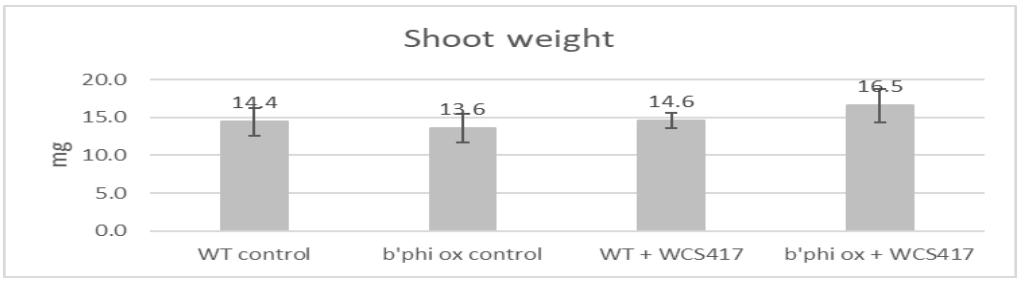
FIGURE 24: First B'PHI 16 Bacteria/chlorophyll experiment.

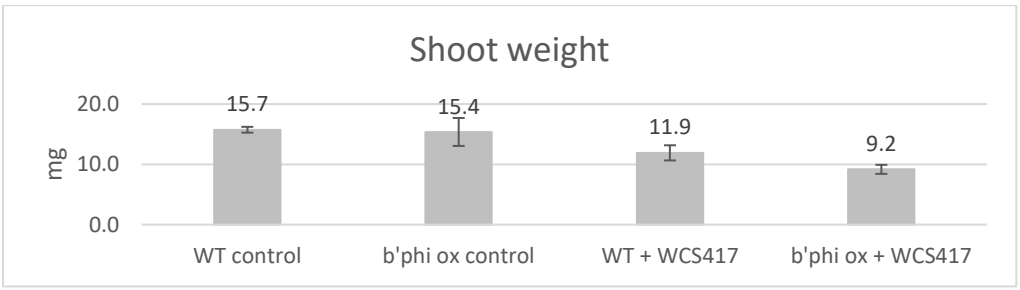
Table 22: Data for Harvested Treated and Untreated Wild Type and Mutants of *A. thaliana* inoculated with Pseudomonas WCS 417 on LB agar medium. (1st B'Phi 16 experiment).

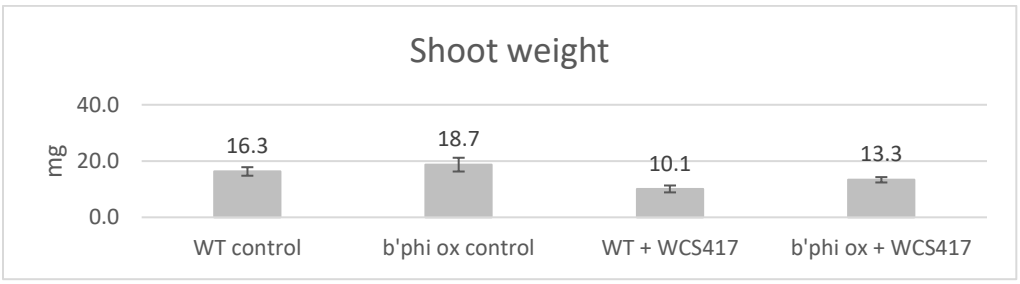
Sample ID	Shoot Weight	Absorbance	Root Weight	Root Length				
				Plant1	Plant2	Plant3	Plant4	Plant5
WT C1	68.3	0.163	18	101.186	103.571	57.827	121.772	70.087
WT C2	81.6	0.323	27.7	97.22	99.635	106.138	103.54	99.669
WT C3	94.6	0.217	23.3	47.982	90.858	55.406	100.352	91.678
B'phi C1	92.3	0.32	22.7	98.41	96.9	103.968	100.985	0
B'phi C2	73	0.207	15.6	108.659	114.818	87.784	84.909	96.85
B'phi C3	74	0.318	23.2	101.771	100.539	104.611	105.507	0
WT T1	38.3	0.179	17.6	23.78	21.992	28.532	16.849	32.66
WT T2	54.9	0.138	11	26.661	25.52	24.111	29.417	27.1
WT T3	58.1	0.176	16.4	24.839	20.231	30.394	32.869	29.135
B'phi T1	68.1	0.257	18	33.403	32.877	35.361	27.06	30.797
B'phi T2	57.8	0.313	22.8	37.623	32.071	32.596	25.348	34.793
B'phi T3	74.1	0.265	27.2	35.771	33.49	32.975	25.847	27.05

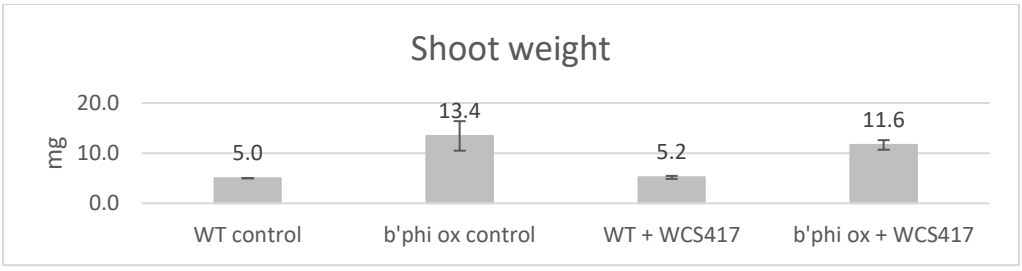
In the first three experiments in Shoot weight (1-3rd B'phi 13 graphs) for Wild type control and B'phi control, the Wild type control fared better than the B'phi 13 control plants. In the last two experiments, (1st and 2nd B'phi 16 graphs), the B'phi 16 control fared better than the WT control plants.





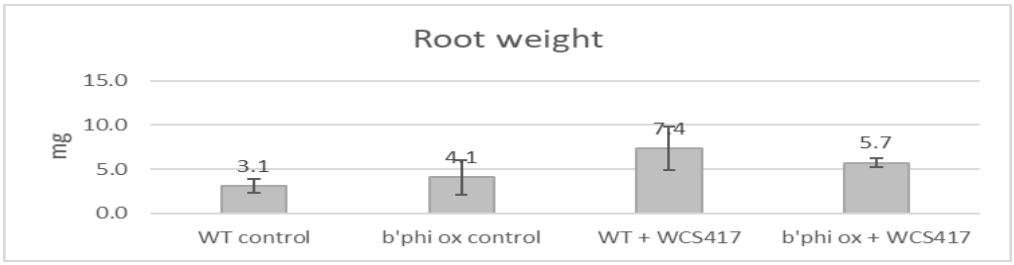


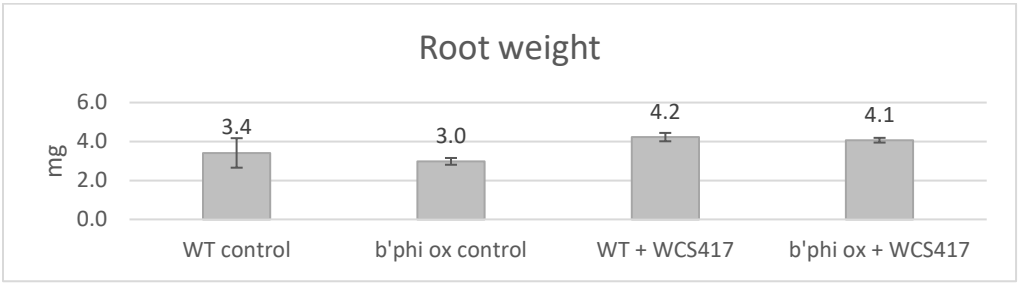


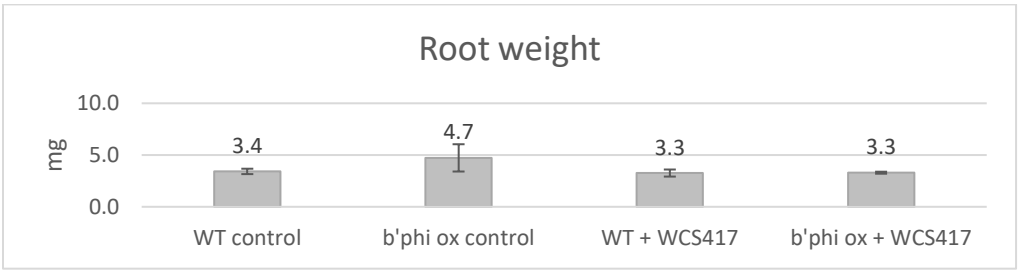


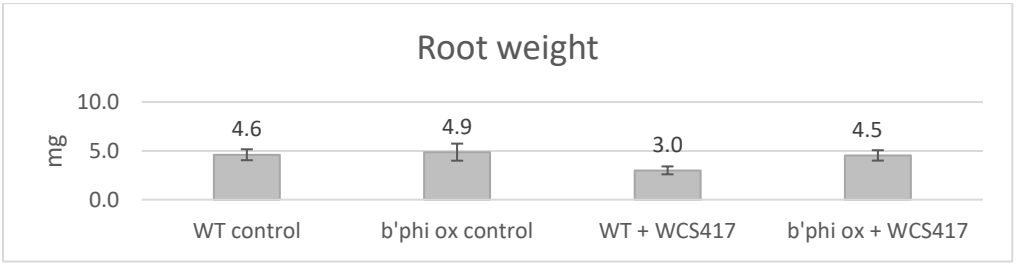
FIGURES A-E: Shoot Weight for B'phi 13(1), B'phi 13(2), B'phi 13(3), B'phi 16 (1), B'phi 16 (2)

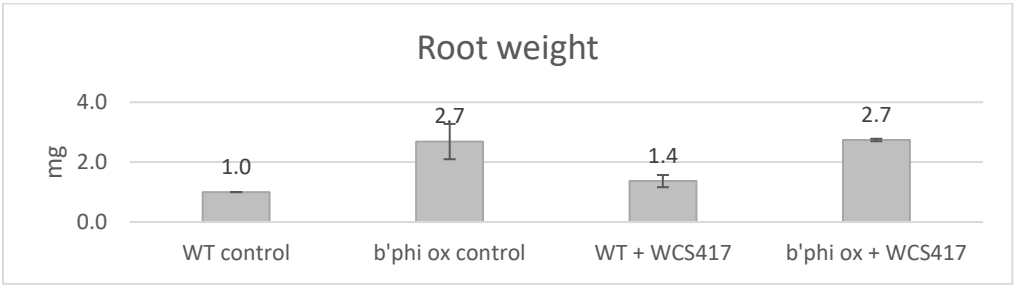
Comparing Wild type control and B'phi control plants in Root weight experiment, it is observed that B'phi control had bigger root weights than the WT control plants (1st and 3rd B'phi 13 graphs) except for a minor difference in the second graph representing B'phi 13 experiment (2nd graph) where the wild type had bigger root weight, this could also be due to experimental error like leaves drying out a bit before being measured . For Wild type-*PseudomonasWCS417(WCS417)* B'phi-*WCS417* the results are not constant, in the first graph (1st B'phi 13 experiment) and second graph (2nd B'phi 13 experiment) the WT-*WCS417* had bigger root weight then they were the same in the third graph (3rd B'phi 13 experiment) and in the last two graphs (1st B'phi 16 and 2nd B'phi 16 experiments), the B'phi-*WCS417* had bigger root weight than the WT-*WCS417*.





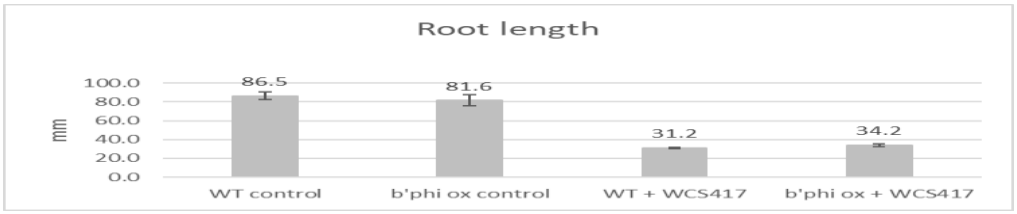


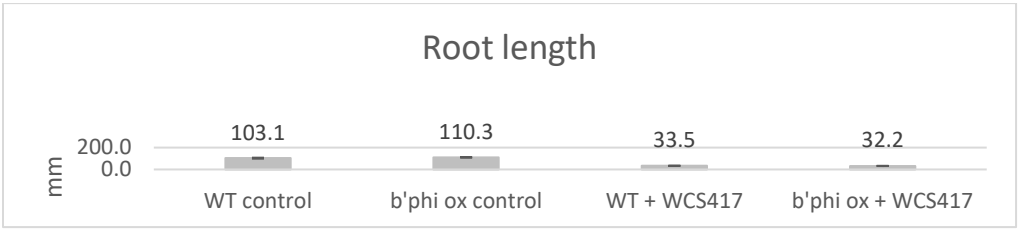


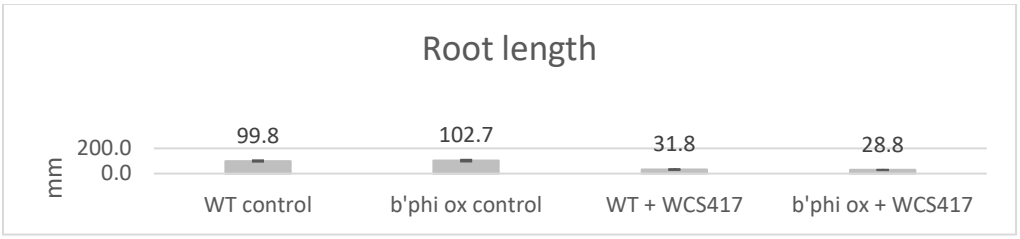


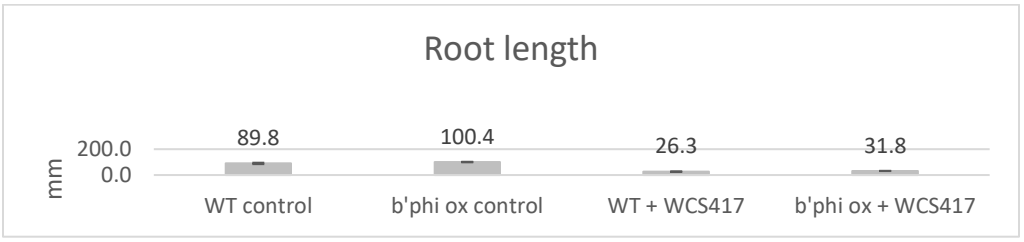
FIGURES A-E: Root Weight B'phi 13(1), B'phi 13(2), B'phi 13(3), B'phi 16 (1), B'phi 16 (2)

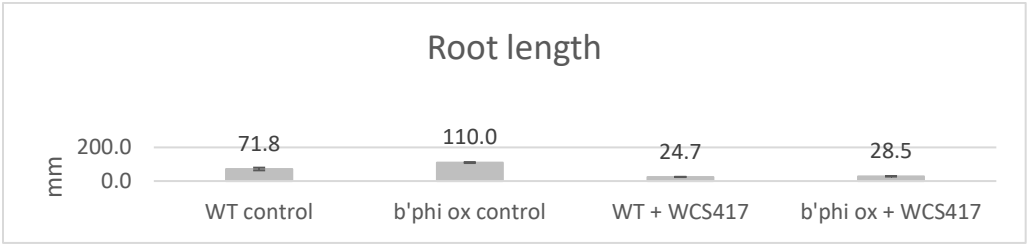
The Root length of the Control plants treatment both Wild type and Mutant plants, showed that the control plants treatment in both Wild type and Mutant plants were longer than that of the Mutant plants and this was observed in all five experiments. In the Wild type and Mutant plants with *Pseudomonas WCS 417*, *Pseudomonas WCS 417* caused alteration of the root structure. *Pseudomonas* reduced primary root length and increased the of thickness of the lateral roots.





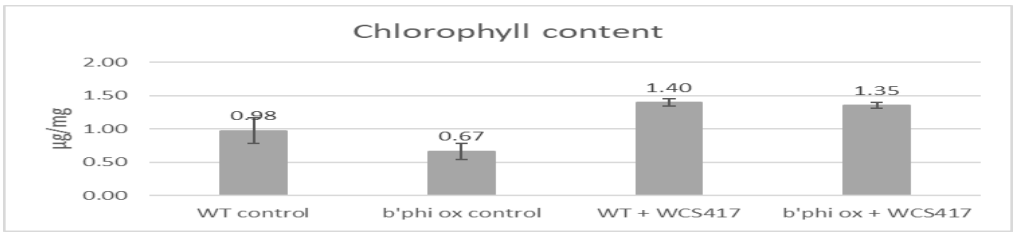


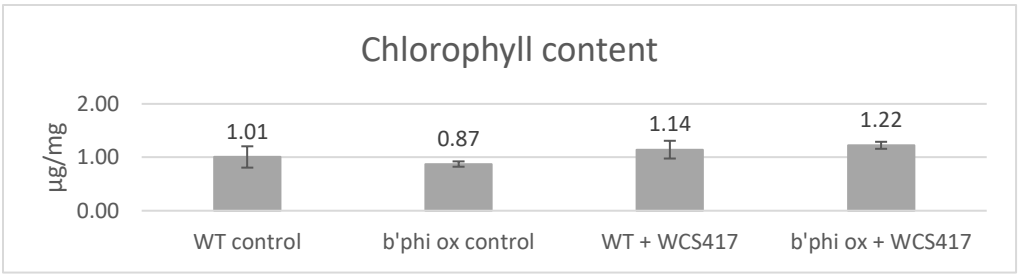


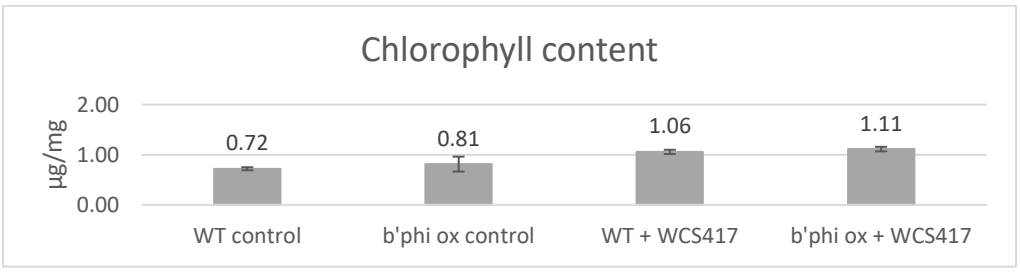


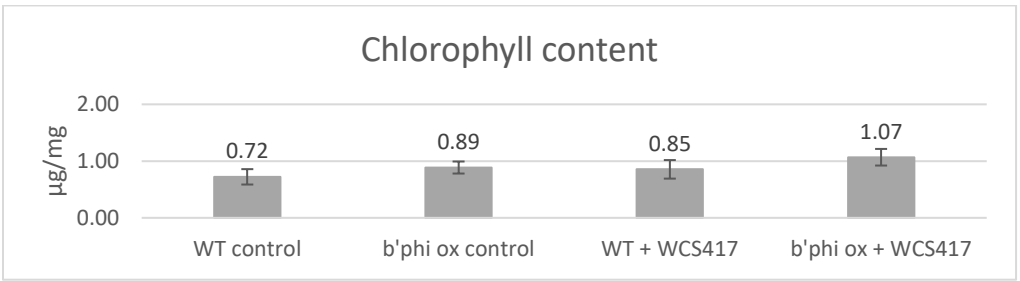
FIGURESA-E: Root Length B'phi 13(1), B'phi 13(2), B'phi 13(3), B'phi 16 (1), B'phi 16(2)

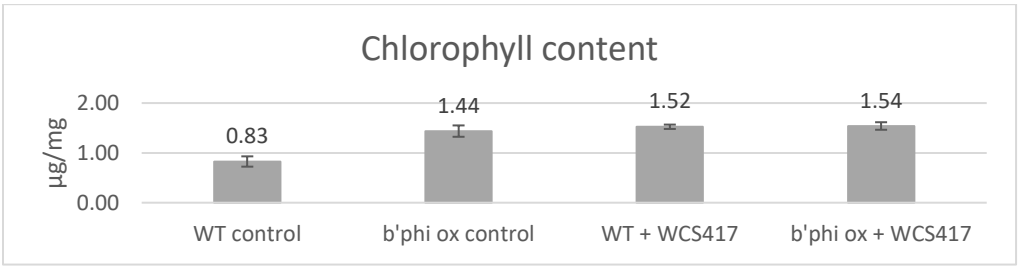
For chlorophyll content this parameter was constant for Wild type and Mutant Plants, the B'phi 13 and 16 plant had higher chlorophyll content than the Wild type plants in Both control plants and plants treated with *Pseudomonas*.











FIGURES A-E: Chlorophyll B'phi 13(1), B'phi 13(2), B'phi 13(3), B'phi 16 (1), B'phi 16 (2)

In all 5 sets of experiment, it was observed that Root length and Chlorophyll content were the constant variables throughout the experiment.

3.5 SALT STRESS for B'phi13 and 16 Wild Type *A. thaliana* Plants.

Plants exposed to high salinity develop severe growth defects and typical symptoms such as chlorosis, necrosis, accelerated senescence, reduced growth, and succulent leaves (Hanaoka, Noda et al. 2002).The main reasons therefore are that high salinity induces osmotic stress in plants due to the reduction of the water potential of the soil and the excessive uptake and accumulation of ions such as Na^+ and Cl^- , which interfere negatively with plant metabolism. Osmotic stress induced by high salinity induces the closure of stomata which decreases internal CO_2 concentrations. This situation will decrease the ability of the Calvin cycle to reduce CO_2 and will lead to the exhaustion of the primary electron acceptor NADP and to the block of the electron transport to NADP in illuminated chloroplasts. Subsequently electrons will be transferred to O_2 causing the formation of Reactive Oxygen Species (ROS) in chloroplasts (Chen, Yin et al. 2012). High O_2 and low CO_2 levels induced by salt stress will also favor photorespiration which leads to the formation of hydrogen peroxide ($H2O_2$) in peroxisomes (Abogadallah, Serag et al. 2010). Thus, depending on the severity and duration of the salt stress large amounts of ROS can accumulate inside the plant and if not detoxified will lead to oxidative stress in plants during drought stress (Cruz de Carvalho 2008). ROS oxidize membrane components and proteins and lead to the degradation of nucleic acids, lipids, pigments, membranes, proteins, RNA, and DNA, causing mutation and eventually cell death (Kong and Lin 2010).

In the experiment of Compartment Specific Changes of the Antioxidative Status in *A. thaliana* during Salt Stress by (Koffler, Luschin-Ebengreuth et al. 2014). First visible symptoms of salt stress could be observed when plants were exposed to salt stress for 6 days. At this point all plants had developed chlorosis. Additionally, both mutants showed necrosis on some leaves. Twelve days after the beginning of salt treatment all plants showed chlorosis, necrosis on some leaves, and reduced growth. Additionally, leaves appeared smaller, curled, and thicker, biomass was strongly decreased by salt stress (Koffler, Luschin-Ebengreuth et al. 2014).

The Salt Stress Experiment was done in 4 sets of (two B'phi 13 experiment and two B'phi 16 experiments). The experiments were repeated to check for reproducibility and to compare the results. The parameters analysed here are Shoot weight Root weight, Root length and Chlorophyll.

Here, 15 petri dishes were prepared after transferring one-week seedlings from 1% MS solution that have been stratified and kept under continuous light 16 hrs./8 hrs. for 12 days. 5M NaCl was used at five different salt concentrations, in 0.5%, ½ MS solution with pH 5.8. The concentrations are 0 mM, 50 mM, 100 mM, 150 mM, 200 mM.

For this experiment, 3 plates were prepared for each concentration with 3 Wild type plants and 3 Mutant plants in each petri dish, for better comparison the WT and Mutant plants must be grown in the same petri dish (Weigel and Glazebrook 2002).The 0 mM concentration treatment set was the control for this experiment.

Only, the first three concentrations were observed, 0 mM, 50mM and 100 mM. This was done so because at 150 mM and 200 mM, most of the plants both Wild type and B'phi plants had withered and developed chlorosis like in the experiment of (Koffler, Luschin-Ebengreuth et al. 2014). with either very poor survival response or they didn't survive at all, making it difficult to analyse their shoot weight, root weight, root length and chlorophyll. Chlorosis is a condition in which leaves produce insufficient chlorophyll, chlorotic leaves are pale, yellow, or yellow-white.

Salt Stress Pictures for B'phi13 and 16 Wild Type *A. thaliana* Plants.

Only two pictures and tables of the experiments of *A. thaliana* Salt Stress are represented her one from the B'phi 13 group and the other from the B'phi 16 group. Please refer to the appendix for the pictures and tables of the other two experiments.

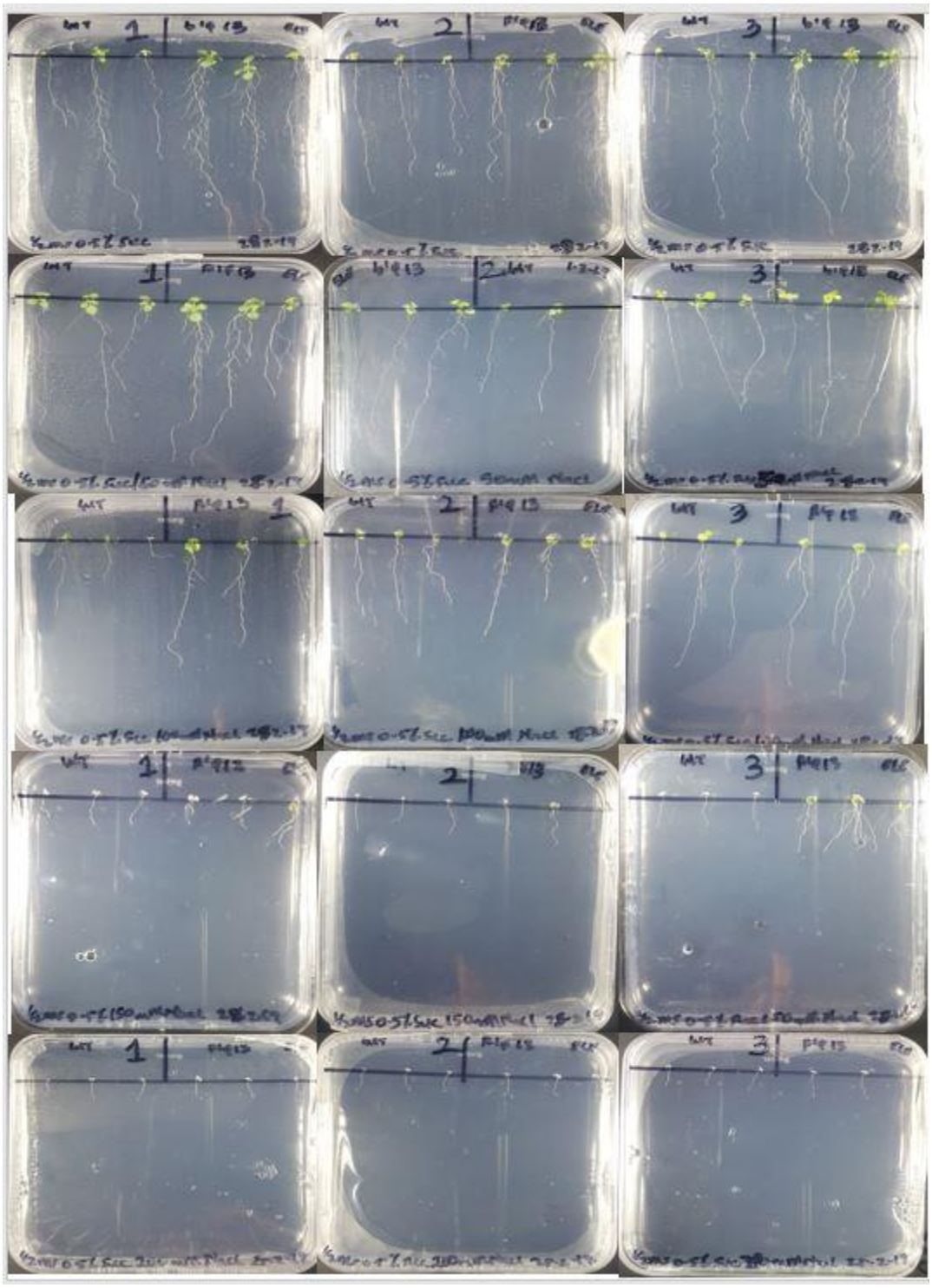


FIGURE 25: First B'PHI 13 Salt Stress-chlorophyll experiment.

Table 23: Data for Harvested Salt Stressed Treated and Untreated Wild Type and Mutants of *A. Tthaliana*. (First B'Phi 13 experiment).

Sample ID (0mM)	**Shoot Weight**	**Absorbance**	**Root Weight**	**Root Length**		
WT C1	9.9	0.088	2.2	40	69	35
WT C2	8.6	0.035	2.1	96	57	86
WT C3	10.2	0.076	0.9	55	48	29
B'phi 1	25.2	0.426	7.6	103	68	93
B'phi 2	29.3	0.167	4.7	92	42	68
B'phi 3	32	0.284	7.8	89	87	93
Sample ID (50mM)						
WT C1	22.1	0.346	3.1	64	30	48
WT C2	17.4	0.176	1.5	71	31	58
WT C3	23.1	0.140	2.1	50	27	59
B'phi 1	43.9	0.444	5.3	85	31	42
B'phi 2	49.5	0.533	4.9	90	31	74
B'phi 3	41.5	0.218	2.5	50	57	79
Sample ID (100Mm)						
WT C1	9	0.049	0.4	36	40	24
WT C2	13.3	0.095	1.9	20	43	64
WT C3	21.6	0.109	1.9	17	39	51
B'phi 1	25.7	0.331	3.6	60	46	70
B'phi 2	27.5	0.221	2.3	50	29	72
B'phi 3	32.1	0.177	5.2	33	31	57

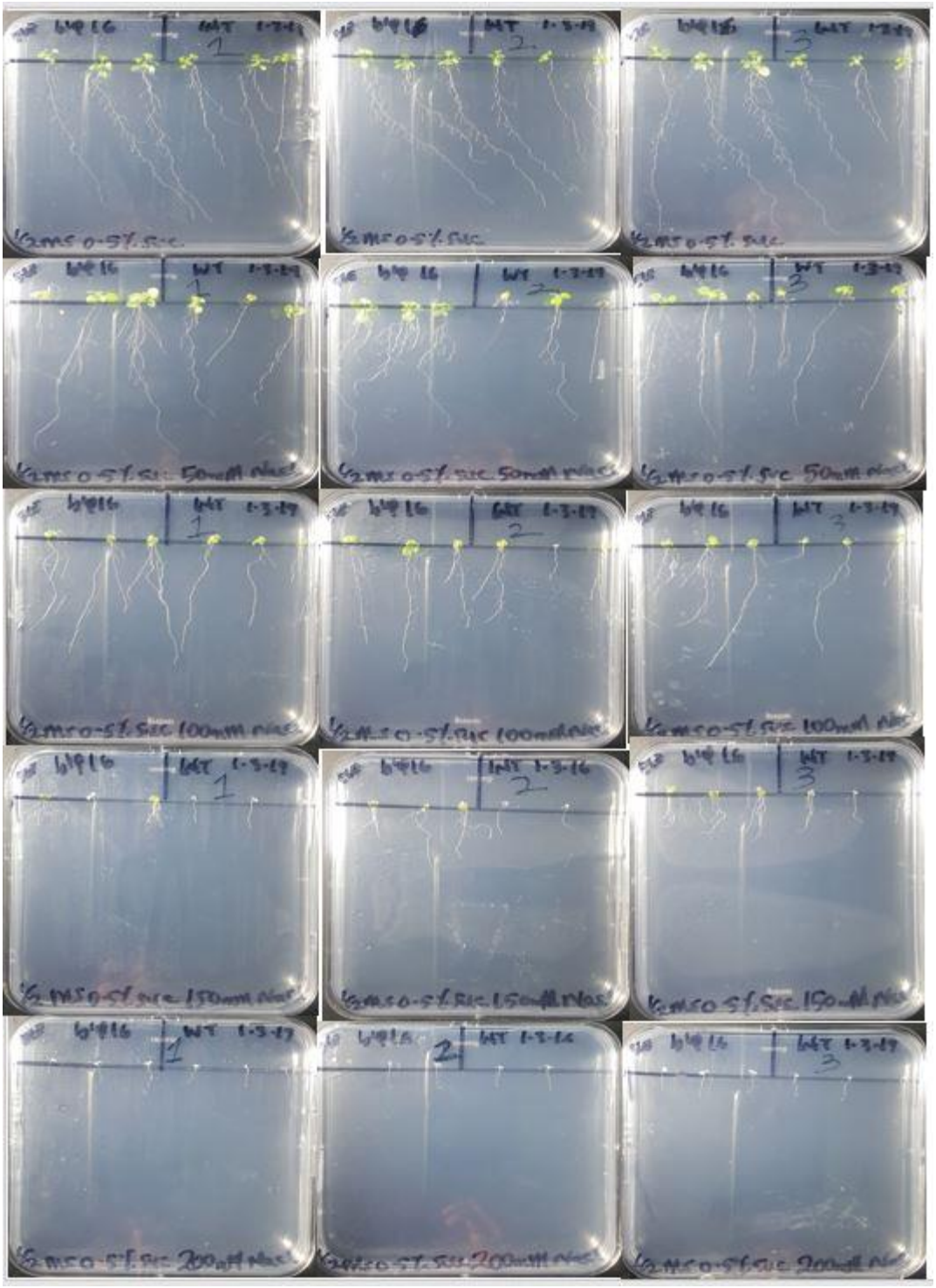
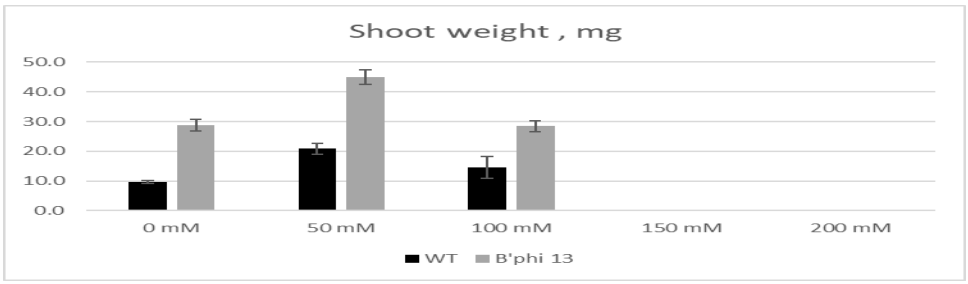


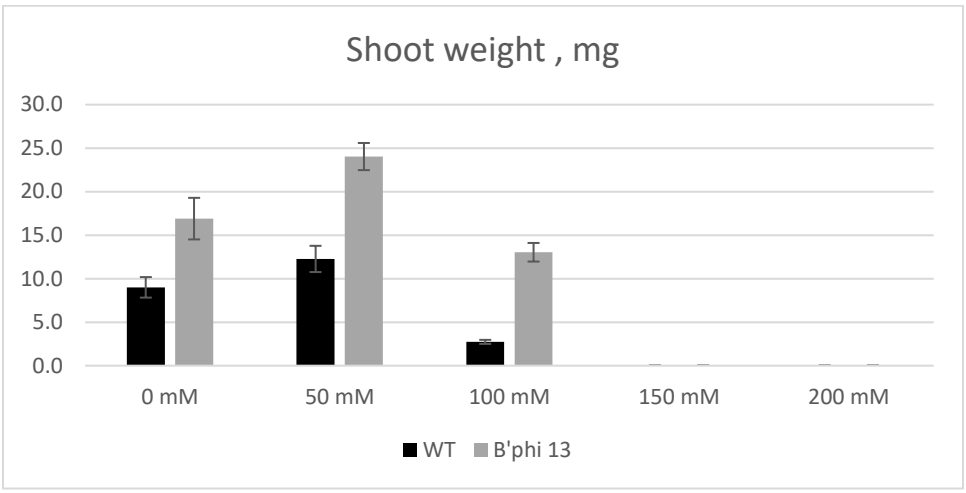
FIGURE 26: First B'PHI 16 Salt Stress-chlorophyll experiment.

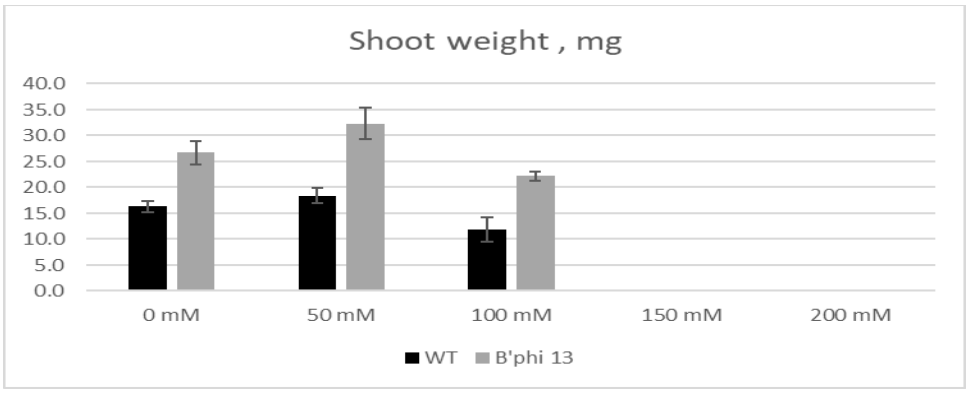
Table 24: Data for Harvested Salt Stressed Treated and Untreated Wild Type and Mutants of *A. thaliana*. (1st B'Phi 16 experiment).

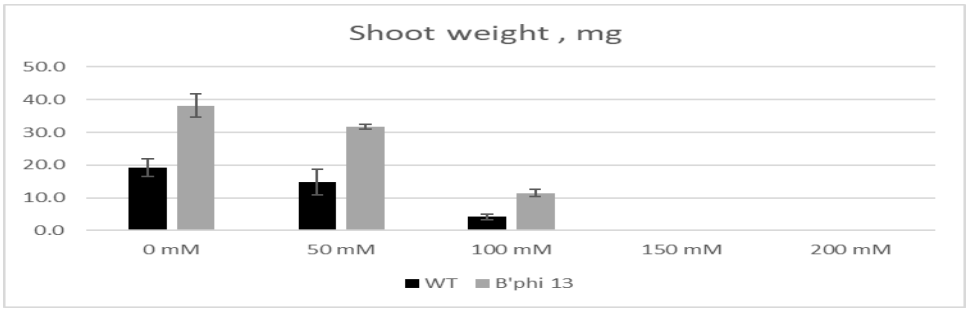
Sample ID (0mM)	Shoot Weight	Absorbance	Root Weight	Root Length		
WT C1	17.7	0.326	2.1	57	72	63
WT C2	17	0.318	2.1	65	56	42
WT C3	14.2	0.291	2.4	76	52	66
B'phi 1	22.3	0.724	3.1	79	93	90
B'phi 2	27.4	0.881	5.7	92	64	94
B'phi 3	30.3	0.725	5.6	84	88	96
Sample ID (50mM)						
WT C1	20.8	0.279	3	63	30	75
WT C2	15.7	0.289	1.4	40	67	66
WT C3	18.7	0.266	1	56	43	61
B'phi 1	34.7	0.614	5.8	28	84	61
B'phi 2	35.9	0.838	6.5	90	58	74
B'phi 3	26.2	0.552	3.4	87	71	43
Sample ID (100Mm)						
WT C1	16.1	0.113	2.3	54	42	17
WT C2	11.1	0.056	1.6	42	19	57
WT C3	8.3	0.075	0.9	41	40	33
B'phi 1	20.6	0.228	4	50	54	46
B'phi 2	22.2	0.34	1.6	57	65	45
B'phi 3	23.7	0.328	3.6	59	44	64

For salt stress shoot weight plants, the result for shoot weight is constant in both B'phi 13 and 16 plants across the observed concentrations (0 mM, 50 mM and 100 mM) these mutant plants had bigger shoot weights than the Wild type plants.



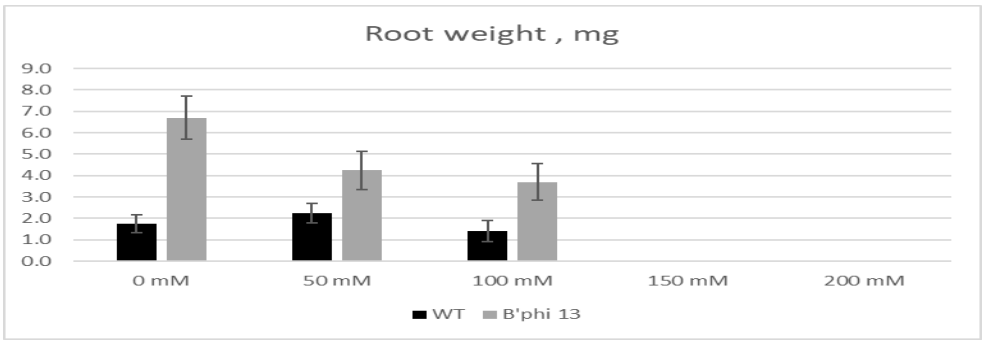


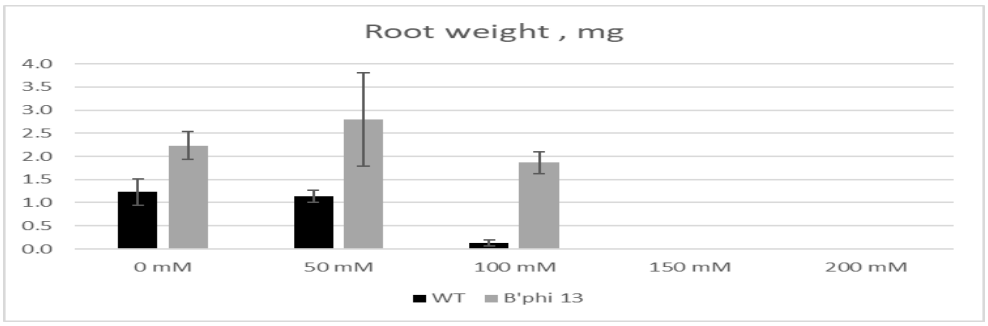


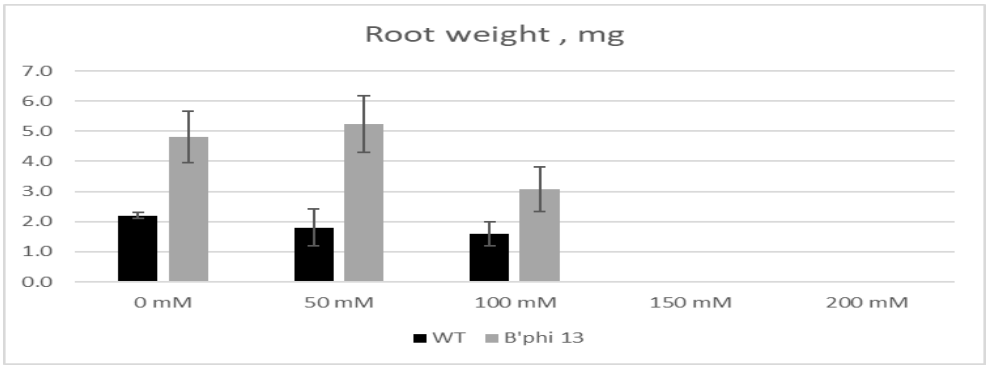


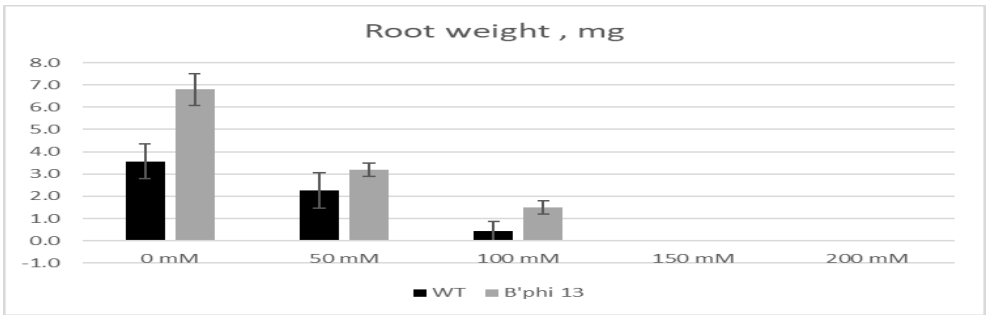
FIGURES A-E: Shoot Weight B'phi 13(1), B'phi 13(2), B'phi 13(3), B'phi 16 (1).

Root weight just like shoot weights were constant for both B'phi 13 and 16 plants and Wild type plants across the observed concentrations. The root weights for the B'phi 13 and 16 plants where bigger than the Wild type plants root weights.



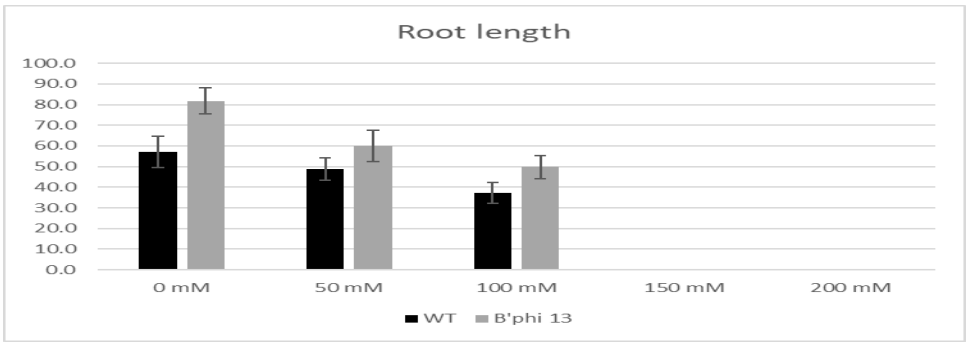


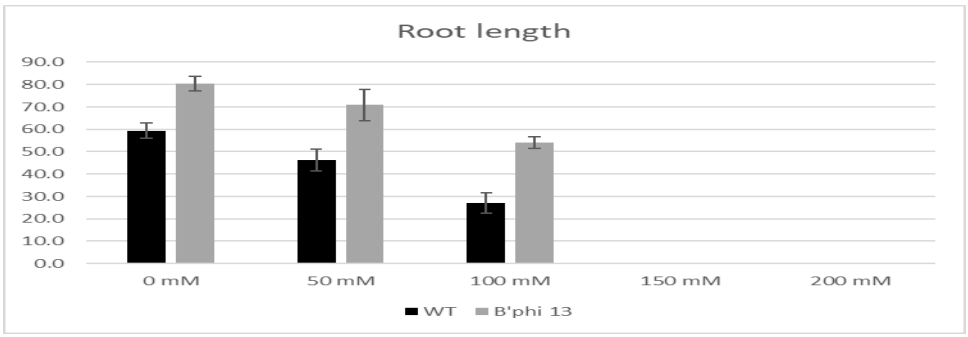


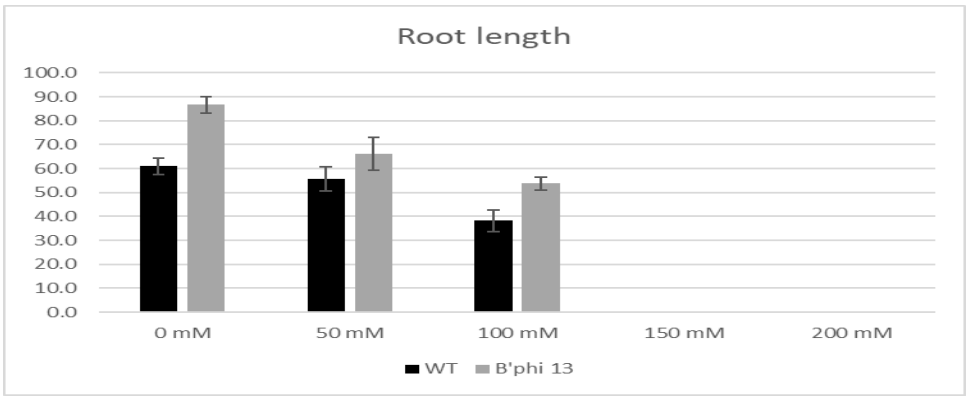


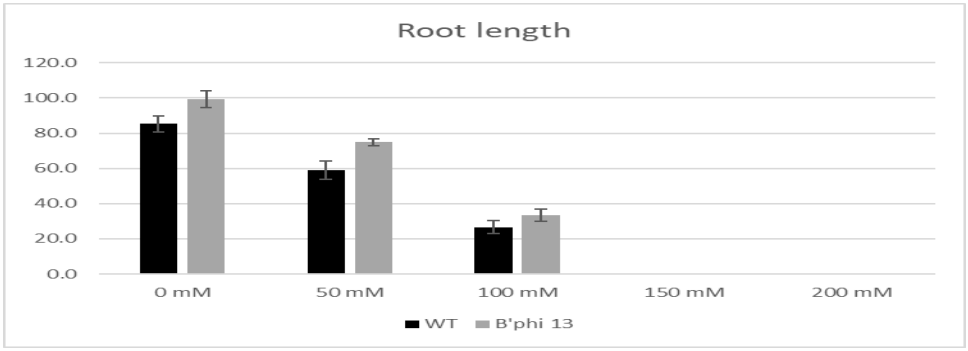
FIGURES A-E: Root Weight B'phi 13(1), B'phi 13(2), B'phi 13(3), B'phi 16 (1).

Root length is also constant for both sets of plants. The B'phi 13 and 16 Mutant plants had longer root lengths than the wild type plants across the observed concentrations.



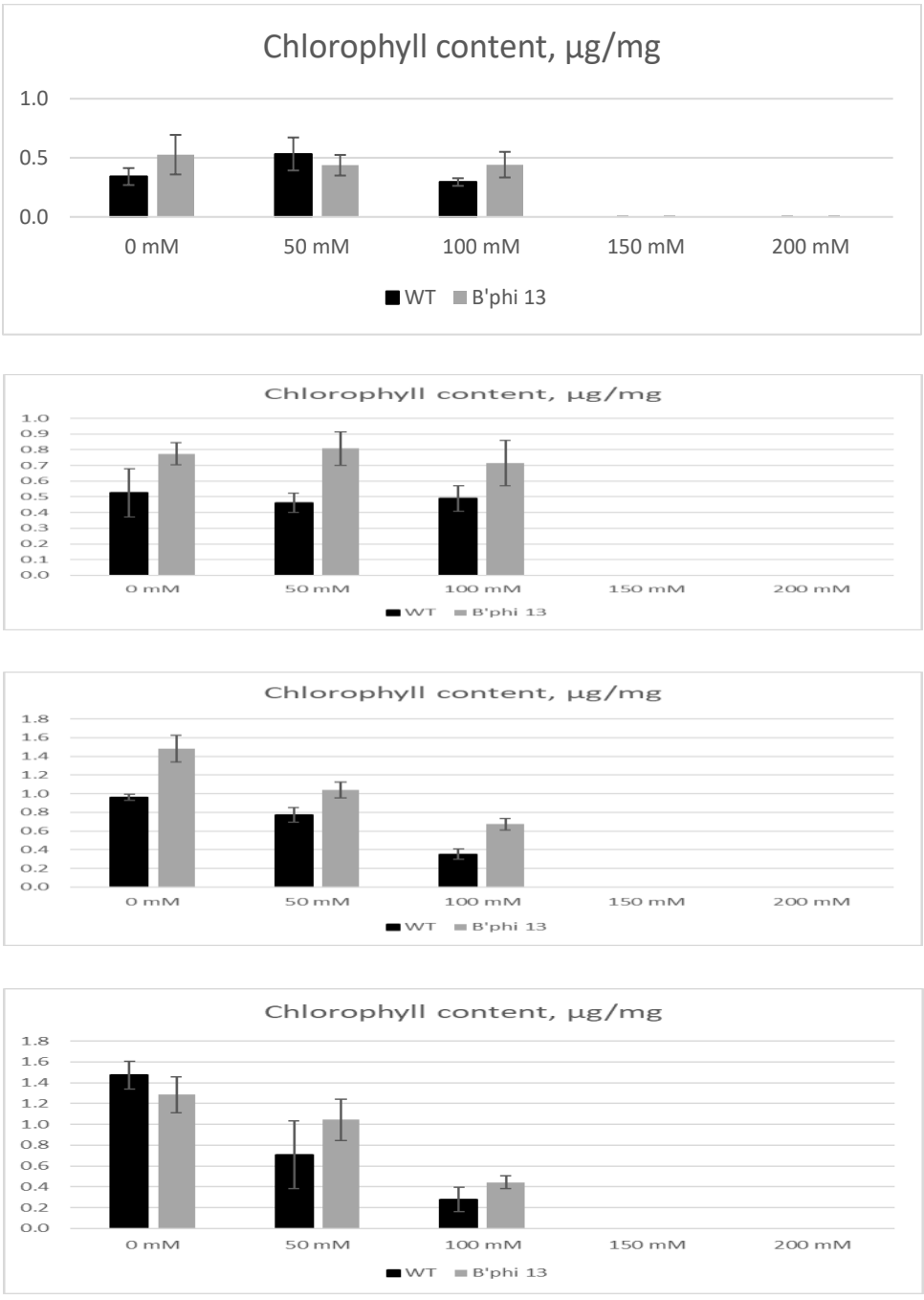






FIGURES A-E: Root Length B'phi 13(1), B'phi 13(2), B'phi 13(3), B'phi 16 (1), B'phi 16 (2).

In the chlorophyll assay analysis, The B'phi 13 and 16 plants had higher chlorophyll content than the Wild type plants except in two cases where B'phi 13 (1) and 16 (2) (1st and last graph under chlorophyll graphs for salt stress) had lower chlorophyll content at concentrations 50 mM and 0 mM respectively, this might have occurred during transplanting of 1 week old seedlings to the salt medium (random selection of not very healthy seedlings).



FIGURES A-E: Chlorophyll Content B'phi 13(1), B'phi 13(2), B'phi 16 (1), B'phi 16 (2).

After all the sets of data was gathered for all 5 sets of salt experiments, it was observed that for Shoot weight, Root weight, Root length that the B'phi 13 and16 plants had heavier Shoot weight, heavier Root weight and Longer Root length (in the three different concentrations observed) than the Wild type plants. So, generally the B'phi plants fared better than the Wild type plants in these parameters.

3.6 POLYMERASE CHAIN REACTION EXPERIMENT (PCR).

Strong constitutive promoters can deliver a high-level expression of transgenes to almost all tissues and development stages in plants, which is particularly useful for the expression of herbicide tolerance, insect resistance and selectable marker genes (Jiang, Zhang et al. 2018). The constitutive promoter in this thesis work is 35S CaMV.

The B'phi gene was fused with the 35S CaMV promoter for constitutive expression, but expression needs to be checked. First, ten plants from the first *A. thaliana* B'phi 13 seeds (50) sown were tested for B'phi expression. Also, the 2nd set of B'phi 13 seeds (50) and 50 WT sown were tested for B'phi gene expression.

Two sets of experiments were carried for Genotyping. This is because two sets of *A. thaliana* B'phi 13 mutant plants were sowed in soil and had to be tested for the presence of gene of insert.

3.6.1 1st Genotyping Experiment.

In the first genotyping experiment, two experiments were because there was contamination in the Wild type 34 sample the first time the experiment was done. Another experiment had to be done to check Wild type 34 to make sure that the expected result was gotten and ascertain if it was during the experiment or the sample itself was contaminated.

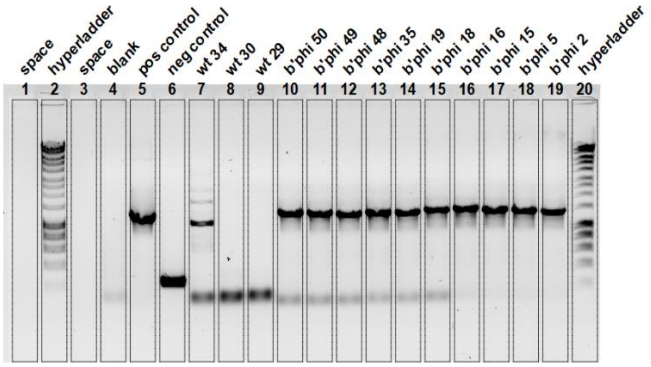


FIGURE 27: PCR RESULT FOR FIRST EXPERIMENT.

For the first experiment, there are 20 wells on the gel. The first well is empty, the second well has the first loaded hyper ladder. The wild type plant samples on the gel are in wells 7 to 9 in this order WT 34, 30, 29. The B'phi plant samples are in wells 10 to 19 in the following order B'phi 50, 49, 48, 35, 19, 18, 16, 15, 5, 2. Finally, in well 20 another hyper ladder sample.

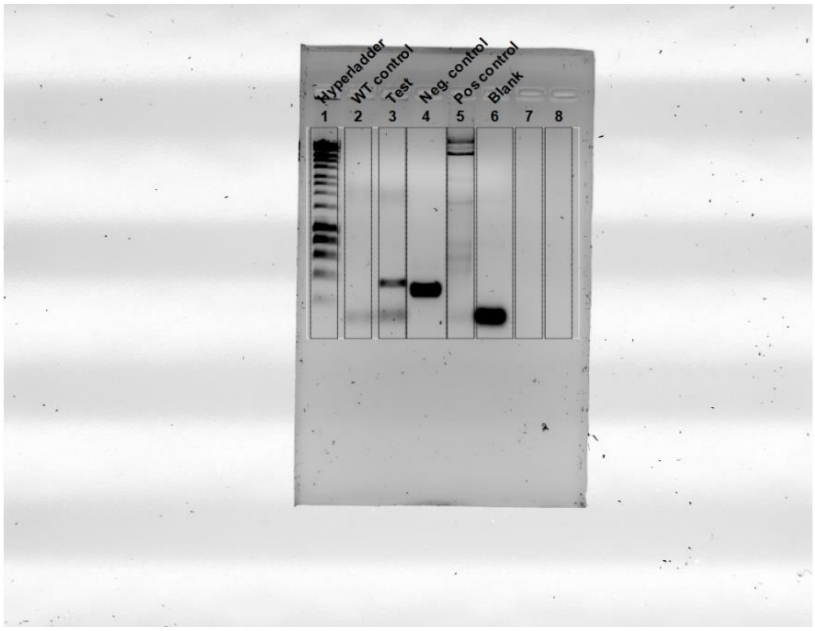


FIGURE 28: PCR RESULT FOR THE SECOND EXPERIMENT.

For the second experiment, there are 8 wells, the first well is the hyper-ladder, the second is the Wild Type 34 re-tested again because of contamination in the firs experiment. The third well (TEST) is a different test not related to this experiment, the fourth well is the negative control, the fifth is positive control and the sixth well is the blank. The last two wells are empty.

3.6.2 SECOND GENOTYPING

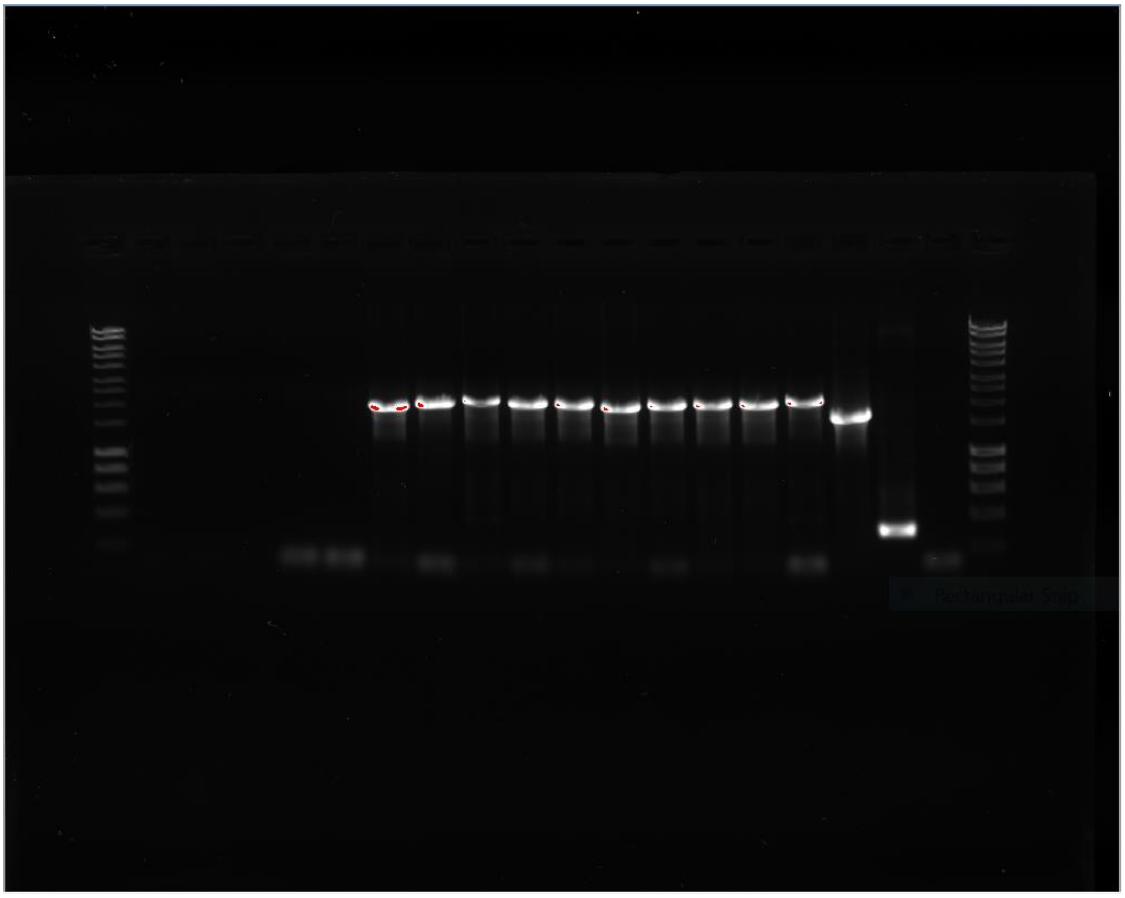


FIGURE 29: Original picture of gel for PCR experiment checking for gene of insert (b'phi) in 10 transformed and five wild type *A. thaliana* plant (Genotyping).

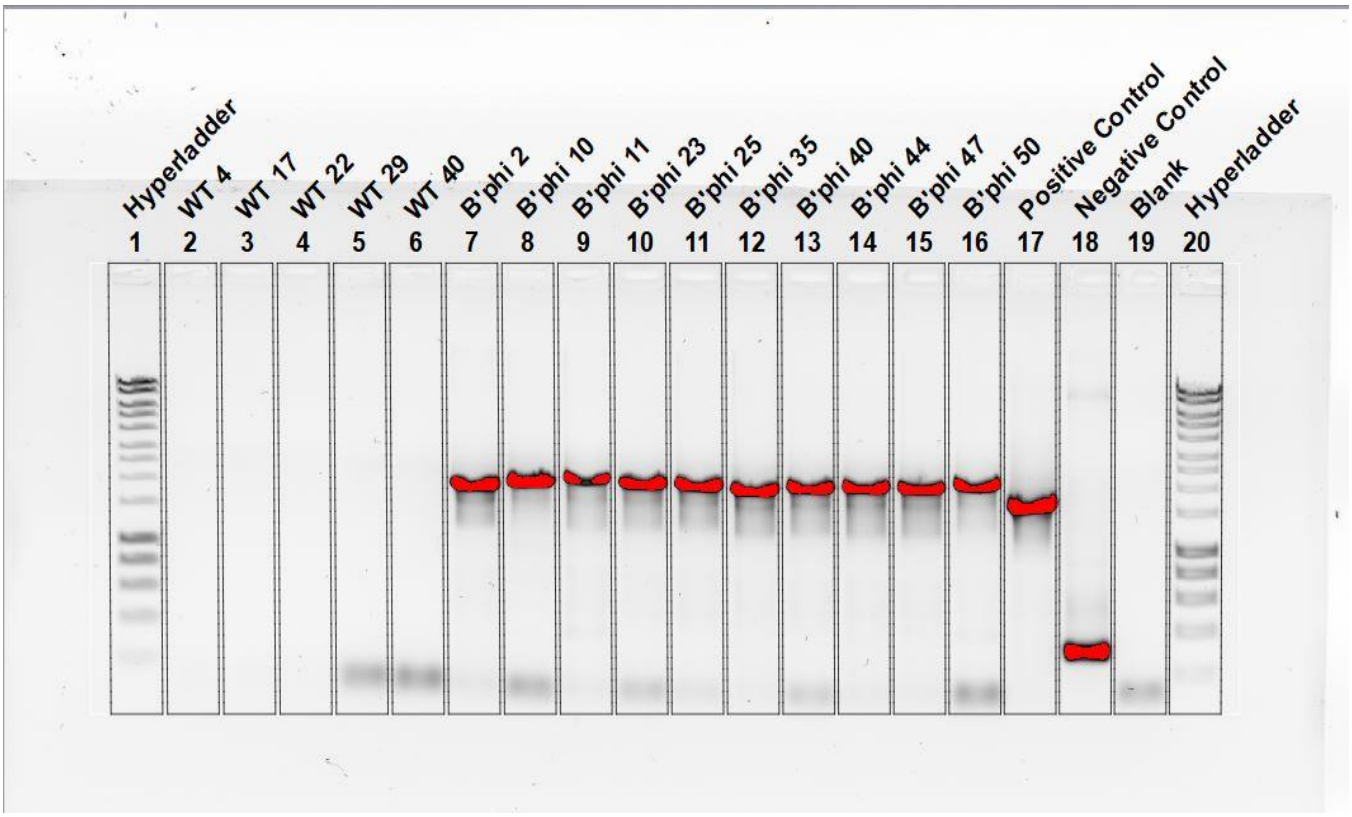


FIGURE 30: PCR experiment checking for gene of insert (B'Phi) in 10 transformed and five wild type *A. thaliana* plant (Genotyping).

There are 20 wells on the gel. The first well is empty, the first well has the first loaded **hyper ladder**. The wild type plant samples on the gel are in wells 2 to 7 in this order **WT 4,17,22,29,40**. The B'phi plant samples are in wells 8 to 16 in the following order **B'Phi 10, 11, 23, 25, 35, 40,44, 47,50.** In well 17 is the **positive control,** well 18 **is negative control,** well 19 is the **blank** samples. Finally, in well 20 another **hyper ladder** sample.

For all the genotyping experiments, the **blank** was just master mix, the **negative control** is the empty vector pBA002. The **positive control** is the B'phi plasmid.

3.7 GENE EXPRESSION ANALYSIS.

Reverse transcription PCR, or RT-PCR, allows the use of RNA as a template. An additional step allows the detection and amplification of RNA. The RNA is reverse transcribed into complementary DNA (cDNA), using reverse transcriptase. The quality and purity of the RNA template is essential for the success of RT-PCR. The first step of RT-PCR is the synthesis of a DNA/RNA hybrid. Reverse transcriptase also has an RNase H function, which degrades the RNA portion of the hybrid. The single stranded DNA molecule is then completed by the DNA-dependent DNA polymerase activity of the reverse transcriptase into cDNA. The efficiency of the first-strand reaction can affect the amplification process. From here on, the standard PCR procedure is used to amplify the cDNA. The possibility to revert RNA into cDNA by RT-PCR has many advantages. RNA is single-stranded and very unstable, which makes it difficult to work with. Most commonly, it serves as a first step in qPCR, which quantifies RNA transcripts in a biological sample.

Five plant samples of *A. thaliana* were used (50 mg each), two Wild type (WT 4 and WT 17) and three Mutant B'phi 13 transgenic plants (B'phi 44, 47 and 50). Genotyping for these plants was done prior to check gene of insert prior to this experiment and the result was positive for the gene.

Time of Harvesting: 11:15

Date of Harvesting: 11th January 2019.

Table 25: Weight of harvested five samples.

Name of Samples	Weight of empty tubes without cover.	Expected weight of tube + 50 mg.	Real weight + tube
WT 4	1.2193	1.2693	1.2701
WT 17	1.1637	1.2137	1.2104
B'phi 44	1.2021	1.2581	1.2478
B'phi 47	1.1879	1.2378	1.2430
B'phi 50	1.2127	1.2627	1.2590

Isolation and purification of these plants' samples RNA was done, and their quality checked on Nanodrop. The concentration of all the RNA samples was good. The quality of all other RNA was good (above 1.7 – requirement for better results when sequencing by Microsynth SEQLAB) except for B'phi 44 and B'phi 47 with quality of 0.81 and 0.62 respectively, these might be as a result of ethanol interference that might still be left in the sample after the experiment of RNA isolation and purification.

Table 26: RNA Quality and Concentration by Nanodrop.

Samples	**ng/ml**	**A260/280**	**A260/230**
WT4	925.9	2.17	2.00
WT 17	605.1	2.15	1.85
B'phi 44	290.9	2.13	0.81
B'phi 47	379.3	2.16	0.62
B'phi 50	299.4	2.13	2.28

These samples (RNA) were diluted to a concentration of 1µg/10 µl (100 ng/ µl) prior to cDNA experiment.

Reverse transcription protocol for SuperScript™ IV VILO™ Master Mix (without ezDNase enzyme treatment).

1.**Prepare RT and No RT**
Control reaction mixes
(on ice)

The following components were added to an empty RNase-free tube on ice.

Table 27: Components of RT and No RT mixtures.

Component	RT reaction	No RT Control reaction
SuperScript™ IV VILO™ Master Mix	2 µl	-
SuperScript™ IV VILO™ No RT Control	-	4 µl
Template RNA (1 pg to 2.5 µg total RNA)	varies	varies
Nuclease-free Water	To 10 µl	To 10 µl

These RNA samples were diluted 1:1 prior to cDNA synthesis experiment. In the course of cDNA, two reaction mixes were made using the kit Reverse transcription protocol for SuperScript™ IV VILO™ Master Mix (without ezDNase enzyme treatment). The reaction mixes are Reverse Transcriptase (RT) reaction mixture and No Reverse Transcriptase (NRT) Reaction mixture. This just means the Reverse Transcriptase Reaction mixture has the enzyme Reverse Transcriptase n the mixture while the other No Reverse Transcriptase Reaction Mixture has no reverse Transcriptase in the mixture therefore, the Reverse Transcriptase enzyme is absent, and this mixture serves as the control.

Table 28: Reverse Transcriptase Reaction.

Sample	Concentration	Master Mix	RT Control	Total RNA	Nuclease free water
WT 4	925.9	2	-	1.1	6.9
WT 17	605.1	2	-	1.7	6.3
B'phi 44	290.9	2	-	3.4	4.6
B'phi 47	379.3	2	-	2.6	5.4
B'phi 50	299.4	2	-	3.3	4.7

These samples (cDNA) were diluted to a concentration of 50 μg/ul 1:1 by nuclease free water prior to quantitative PCR (RT-qPCR)

Table 29: No Reverse Transcriptase Reaction.

Sample	Concentration	Master Mix	RT Control	Total RNA	Nuclease free water
WT 4	925.9	-	2	1.1	6.9
WT 17	605.1	-	2	1.7	6.3
B'phi 44	290.9	-	2	3.4	4.6
B'phi 47	379.3	-	2	2.6	5.4
B'phi 50	299.4	-	2	3.3	4.7

Two primers were used - **Short reverse and Short forward primers** and the **Complete reverse and Short forward primers** for quantitative PCR (RT-qPCR).

The following samples were ran on the gel for each primer set: No Reverse Transcriptase WT 4, WT 17, B'phi 44, 47, 50, also for Reverse Transcriptase WT 4, 17, B'phi 44, 47, 50., then pure RNA samples RNA WT 4, 17, B'phi 44, 47, 50.

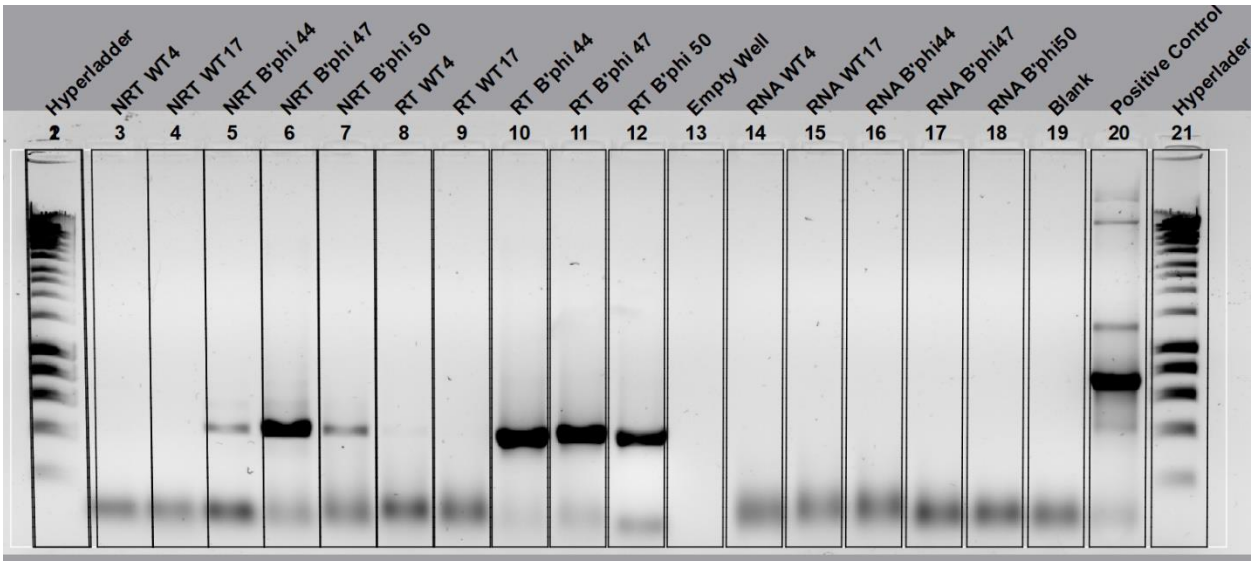


FIGURE 31: SHORT REVERSE SHORT FORWARD PRIMERS.

For the first primer, **Short reverse and Short forward Primers**, all the RNA samples, RNA WT 4, 17, B'phi 44, 47, 50, were clean and there was no contamination, which means during the RNA isolation and purification experiments all genomicDNA were removed. For the RT samples, RT WT 4 and 17 gels were clean and free of contamination and the expected product of 400 bp was gotten for the B'phi 44, 47, 50, when the band of the product was matched against the hyper ladder which is the guide to know if the expected product is what we obtain from our gel bands. Finally, for **NRT samples** WT 4, 17, B'phi 44, 47, 50, here, the WT 4 and 17 gels are clean and free of contamination and no bands is seen which is to be expected because there were Wild type samples with no gene of insert and the B'phi 47 has a thick band there is likely the presence of gDNA and the gels of b'phi 44 and 50 are clean with faint bands, the explanation for the faint bands here could be because the cycle was ran at the maximum number of cycles (40) and the primers must have picked up different products because of the long running time in the machine thereby giving bands on the gel. A minus Reverse Transcription control (-RT control i.e. no RT)) was included in this RT-qPCR experiment to test for contaminating DNA (such as genomic DNA or PCR product from a previous run). Such a control contains all the reaction components except for the reverse transcriptase. Reverse transcription should not occur in this control, so if PCR amplification is seen, it is most likely derived from contaminating DNA.

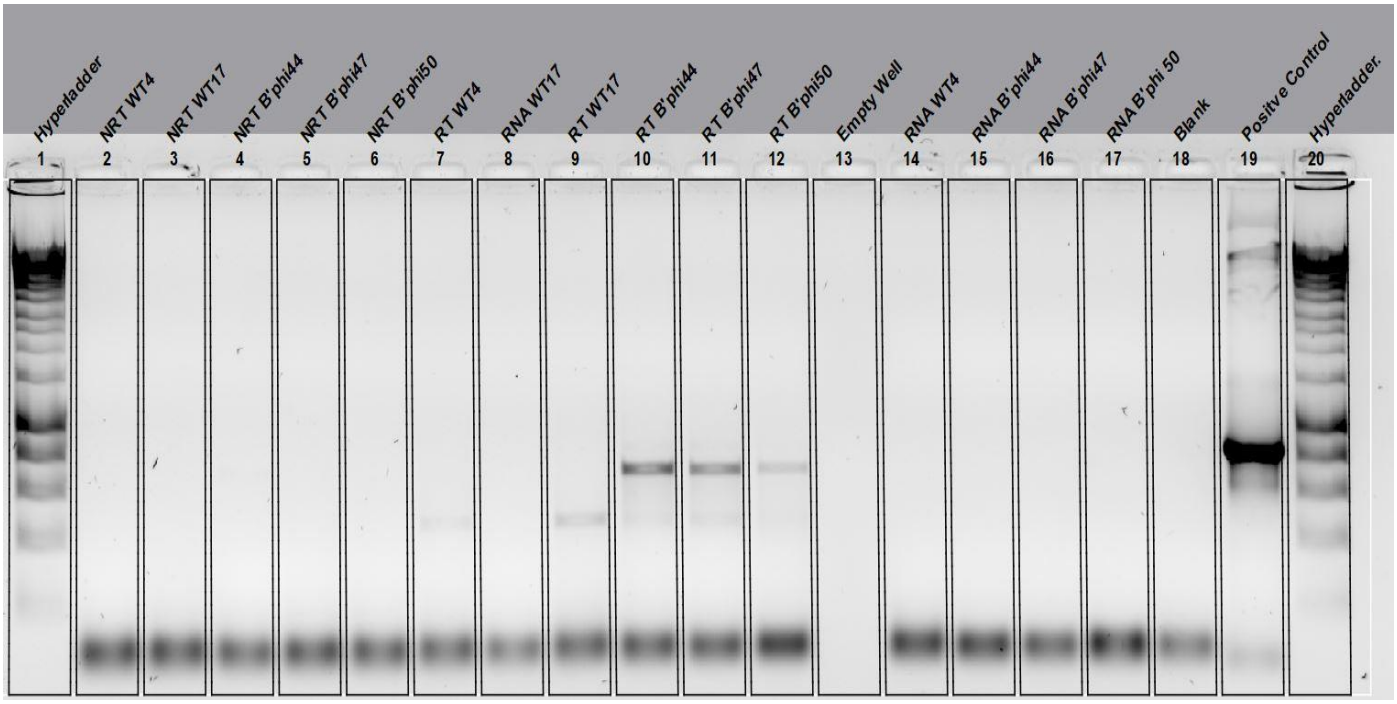


FIGURE 32: COMPLETE REVERSE AND COMPLETE FORWARD PRIMERS.

The second Primer, **Complete reverse and Short forward primers**, all the RNA samples, RNA WT 4, 17, B'phi 44, 47, 50, were clean and there was no contamination, which means during the RNA isolation and purification experiments all genomicDNA were removed. For the RT samples, RT WT 4 and 17 gels were clean and free of contamination also, they had no bands because they have no gene of insert and the expected product of 900 bp was gotten for the B'phi 44, 47, 50, when the band of the product was matched against the hyper ladder and our positive control which is the guide to know if the expected product is what we obtain from our gel bands. Finally, for NRT samples WT 4, 17, B'phi 44, 47, 50, here, the WT 4 and 17 gels are clean and free of contamination and no bands is seen in the NRT , B'phi 44, 47, 50 wells, this is good because for NRT samples which is devoid of Reverse Transcriptase, there should be no transcribing therefore no bands.

3.8 RESULTS FOR SEQUENCING.

DNA sequencing is the process of determining the sequence of nucleotide bases (As, Ts, Cs, and Gs) in a piece of DNA. Today, with the right equipment and materials, sequencing a short piece of DNA is relatively straightforward. Determining the order of nucleic acid residues in biological samples is an integral component of a wide variety of research applications. Over the last fifty years large numbers of researchers have applied themselves to the production of techniques and technologies to facilitate this feat, sequencing DNA and RNA molecules. This timescale has witnessed tremendous changes, moving from sequencing short oligonucleotides to millions of bases, from struggling towards the deduction of the coding sequence of a single gene to rapid and widely available whole genome sequencing. The order of nucleic acids in polynucleotide chains ultimately contains the information for the hereditary and biochemical properties of terrestrial life. Therefore, the ability to measure or infer such sequences is imperative to biological research (Heather and Chain 2016).

Sanger sequencing was used to determine the sequence of nucleotides of the B'phi gene, In **Sanger sequencing**, the target DNA is copied many times, making fragments of different lengths. Fluorescent "chain terminator" nucleotides mark the ends of the fragments and allow the sequence to be determined.

Another PCR was done using the primers **Short reverse and Short forward primers** and the **Complete reverse and Short forward primers.** Instead of the usual 10 µl PCR sample in each well, 50 µl of PCR sample was put in the well after running in the PCR machine. After electrophoresis, product band on the gel was cut for each primer under Ultraviolet light. The gels were placed in different tubes an purified using the GFX™ PCR DNA and Gel Band Purification Kit and the concentration of the PCR products were measured on Nanodrop. The concentration of the Short reverse and Short forward primers was higher than the Complete reverse and Short forward primers because there was little error of cutting out too much gel with the band of interest.

TABLE 30: Concentration of purified PCR products on gel for sequencing.

Their quality was not up to 1.7 ng/µl which is the recommended quality. After purification of the agarose gel, concentration of the PCR products was measured on nanodrop.

Samples	**Fragment size**	**ng/µl**	**A260/280**	**A260/230**
Complete forward/Short reverse	900 bp	32.1	1.92	0.17
Short forward/Short reverse.	400 bp	141.7	1.81	1.01

TABLE 31: Sample for sequencing concentration requirements by Microsynth SEQLAB.

Before samples were sent to Microsynth SEQLAB, Germany gave the following requirements for best sequencing results.

Template	**Concentration**
Plasmid	40-100 ng/ µl
PCR (purified and unpurified products)	General Rule: 1.5 ng/ µl
PCR 1000-2000 bp	30 ng/ µl
PCR 500-1000 bp	15 ng/ µl
PCR 250-500 bp	7.5 ng/ µl
PCR 100-250 bp	4 ng/ µl
Primer	**Concentration**
As separate tube (enclosed)	10µM
Premixed	2 µM

TABLE 32: Calculation of sample concentration requirement for sequencing.

Using the concentration of the samples from nanodrop, final concentration, volume of each sample to be sent plus the volume of primer, the volume needed to prepare the mix for the sample to be sent for sequencing was calculated.

Concentration requirements (Microsynth SEQLAB) for sample being sent for sequencing was prepared.

Fragment size	**Water**	**Sample**	**DNA**	**Primers (for)**	**Primers (rev)**
900 bp	5 µl	7 µl	**Short forward**	3 µl	-
900 bp	5 µl	7 µl	**Complete reverse**	-	3 µl
Fragment size	**Water**	**Sample**	**DNA**	**Primers (for)**	**Primers (rev)**
400 bp	11 µl	1 µl	**Short forward**	3 µl	-
400 bp	11 µl	1 µl	**Short reverse**	-	3 µl

4.DISCUSSION.

4.1 BASTA SELECTION OF B'PHI MUTANTS.

Genetic transformation is one of the most widely used technique in crop improvement. Plants can be transformed by using Agrobacterium-based T-DNA vectors or by direct uptake of DNA. However, for the selection of transformed cells or tissues, selectable marker genes are required (Nada 2016).

The use of herbicides to reduce loss in crop yield due to weeds has become an integral part of agriculture. It is important for humans and animals that herbicides on crops used are safe for them and highly effective on weeds. Basta, is one of the most effective herbicides that works by inhibiting specific amino acid biosynthesis pathways in plants. Basta, does not distinguish between weeds and crops, to protect plants from herbicides like Basta, these plants are modified to become resistant to Basta, allowing their selective use for crop protection (D'Halluin, De Block et al. 1992).

From the above analysis the transgenic seedlings fused with a gene resistant to Basta B'phi 13, B'phi 16 and 20, fared well when compared to other plants. The B'phi 13 fared best followed by B'phi 16 and then B'phi 20.

The Wild type plant with Basta's survival response was poor because it lacked the gene resistant to Basta hence it was easily and directly attacked by Basta leading to poor yield. The Wild type without Basta is the control treatment set of this experiment therefore, it grew normally and had a good survival rate compared to the Wild type treatment set with Basta but not better than the transgenic B'phi plants fused with gene resistant to Basta B'phi 13, 16 and 20.

The B'phi 13, 16 and 20 plants grew better on the growth agar with Basta because they have the gene which gives them a better resistance to Basta than the Wild type plant with and without Basta. The plants B'phi 13, 16 and 20 did not segregate as regards to Basta resistance therefore they were homozygous.

4.2 FLOWERING EXPERIMENTS.

1st Flowering Experiment.

Generally, from the analyzed results in the results section, the flowering in the Mutant type plants was faster than the Wild Type Plants.

The final observation, on each day of observation plants that was observed to have flowered on the same day had the same number or almost the same number of Cauline and Rosette leaves at the time as seen in the table for both Wild Type and Mutant Plants.

2nd Flowering Experiment.

The difference in flowering between the Wild type and Mutant plants was not too far apart but, the Mutant plants, flowered faster.

In this second flowering experiment, it was also observed that the b'phi plants flowered faster than the wild type plants just like the first flowering experiment. Hence, the two flowering experiments done are comparable when taking into cognizance the flowering rate between Wild type plants and Mutant plants.

4.3 Chlorophyll Assay for Bacteria inoculation.

The Root length of the Control plants treatment both Wild type and Mutant plants, showed that the control plants treatment in both Wild type and Mutant plants were longer than that of the Mutant plants and this was observed in all five experiments. In the Wild type and Mutant plants with *Pseudomonas WCS 417*, *Pseudomonas WCS 417* caused alteration of the root structure. *Pseudomonas* reduced primary root length and increased the number of thickness of the lateral roots as seen in the experiment of *WCS 417* Promoting LR Formation (*WCS 417* Promotes LR Formation) (Zamioudis, Mastranesti et al. 2013). the data of their experiment indicate that *WCS 417* promotes LR formation by stimulating both LR initiation and LR outgrowth. One of the most prominent *WCS 417*-mediated morphological alterations in the root system architecture is the stimulation of LR formation and root hair like In the experiment of *WCS 417* Promoting LR Formation (*WCS 417* Promotes RH Development (Zamioudis, 2013). In addition to positive effects on LR formation, *WCS 417* has a strong impact on RH development, as evidenced by the increased RH density and length in *WCS 417*-treated roots The shoot size seemed to increase in

size too. These observations are because of side by side comparison of Control treatment set of plants for Wild type and Mutants and the *Pseudomonas* treated set of plants for Wild type and Mutants. Also, by the analysing the graph for Root length of all the plants.

In the experiment of Chlorophyllase activity and chlorophyll content in wild and mutant plants of *A. thaliana* by (Todorov, Karanov et al. 2003). The activity of chlorophyllase in WT was higher than in ethylene insensitive mutant (eti 5) of *A. thaliana* (L) Heynh plants during vegetative period. Chlorophyllase (Chlase), catalyses the hydrolysis of chlorophyll into chlorophyllide (Childe) and phytol, suggesting a key role in Chlorophyll catabolism (Todorov, Karanov et al. 2003). In my work here, For the chlorophyll assay experiment, it was observed that the chlorophyll content of the Wild type and Mutant treated with *Pseudomonas WCS 417* were higher than the chlorophyll content of the control treatments of Wild type and Mutant plants. This observation can be seen on the graph showing results for chlorophyll assay. This shows that just like the experiment by (Todorov, Karanov et al. 2003) the chlorophyllase activity (catalyzing hydrolysis of chlorophyll into chlorophyllide (Childe) and phytol) in WT *A. thaliana* plant was higher (hence the lower concentration of chlorophyll in WT) than in the B'phi Mutant *A. thaliana* plant.

4.4 SALT STRESS EXPERIMENT AND CHLOROPHYLL ASSAY.

The results seen in the Shoot weight and Root weight parameters for Salt Stress is different than those observed for these same parameters in the bacteria inoculation experiments where the Shoot weight, Root weight were not constant or consistent. It was observed in Salt Stress experiment that for Shoot weight, Root weight, Root length that the B'phi 13 and16 plants had heavier Shoot weight, heavier Root weight and Longer Root length (in the three different concentrations observed) than the Wild type plants (there was no reduction in primary root length or increase in the thickness of the lateral roots unlike in the bacteria inoculation experiment where the Wild type and Mutant control plants had longer roots). So, generally the B'phi plants fared better than the Wild type plants in these parameters.

In the chlorophyll assay analysis, The B'phi 13 and 16 plants had higher chlorophyll content than the Wild type plants except in two cases where B'phi 13 (1) and 16 (2) (1st and last graph under chlorophyll graphs for salt stress) had lower chlorophyll content at concentrations 50 mM and 0 mM respectively, this might have occurred during transplanting of 1 week seedlings to the salt medium (random selection of not very healthy seedlings). Except for these- lower chlorophyll content in two different Mutant plants at two different concentration which could have been caused by experimental error like the one mentioned above, generally it can be said that in the Salt stress experiment. The Mutant plant fared better having a better survival response than the Wild type plants.

As stated earlier the Wild type and Mutant plants in the other two concentrations, 150 mM and 200 mM were just observed phenotypically, as their survival response was poor, or the plants were completely dead. Observation still showed that the B'phi 13 and 16 plants survived better -having more green plants than the Wild type plants, most of the wild type plants turned white showing the effect of the high salt concentration on them. Fewer B'phi 13 and 16 plants developed chlorosis compared to the Wild Type plants. The reason why the Wild Type plants had lower chlorophyll content than the Mutant plants, because the plants were more affected by chlorosis. The Mutant plants might have fared better because of the B'phi gene helping them have a better survival response.

4.6 PCR EXPERIMENTS.

In the first experiment, the expected PCR amplicon with gene for our experiment is 1486 bp as seen in Table 8. Using the Hyper-ladder 10 Kb (Bioline) used to determine size of PCR products in FIGURE 6, to compare result from the PCR experiment shows that the expected product (the gene) is present.
Therefore, the result is positive.

In the second experiment, WT 34 did not give product which is what was expected because it is not a mutant plant, but control of the mutant B'Phi of *A. thaliana* plant.

The blank was just master mix, the negative control is the empty vector pBA002. The positive control is the B'phi plasmid.

2nd Genotyping Experiment.

the expected PCR amplicon with gene for our experiment is 1486 bp as seen in Table 13. Using the Hyper-ladder 10 Kb (Bioline) used to determine size of PCR products in FIGURE 6, to compare result from the PCR experiment shows that the expected product (the gene) is present. Therefore, the result is positive.

The ten plants in each experiment showed highly positive PCR for the inserted B'phi gene.

4.7 GENE EXPRESSION ANALYSIS.

Reverse transcription PCR, or RT-PCR, allows the use of RNA as a template. An additional step allows the detection and amplification of RNA. The RNA is reverse transcribed into complementary DNA (cDNA), using reverse transcriptase. The quality and purity of the RNA template is essential for the success of RT-PCR. The first step of RT-PCR is the synthesis of a DNA/RNA hybrid. Reverse transcriptase also has an RNase H function, which degrades the RNA portion of the hybrid. The single stranded DNA molecule is then completed by the DNA-dependent DNA polymerase activity of the reverse transcriptase into cDNA. The efficiency of the first-strand reaction can affect the amplification process. From here on, the standard PCR procedure is used to amplify the cDNA. The possibility to revert RNA into cDNA by RT-PCR has many advantages. RNA is single-stranded and very unstable, which makes it difficult to work with. Most commonly, it serves as a first step in qPCR, which quantifies RNA transcripts in a biological sample.

Five plant samples of *A. thaliana* were used (50 mg each), two Wild type (WT 4 and WT 17) and three Mutant B'phi 13 transgenic plants (B'phi 44, 47 and 50). Genotyping for these plants was done prior to check gene of insert prior to this experiment and the result was positive for the gene.

Isolation and purification of these plants' samples RNA was done, and their quality checked on Nanodrop. The concentration of all the RNA samples was good. The quality of all other RNA was good (above 1.7 – requirement for better results when sequencing by Microsynth SEQLAB) except for B'phi 44 and B'phi 47 with quality of 0.81 and 0.62 respectively, these might be as a result of ethanol interference that might still be left in the sample after the experiment of RNA isolation and purification.

These RNA samples were diluted 1:1 prior to cDNA synthesis experiment. In the course of cDNA, two reaction mixes were made using the kit Reverse transcription protocol for SuperScript™ IV VILO™ Master Mix (without ezDNase enzyme treatment). The reaction mixes are Reverse Transcriptase (RT) reaction mixture and No Reverse Transcriptase (NRT) Reaction mixture. This just means the Reverse Transcriptase Reaction mixture has the enzyme Reverse Transcriptase n the mixture while the other No Reverse Transcriptase Reaction Mixture has no reverse Transcriptase in the mixture therefore, the Reverse Transcriptase enzyme is absent, and this mixture serves as the control.

The RT and NRT mixtures were diluted to a concentration of 50 µg/ul 1:1 by nuclease free water prior to quantitative PCR (RT-qPCR).

Two primers were used - **Short reverse and Short forward primers and the Complete reverse and Short forward primers** for quantitative PCR (RT-qPCR).

The following samples were ran on the gel for each primer set: No Reverse Transcriptase WT4, WT17, B'phi 44, 47, 50, also for Reverse Transcriptase WT 4, 17, B'phi 44, 47, 50., then pure RNA samples RNA WT 4, 17, B'phi 44, 47, 50.

The second Primer, **Complete reverse and Short forward primers**, all the RNA samples, RNA WT 4, 17, B'phi 44, 47, 50, were clean and there was no contamination, which means during the RNA isolation and purification experiments all genomicDNA were removed. For the RT samples, RT WT 4 and 17 gels were clean and free of contamination also, they had no bands because they have no gene of insert and the expected product of 900 bp was gotten for the B'phi 44, 47, 50, when the band of the product was matched against the hyper ladder and our positive control which is the guide to know if the expected product is what we obtain from our gel bands. Finally, for NRT samples WT 4, 17, B'phi 44, 47, 50, here, the WT 4 and 17 gels are clean and free of

contamination and no bands is seen in the NRT , B'phi 44, 47, 50 wells, this is good because for NRT samples which is devoid of Reverse Transcriptase, there should be no transcribing therefore no bands. In the PCR machine these primers (**Complete reverse and Short forward primers)** and samples was set to run for 35 cycles, the results of the gels showed clean uncontaminated and expected results. The number of cycles might be the reason why the gels here are a little cleaner and free of contamination compared to the Short reverse and Short forward primers because even though the Complete reverse and short forward primers have a longer fragment (900 bp) than the Short reverse and Short forward primers (400 bp) with a shorter cycle the bands on the gel were clean and free of gDNA or contamination. Showing that 35 cycles was appropriate even for a primer with longer fragment without picking up different products and showing unnecessary bands.

It is not enough sometimes to just genotype the Transgenic plants, because it might be a positive result that the gene of insert is present, but it cannot be ascertained if the gene like in this thesis work B'phi gene is expressed even when positive for genotyping. Hence, the necessity of this Gene expression experiment. Comparing at the WT and Mutant plants in this experiment the expression level was constant across all Reverse Transcriptase Mutant plants as against the Reverse Transcriptase Wild Type plants, showing there was expression of our gene of interest (B'phi) in the Mutant plants.

4.8 SEQUENCING.

Bioinformatics method was used to check and confirm if the gene that was sent for sequencing was the B'phi gene from tomato plant that was transformed into *A. thaliana* (the model plant for this thesis). After preparation of the sample by Microsynth SEQLAB requirement, the received sequence result from the laboratory was blasted against the tomato B'phi gene on National Centre for Biotechnology Information (NCBI). After blasting, the sequenced gene was the same as the B'phi gene (gene of interest) from tomato plant that was transformed into *A. thaliana.* Clustal omega was used to present the result of the sequence gotten after blasting on NCBI.

After blasting the gene was represented in Clustal Omega form. A positive result was not only gotten after genotyping, also, the B'phi gene was expressed in the gene expression analysis and it

was also ascertained by sequencing that the B'phi gene is what was being worked with all along in this thesis.

4.9 FUTURE PROSPECTIVE

From the results in this thesis, it shows from Flowering, Bacteria inoculation and Salt stress experiments that the gene B'phi plays a vital role in plant survival response. This is because from analysis, statistics and observations the Mutant *A. thaliana* fared better in stressful conditions than the Wild type plants that do not have the B'phi gene. One of the examples is in my second flowering experiment. Both the 50 Wild type plants and 50 Mutant plants were attacked by aphids, but 9 of the wild type plants could not make it but just 2 of the B'phi plants had poor response. Also, for salt stress the transgenic plants had a better survival response. In the flowering experiment the Mutant plants flowered faster, this is good for food crops or economically important crops as flowering means the plants are about to produce fruits, or food. Good survival response to stress and environmental or external forces is important for plant development in order to withstand these forces, especially for food crops so they can reach their potential, like for food production. Plants, or food crops are faced with different kind of stress or external forces, it would be great to further investigate the B'phi gene on plants like food crops (economically important crops), this is because they are more of economic importance and provide food for the population so as to eradicate famine that is already ravishing some parts of the world and increase food production that is beginning to decline because of global warming, human factors (activities we do that are harmful to plants).

Food shortage for some time now, has been a great challenge around the world and helping plants to survive whatever environmental or external stress that hits them will save crops and the population at large.

Also, more work should be done to know how the B'phi gene regulates the plant's cell activity and what it interacts with in the cell to support the plant for better survival response.

5. APPENDIX.

FLOWERING EXPERIMENT.

Pictures of Wild Type and b'phi 13 *A. thaliana* plants for 1st flowering Experiment.

FIGURE 33: Wild Type Plants (1-25), taken on the 11.10.2018.

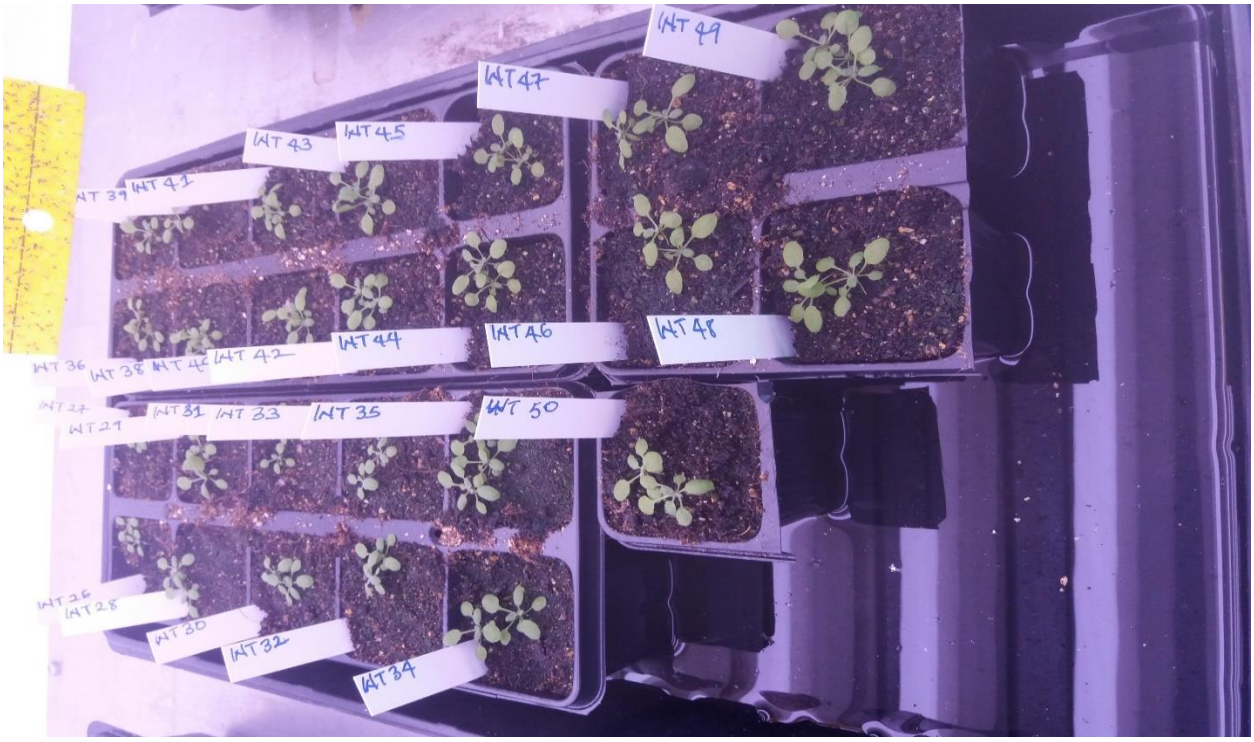


FIGURE 34: Wild Type Plants (26-50), taken on the 11.10.2018.

FIGURE 35: Mutant Type Plants (1-25), taken on the 11.10.2018.

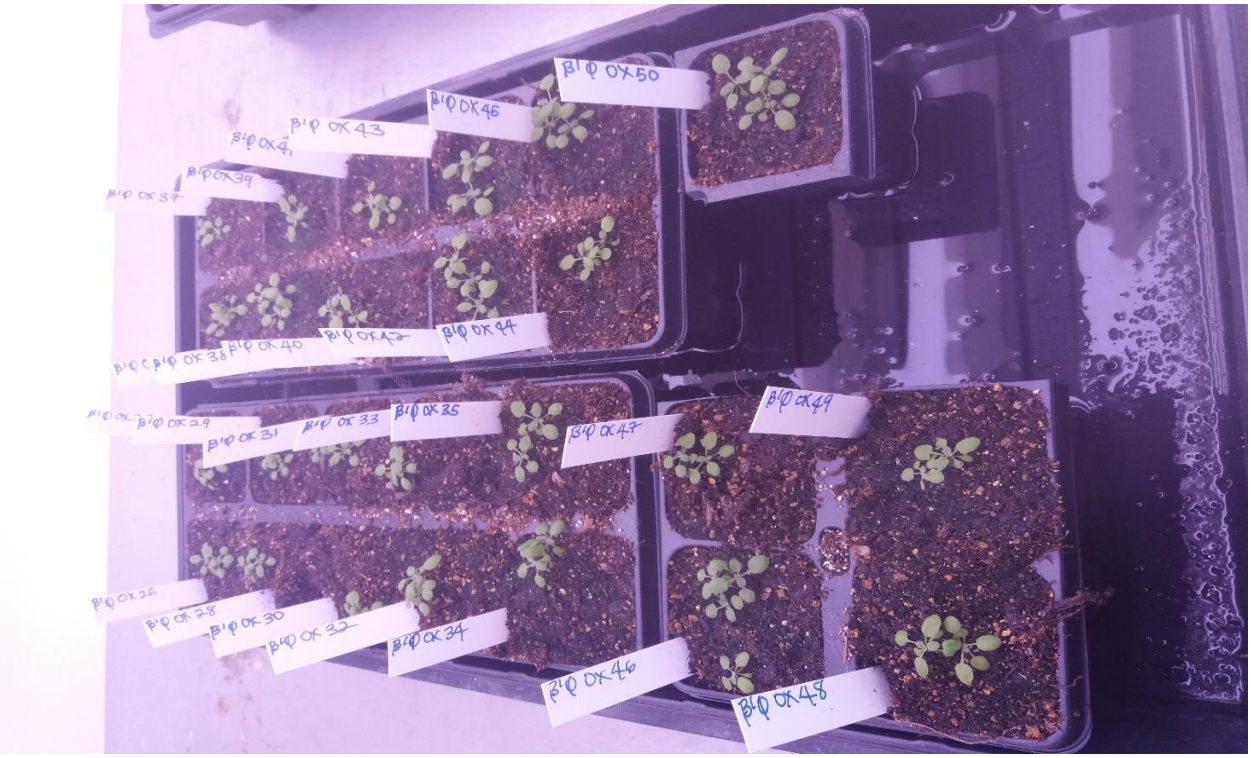


FIGURE 36: Mutant Type Plants (26-50), taken on the 11.10.2018.

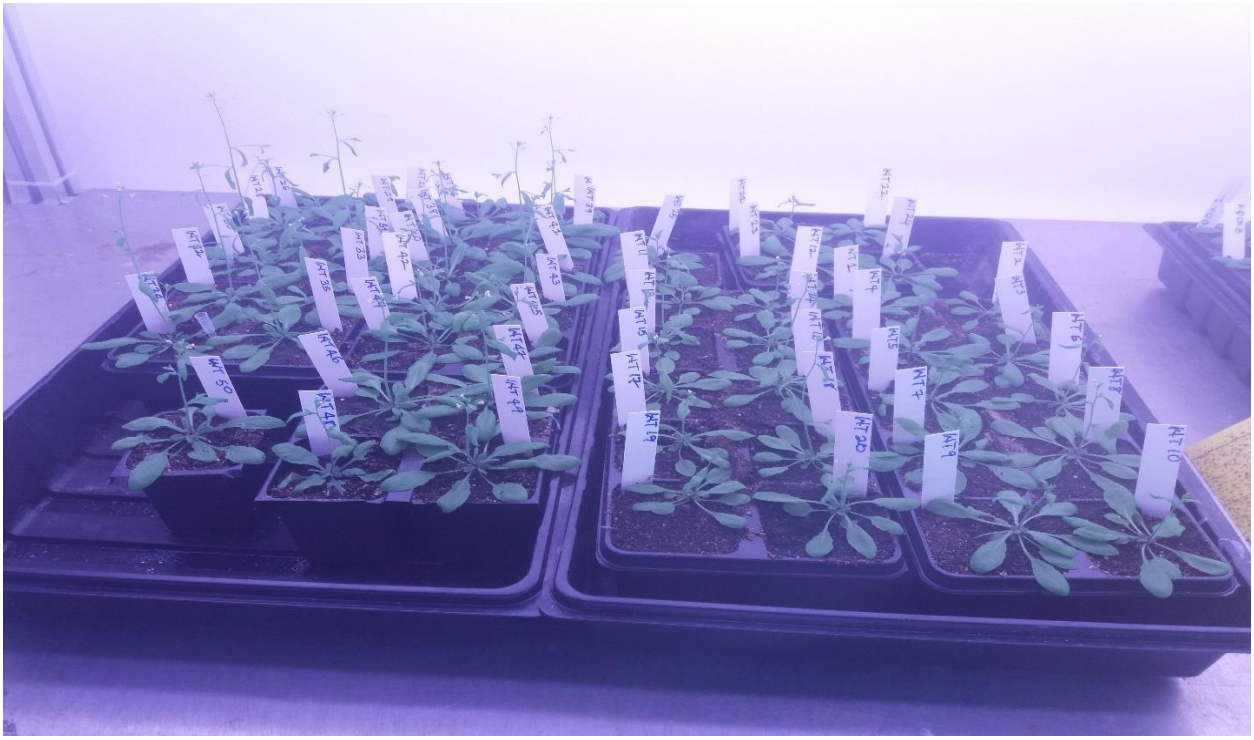


FIGURE 37: Wild Type numbers (1-50), taken on the 25.10.2018 (flowering had started here but was not the first day flowering was observed).

FIGURE 38: Treated Type Plants numbers (1-50), taken on the 25.10.2018 (flowering had started here but was not the first day flowering was observed).

FIGURE 39: Wild Type numbers (1-50), taken on the 30.10.2018 (Last day of flowering observation).

FIGURE 40: Treated Type Plants numbers (1-50), taken on the 30.10.2018 (last day for flowering observation).

Table 33: Flowering Statistics for *A. thaliana* Wild Type Plants for 1st flowering experiment.

No of Rows	22.10.18	Rosette/ Cauline Leaves	24.10.18	Rosette/ Cauline Leaves	26.10.18	Rosette/ Cauline Leaves	30.10.18	Rosette/ Cauline Leaves
1	WT 28	12/4	WT 12	11/3	WT 1	12/3	WT 2	19/9
2	WT 29	9/3	WT 13	10/3	WT 3	12/4	WT 5	22/11
3			WT 16	9/3	WT 4	14/3	WT 22	18/4
4			WT 18	10/3	WT 6	11/3	WT 26	20/9
5			WT 28	12/4	WT 7	12/4		
6			WT 31	10/4	WT 8	12/4		
7			WT 33	10/4	WT 9	11/4		
8			WT 35	11/4	WT 10	11/3		
9			WT 37	10/4	WT 11	11/3		
10			WT 38	9/3	WT 12	11/3		
11			WT 40	9/3	WT 14	11/3		
12			WT 41	12/3	WT 15	12/4		
13			WT 42	9/3	WT 17	12/3		
14			WT 43	10/4	WT 19	11/3		
15			WT 46	11/3	WT 20	11/3		
16			WT 47	10/3	WT 21	12/4		
17			WT 50	10/3	WT 23	12/3		
18					WT 24	11/3		
19					WT 25	11/3		
20					WT 27	14/4		
21					WT 32	16/6		
22					WT 36	12/3		
23					WT 39	10/4		
24					WT 44	14/4		
25					WT 45	14/5		
26					WT 48	11/4		
27					WT 49	16/6		

Total number of Plants	**2**		**17**		**27**		**4**	

Table 34: Summary of the table above, flowering number statistics for Wild and Mutant Type B'ϕ (phi) 13 *A. thaliana* Plants.

Dates of Observation.	**Number of WT Plants**	**Number of b'ϕ Plants**
22.10.18	2	7
24.10.18	19	28
26.10.18	46	47
30.10.18	50	50

Table 35: For Treated *A. thaliana* (B'phi) Plants.

No of Rows	22.10.18	Rosette/ Cauline Leaves.	24.10.18	Rosette/ Cauline Leaves.	26.10.18	Rosette/ Cauline Leaves.	30.10.18	Rosette/ Cauline Leaves.
1	B'ϕ 2	9/4	B'ϕ 1	11/4	B'ϕ 4	12/5	B'ϕ 8	26/13
2	B'ϕ 5	9/3	B'ϕ 3	12/4	B'ϕ 10	11/3	B'ϕ 37	23/11
3	B'ϕ 15	9/4	B'ϕ 6	12/4	B'ϕ 11	12/3	B'ϕ 22	25/14
4	B'ϕ 16	9/3	B'ϕ 7	12/4	B'ϕ 17	12/4		
5	B'ϕ 18	9/4	B'ϕ 9	11/4	B'ϕ 24	11/4		
6	B'ϕ 19	9/4	B'ϕ 12	11/4	B'ϕ 25	11/3		
7	B'ϕ 35	9/4	B'ϕ 13	12/5	B'ϕ 26	11/3		
8			B'ϕ 14	20/4	B'ϕ 33	11/4		
9			B'ϕ 20	11/4	B'ϕ 36	11/4		
10			B'ϕ 21	11/4	B'ϕ 38	14/4		
11			B'ϕ 23	11/4	B'ϕ 39	16/5		
12			B'ϕ 27	11/3	B'ϕ 41	12/3		
13			B'ϕ 28	10/4	B'ϕ 42	13/3		
14			B'ϕ 29	11/4	B'ϕ 43	12/4		
15			B'ϕ 30	12/3	B'ϕ 45	11/4		
16			B'ϕ 31	10/4	B'ϕ 46	12/4		
17			B'ϕ 32	12/4	B'ϕ 47	14/4		
18			B'ϕ 34	10/4	B'ϕ 49	12/2		
19			B'ϕ 40	10/3	B'ϕ 50	16/5		
20			B'ϕ 44	11/3				
21			B'ϕ 48	10/3				
Total Number of Plants.	**7**		**21**		**19**		**3**	

Table 36: Summary of the Table Above, Flowering Percentage Statistics for Wild and Mutant Type B'ϕ *A. thaliana* Plants.

Dates of Observation.	**Percentage of WT Plants (%).**	**Number of b'ϕ Plants (%)**
22.10.18	4	14
24.10.18	38	56
26.10.18	92	94
30.10.18	100	100

Pictures of Wild Type and b'phi 13 *A. thaliana* plants for 2nd flowering Experiment.

Figure 41: B'phi 13 plants (Numbers 1-25).

FIGURE 42: Wild type plants (Numbers 25-50).

Table 37: Flowering observation for 2nd experiment of b'phi 13 and wild type *A. Thaliana* plants.

DATES	WT	B'PHI 13
11.02.19	4	7
12.02.19	7	10
13.04.19	16	17
14.02.19	17	19
15.02.19	17	29
16.02.19	17	29
17.02.19	17	29
18.02.19	17	29
19.02.19	22	30
21.02.19	23	30
21.02.19	23	30
22.02.19	38	48
28.02.19	41	48
TOTAL	**41**	**48**

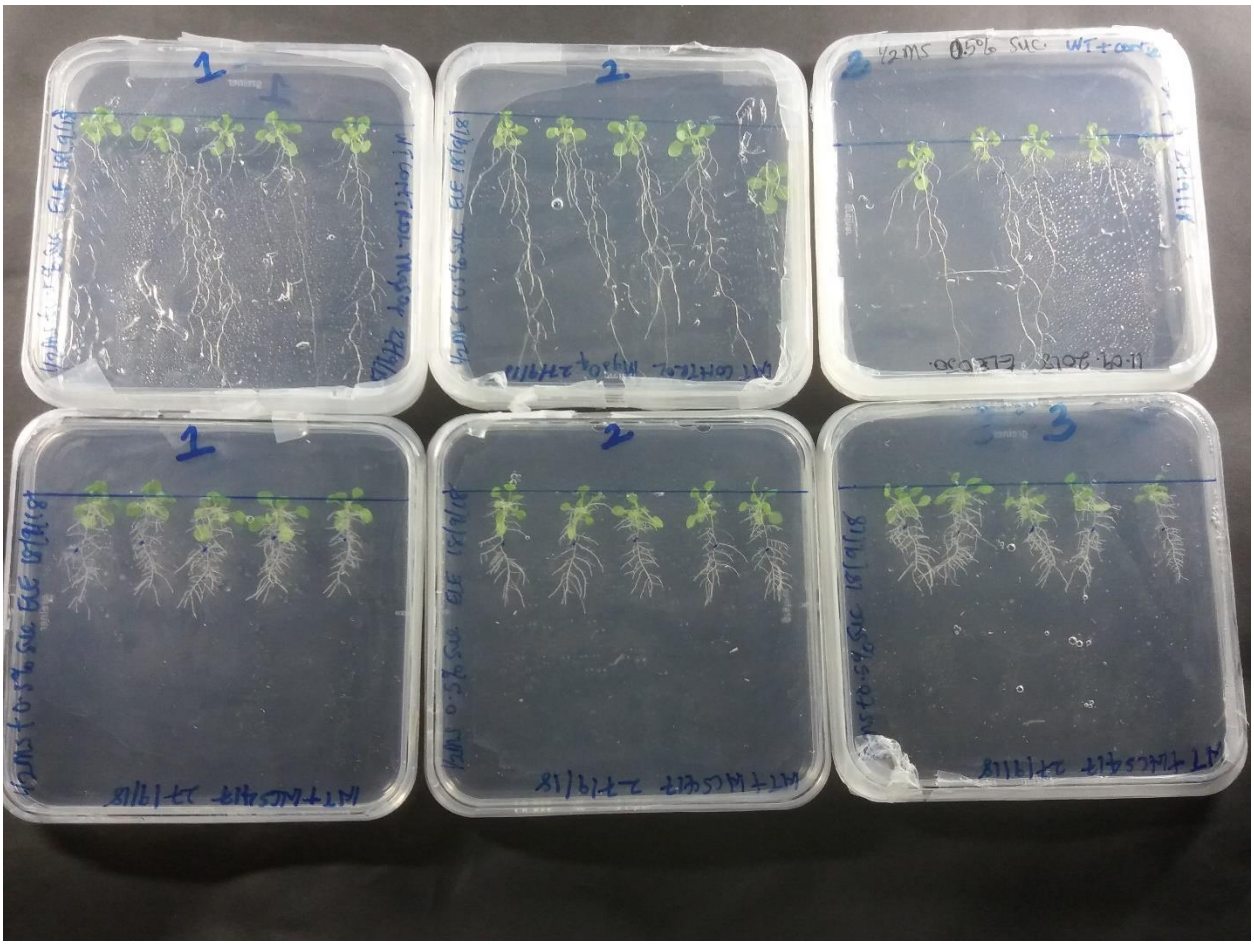


FIGURE 43: Plates of wild type *A. Thaliana* untreated (control) and treated (*Pseudomonas WCS 417).*

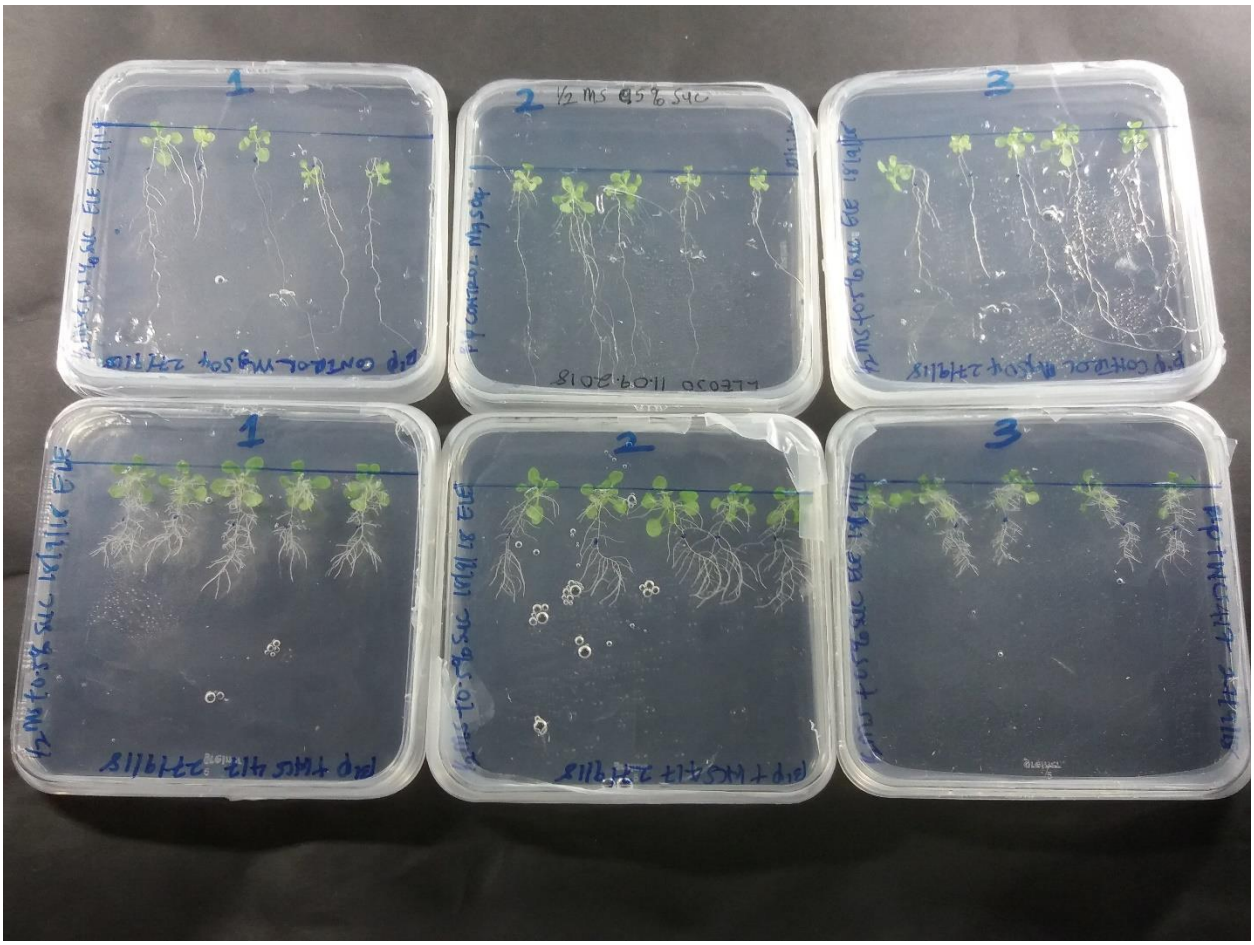


FIGURE 44: Plates of mutants *A. Thaliana* untreated (control) and treated (Pseudomonas WCS 417).

Table 38: Data for Harvested Treated and Untreated Wild Type and Mutants of *A. Thaliana* inoculated with *Pseudomonas WCS 417* on LB agar medium. (First B'Phi 13 experiment).

Sample ID	Shoot Weight	Absorbance	Root Weight	Root Length				
				Plant1	Plant2	Plant3	Plant4	Plant5
WT C1	71.4	0.354	14	100.57	98.94	98.843	89.677	92.467
WT C2	88.2	0.377	22.6	90.599	98.688	91.014	94.426	54.996
WT C3	56.6	0.138	09.7	76.03	90.478	85.422	81.394	54.648
B'phi C1	49.4	0.143	6.3	94.276	38.674	97.288	75.957	95.552
B'phi C2	75.6	0.132	39.4	84.504	78.107	49.15	122.405	35.174
B'phi C3	78.4	0.262	15.2	91.851	49.15	122.405	103.367	84.36
WT T1	83	0.434	41.3	33.525	31.65	35.174	29.818	25.682
WT T2	67.2	0.370	14	33.189	34.273	26.208	32.443	30.752
WT T3	68.1	0.408	25.1	28.052	35.138	31.423	32.67	27.854
B'phi T1	92.1	0.505	32.9	30.783	33.365	37.942	31.333	33.135
B'phi T2	95.4	0.484	25	39.134	39.877	43.242	38.736	40.542
B'phi T3	60.7	0.342	23.8	21.131	29.141	31.816	30.137	31.987

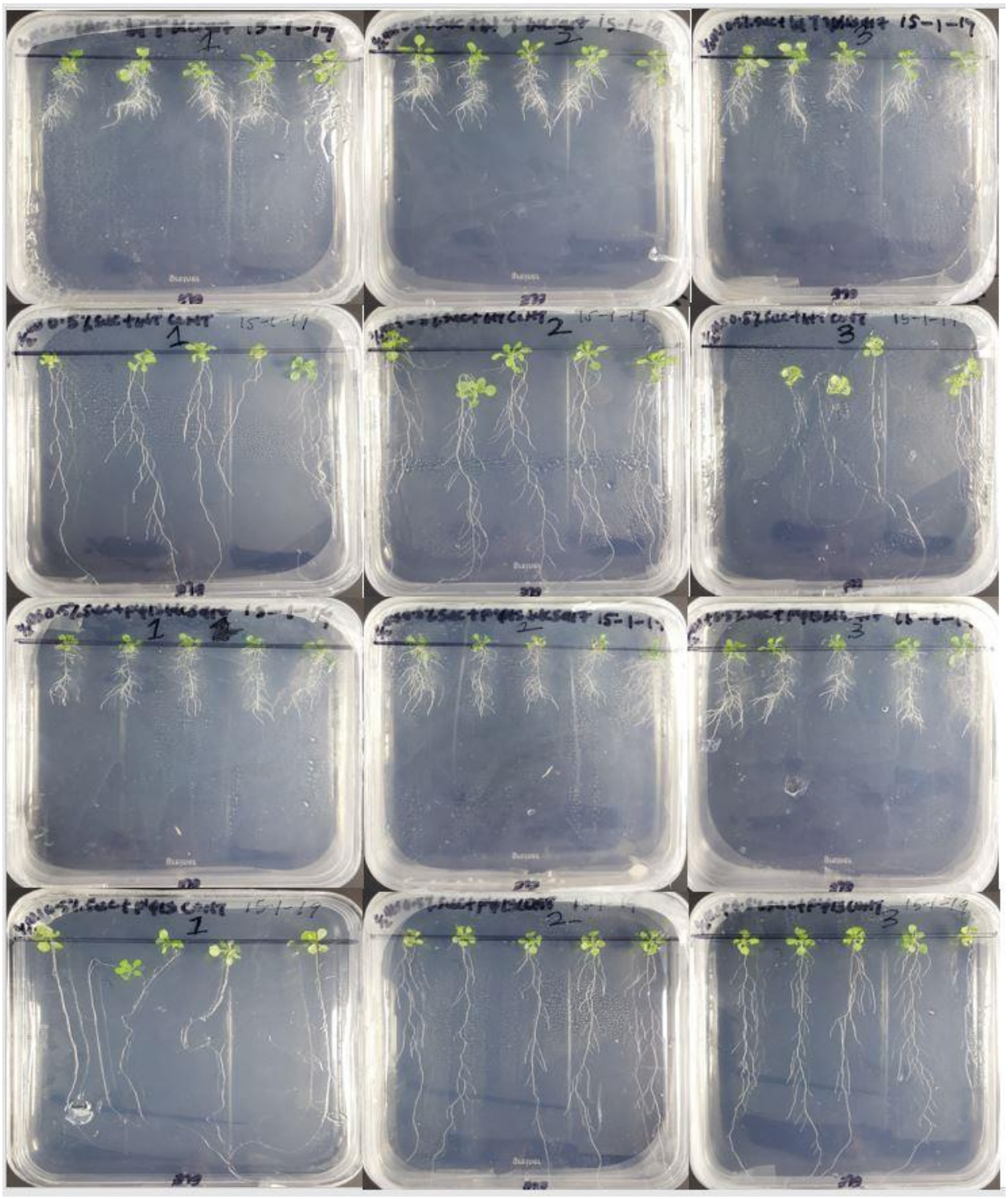


FIGURE 45: Third B'Phi 13 Bacteria/ chlorophyll experiment.

Table 39: Data for Harvested Treated and Untreated Wild Type and Mutants of *A. thaliana* inoculated with *Pseudomonas WCS 417* on LB agar medium. (3rd B'Phi 13 experiment).

Sample ID	Shoot Weight	Absorbance	Root Weight	Root Length				
				Plant1	Plant2	Plant3	Plant4	Plant5
WT C1	75.5	0.199	14.8	78.478	77.917	102.573	123.016	95186
WT C2	83.4	0.243	17.2	124.143	128.702	56.609	107.389	82.443
WT C3	77.1	0.235	19.3	114.029	105.431	93.543	108.606	99.372
B'phi C1	63.4	0.172	17.2	116.65	120.102	125.674	106.417	21.122
B'phi C2	99.9	0.442	36.8	97.828	129.917	113.919	96.145	102.473
B'phi C3	67.2	0.173	16.9	118.601	99.134	98.849	102.473	111.253
WT T1	68.2	0.264	15.4	29.442	29.869	34.502	32.448	32.818
WT T2	63	0.278	19.6	34.697	27.792	35.16	33.884	27.275
WT T3	47.3	0.205	13.9	33.697	32.564	27.967	32.106	33.313
B'phi T1	41.3	0.189	17	31.026	17.835	23.664	25.389	26.626
B'phi T2	53.3	0.217	16.9	36.955	27.611	30.366	36.898	28.666
B'phi T3	42.9	0.199	15.5	31.516	28.238	30.641	29.879	27.229

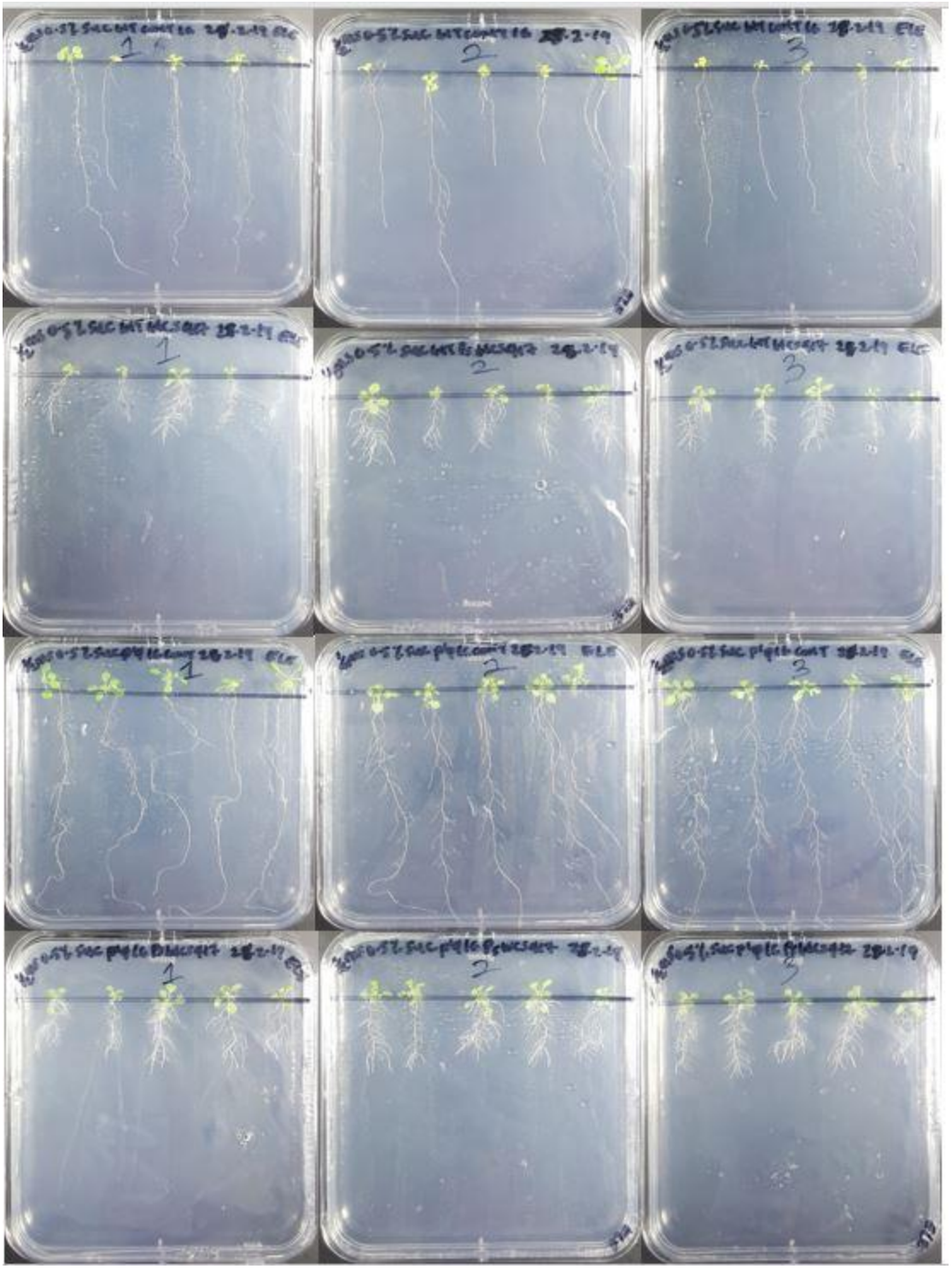


FIGURE 46: Second B'Phi 16 Bacteria/ chlorophyll experiment.

Table 40: Data for Harvested Treated and Untreated Wild Type and Mutants of *A. thaliana* inoculated with *Pseudomonas WCS 417* on LB agar medium. (2nd B'Phi 16 experiment).

Sample ID	Shoot Weight	Absorbance	Root Weight	Root Length				
				Plant1	Plant2	Plant3	Plant4	Plant5
WT C1	23.2	0.086	5	99.865	54.385	85.583	87.289	53.712
WT C2	27.4	0.079	5	63.334	97.574	39.922	35.792	100.782
WT C3	20.3	0.019	2.2	70.293	54.13	56.886	46.992	80.073
B'phi C1	42	0.234	9.2	134.666	114.034	122.802	122.088	116.738
B'phi C2	53.3	0.268	12	117.255	100.021	103.013	104.387	103.989
B'phi C3	47.1	0.31	19.1	106.91	106.004	105.647	99.922	99.946
WT T1	14.6	0.088	4.8	25.853	19.035	26.361	21.662	23.345
WT T2	28.8	0.167	7.8	28.517	23.999	26.945	26.578	28.971
WT T3	24.7	0.158	7.9	26.119	25.042	26.541	20.786	20.435
B'phi T1	58.6	0.394	14	25.592	23.338	27.816	29.522	28.018
B'phi T2	49.7	0.296	13.3	31.673	29.206	25.267	29.505	28.18
B'phi T3	66.2	0.379	13.8	29.831	31.899	30.209	29.776	27.427

SALT STRESS for B'phi13 and 16 Wild Type *A. thaliana* Plants

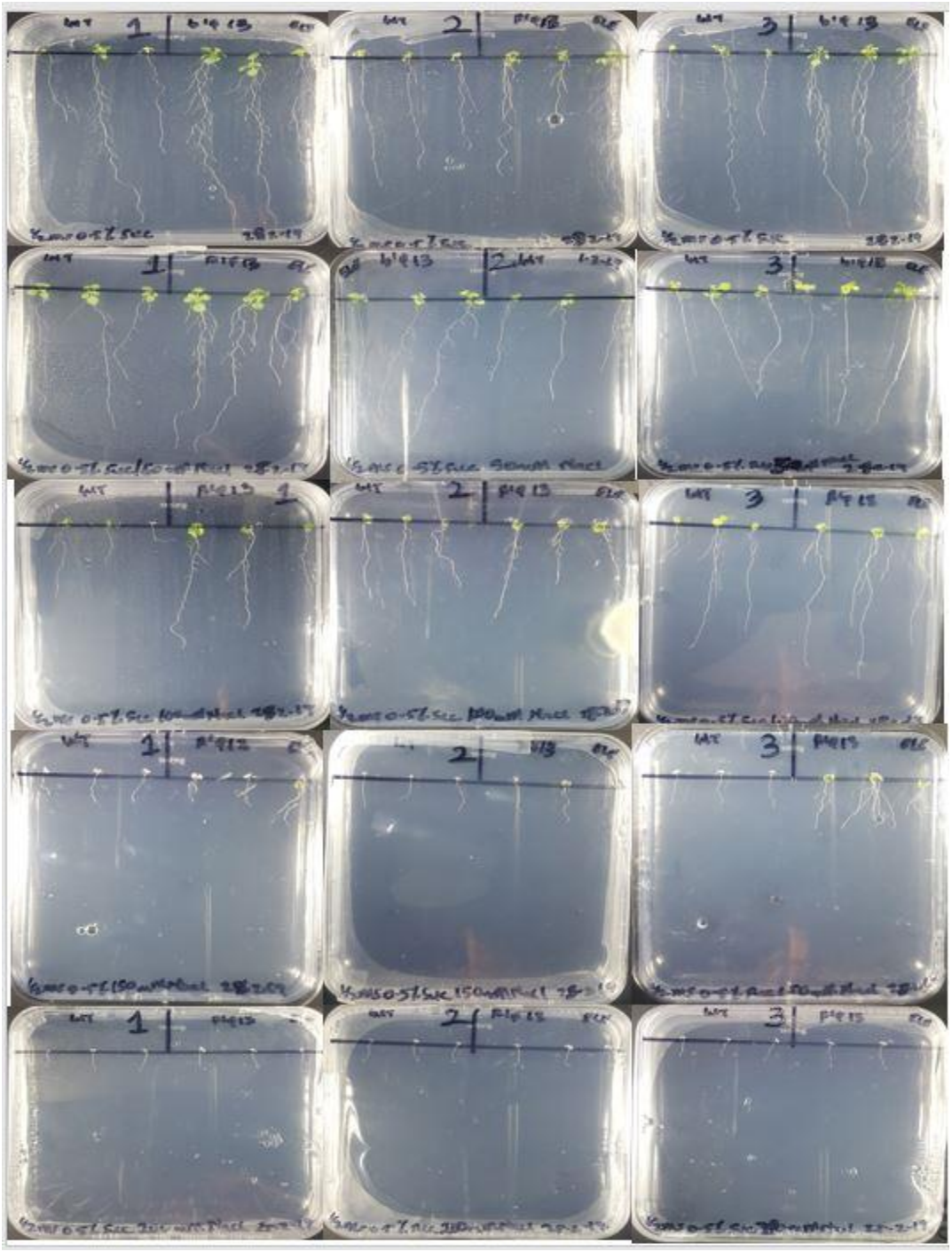


FIGURE 47: First B'Phi 13 Salt Stress-chlorophyll experiment.

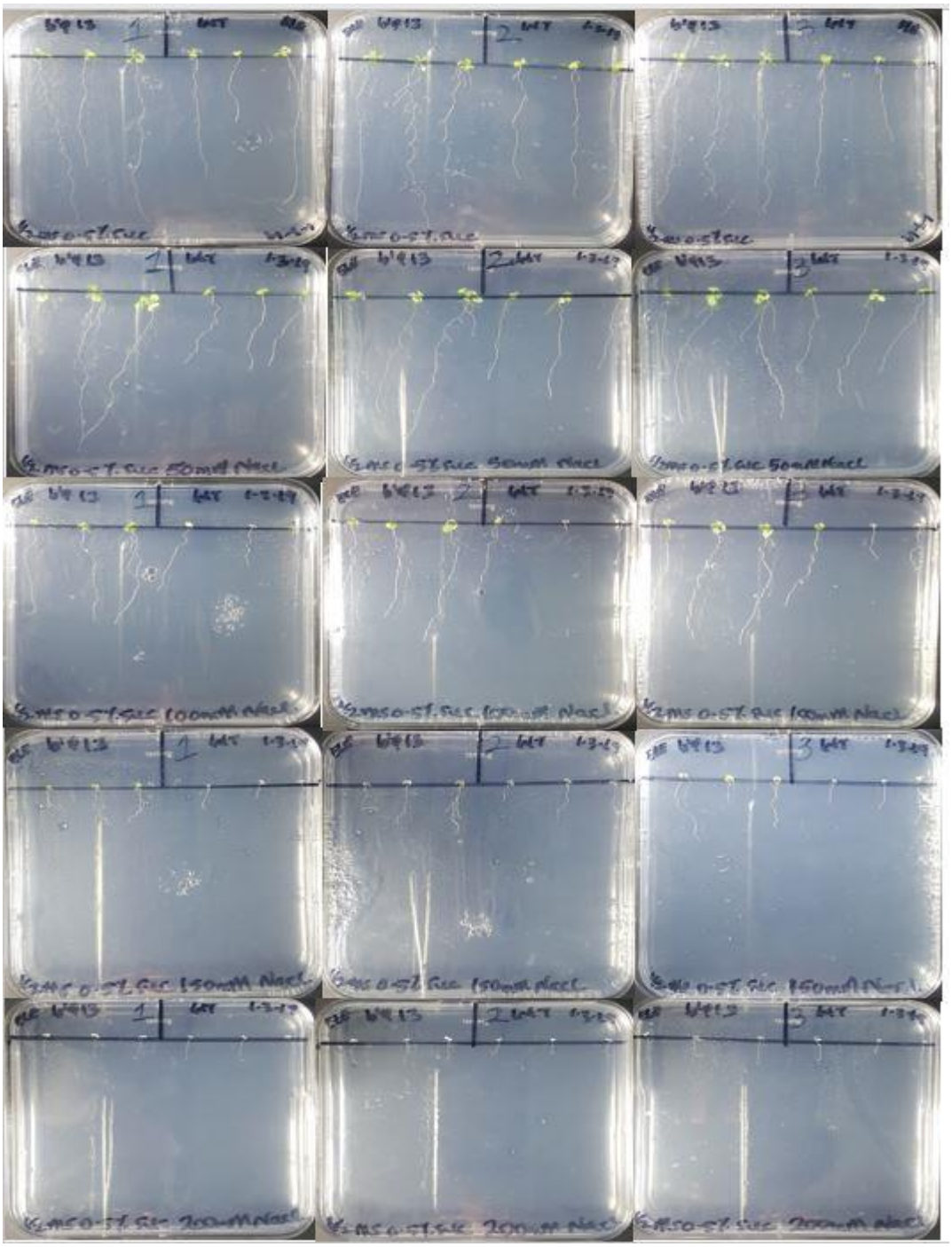


FIGURE 48: Second B'Phi 13 Salt Stress-chlorophyll experiment.

Table 41: Data for Harvested Salt Stressed Treated and Untreated Wild Type and Mutants of *A. thaliana*. (2nd B'Phi 13 experiment).

Sample ID (0mM)	Shoot Weight	Absorbance	Root Weight	Root Length		
WT C1	7.4	0.121	0.9	64	62	65
WT C2	11.3	0.102	1.8	38	70	44
WT C3	8.3	0.05	1	82	76	34
B'phi 1	15.3	0.257	1.8	90	89	78
B'phi 2	21.6	0.272	2.1	58	91	74
B'phi 3	13.8	0.233	2.8	90	72	81
Sample ID (50mM)						
WT C1	9.8	0.095	1	45	46	45
WT C2	15	0.104	1	43	52	56
WT C3	12	0.132	1.4	42	40	47
B'phi 1	27.1	0.529	4.8	68	69	41
B'phi 2	22	0.364	2	88	66	74
B'phi 3	23	0.281	1.6	80	85	67
Sample ID (100Mm)						
WT C1	2.8	0.033	0.2	35	40	46
WT C2	2.3	0.015	0	18	5	17
WT C3	3.1	0.034	0.2	35	18	29
B'phi 1	14	0.142	1.5	50	55	50
B'phi 2	14.2	0.181	1.8	5	48	59
B'phi 3	10.9	0.216	2.3	52	64	60

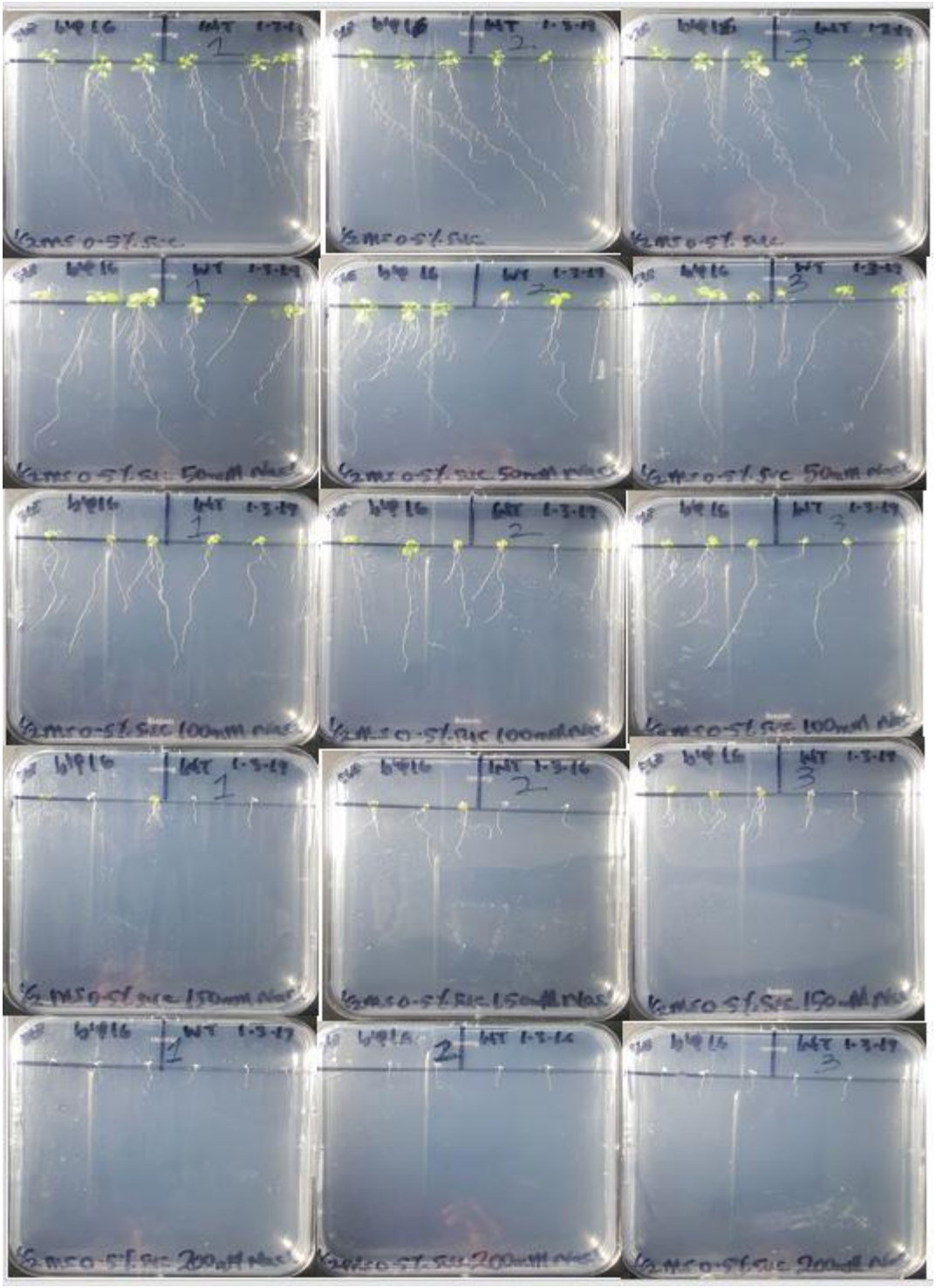


FIGURE 49: First B'Phi 16 Salt Stress-chlorophyll experiment.

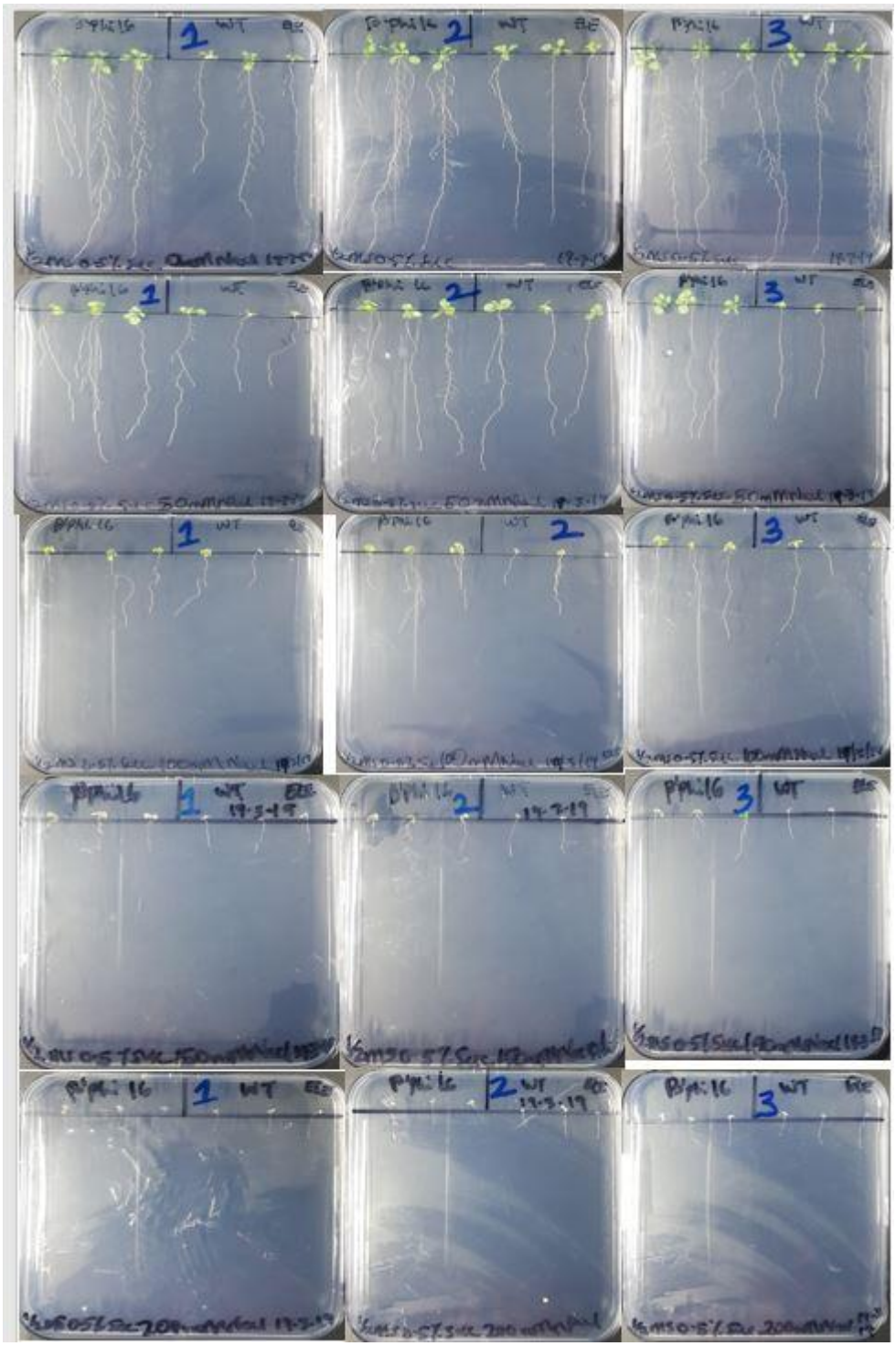


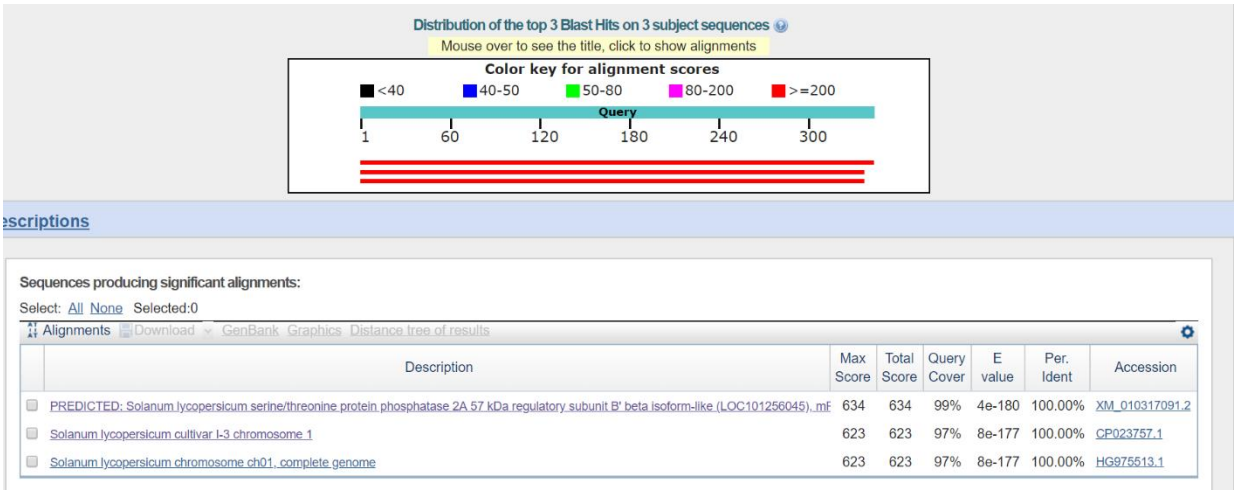
FIGURE 50: Second B'Phi 16 Salt Stress-chlorophyll experiment.

Table 42: Data for Harvested Salt Stressed Treated and Untreated Wild Type and Mutants of *A. thaliana*. (2nd B'Phi 16 experiment).

Sample ID (0mM)	Shoot Weight	Absorbance	Root Weight	Root Length		
WT C1	15.5	0.378	2.6	57	85	98
WT C2	17.8	0.537	3	97	84	98
WT C3	24.6	0.825	5.1	82	71	96
B'phi 1	44.5	0.858	7.7	62	108	102
B'phi 2	37.6	0.992	7.3	101	103	107
B'phi 3	32.5	1.012	5.4	102	100	108
Sample ID (50mM)						
WT C1	13.2	0.157	1.8	73	88	60
WT C2	22.4	0.586	3.8	41	68	48
WT C3	8.8	0.037	1.2	44	63	47
B'phi 1	30.4	0.402	2.6	62	75	79
B'phi 2	32.2	0.848	3.4	79	80	75
B'phi 3	32.5	0.741	3.6	69	75	75
Sample ID (100Mm)						
WT C1	5.1	0.022	1.8	36	17	45
WT C2	2.6	0.006	3.8	19	32	16
WT C3	4.9	0.049	1.2	34	13	28
B'phi 1	10.1	0.112	2.6	15	20	40
B'phi 2	13.7	0.092	3.4	43	43	31
B'phi 3	10.5	0.092	3.6	33	29	46

All have same results after Blast NCBI

https://www.ncbi.nlm.nih.gov/nucleotide/XM_010317091.2?report=genbank&log$=nuclalign&blast_rank=1&RID=ENHFX9JZ014 AS FIRST RESULTS



>Eleojo_long_For_B'phi short forward

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CLUSTAL O (1.2.4) multiple sequence alignment

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Ele     ------------------------------------------------------------        0
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Ele     ------------------------------------------------------------        0
B'      CTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACTGTCACCCCTT        180

Ele     ------------------------------------------------------------        0
B'      TTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCATTCTGCCGTC        240

Ele     ------------------------------------------------------------        0
B'      TCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTGGCCACATTTG        300

Ele     ------------------------------------------------------------        0
B'      CAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGAATCGCTTCGT        360

Ele     ------------------------------------------------------------        0
B'      ATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATCTGAAGATCAA        420

Ele     ------------------------------------------------------------        0
B'      AGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTTAACATTCTAC        480

Ele     ------------------------------------------------------------        0
B'      AGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTTCGAGACTGAT        540

Ele     ------------------------------------------------------------        0
B'      CAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTATAAATGGATTT        600

Ele     ----TTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT        56
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B'      AGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT      660
            ********************************************************

Ele     AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG      116
B'      AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG      720
        ************************************************************

Ele     CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT      176
B'      CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT      780
        ************************************************************

Ele     ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT      236
B'      ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT      840
        ************************************************************

Ele     GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTACCAAGTGTTTA      296
B'      GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTACCAAGTGTTTA      900
        ************************************************************

Ele     AACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAATGAGCAATTC      356
B'      AACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAATGAGCAATTC      960
        ************************************************************

Ele     TGGAAGATGTTATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTAGTGGAAGGGATGGAG      416
B'      TGGAAGATGTTATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTAGTGGAAGGGATGGAG      1020
        ************************************************************

Ele     AAGAACTTGCAAGGACATTGGAGCAAAAGTGTTAAGCAATTGACGGAGAATGTGAAGGGA      476
B'      AAGAACTTGCAAGGACATTGGAGCAAAAGTGTTAAGGAATTGACGGAGAATGTGAAGGGA      1080
        ********************************** ***********************

Ele     ATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCTACAGCTTGAAATCCAA      536
B'      ATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCTACAGCTTGAAATCCAA      1140
        ************************************************************

Ele     GAAGCCAGTGAACGCATAGAAGAGATGAGAAGGAAAGAAATTTGGGAAAAAATTGAAAAT      596
B'      GAAGCCAGTGAACGCATAGAAGAGATGAGAAGGAAAGAAATTTGGGAAAAAATTGAAAAT      1200
        ************************************************************

Ele     GCAGCAATGTGAACGTA        613
B'      GCAGCAATGTGA-----        1212
        ************
```

>Eleojo_long_Rev_B'phi complete reverse

TTTCAAGCTGTAGAAGGCACTTGGAATAGAGAAATGGTGCTAGAGCTTCCAGCATTCCCTTCACATTCTCCGTC
AATTGCTTAACACTTTTGCTCCAATGTCCTTGCAAGTTCTTCTCCATCCCTTCCACTAGAACTGGAAAGACTTC
TTCCATTGCTTGTGATAACATCTTCCANAATTGCTCATTGTTCCACACATACAATGCGCGTTCAGCAACCTGCG
AGTTCCAACTGTTTAAACACTTGGTAATTTTGGTGCACAAAGGCAGGGCAAGTTCCTTGTACAATTGTGGATCA
ACAGTCTCCACAAGTTCTTCCAATTCACCGATAAACAGAACTTCCTTCTGGCAATTGGTAATTGGCCAGTACTT
CAATATGCCTCTTATAACAACCTCACCAAGCTCAGGCTCTTTTTGCACAAACTGAGATACACAATAAGTCAACT
GCCTATGATAAACTTGCATCCCTTTTGGTTTATGCAAAGGGACAAGAACTCTATTCAAGAAAAACTTGTGTTCT
TCTTTCAAAGGAACACTAAATCCATTTATAATTGTGCCCCATATTTCAAGAAGCTCTCCGATTCCAGGGG

```
Ele     ------------------------------------------------------------        0
B'      ATGACAAATTTTCTTGATTCTGAGACAGAGGAAATGCTTTCTGTAATATCTTACTGTTCT        60

Ele     ------------------------------------------------------------        0
B'      TTCACTGATCCACAAGAATCCCCTTCACTACAAGATTTAAAGAGGCTAAAACTCATCCAA        120

Ele     ------------------------------------------------------------        0
B'      CTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACTGTCACCCCTT        180

Ele     ------------------------------------------------------------        0
B'      TTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCATTCTGCCGTC        240

Ele     ------------------------------------------------------------        0
B'      TCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTGGCCACATTTG        300

Ele     ------------------------------------------------------------        0
B'      CAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGAATCGCTTCGT        360

Ele     ------------------------------------------------------------        0
B'      ATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATCTGAAGATCAA        420
```

```
Ele     ------------------------------------------------------------        0
B'      AGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTTAACATTCTAC        480


Ele     ------------------------------------------------------------        0
B'      AGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTTCGAGACTGAT        540


Ele     -------CCCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTATAAATGGATTT        53
B'      CAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTATAAATGGATTT        600
               **************************************************


Ele     AGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT        113
B'      AGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT        660
        ************************************************************


Ele     AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG        173
B'      AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG        720
        ************************************************************


Ele     CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT        233
B'      CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT        780
        ************************************************************


Ele     ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT        293
B'      ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT        840
        ************************************************************


Ele     GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTACCAAGTGTTTA        353
B'      GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTACCAAGTGTTTA        900
        ************************************************************


Ele     AACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAATGAGCAATTN        413
B'      AACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAATGAGCAATTC        960
        ************************************************************


Ele     TGGAAGATGTTATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTAGTGGAAGGGATGGAG        473
B'      TGGAAGATGTTATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTAGTGGAAGGGATGGAG        1020
        ************************************************************
```

```
Ele       AAGAACTTGCAAGGACATTGGAGCAAAAGTGTTAAGCAATTGACGGAGAATGTGAAGGGA       533
B'        AAGAACTTGCAAGGACATTGGAGCAAAAGTGTTAAGGAATTGACGGAGAATGTGAAGGGA       1080
          ********************************** **********************

Ele       ATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCTACAGCTTGAAA-----       588
B'        ATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCTACAGCTTGAAATCCAA       1140
          ***************************************************

Ele       ------------------------------------------------------------       588
B'        GAAGCCAGTGAACGCATAGAAGAGATGAGAAGGAAAGAAATTTGGGAAAAAATTGAAAAT       1200


Ele       ------------     588
B'        GCAGCAATGTGA     1212
```

>Eleojo_short_For_B'phi Short new forward

CAATTATAAATGGATTTAGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTG
CATAAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTGCAAAAAGAGCC
TGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATTACCAATTGCCAGAAGGAAGTTCTGT
TTATCGGTGAATTGGAAGAACTTGTGGAGACTGTTGATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGC
ACCAAAATTACCAAGTGTTTAAACAGTTGGAACTCGCAGGTTGCTGAC

```
CLUSTAL O(1.2.4) multiple sequence alignment


Ele       ------------------------------------------------------------       0
B'        ATGACAAATTTTCTTGATTCTGAGACAGAGGAAATGCTTTCTGTAATATCTTACTGTTCT       60


Ele       ------------------------------------------------------------       0
B'        TTCACTGATCCACAAGAATCCCCTTCACTACAAGATTTAAAGAGGCTAAAACTCATCCAA       120


Ele       ------------------------------------------------------------       0
B'        CTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACTGTCACCCCTT       180


Ele       ------------------------------------------------------------       0
B'        TTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCATTCTGCCGTC       240
```

```
Ele     ------------------------------------------------------------        0
B'      TCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTGGCCACATTTG        300

Ele     ------------------------------------------------------------        0
B'      CAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGAATCGCTTCGT        360

Ele     ------------------------------------------------------------        0
B'      ATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATCTGAAGATCAA        420

Ele     ------------------------------------------------------------        0
B'      AGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTTAACATTCTAC        480

Ele     ------------------------------------------------------------        0
B'      AGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTTCGAGACTGAT        540

Ele     ------------------------------------------CAATTATAAATGGATTT        17
B'      CAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTATAAATGGATTT        600
                                                 *****************

Ele     AGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT        77
B'      AGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT        660
        ************************************************************

Ele     AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG        137
B'      AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG        720
        ************************************************************

Ele     CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT        197
B'      CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT        780
        ************************************************************

Ele     ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT        257
B'      ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT        840
        ************************************************************
```

```
Ele      GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTACCAAGTGTTTA       317
B'       GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTACCAAGTGTTTA       900
         ************************************************************

Ele      AACAGTTGGAACTCGCAGGTTGCTGAC---------------------------------       344
B'       AACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAATGAGCAATTC       960
         *************************

Ele      ------------------------------------------------------------       344
B'       TGGAAGATGTTATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTAGTGGAAGGGATGGAG       1020

Ele      ------------------------------------------------------------       344
B'       AAGAACTTGCAAGGACATTGGAGCAAAAGTGTTAAGGAATTGACGGAGAATGTGAAGGGA       1080

Ele      ------------------------------------------------------------       344
B'       ATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCTACAGCTTGAAATCCAA       1140

Ele      ------------------------------------------------------------       344
B'       GAAGCCAGTGAACGCATAGAAGAGATGAGAAGGAAAGAAATTTGGGAAAAAATTGAAAAT       1200

Ele      ------------     344
B'       GCAGCAATGTGA     1212
```

>Eleojo_short_Rev_B'phi short new reverse

TTTGGTGCACAAAGGCAGGGCAAGTTCCTTGTACAATTGTGGATCAACAGTCTCCACAAGTTCTTCCAATTCAC
CGATAAACAGAACTTCCTTCTGGCAATTGGTAATTGGCCAGTACTTCAATATGCCTCTTATAACAACCTCACCA
AGCTCAGGCTCTTTTTGCACAAACTGAGATACACAATAAGTCAACTGCCTATGATAAACTTGCATCCCTTTTGG
TTTATGCAAAGGGACAAGAACTCTATTCAAGAAAAACTTGTGTTCTTCTTTCAAAGGAACACTAAATCCATTTA
TAATTGTGCCCCATATTTCAAGAAGCTCTCCGATTCCAGGGTGCCTTTGGTC

```
Ele      ------------------------------------------------------------       0
B'       ATGACAAATTTTCTTGATTCTGAGACAGAGGAAATGCTTTCTGTAATATCTTACTGTTCT       60
```

```
Ele     ------------------------------------------------------------        0
B'      TTCACTGATCCACAAGAATCCCCTTCACTACAAGATTTAAAGAGGCTAAAACTCATCCAA        120

Ele     ------------------------------------------------------------        0
B'      CTCCTTTCCATCATCAAGACTCTCATTAGACCACTTGATGATCAAGTACTGTCACCCCTT        180

Ele     ------------------------------------------------------------        0
B'      TTCATAATGTTGTCATCTAATCTTTTTAGGCCTCTCCCTCCACCAATTCATTCTGCCGTC        240

Ele     ------------------------------------------------------------        0
B'      TCAGTATTACTGGATGACGATGATCTTATCAGCAATCCAACACCCTCCTGGCCACATTTG        300

Ele     ------------------------------------------------------------        0
B'      CAAATAGTTTACGACATTTTCCTCAGGATTGTCAGTAGAACAAGTGTTGAATCGCTTCGT        360

Ele     ------------------------------------------------------------        0
B'      ATCTACATAGACCATGCTTTCCTCCTTAGTCTCCTCACGTTGTTCCAATCTGAAGATCAA        420

Ele     ------------------------------------------------------------        0
B'      AGAGAACGCGACAACTTAAAGAATGTGTTCCACAGAATCTATTCAAAGTTAACATTCTAC        480

Ele     ----------------------------------------------------------GAC        3
B'      AGACCATTCATGAGAAAGACTATGCATGATGTATTCTTGCACTATGTTTTCGAGACTGAT        540
                                                                  **

Ele     CAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTATAAATGGATTT        63
B'      CAAAGGCACCCTGGAATCGGAGAGCTTCTTGAAATATGGGGCACAATTATAAATGGATTT        600
        ************************************************************

Ele     AGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT        123
B'      AGTGTTCCTTTGAAAGAAGAACACAAGTTTTTCTTGAATAGAGTTCTTGTCCCTTTGCAT        660
        ************************************************************

Ele     AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG        183
```

```
B'      AAACCAAAAGGGATGCAAGTTTATCATAGGCAGTTGACTTATTGTGTATCTCAGTTTGTG      720
        ************************************************************

Ele     CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT      243
B'      CAAAAAGAGCCTGAGCTTGGTGAGGTTGTTATAAGAGGCATATTGAAGTACTGGCCAATT      780
        ************************************************************

Ele     ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT      303
B'      ACCAATTGCCAGAAGGAAGTTCTGTTTATCGGTGAATTGGAAGAACTTGTGGAGACTGTT      840
        ************************************************************

Ele     GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAA----------------      348
B'      GATCCACAATTGTACAAGGAACTTGCCCTGCCTTTGTGCACCAAAATTACCAAGTGTTTA      900
        *****************************************

Ele     ------------------------------------------------------------      348
B'      AACAGTTGGAACTCGCAGGTTGCTGAACGCGCATTGTATGTGTGGAACAATGAGCAATTC      960


Ele     ------------------------------------------------------------      348
B'      TGGAAGATGTTATCACAAGCAATGGAAGAAGTCTTTCCAGTTCTAGTGGAAGGGATGGAG      1020


Ele     ------------------------------------------------------------      348
B'      AAGAACTTGCAAGGACATTGGAGCAAAAGTGTTAAGGAATTGACGGAGAATGTGAAGGGA      1080


Ele     ------------------------------------------------------------      348
B'      ATGCTGGAAGCTCTAGCACCATTTCTCTATTCCAAGTGCCTTCTACAGCTTGAAATCCAA      1140


Ele     ------------------------------------------------------------      348
B'      GAAGCCAGTGAACGCATAGAAGAGATGAGAAGGAAAGAAATTTGGGAAAAAATTGAAAAT      1200


Ele     ------------    348
B'      GCAGCAATGTGA    1212
```

FIGURE 51: GENE SEQUENCE RESULT AFTER BLASTING IN NCBI AND RESULT PRESENTED IN CLUSTAL OMEGA FORMAT.

6. REFERENCES.

Abogadallah, G. M., M. M. Serag and W. P. Quick (2010). "Fine and coarse regulation of reactive oxygen species in the salt tolerant mutants of barnyard grass and their wild-type parents under salt stress." Physiol Plant **138**(1): 60-73.

Balasubramanian, S. and D. Weigel (2006). "Temperature Induced Flowering in Arabidopsis thaliana." Plant Signal Behav **1**(5): 227-228.

Barford, D., A. K. Das and M.-P. Egloff (1998). "The structure and mechanism of protein phosphatases: insights into catalysis and regulation." Annual review of biophysics and biomolecular structure **27**(1): 133-164.

Charpentier, M., J. Sun, J. Wen, K. S. Mysore and G. E. Oldroyd (2014). "Abscisic acid promotion of arbuscular mycorrhizal colonization requires a component of the PROTEIN PHOSPHATASE 2A complex." Plant physiology **166**(4): 2077-2090.

Chen, S., C. Yin, R. J. Strasser, Govindjee, C. Yang and S. Qiang (2012). "Reactive oxygen species from chloroplasts contribute to 3-acetyl-5-isopropyltetramic acid-induced leaf necrosis of Arabidopsis thaliana." Plant Physiol Biochem **52**: 38-51.

Cho, U. S. and W. Xu (2007). "Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme." Nature **445**(7123): 53.

Conrath, U., G. J. Beckers, V. Flors, P. García-Agustín, G. Jakab, F. Mauch, M.-A. Newman, C. M. Pieterse, B. Poinssot and M. J. Pozo (2006). "Priming: getting ready for battle." Molecular Plant-Microbe Interactions **19**(10): 1062-1071.

Cruz de Carvalho, M. H. (2008). "Drought stress and reactive oxygen species: Production, scavenging and signaling." Plant Signal Behav **3**(3): 156-165.

D'Halluin, K., M. De Block, J. Denecke, J. Janssens, J. Leemans, A. Reynaerts and J. Botterman (1992). "The bar gene as selectable and screenable marker in plant engineering." Methods Enzymol **216**: 415-426.

De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, N. R. Movva, C. Thompson, M. Van Montagu and J. Leemans (1987). "Engineering herbicide resistance in plants by expression of a detoxifying enzyme." The EMBO journal 6(9): 2513-2518.

Farkas, I., V. Dombradi, M. Miskei, L. Szabados and C. Koncz (2007). "Arabidopsis PPP family of serine/threonine phosphatases." Trends in plant science **12**(4): 169-176.

Hanaoka, H., T. Noda, Y. Shirano, T. Kato, H. Hayashi, D. Shibata, S. Tabata and Y. Ohsumi (2002). "Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene." Plant Physiol **129**(3): 1181-1193.

Hardie, D. (1999). "Plant protein serine/threonine kinases: classification and functions." Annual review of plant biology **50**(1): 97-131.

Heather, J. M. and B. Chain (2016). "The sequence of sequencers: The history of sequencing DNA." Genomics **107**(1): 1-8.

Hoagland, D. R. and D. I. Arnon (1950). "The water-culture method for growing plants without soil." Circular. California agricultural experiment station **347**(2nd edit).

Janssens, V. and J. Goris (2001). "Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling." Biochemical Journal **353**(3): 417-439.

Jiang, P., K. Zhang, Z. Ding, Q. He, W. Li, S. Zhu, W. Cheng, K. Zhang and K. Li (2018). "Characterization of a strong and constitutive promoter from the Arabidopsis serine

carboxypeptidase-like gene AtSCPL30 as a potential tool for crop transgenic breeding." BMC Biotechnology **18**(1): 59.

Johanson, U., J. West, C. Lister, S. Michaels, R. Amasino and C. Dean (2000). "Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time." Science **290**(5490): 344-347.

Kang, G. R., H. Y. Song and D. S. Kim (2014). "Toxicity and Effects of the Herbicide Glufosinate-Ammonium (Basta) on the Marine Medaka Oryzias dancena." Fisheries and aquatic sciences **17**(1): 105-113.

Kataya, A. R., B. Heidari, L. Hagen, R. Kommedal, G. Slupphaug and C. Lillo (2015). "Protein phosphatase 2A holoenzyme is targeted to peroxisomes by piggybacking and positively affects peroxisomal β-oxidation." Plant physiology **167**(2): 493-506.

Kataya, A. R., B. Heidari and C. Lillo (2015). "Protein phosphatase 2A regulatory subunits affecting plant innate immunity, energy metabolism, and flowering time–joint functions among B'η subfamily members." Plant signaling & behavior **10**(5): e1026024.

Koehorst-van Putten, H., E. Sudarmonowati, M. Herman, I. Pereira-Bertram, A. Wolters, H. Meima, N. De Vetten, C. Raemakers and R. Visser (2012). "Field testing and exploitation of genetically modified cassava with low-amylose or amylose-free starch in Indonesia." Transgenic research 21(1): 39-50.

Koffler, B. E., N. Luschin-Ebengreuth, E. Stabentheiner, M. Müller and B. Zechmann (2014). "Compartment specific response of antioxidants to drought stress in Arabidopsis." Plant Science **227**: 133-144.

Kong, Q. and C. L. Lin (2010). "Oxidative damage to RNA: mechanisms, consequences, and diseases." Cell Mol Life Sci **67**(11): 1817-1829.

Koornneef, M., C. Hanhart and J. Van der Veen (1991). "A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana." Molecular and General Genetics MGG **229**(1): 57-66.

Kopertekh, L., I. Broer and J. Schiemann (2009). "Developmentally regulated site-specific marker gene excision in transgenic B. napus plants." Plant cell reports 28(7): 1075-1083.

Leason, M., D. Cunliffe, D. Parkin, P. J. Lea and B. J. Miflin (1982). "Inhibition of pea leaf glutamine synthetase by methionine sulphoximine, phosphinothricin and other glutamate analogues." Phytochemistry **21**(4): 855-857.

Luan, S. (2003). "Protein phosphatases in plants." Annual review of plant biology **54**(1): 63-92.
McCourt, P. and C. Benning (2010). "Arabidopsis: A rich harvest 10 years after completion of the genome sequence." The Plant Journal **61**(6): 905-908.

McCourt, P. and C. Benning (2010). "Arabidopsis: A rich harvest 10 years after completion of the genome sequence." The Plant Journal 61(6): 905-908.

McFeeters, R. F., C. O. Chichester and J. R. Whitaker (1971). "Purification and properties of chlorophyllase from Ailanthus altissima (Tree-of-Heaven)." Plant physiology 47(5): 609-618.

Meinke, D. W., J. M. Cherry, C. Dean, S. D. Rounsley and M. Koornneef (1998). "Arabidopsis thaliana: a model plant for genome analysis." Science **282**(5389): 662-682.

Molina, R. and J. Trappe (1984). Mycorrhiza management in bareroot nurseries. Forestry nursery manual: Production of bareroot seedlings, Springer**:** 211-223.

Nada, R. M. (2016). "Novel recombinant binary vectors harbouring Basta (bar) gene as a plant selectable marker for genetic transformation of plants." Physiology and molecular biology of plants **22**(2): 241-251.

Padgette, S. R., K. H. Kolacz, X. Delannay, D. Re, B. LaVallee, C. Tinius, W. Rhodes, Y. Otero, G. Barry and D. Eichholtz (1995). "Development, identification, and characterization of a glyphosate-tolerant soybean line." Crop science 35(5): 1451-1461.

Page, D. R. and U. Grossniklaus (2002). "The art and design of genetic screens: Arabidopsis thaliana." Nature Reviews Genetics **3**(2): 124.

Pieterse, C. M., S. C. Van Wees, J. A. Van Pelt, M. Knoester, R. Laan, H. Gerrits, P. J. Weisbeek and L. C. Van Loon (1998). "A novel signaling pathway controlling induced systemic resistance in Arabidopsis." The Plant Cell **10**(9): 1571-1580.

Reeves, P. H. and G. Coupland (2000). "Response of plant development to environment: control of flowering by daylength and temperature." Current opinion in plant biology **3**(1): 37-42.

Reeves, P. H. and G. Coupland (2001). "Analysis of flowering time control in Arabidopsis by comparison of double and triple mutants." Plant Physiology **126**(3): 1085-1091.

Schwachtje, J., S. Karojet, I. Thormählen, C. Bernholz, S. Kunz, S. Brouwer, M. Schwochow, K. Köhl and J. T. van Dongen (2011). "A naturally associated rhizobacterium of Arabidopsis thaliana induces a starvation-like transcriptional response while promoting growth." PLoS One **6**(12): e29382.

Secko, D. (2003). "Protein phosphorylation: a global regulator of cellular activity." Sci. Creative Quarterly 3.

Serra Camprubí, Q. (2014). "The Role of histone H1 in Alzheimer's disease: the design of a research project."

Strack, S., R. Ruediger, G. Walter, R. K. Dagda, C. A. Barwacz and J. T. Cribbs (2002). "Protein Phosphatase 2A Holoenzyme Assembly IDENTIFICATION OF CONTACTS BETWEEN B-

FAMILY REGULATORY AND SCAFFOLDING A SUBUNITS." Journal of Biological Chemistry **277**(23): 20750-20755.

Todorov, D., E. Karanov, A. Smith and M. Hall (2003). "Chlorophyllase activity and chlorophyll content in wild and mutant plants of Arabidopsis thaliana." Biologia plantarum **46**(1): 125-127.
Weigel, D. and J. Glazebrook (2002). Arabidopsis: a laboratory manual, CSHL Press.

UK, P. A. N. (2001). "Health and environmental impacts of glufosinate ammonium."

Weigel, D. and J. Glazebrook (2002). Arabidopsis: a laboratory manual, CSHL Press.

Xu, Y., Y. Xing, Y. Chen, Y. Chao, Z. Lin, E. Fan, W. Y. Jong, S. Strack, P. D. Jeffrey and Y. Shi (2006). "Structure of the protein phosphatase 2A holoenzyme." Cell **127**(6): 1239-1251.

Zamioudis, C., P. Mastranesti, P. Dhonukshe, I. Blilou and C. M. Pieterse (2013). "Unraveling root developmental programs initiated by beneficial Pseudomonas spp. bacteria." Plant Physiology **162**(1): 304-318.

Zamioudis, C. and C. M. Pieterse (2012). "Modulation of host immunity by beneficial microbes." Molecular Plant-Microbe Interactions **25**(2): 139-150.

Zhong, G.-w., P. Jiang, W.-r. Qiao, Y.-w. Zhang, W.-f. Wei and L. Lu (2014). "Protein phosphatase 2A (PP2A) regulatory subunits ParA and PabA orchestrate septation and conidiation and are essential for PP2A activity in Aspergillus nidulans." Eukaryotic cell **13**(12): 1494-1506.

https://tuitiontube.com/enzymes-phosphorylation-dephosphorylation/

https://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?Dopt=s&uid=84468

http://www.blossombio.com/pdf/products/UG_28951562AA.pdf

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013358_Phire_Plant_Direct_PCR_UG.pdf

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FsuperscriptIV_VILO_master_mix_UG.pdf&title=UHJvdG9jb2w6IFN1cGVyU2NyaXB0IElWIFZJTE8gT

https://www.bioline.com/us/hyperladder-1kb.html

https://www.microsynth.seqlab.de/sample-requirements-578.html