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Molecular Analysis of Arabidopsis thaliana Genes Involved in Stress Response

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Abstract

Arabidopsis thaliana is an excellent model plant to study various plant processes, including plant's response to environment, its interactions with other organisms, etc. In this study I characterized eight *Arabidopsis* genes that are likely to regulate plant's responses to biotic and abiotic stresses. Microarray analysis was done previously in our lab to identify *Arabidopsis* genes that are differentially expressed in response to various biotic and abiotic stresses and several plant hormones. The biotic stresses include bacterial, fungal and viral pathogens (both virulent and avirulent strains), sucking and chewing insects. The abiotic stresses include chemicals that induce variety of oxidative stresses (paraquat, 3AT, mixture of glucose/glucose oxidase, and mixture of xanthine/xantine), wounding, heat, cold, freezing, senescence, drought, flooding and salt. The plant hormones include IAA (auxin), 2,4-D (synthetic auxin), BA (cytokinin), GA (gibberellic acid) , ABA (abscisic acid) , ACC (ethylene precursor), JA (jasmonic acid), BR (brassinosteroid), and SA (salicylic acid). The results of these experiments were used to construct Arabidopsis Stress Microarray Database (ASMD). From this database, eight genes that were considerably up regulated in a number of different stress treatments were identified. To determine the role of these genes in regulating stresses, I constructed transgenic plants that over- or under-express the target genes.

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Acknowledgements

Deciding to become involved with research here at Syracuse University was one of the best choices that I made during my four years as an undergraduate. Learning about science in a place that truly encourages students to 'experience' biology has allowed me to fully extract all that I can out of my education. Jacques Yves Cousteau once asked, "What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on." Dr. Ramesh Raina is a man who is interested in the functionality of nature and is enthusiastic about his work. It has been extremely motivating to be in the company of such an understanding and hard working person. He has given me a forum from which I have constructed a bank of knowledge and experience, for this I will be forever grateful. I am so fortunate to have studied in an environment where eager graduate students and post-doctorate staff are willing to lend a hand and divulge their knowledge. One of these people I would like to thank personally is Aditya Dutta who was given the task of teaching this undergraduate everything she needs to know about molecular biology, helping her to unravel some of the intricacies in the field, and aiding her in the drafting of her thesis. Not only did he do all of these things meticulously, but he became a friend. I am honored to have studied alongside such amazing scientists. Thanks to both of you and the rest of the Raina lab for your support over these last two difficult years.

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Introduction

Learning about the manner in which plants function can provide a variety of information that will lead scientists to the answers of some serious issues plaguing our world today. Plants are of great economic importance and the UN reported that the annual crop loss due to disease was estimated at 100 billion dollars. Theoretically, if scientists could eliminate this loss there would be sufficient food to feed the world.

Arabidopsis thaliana is a model organism in plant biology. It is a small flowering plant. *Arabidopsis* is a member of the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish. It offers important advantages for basic research in genetics and molecular biology. Studies include response to the environment, the manner in which it interacts with other organisms, and its recognition, signaling, and transcriptional regulation. In the year 2000, *Arabidopsis thaliana* became the first plant genome to be sequenced, four years ahead of schedule. *Arabidopsis* has a small genome, only 125 million base pairs which allow researchers to make changes to it in order to study its variety of functions. It has a rapid generation time, about five to six weeks under optimal conditions, and a small chromosome number making it is easy to genetically analyze. *Arabidopsis* produces about ten thousand seeds per plant which is quite a high fecundity. It is very easy to cross and to self fertilize, and it grows well in controlled conditions, be that media plates or soil. Finally, plant transformation in *Arabidopsis* is routine, using *Agrobacterium tumefaciens* to transfer DNA to the plant genome.

To uncover information about *Arabidopsis* signaling it is important to be knowledgeable about what can harm it. The signaling processes of *Arabidopsis thaliana* is contingent upon the pathogen or infiltrate being studied. The term pathogen is derived from the Greek word, pathos, meaning that which produces suffering. It is a biological agent that causes disease or illness to a host.

Pseudomonas syringae is an antagonist bacterium originally isolated from the apple leaf. It causes disease in a wide range of plants and crops and is a model organism in plant pathology. *Pseudomonas syringae,* isolated from the tomato, is the strain used in the lab for this set of experiments.

When a plant is attacked by a pathogen there can be two outcomes. The plants may be resistant or susceptible to the pathogen, which then causes disease. Both these instances involve cell death and activation of defense mechanisms. In the case when the plant is resistant, cell death is localized at the site of infection and defense pathways are strongly induced. In the second case, cell death occurs once disease has set in, *i.e.,* at a later time and after very low levels of defense gene activation has taken place that was not able to overpower the invading pathogen. This leads to eventual death of plant tissue. There are many individual genes called resistance genes that control the plant's resistance (Bent, 1996).

Successful host resistance against pathogen involves identification of an avirulent gene on the pathogen by the resistance gene on the plant. Absence of either of these, leads to non-perception of the pathogen by the host and disease results. Thus, one gene on the pathogen is identified by one gene on the plant,

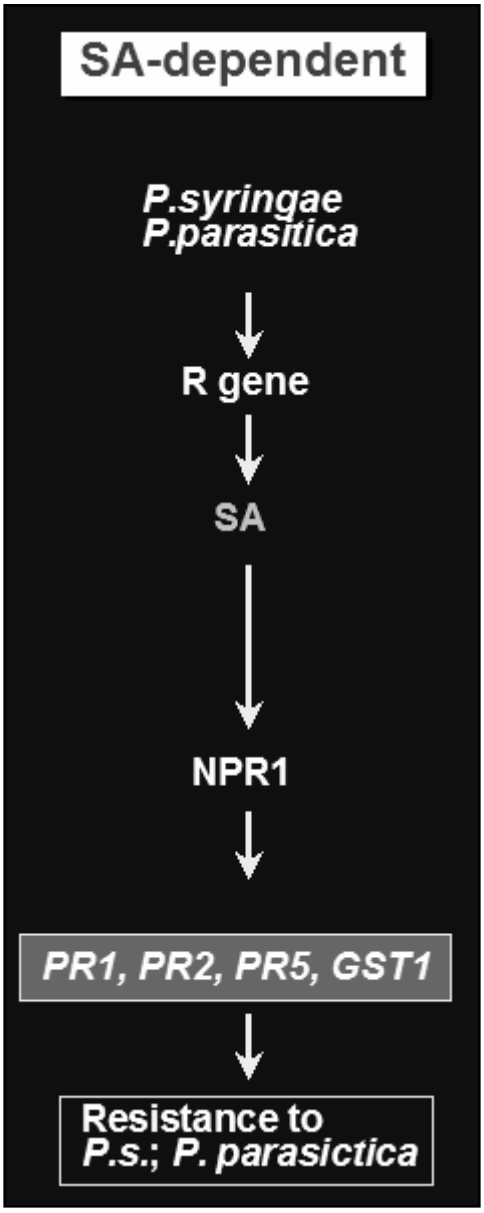
leading to gene-for-gene resistance, which produces an extremely robust defense response.

Plants such as *Arabidopsis thaliana* have two different forms of defense mechanisms. The first is the pre-existing defense which encompasses structural protection and chemical excretions that aid the plant in fighting infection. *Arabidopsis* has a strong cuticle covered in a waxy coating. Some of these excretions change the pH of the plant which doesn't allow the fungus to grow. The second type of defense mechanism is induced defense which includes phenomenon such as the hypersensitive response (HR) and systematic acquired resistance (SAR). Hypersensitive response is an inducible plant defense mechanism characterized by programmed cell death. It works to contain the spread of infection by inducing rapid localized cell death at site of infection. Hypersensitive response is characterized as an incompatible interaction involving an elicitor and a receptor gene product. When HR is induced, systemic acquired resistance (SAR) is established in the tissue that is uninfected (Ryals et al., 1996). Systematic acquired resistance is a mechanism of induced defense that requires salicylic acid (SA). This particular method is currently being explored for crop protection. HR and SAR work together to contain the spread of infection. HR is a fast acting response that notifies SAR, which makes the plant resistant to any further infection.

Three main signaling molecules are involved in bringing about defense against pathogens. These signaling molecules are salicylic acid, jasmonic acid and ethylene, (Figure 1A). The salicylic acid pathway is mediated by salicylic acid

and provides defense against mainly biotrophic organisms. Its marker genes are *PR1* and *PR5*. Ethylene and jasmonic acid work together both synergistically and antagonistically to bring about resistance against necrotrophic organisms and is identified by presence of genes such as *PDF1.2*. *(Figure 1B)*

A.



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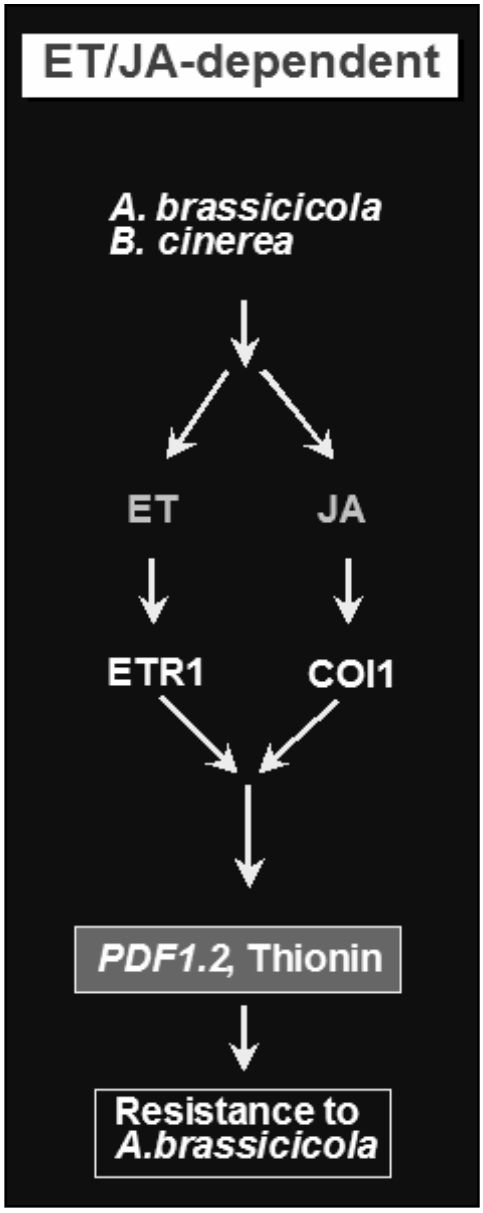

C.

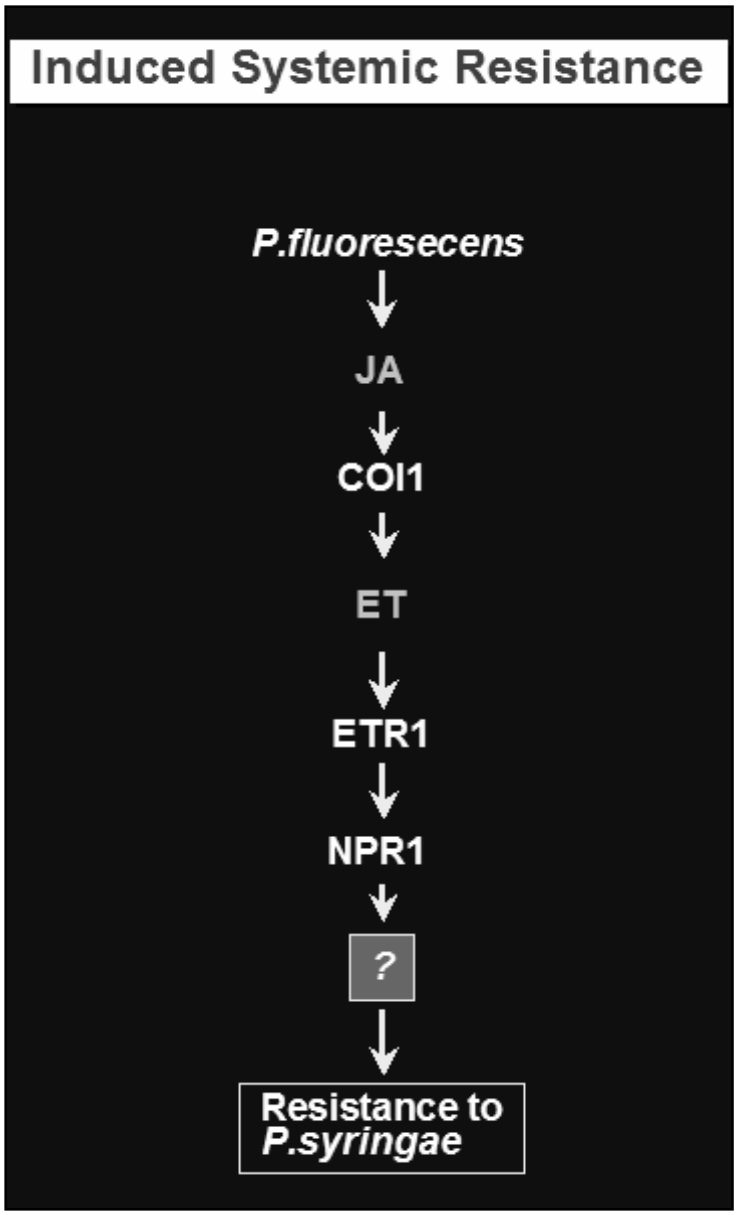

Figure 1: Defense signaling pathways in *Arabidopsis*
A. SA- dependent signaling
B. ET/JA-dependent signaling
C. Induced Systematic Resistance signaling

Other than the two pathways mediated by salicylic acid, ethylene and jasmonic acid a third pathway involved components from both pathways (Figure

1C). This is the induced systemic resistance pathway. It is activated by non-pathogenic bacteria but provides resistance against pathogenic bacteria.

The study of the intricacies of the plant defense mechanism is best understood by understanding the players involved in bringing about this process. To that end the *Arabidopsis* Stress Microarray Database (ASMD) was generated in our lab to study the response of *Arabidopsis thaliana* against biotic stresses like insects, fungi, bacteria and abiotic stresses like temperature, salt, wounding, and senescence. Response against oxidative stress and various defense signaling hormones was also tested. Based on their expression analysis for various stress factors, eight genes were identified for further analysis.

Table 1: MIPS ID and Gene Function

Gene ID	*General Description*
At5g35735	Auxin responsive family protein
At1g27730	Salt tolerance zinc finger protein
At3g07390	Auxin responsive protein
At3g22600	Lipid transfer protein family protein
At4g12230	Thioesterase family protein
At4g25030	Expressed protein (function unknown)
At5g25260	Expressed protein (function unknown)
At5g64310	Arabinogalactan protein

Overexpression and underexpression constructs were made to study role of these genes in the wild type plant. Underexpression analysis utilized both T-DNA insertional mutants and RNAi knockdown of the gene of interest.

Materials and Methods

DNA Isolation

1. Wild type (WT) *Arabidopsis thaliana* plants were grown for approximately 5-6 weeks or until a significant amount of tissue could be collected. Individual pots were labeled and tissue was collected in 14 mL tubes placed in a container filled with liquid nitrogen. Each sample, one at a time, was transferred into ceramic mortars and liquid nitrogen was added. The tissue was ground with the corresponding ceramic pestle until a fine powder remained.

2. One Hundred mg of the ground tissue was transferred in a microfuge tube. 300 µL of Plant DNAZOL® (Invotrogen, Carlsbad, CA) was added to this tube. These contents were mixed and incubated at 25 °C for 5 minutes on a shaker. 300 µL of chloroform was added to this mixture and then vortexed vigorously. The tube was incubated at 25 °C for another 5 minutes on the shaker. The tube was centrifuged at 12000 x g for 10 minutes. The pellet was discarded and the aqueous layer was transferred into a new sterile microfuge tube. 225 µL of 100% ethanol was added to the supernatant and the tube was inverted 6-8 times to mix. It was then incubated at room temperature for 5 minutes and centrifuged at 5000 x g for 4 minutes. The supernatant was discarded and the pellet was kept.

3. One volume of Plant DNAZOL was added to 0.5 volumes of 100% ethanol to create the wash mixture. 300 µL of this mixture was added to the pellet and vortexed. It was then incubated at room temperature for 5 minutes. The tube was centrifuged at 5000 x g for 4 minutes. The liquid wash solution was removed leaving the DNA. 300 µL of 75% ethanol was added to the resulting DNA and it

was vortexed. This sample was centrifuged at 5000 x g for 4 minutes one last time and the supernatant was discarded. The pellet was dried then 50 µL of double distilled water was added.

RNA Isolation

1. Individual pots were labeled and tissue was collected in 14 mL tubes placed in a container filled with liquid nitrogen. Each sample, one at a time, was transferred into ceramic mortars and liquid nitrogen was added. The tissue was ground with the corresponding ceramic pestle until a thin powder remained. 100 mg of the ground tissue was quickly transferred to a microfuge tube and TRIZOL® was added (Invitrogen, Carlsbad, CA). This mixture was incubated at room temperature for 5 minutes. 200 µL of chloroform was added to the incubated sample and it was mixed well for 15 seconds. It was then incubated at room temperature for 3 more minutes and centrifuges at 12000 x g at 4°C for 15 minutes. The aqueous layer was carefully removed and transferred to a new sterile microfuge tube containing 500 µL of isopropyl alcohol. The contents were mixed 2 to 3 times by inverting the tube and the tube was incubated at room temperature for 10 minutes. Later it was centrifuged at 12000 x g at 4 °C for 10 minutes and the supernatant was discarded. The resulting RNA pellet was washed with 75% ethanol 2 times and the ethanol was removed. The pellet was dried and dissolved in 10 µL of RNase-free sterile water. The final product was stored at -80 °C.

RT-PCR

1. Two µg of the RNA was reverse transcribed to cDNA. Each reaction contained 2 µg of total RNA, 1X reaction buffer, 1 mM of dNTP, 2.5 µM hexamer primers, 2 U RNAse inhibitor, and 2 U AMV reverse transcriptase, all adding up to 20 µL. This reaction was incubated at 42°C for 2 hours. Using specific primers and 15 ng of the reverse transcribed cDNA, a 50 µL PCR reaction was performed.

Amplification by PCR

Included in each of the PCR tubes was; DNA, Primer 1, Primer 2, Buffer, dNTP, ddH20, and Red *Taq*™ polymerase.

Table 2: PCR Protocol

PCR STEPS	TIME	TEMPERATURE (°C)
Initial denaturation step	5 min	95
Denaturation	30 sec	94
Annealing	30 sec	60
Extension	1-2 min	72
# of Cycles	25-30 cycles	--
Final Extension	10 min	72
Hold	Forever	10

After the PCR the products were electrophoresed on a 1% agarose gel. These PCR products were stored at -20 °C for future use.

Plant Growth

Arabidopsis plants were grown in soil (metro-Mix 360; Scotts Company, Marysville, OH) in the growth chambers. These chambers create an environment of 25 °C in the day and 23 °C in the night. The chambers also create an area of relative humidity of 60%-70%. The plants are exposed to a photosynthetic photon flux density of 100 to 150 µmol m^2 sec^1 with a 10 hour photoperiod. Soil was autoclaved for 1 hour using 8 L of soil for every 4 L of water.

Cloning in TOPO Vector

Using the pCR2.1 TOPO® TA Cloning Kit the previous PCR products are ligated into the TOPO® vector (Invitrogen, Carlsbad, CA). 3 µL ddH2O, 1 µL PCR product, and 1 µL pCR2.1 TOPO® TA cloning vector are added together in a new sterile tube. It was then incubated at room temperature for 5 minutes.

Digesting using Restriction Enzymes

1. Digestion reaction was carried out in a sterile microfuge tube in a 50 µL using 5 µg plasmid DNA, 5 µL 10X Buffer, and 5-10 units of the restriction enzymes. These enzymes were purchased from New England Biolabs (Beverly, MA). After adding all of the necessary components the reaction was incubated at the appropriate temperature.

2. In order to create blunt-end DNA, the digested DNA was treated with DNA Polymerase I Large Fragment (Klenow) (New England Biolabs, Beverly,

MA). 33 µM of each dNTP and 1 U of Klenow per µg of DNA was added to fill in the 5' overhang post digestion. This was then incubated at 25 °C for 15 minutes. To terminate the reaction, EDTA was added and the sample was heated at 75 °C for 20 minutes.

Purifying DNA Fragment using Agarose Gel

1. A low melting 0.8% agarose gel was poured and the digested DNA was run on it and visualized by Ethidium Bromide staining and UV illumination. Using a razor blade the gel piece containing the DNA was excised with the help of ultraviolet light. This piece of gel was transferred into a sterile microfuge tube which was then incubated in the 65 °C water bath. 0.1 volumes of 5 M NaCl were added to the melted gel with the desired band and it was mixed well. An equal volume of Tris-saturated phenol was added and the tube was mixed again. This extraction process was repeated again and then 2 volumes of ethanol were added to the mixture. The tube was then incubated in -20 °C for 1 hour. It was then centrifuged at 12000 x g at 4 °C for 10 minutes. The supernatant was discarded and to the pellet 70% ethanol was added. The tube was centrifuged at 12000 x g at 4 °C for 2 minutes. The pellet was dried and then resuspended in 10 µL of ddH_2O. The final product was stored at -80 °C.

Isolation of the Plasmid

1. Using the QIAprep Spin Miniprep Kit, (QIAGEN, Valencia, CA), the plasmid DNA was isolated from the *E. coli* cells. 1 mL of the bacterial culture

was transferred to a sterile microfuge tube and centrifuged at 10000 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 250 µL of Buffer P1. This mixture was vortexed and 250 µL of Buffer P2 was added. The contents were mixed by inverting 4-6 times and let to sit at room temperature for 3 minutes. 350 µL of N3 Buffer was added and the contents were mixed by inverting the tube another 4-6 times. It was then centrifuged at 12000 x g for 10 minutes.

2. The supernatant was transferred to a QIAprep Spin Column and centrifuged at 12000 x g for 1 minute. The liquid in the column was decanted and 750 µL of Buffer PE was added to the column. The tube was again centrifuged at 12000 x g for 1 minute and the resulting liquid was decanted. The tube was then centrifuged once more at 12000 x g for 2 minutes and the column was transferred to a new sterile microfuge tube. 50 µL of ddH_2O was added to the center of the column and let to stand for 1 minute. The column that is now in the microfuge tube was then centrifuged at 12000 x g for 1 minute and then discarded leaving the microfuge tube with the plasmid DNA. This tube was stored at -20 °C and the correct insert was confirmed by sequencing.

Cloning in Binary Vector

1. Using a binary vector, pSR3000, the insert DNA was ligated using T4 DNA Ligase (New England Biolabs). Included in each ligation were 50 mM Tris Buffer (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 100 ng vector, 30 ng insert, and 1 U of T4 DNA Ligase. This mixture was incubated at 16 °C for approximately 16

hours. pSR3000 is a 10 kb vector derived from pCAMBIA 2301. It has the *nptII* gene which provides resistance against kanamycin. It has a constitutive strong 2x35S promoter driving transcription of the inserted gene.

E .coli **Transformation**

1. The process of *E. coli* transformation involves the One Shot® TOP10 protocol, (Invitrogen, Carlsbad, CA), and the Cell Porator (Gibco BRL-Life Technologies, Grand Island, NY). *E. coli* Electrocompetent cells were added to the ligation mixture and transformed using by high-voltage electroporation. These transformed cells were plated using glass beads on LB (Luria Bertani) agar plates. These plates also contained necessary antibiotics for selection. The plates were incubated at 37 °C overnight. The following day (24 hours later) colonies were inoculated in 1 mL liquid LB and selected antibiotics. The tubes were placed in the 37 °C shaker overnight. Colony PCR amplification was used to check the clones with correct inserts.

Agrobacterium **Transformation**

1. One µg of the plasmid was transferred to a microfuge tube containing 100 µL of *Agrobacterium* competent cells that were thawed on ice. This combination was quickly placed in liquid Nitrogen for 5 minutes. The tube was then placed in the 37 °C water bath for 25 minutes (the lids open for 5 minutes and the lids closed for 20 minutes). In the hood 1 mL of liquid LB was added to a 14 mL bacteria tube. The cells were transferred to this LB broth and incubated at 28 °C

for 2 hours in the shaker set at 150 rpm. The cells were then centrifuged at 6000 rpm for 5 minutes and the resulting supernatant was discarded and the pellet resuspended in 100 µL of LB. This mixture was plated on LB Agar plates containing the correct antibiotics. The plates were placed in the 28 °C incubator overnight (24 hours). PCR was utilized to confirm that the correct constituents were present in the positive *Agrobacterium* clones. Colonies were selected and inoculated in 1 mL of liquid LB and antibiotics were again added. This inoculation was incubated in the 28 °C shaker for 24 hours.

Plant Transformation

1. To transform the gene of interest into the *Arabidopsis* plant the floral dip method was used (Clough and Bent, 1998). The previously inoculated sample was used to inoculate 100 mL of liquid LB with antibiotics. This new larger mixture was also grown in the 28 °C shaker for 24 hours. The resulting culture was centrifuged at 6000 rmp at 20 °C for 7 minutes The pellet was resuspended in 200 mL 5% sucrose and 0.015% silwet L-77.

2. The flowering Arabidopsis Col-0 plants were dipped in the *Agrobacterium* suspension. The dip included all of the above ground parts of the plant and lasted approximately 30 seconds. The plants were swirled in the liquid, ensuring coverage while being cautious not to break the stem. Plants were placed in the dark room under a cover to maintain high humidity for 24 hours. The plants were then moved to the long-day were they could be exposed to normal growth conditions. “Long-day” and “short-day” refers to the amount of time the plants

are exposed to light in the controlled conditions. "Short-day" is 10 hours of light per day. This is optimal for seedling growth. "Long-day" is 16 hours of light per day. This floral dipping was repeated 2 more times at an interval of 1 week between dips.

Seed Collection and Transgenic Plant Identification

1. When the plants began to produce seeds they were allowed to dry out. It takes about 9 weeks for the seeds to form after the 3rd dip. The seeds were collected and dried out in room temperature for 5 days. The seeds were transferred into 14 mL bacteria tubes and 5 mL of 20% Bleach along with 1 drop of 20% SDS per 10 mL of 20% Bleach was added. This tube was vortexed for 15 minutes and the solution was removed. The seeds were sterilized once more before moving forward. The seeds were washed with 1 mL of ddH_2O 3 times. 1 mL of ddH_2O was added and the seeds underwent cold treatment at 4 °C for 48 hours. The seeds were then resuspended using Top Agar and plated on MS plates containing 1% sucrose and 0.8% agar along with antibiotics (Life Technologies, Grand Island, NY). Transgenic plants began to grow on these plates at about 10 days and they were removed using forceps and placed on soil.

Northern Blotting

DEPC Treated H_2O

1. DEPC treated H_2O was created by adding 2 mL diethylpyrocarbonate (DEPC) per liter of double distilled H_2O in the hood. This was stirred for a

minimum of 6 hours or preferable overnight. It was then autoclaved with stir bar in flask and tape was secured around the opening.

10X MOPS Buffer

2. MOPS Buffer cannot be autoclaved so it must be made with DEPC H_2O. 18.5 g MOPS Na-salt (MW=231.2) was added to 10.7 mL of 3 M Na-acetate and 8 mL of 0.5 M EDTA. The pH was adjusted to 7.0 using NaOH (approximately 5 mL per 800 mL). It was then filter sterilized, covered in aluminum foil and stored at 4 °C.

RNA Formaldehyde Gel

3. 83 mL of DEPC H_2O, 10 mL of 10 X MOPS, and 1.2 g of agarose was mixed together in a flask. The agarose was melted in the microwave and allowed to cool before 7mL of formaldehyde was added to it in the hood. The mixture was poured into trays previously cleaned with 100% ethanol. The ends were taped and combs inserted. The gel took 15 to 20 minutes to solidify. If the gel was not ready to be used it was covered with 1 X MOPS buffer to keep it from drying out. Before removing the combs buffer was added to the gel.

Preparing RNA Samples

4. Seven µL of formamide, 4 µL of formaldehyde, 3 µL of 10 X MOPS Buffer, and 2.5 µL of loading dye were added to each RNA sample that was being held on ice. The samples were boiled for 2 to 3 minutes and then immediately

placed on ice for 3 to 5 minutes. The samples were then briefly centrifuged and loaded on the gel. The gel was run for approximately 2 hours at 100 V.

RNA Gel Transfer

5. The gel was placed in a clean white tray with DEPC H_2O and was allowed to soak for 30 minutes. Filters and membranes were prepared while the gel was soaking. 3 Whatman papers the size of the gel, 1 Whatman paper that is approximately 2 inches longer than the gel at either end, but the same width of the gel, and 1 membrane of the size of the gel. A stack of paper towels were folded and a second white tray was filled with DEPC H_2O.

6. When the gel was ready, the casting tray was inverted in the white tray to make a base. It was then filled with 20 X SSC. The long filter was dipped into the SSC and then positioned on top of the gel box so that the ends hang into the SSC. To remove air bubbles a 10 mL pipette was used to roll along the gel. The gel was carefully flipped over and placed on the Whatman filter paper not allowing it to hang over the edges. It was rolled again with the pipette. Water was added to the membrane and it was placed on top of the gel. Once this had been added the gel should not be moved. Saran wrap was placed around the edges, just barely touching the border of the gel, so that the paper towels did not short circuit the capillary action. The membrane was covered with 3 more small Whatman papers which had been previously placed in DEPC H_2O. Air bubbles were removed if necessary. The stack of paper towels were place on top of the pile and weighted down. This was left alone for 16 to 18 hours.

RNA Destaining and Crosslinking

7. Once the transfer was complete, the membrane was removed using forceps and placed in a clean tray containing DEPC H_2O. This was left for 1 minute. The membrane was then placed face up on a paper towel and crosslinked in the UV crosslinker. The membrane was then placed back in the tray and Methylene blue dye was added. This is left until the bands appeared and then the excess was poured off. With a pencil, my name, the date, and the wells were marked, then a picture was taken. This picture was taken using white reflective light and it had to be done quickly so that the membrane did not dry out. The membrane was placed inside a plastic bag between two pieces of Whatman paper and stored at -80 °C.

Blot Stripping Pre-hybridization/ Hybridization

8. 0.5% SDS was prepared and boiled in the microwave. The boiling SDS was poured over the membrane contained in the white tray and left for 2 to 3 minutes or until it cooled. The SDS was then discarded and added again in the same manner. The SDS was then discarded and the membrane was rolled into a clean hybridization tube for pre-hybridization.

9. Hybridization buffer was placed in the 65 °C water bath to thaw. The membranes were rolled into the tubes and 5 mL of hybridization buffer was added. It was then placed in the oven at 55 °C for 2 hours (max 8 hours).

10. To make the probe the ^{32}P is taken out to thaw. 10-12.5 ng of DNA was added to H_2O to bring the volume up to 16.5 µL. The tubes were boiled for 5

minutes then placed immediately on ice for 5 minutes. The tubes were then quickly centrifuged and the following was added:

Table 3: Hybridization Mixture

Buffer	2.5µL
dNTP's (1µL each of dATP, dTTP, dGTP)	3µL
^{32}PdCTP	2.5µL
Klenow	0.5µL
TOTAL	8.5µL

Tubes were immediately placed on 37 °C heating block for 1 hour. After the 1 hour the heating block was turned up to 95 °C and left for 5 minutes. The samples were then immediately placed on ice and spun briefly. They were then kept on ice.

11. The hybridization buffer was thawed in the 65 °C water bath. The hybridization tube was taken out of the oven and the buffer was decanted. 5 to 10 mL of new buffer was added to the tube and the probe was carefully transferred into it. The tube was capped and was checked for leaks. The tube was then placed in the oven overnight.

Washing of Membranes

12. To wash the membranes two solutions were made:

Table 4: Wash Solution

Solution #1	2 X SSC + 0.2% SDS
Solution #2	0.2 X SSC + 0.2% SDS

Solution #2 was warmed in the 65 °C water bath. The membrane was washed with Solution #1 first because it is less stringent. The tubes were removed from the oven and the hybridization buffer was decanted into the liquid radioactive waste jar. 50 mL of Solution #1 was added and the tube was swirled and decanted. Again, 50 mL of Solution #1 was added and placed in the hybridization oven at 65 °C for 15 minutes. The membrane was then washed with Solution #2 which is a more stringent solution. Solution #1 was decanted and 50 mL of warmed Solution #2 was added. It was then placed in the hybridization oven for 15 minutes. This step was repeated once more.

Film Exposure and Development

13. When the washing was completed the membrane was placed on an old, clean film sheet and wrapped tightly so there were no wrinkles. Tape was used to hold the membrane in place. This had to be done quickly so the membrane would not dry out. The counts were checked to determine the exposure time. The film and the membrane were placed in a cassette. In the darkroom, a piece of film was placed in the cassette and later placed in the -80 °C during exposure.

14. When taking the film out of the cassette, the corner was snipped so that it could be oriented properly later.

Results and Discussion

The degree of progress that I have made in the analysis of the eight selected genes varies from gene to gene. In this section I will summarize how far I have gotten with the analysis of each gene.

For the characterization of each gene I proceeded with reverse genetic analysis where I first identified the gene which is found to be differentially expressed within the plant, given certain biotic and abiotic stresses. For the further characterization of the gene I proceed in two ways. The first is the gain-of-function (overexpression analysis) and the second is loss-of-function (underexpression analysis or gene knockout analysis). The hypothesis is that if a gene is critical for resistance, underexpression of that gene will make the plant more susceptible to the plant pathogen, whereas the overexpression of that same gene will increase the plant's resistance against pathogen attack. The process of obtaining this data is as follows:

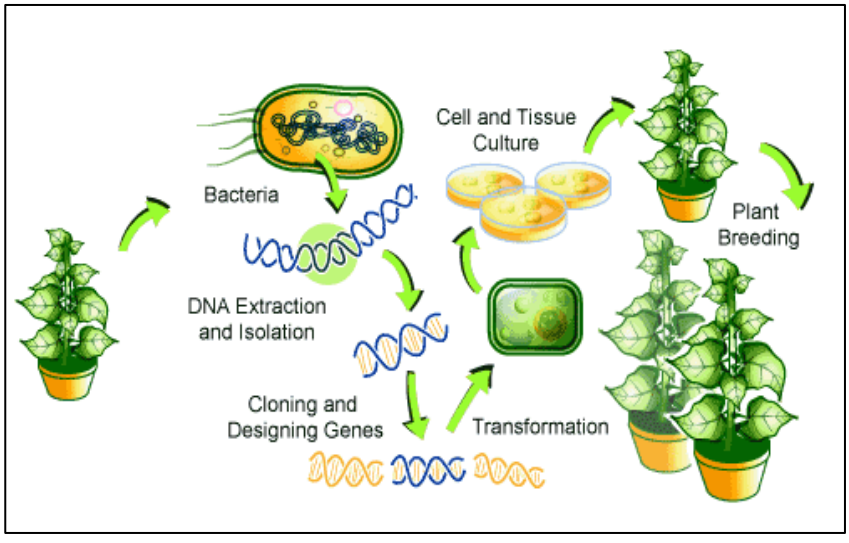

Figure 2: Construction of transgenic plants - General Overview.

Overexpression analysis (Gain-of-function):

For the overexpression analysis I made a construct where the gene was cloned downstream of a constitutively expressed promoter. For my studies I used pSR3000 which has a 35S promoter and multiple cloning sites. Each gene was cloned downstream of this 35S promoter which constitutively expresses the gene of interest. Plants transformed with this construct expressed the gene at higher levels.

Figure 3: Vector used for overexpression of the target gene. The full length gene of interest was amplified by PCR from cDNA and sequenced. This was then cloned into a binary vector (pSR3000).

Underexpression analysis (Loss-of-function):

The function of the gene is either knocked out or it can be expressed at a minimal level. Knockout studies will help us to understand the importance of the presence of these genes in the plant system.

RNAi is a technique where we can guide RNA expression by utilizing double stranded RNA inhibiting the gene expression. The RNAi pathways start when the double stranded RNA is cleaved by dicer enzyme into short double stranded fragments of 20 to 25 bps. One of the two strands, called the guide strand, is incorporated into RNA-induced silencing complex and base pairs with complementary sequence and thereby silences the gene function. The RNAi construct made is driven by the 35S promoter.

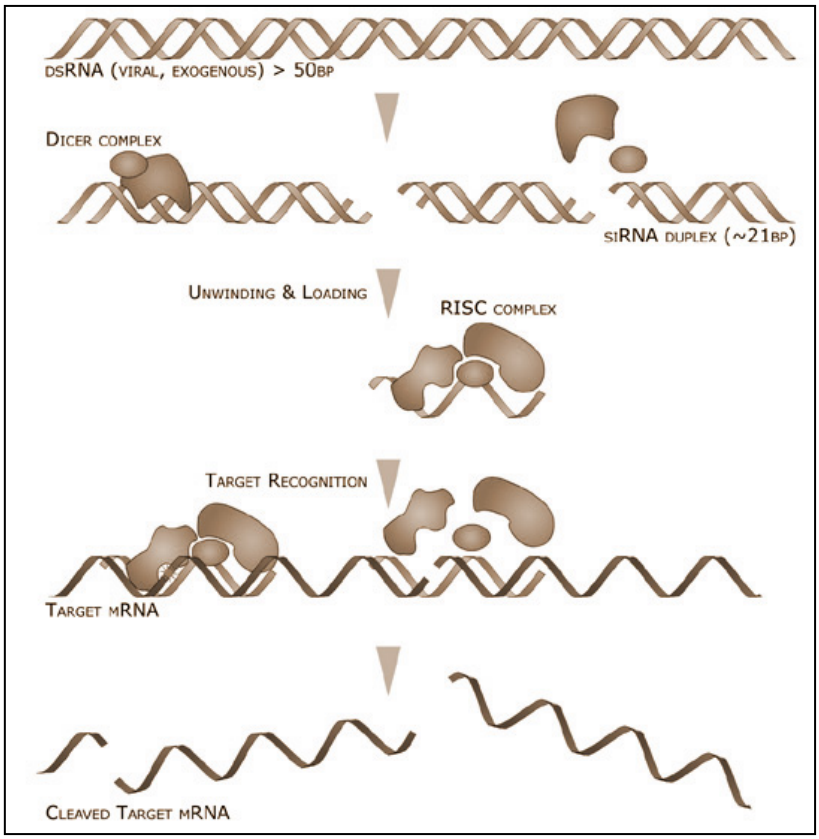

Figure 4: Graphical representation of RNAi mediated knockdown of expression of gene of interest.

Constructs were made as follows to knockdown gene expression via RNAi:

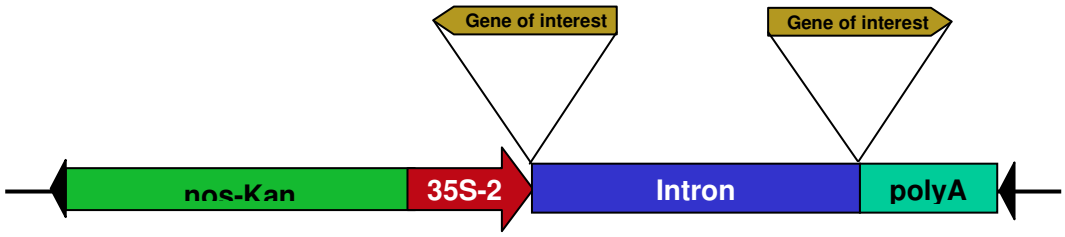

Figure 5: RNAi construct used for underexpression of the target gene ~500 bp 3' fragment of gene of interest was isolated by PCR from cDNA and sequenced. Two copies of the same fragment were cloned in opposite orientation. It is driven by a strong constitutive CaMV 35S promoter.

T-DNA insertion is when a portion of the Ti plasmid is inserted into the genome of the host plant cell. The Ti plasmid is present in agrobacterium tumifasicans. This technique utilizes the natural ability of agrobacterium to insert a piece of DNA into the plant genome upon infection.

Current Project Status

The current status of work for each gene is summarized below. The primer sequences are shown along with the restriction sites that were used in the cloning process. The tables were used to derive important information about each gene before and during the cloning process.

At5g35735:

For overexpression analysis, this gene has been cloned into a full-length T-vector, its sequence has been confirmed, it has been cloned into pSR3000 *(E.coli)*, it has been cloned into agrobacterium, the plant has been transformed, transgenic overexpression lines have been identified and seeds have been collected from four of these lines.

For RNAi analysis, this gene was cloned into a T-vector, its sequence was confirmed, the 1st and 2nd insert was cloned in *E.coli,* it was cloned in agrobacterium, it has been transformed into the plant, and transgenic lines have been selected.

Table 5:

At5g35735 (Gene #1)

Annotated Function	auxin-responsive family protein
Size of Full Length Gene	1215bp
Size of Full Length Protein	405aa
# of Introns	1
# of Exons	2
# of Insertion Lines Available	0

Primer Sequence Used for Amplification:

Overexpression forward primer:

5'AC GGCGCGCC ATG GAC CGA ACA CAA TCT CCA AAG A3'
AscI

Over expression reverse primer:

5'AA ATTTAAAT GTTTAAAC CTA GGC GTC CTG GTG ATG TG3'
SwaI PmeI

RNAi forward primer:

5'TC GGTGACC GGCGCGCC AAG TCT TCG CCG ATC CAA CAT G3'
BstEII AscI

RNAi reverse primer:

5'AA ATTTAAAT GTTTAAAC CTA GGC GTC CTG GTG ATG TG3'
SwaI PmeI

At1g27730:

For overexpression analysis, this gene has been cloned into a full-length T-vector, its sequence has been confirmed, it has been cloned into pSR3000 *(E.coli)*, it has been cloned into agrobacterium, the plant has been transformed, transgenic overexpression lines have been identified and seeds have been collected from four of these lines.

A T-DNA line has been obtained but no futher cloning has been done for this gene.

Table 6:

At1g27730 (Gene #2)	
Annotated Function	salt tolerance zinc finger protein
Size of Full Length Gene	684bp
Size of Full Length Protein	228aa
# of Introns	0
# of Exons	1
# of Insertion Lines Available	7

Primer Sequence Used for Amplification:

Overexpression forward primer:

5'CT GGCGCGCC ATG GCG CTC GAG GCT CTT A3'
AscI

Overexpression reverse primer:

5'AA GGATCC GTTTAAAC TTA AAG TTG AAG TTT GAC CGG AAA G3'
BamHI PmeI

T-DNA:

lines were available from the stock center (SALK_054092). PCR was used to identify knockout lines for this gene.

At3g07390:

For overexpression analysis, this gene has been cloned into a full-length T-vector and its sequence has been confirmed. It will now be cloned into pSR3000 *(E.coli)*.

For RNAi analysis, this gene was cloned into a T-vector, its sequence was confirmed, the 1st and 2nd insert was cloned in *E.coli,* it was cloned in agrobacterium, and it has been transformed into the plant.

Table 7:

At3g07390 (Gene #3)	
Annotated Function	auxin response protein
Size of Full Length Gene	822bp
Size of Full Length Protein	274aa
# of Introns	0
# of Exons	1
# of Insertion Lines Available	1

Primer Sequence Used for Amplification:

Overexpression forward primer:

5'TA GGCGCGCC ATG TCC CTG TGT CTT AAA ATA CCT C3'
AscI

Overexpression reverse primer:

5'AT GGATCC GTTTAAAC TCA GAA AAT AAA AAT AGA ACC CAA
BamHI PmeI
CAA AAC C3'

RNAi forward primer:

5'TC GGTGACC GGCGCGCC GTC GCT TGG GCT ATT AAC CCT A3'
BstEII AscI

RNAi reverse primer:

5'AT GGATCC GTTTAAAC TCA GAA AAT AAA AAT AGA ACC CAA
BamHI PmeI
CAA AAC C3'

At3g22600:

For overexpression analysis, this gene has been cloned into a full-length T-vector and its sequence has been confirmed and it has been cloned into pSR3000 *(E.coli)*.

For RNAi analysis, this gene was cloned into a T-vector, its sequence was confirmed, the 1st and 2nd insert was cloned in *E.coli,* it was cloned in agrobacterium, and it has been transformed into the plant.

Table 8:

At3g22600 (Gene #4)	
Annotated Function	lipid transfer protein family protein
Size of Full Length Gene	513bp
Size of Full Length Protein	171aa
# of Introns	2
# of Exons	3
# of Insertion Lines Available	1

Primer Sequence Used for Amplification:

Overexpression forward primer:

5'TC GGCGCGCC ATG AAA ATG GAA ATG GGT TTA GTG TTC C3'
AscI
Overexpression reverse primer:

5'AT GGATCC GTTTAAAC TCA GAA GAT TGC CAT GTA GGA AAC3'
BamHI SwaI

RNAi forward primer:

5'TC GGTGACC GGCGCGCC GCA TGT CGC CGT GTC TCA A3'
BstEII AscI

RNAi reverse primer:

5'AT GGATCC GTTTAAAC TCA GAA GAT TGC CAT GTA GGA AAC3'
BamHI BstEII

At4g12230:

For overexpression analysis, this gene has been cloned into a full-length T-vector, its sequence has been confirmed, it has been cloned into pSR3000 *(E.coli)*, it has been cloned into agrobacterium, the plant has been transformed, however no transgenic plants were found. Overexpression of this gene is predicted to be lethal.

For RNAi analysis, this gene was cloned into a T-vector, its sequence was confirmed, the 1st and 2nd insert was cloned in *E.coli,* it was cloned in agrobacterium, and it has been transformed into the plant.

Table 9:

At4g12230 (Gene #5)	
Annotated Function	thioesterase family protein
Size of Full Length Gene	1179bp
Size of Full Length Protein	393aa
# of Introns	7
# of Exons	8

# of Insertion Lines Available	4

Primer Sequence Used for Amplification:

Overexpression forward primer:

CC GGCGCGCC ATG AAA GGC GTC TCT TCG ACG
AscI

Overexpression reverse primer:

AA GGATCC GTTTAAAC CTA AAC CAA TTG TCT GGA GAT AAA ACC
BamHI BstEII

RNAi forward primer:

TC GGTGACC GGCGCGCC TGC TTC CGT GGG ATC TCT AC
BstEII AscI

RNAi reverse primer:

AA GGATCC GTTTAAAC CTA AAC CAA TTG TCT GGA GAT AAA ACC
BamHI BstEII

At4g25030:

For overexpression analysis, this gene has been cloned into a full-length T-vector, its sequence has been confirmed, it has been cloned into pSR3000 *(E.coli)*, it has been cloned into agrobacterium, the plant has been transformed, and transgenic plants have been identified.

For RNAi analysis, this gene was cloned into a T-vector, its sequence was confirmed, the 1st and 2nd insert was cloned in *E.coli,* it was cloned in agrobacterium, and it has been transformed into the plant.

Table 10:

At4g25030 (Gene #6)	
Annotated Function	expressed protein (function unknown)

Size of Full Length Gene	1035bp
Size of Full Length Protein	345aa
# of Introns	3
# of Exons	4
# of Insertion Lines Available	2

Primer Sequence Used for Amplification:

Overexpression forward primer:

5'CT GGCGCGCC ATG GAT AAT TGT ACT GGA GAA AAT CCC3'
AscI

Overexpression reverse primer:

5'AA GGATCC GTTTAAAC TTA GCA GAC AAC AGT AGC TGC TC3'
BamHI PmeI

RNAi forward primer:

5'TC GGTGACC GGCGCGCC GCA ACC CCC TCC AGG CAT3'
BstEII BstEII

RNAi reverse primer:

5'AA GGATCC GTTTAAAC TTA GCA GAC AAC AGT AGC TGC TC3'
BamHI PmeI

At5g25260:

For overexpression analysis, this gene has been cloned into a full-length T-vector.

For RNAi analysis, this gene was cloned into a T-vector, its sequence was confirmed, the 1st and 2nd insert was cloned in *E.coli,* it was cloned in agrobacterium, and it has been transformed into the plant.

Table 11:

At5g25260 (Gene #7)	
Annotated Function	expressed protein (function unknown)
Size of Full Length Gene	1392bp
Size of Full Length Protein	464aa
# of Introns	1
# of Exons	2
# of Insertion Lines Available	0

Primer Sequence Used for Amplification:

Overexpression forward primer:

5'GA GGCGCGCC ATG TTC AAG GTT GCA AGA GCA TCA3'
AscI

Overexpression reverse primer:

5'CG GGATCC GTTTAAAC TCA CTT GCT TAG AGT ACC GAT CC3'
BamHI PmeI

RNAi forward primer:

5'TC GGTGACC GGCGCGCC AAA TGA ACG CTT TGA CTC GAA CAG
BstEII AscI
A3'

RNAi reverse primer:

5'CG GGATCC GTTTAAAC TCA CTT GCT TAG AGT ACC GAT CC3'
BamHI PmeI

At5g64310:

For overexpression analysis, this gene has been cloned into a full-length T-vector, its sequence has been confirmed, it has been cloned into pSR3000

(E.coli), it has been cloned into agrobacterium, the plant has been transformed, transgenic overexpression lines have been identified and seeds have been collected from three of these lines.

A T-DNA line has been obtained but no futher cloning has been done for this gene.

Table 12:

At5g64310 (Gene #8)	
Annotated Function	arabinogalactan protein
Size of Full Length CDS	396bp
Size of Full Length Protein	132aa
# of Introns	0
# of Exons	1
# of Insertion Lines Available	3

Primer Sequence Used for Amplification:

Overexpression forward primer:

5'TT GGCGCGCC ATG GCT TTT TCC AAA TCT CTA GTG TTT G3'
AscI

Overexpression reverse primer:

5'GG GGATCC GTTTAAAC CTA GAT AAC CAA AAC GGC AGC AG3'
BamHI PmeI

T-DNA:

Lines available from the stock center (SALK_149861). PCR was used to identify knockout lines for this gene.

cDNAs from the genes were amplified by RT-PCR and ligated into the plasmid TOPO2.1. After confirming that the sequences were correct, the inserts were cut out and ligated into the binary vector pSR3000. Transformants were then checked by PCR to confirm that they had the genes of interest. Figure 6 shows an example of some colony PCR amplifications from samples of representative transformants corresponding to each gene. Each transformant gave a PCR product of the expected size, confirming that they all are the correct transformants.

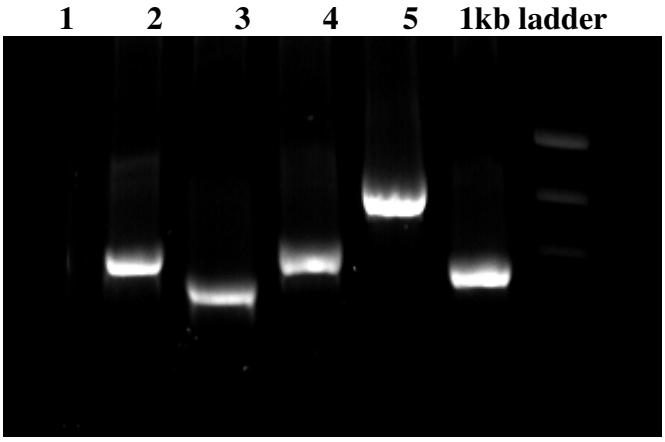

Figure 6: PCR was performed on transformants. This agarose gel picture shows that the 1st insert has been cloned into pSR3000. Lane 1 is gene At3g07390, lane 2 is gene At3g22600, lane 3 is gene At4g12230, lane 4 is At4g25030, and lane 5 is gene At5g25260.

Positive transformants were inoculated overnight in liquid growth media and recombinant plasmids (pSR3000+1st insert) were isolated. Figure 7 shows an example of some isolated recombinant plasmids.

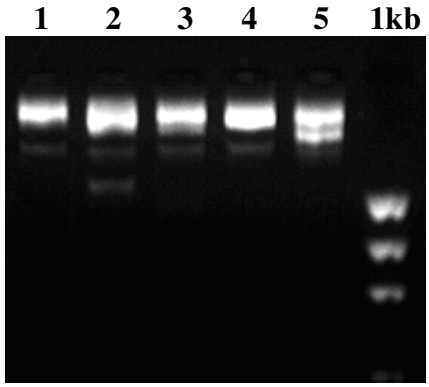

Figure 7: pSR 3000 containing 1st insert was isolated. Lane 1 is gene At3g07390, lane 2 is gene At3g22600, lane 3 is gene At4g12230, lane 4 is At4g25030, and lane 5 is gene At5g25260.

Inserts were cut out from recombinant TOPO2.1 using different enzyme sets. The recombinant vector (pSR3000+1st insert) was also digested with the same enzyme pairs. The digestion mix was purified by phenol-chloroform extraction. Figure 8 shows a representative sample of of inserts and vectors digested and purified for ligation.

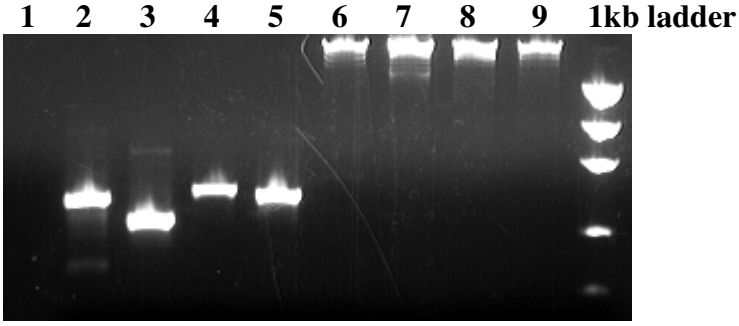

Figure 8: The second insert and vector (pSR3000 + 1st insert) were digested, the samples were purified and this is the resulting gel. 1, 2, 3, and 4 are the digested inserts and 5, 6, 7, and 8 are the digested vectors. Lane 1 and lane 6 are gene At3g07390, lane 2 and lane 7 are gene At3g22600, lane 3 and lane 8 are gene At4g12230, lane 4 and lane 9 are gene At5g25260.

Transformants were then checked by PCR to confirm that they had both 1st and 2nd inserts from genes of interest. Figure 9 shows an example of some colony PCR amplifications from samples of representative transformants corresponding to each gene. Each transformant gave a PCR product of the expected size, confirming that they all are the correct transformants.

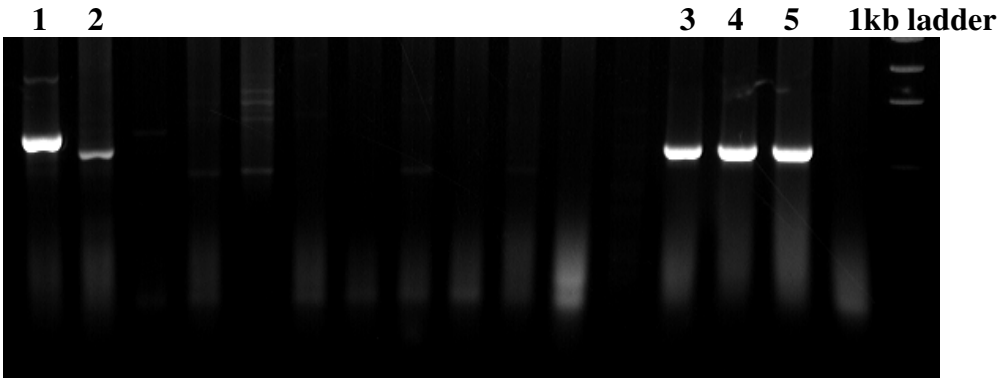

Figure 9: PCR was performed on transformants. This agarose gel picture shows that the 2nd insert has been cloned into pSR3000. 1 and 2 are from the RNAi line from gene At3g07390 and 3, 4, and 5 are from the RNAi line from gene At3g22600.

Recombinant plasmid (pSR3000+1st insert+2nd insert) was isolated from *E.coli* and transformed into *Agrobacterium*. Figure 10 is a representative sample of some of the recombinant plasmid isolated. Transformation into *Agrobacterium* is necessitated because of its ability to transfer DNA into plants.

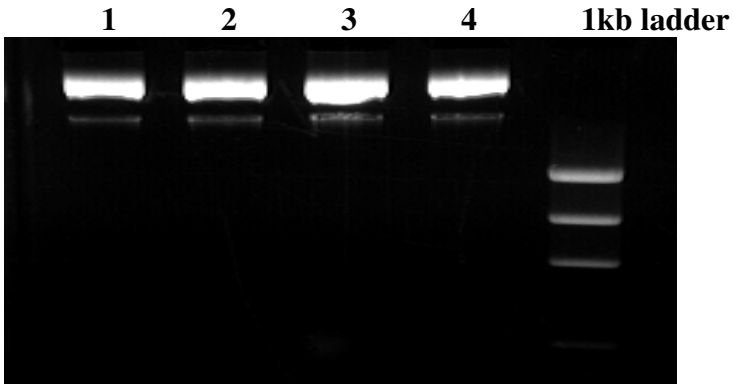

Figure 10: pSR3000 containing 1st and 2nd insert was isolated. Label lanes and write which sample they have. Lane 1 is gene At3g07390, lane 2 is gene At3g22600, lane 3 is gene At4g12230, lane 4 is gene At5g25260.

Agrobacterium transformants were then checked by PCR to confirm that they had both 1st and 2nd inserts from genes of interest. Figure 11 shows an example of some colony PCR amplifications from samples of representative transformants corresponding to each gene. Each transformant gave a PCR product of the expected size, confirming that they all are the correct transformants.

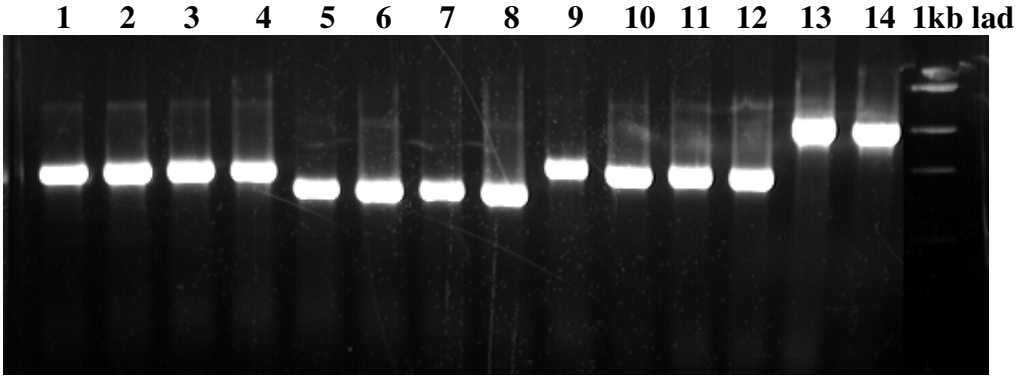

Figure 11: PCR was performed on Agrobacterium transformants. This agarose gel picture shows that the 1st and 2nd insert have been cloned into pSR3000 in Agrobacterium. Lanes 1, 2, 3, and 4 correspond to gene At3g07390, lanes 5, 6, 7, and 8 correspond to gene At3g22600, lanes 9, 10, 11, 12 correspond to gene At4g12230, and lanes 13 and 14 correspond to gene At4g25030.

I have included this table to show the current cloning stage of each gene. Because each gene acts so differently and special materials are available for each, such as T-DNA insertion lines, the genes are often not in the same stage.

Table 13: Current stage in the cloning process

Gene ID	*OX*	*RNAi/ T-DNA*
At5g35735	Seeds have been collected from four of these lines	Transgenic lines have been selected
At1g27730	Seeds have been collected from four of these lines	T-DNA line has been obtained
At3g07390	Sequence has been confirmed	Transformed into the plant
At3g22600	Cloned into pSR3000 *(E.coli)*	Transformed into the plant
At4g12230	No transgenic plants were found	Transformed into the plant
At4g25030	Transgenic plants have been identified	Transformed into the plant
At5g25260	Cloned into a full-length T-vector	Transformed into the plant
At5g64310	Seeds have been collected from three of these lines	T-DNA line has been obtained

During my 2 years of research working with these 8 genes, which were expected to be involved in plant defense, I wanted to study the role of gene function. The way to study is to overexpress and underexpress each gene. Overexpression and underexpression lines were made and these constructs will form the backbone of future studies to decipher the role of each of these genes in the plant. In my research I laid the foundation and now these lines will be taken and studied in detail to measure plant-pathogen interaction.

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