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Characterization of Hypersensitive Response Related Genes of Arabidopsis thaliana

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Abstract

Like most complex living organisms, plants have many mechanisms to prevent disease by microbial pathogens. One of the most important and well developed defense systems that involve recognition, identification and systematic response is the hypersensitive response.

The hypersensitive response is a complex, early defense response against pathogens that causes necrosis and cell death at the site of infection to restrict the spread of pathogen. Hypersensitive response is a type of programmed cell death, and its activation usually happens when the plant recognizes a pathogen through an elicitor. This recognition triggers a series of signal transductions events which end in the expression of several defense-associated genes. The result of those responses causes death of plant cells and the formation of local lesions, creating barriers and hostile environment to inhibit the spread of infection.

The work in our laboratory has identified several hundred *Arabidopsis* genes that are differentially expressed in response to pathogen infection. In addition to regulating defense against pathogens, some of these genes are likely to be involved in regulating other plant physiological processes. To determine the role of these genes in regulating defense against pathogens and other biotic and abiotic stresses, I have analyzed responses to knockout mutants of several of these genes to a variety of biotic and abiotic stresses. My results suggest that many of the pathogen defence-related genes are also involved in regulating other biotic and abiotic stresses.

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Acknowledgement

In the past two and half years under the benefit of Ruth Meyer Undergraduate Research Award, I was very fortunate to have conducted undergraduate research and wrote a senior thesis. During the process I have met many gracious individuals that provided me with support and guidance. I would like to first thank Drs. John Belote and Larry Wolf, for being my honors research advisors, providing the grass-root support for my participation in the honors program. I want to thank Dr. Scott Erdman, for accepting the responsibility for proof reading my thesis. I need to express my utmost gratitude for Drs. Ramesh Raina and Surabhi Raina, for giving me the opportunity to do research in their laboratory. I thank all those who helped me to master the techniques necessary for performing all my experiments. I'm grateful for having so many Post-doctoral researchers and graduate students donating their time and knowledge so that I as a biochemistry student could learn beyond what is taught in the classroom. Needless to say, through the positive environment you collectively created, I've learned so much.

I also need to recognize how the other undergraduate student researchers supported each other so that the whole process of scholastic production was filled with energy and adventure. Through the path made available by the honors program, I was able to broaden the scope of understanding, not only in research field that I was involved in, but also in many other areas. It is very comforting to have so many individuals at ages working under the same roof.

Lastly, I would like to express my gratitude for all the staff working for our soon to be replaced biological research facility. I thank them for making the best use out of all the hardware and research equipment in the building, for making sure all our supplies were delivered promptly. Let me also not forget, that my family supported and gave encouragement for all the things I've done. I'll treasure my experience here and use it to guide my future as a scientist, hoping to benefits for our society.

Introduction

Programmed cell death (PCD) is the active process of cell death, which occurs during development and in response to environmental cues (Breenberg, 1996). PCD is controlled genetically and has characteristic features of the apoptotic cell death in animal cells, such as cell shrinkage, cytoplasmic and nuclear condensation, chromatin condensation, and DNA fragmentation (Vranova et al)

PCD in plants serve three important functions. The first function involves xylogenesis. Xylogenesis is the formation of water and nutrient conducting tubes that's part of the vascular system. The function of PCD in the case of xylogenesis is to create tracheary xylem elements that function as long conducting tubes to bring water from the roots to the rest of the plants and give the plant mechanical support. The second function involves many stages of plant reproduction. In Maize, sex determination involves the selective killings of female reproductive primordia in order for the male floral structures to develop in the tassel. PCD is important in plant senescence. There are two distinct processes for senescence in plants; one involves the aging of various tissues and organs as plant matures, the other involves death of plant tissue (such as flower) that occurs after fertilization. The last function, which is the subject of my work, is involvement of the PCD in pathogen defense. Plants can recognize certain pathogens and activate defenses that result in the limitation of pathogen growth at the site of infection. One dramatic hall-mark of the resistance response is the induction of a localized cell death in the forms of lesion at the site of infection (Breenberg, 1996).

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These defense related responses are called hypersensitive response (HR). HR cell death is a PCD response that bears features of the apoptotic cell death processes that occur in other metazoan organism. The proposed roles for cell death in HR include mass release of antimicrobial enzymes and metabolites into the extracellular matrix, the elimination of a cell that the pathogen is exploiting for life support, and the release of signals that activate defense in neighboring and distant cells. HR cell death can also be a side effect caused by exceptionally strong activation of signaling responses such as ion channel gating or oxidative burst or by the extensive build-up of toxic antimicrobial compounds within the cell (Yu et al, 1998).

Our study concentrates on the genetic basis of HR as it typically occurs in "gene-for-gene" plant-pathogen interactions in which resistance in host is controlled by parasite-specific resistance (*R*) genes that have to be "matched by avirulence (*Avr*) genes in the pathogen. However, many defense responses can also be triggered by bacteria or by fungal or plant products in the absence of cell death. Alternatively, similar responses may accompany cell death caused by known biocides such as heavy metals, by the expression of a bacterial ribonuclease in transgenic plants, or by certain types of physical damage (Heath, 1998). One particular bacterial foliar pathogen, *Pseudomonas syringae*, has been intensively used to determine the molecular and physiological basis of plant–bacterial interactions. In *Arabidopsis* that has *R*-gene-mediated resistance, *P. syringae* can induce HR and stop the growth and spread of the disease causing bacteria. The actual mechanism by which bacterial growth is promoted during

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pathogenesis and restricted during the HR is currently not well understood. However, several well studied model systems for bacterial-plant interactions have been developed based on *P. syringae pv. tomato* strain DC3000 (*Pst* DC3000) and *Arabidopsis*. Pathogenic *Pst* DC3000 can also be converted into avirulent form by introducing one of several avirulence genes (Wright and Beattie, 2004).

In *Arabidopsis*, the accumulation of salicylic acid (SA) is associated with the development of HR and it is a direct inducer of disease resistance. Although SA can interfere with viral replication and systemic movement inside plant tissue and thus can directly suppress spreading through a process unrelated to PCD, SA is known to influence the rate of HR-like lesion formation (Alvare, 2000). A family of receptor-like protein kinases (RLKs) containing cysteine-rich repeats in their extracellular domains are induced by pathogen infection and are beleived to play an important role in eliciting HR. There are more than 400 membrane-associated RLKs that may play roles in plant cell-cell and cell-environment communications (Chen et al, 2004). Figure 1 features a more detailed model of SA signal system.

SA is also known for its involvement in the protection against heat-induced oxidative damage in *Arabidopsis* (Larkindale and Knight, 2002). Heat stress response usually involves the production of specific families of proteins known as heat shock proteins (HSPs). Not much is known about the nature of heat shock responses and their pathways, but there is considerable evidence that oxidative stress induces pathways resulting in accumulation of some HSPs (Dat et

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al., 1998). There is currently, however, no evidence that SA directly induces HSP gene transcription in plants (Larkindale and Knight, 2002).

In addition to heat stress, SA is also responsible for plant response to salt stress, especially in the case of *Arabidopsis* seedling germination. SA increases the oxidative damage generated by NaCl and thus causes seedling lethality. It has also been shown that NaCl treatment does not induce *PR1* gene expression, a marker that frequently accompanies SA accumulation (Borsani et al, 2001).

The idea that oxidative burst might be an integral part of HR immediately arises the importance for finding genes and their expressed proteins that are associated with oxidative stress response. Active oxygen species (AOS) such as superoxide radical, hydrogen peroxide, and hydroxyl radicals are generated in plants at the plasma membrane level or extracellularly in the apoplast. NADPH-dependent oxidase (NADPH oxidase) of plasma membranes has recently been regarded as a source of AOS for the oxidative burst that accompanies pathogen incompatible interactions (Vranova et al, 2002). Hydrogen peroxide has also been shown to play an integral role in signaling cellular responses. In tobacco cells that undergo HR upon incompatible inteaction, strong hydrogen peroxide accumulation occurs. In Arabidopsis, exogenous hydrogen peroxide (>5 mM) initiates an active cell death pathway. Moreover, most evidence in the literature indicates that hydrogen peroxide functions downstream of SA (Vranova et al, 2002). Paraquat (PQ) is known to cause the formation of superoxide anions and singlet oxygens, thus inducing oxidative stress in plant cells (Taji et al, 2004). By

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using PQ to produce oxidative stress we can study the relationship of various genes with HR.

Table 1a shows a list of genes and their available T-DNA insertion lines used for stress treatment. T-DNA insertion lines are available from various source and they can be massively generated using T-DNA (transfer DNA) of *Agrobacterium tumefaciens*. This method allows genes tagging to be massively introduced into desired known DNA sequences to both mutate the gene (knocking them out) and to determine the site of insertion via PCR of the flanking plant DNA (Feldmann, 1991).

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The gene AT1G67360 has very little current literature on any of its possible functions other than that it encodes for a stress-related protein belonging to the rubber elongation factor family (Martinez and Chrispeels, 2003; Wong et al, 2006). It is also upregulated by 12-oxo-phytodienoic acid and mechanical wounding (Taki et al, 2005). Gene AT1G51800 according to its sequence homology could be an endomembrane protein kinase (Osakabe et al, 2005). Gene AT1G26390 might be involved in electron transport system as its sequence homology indicates it is in the endomembrane system and its list of possible functions includes electron carrier activity (Armengaud et al, 2004). AT4G33010 has been shown to be upregulated in salt cress grown at normal conditions (Taji et al, 2004). It has also been identified as a glycine dehydrogenase, possibly involved in the mitochondrion (Rohde et al, 2004). AT3G14210 is predicted to be a myrosinase-associated protein (MyAP) containing a lipase/acel hydrolase-like motif. AT3G14210 is a semidominant quantitative trait locus (QTL) which

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has an epistatic effect on the Epithio specifier gene. It represses nitrile formation and favors isothiocyanate production during glucosinolate hydrolysis (Zhang et al, 2006). Its product is identified as a myrosinase-associated protein (Carter et al, 2004). This gene is also found to be significantly up regulated by *Arabidopsis* bHLH and MYB, which both function as transcriptional activators in abscisic acid signaling (Abe et al, 2002). AT2G45180 encodes a protein that belongs to a protease inhibitor LTP family (Arteaga-Vazquez et al, 2006). It is also one of the most frequently expressed proteins in *Arabidopsis* leaf tissue (Robinson et al, 2004). This gene in the *Arabidopsis* genome also features an ACTCAT sequence in its promoter region, which is a *cis*-acting element for Proline- and Hypo osmolarity-Responsive Expression of the ProDH Gene Encoding Proline Dehydrogenase (Satoh et al, 2002). AT2G40000 is a stress related gene that encodes a putative nematode-resistance protein involved in cell rescue, defense, and virulence. It is moderately expressed in *Arabidopsis* under phosphate starving conditions (Hammond et al, 2003). It is heavily induced following wounding, moderately induced by abscisic acid (ABA), regulated by indole-3-acetic acid (IAA), and up regulated by iron deficiency (Guan and Nothnagel, 2004; Leonhardt et al, 2004; Goda et al, 2004; Colangelo and Guerinot, 2004). AT2G29350 is a gene that encodes a short-chain alcohol dehydrogenase. Studies have shown that it is highly expressed after oviposition by Pierid butterflies and feeding of Phloem-feeding insects (Little et al, 2007; Kempema et al, 2007). Lastly, AT3G01290 is a band 7 protein that is induced during HR. It is also slightly induced by hydrogen peroxide (Davletova et al, 2005).

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Table 1b show the expression profiles of the gene listed in table 1a. This data was retrieved from Genevestigator. The first goal of my research was to determine the role of the genes listed in table 2 in regulating biotic stress caused by *Pseudomonas syringae* and abiotic stresses cause by heat, salt, and PQ. These studies include identification of homozygous T-DNA knockout lines of these genes. These characterizations can then be used to assess the degree of relevance of each gene to HR. These studies should help us better understand the roles of these genes in regulating HR and defense against pathogens and provide directions and ideas for further experiments for characterization of these genes.

Material and Methods

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LB (Luria – Bertani) Broth/Agar

I typically made 400ml of LB broth by mixing 4g of tryptone, 2g of yeast extract, and 4g of NaCl, and bring solution to 400ml of total volume with distilled and deionized water. I made the same LB agar by adding 3.2g of bacto agar. I autoclaved the media solution under high temperature pressure conditions.

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Deleted: . The numerical values representing gene expressions are the quantitative changes in the levels of expression on log scales compared to the genes' expressions under normal conditions. Values greater than 2 are highlighted in red and values less than –2 are highlighted in blue to indicate significant up regulations or down regulations.¶

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MS (Murashige & Skoog) media

I made 400ml of MS agar by mixing 1.73g of MS salts with 4g of sucrose (1% sucrose, can be removed to reduce chances of fungal contamination). I bought the solution close to 400ml with distilled deionized water, with constant stirring, adjusted the pH to 5.7 with NaOH. After pH is properly adjusted, I bought the volume to 400ml and mixed it with 3.2g of bacto agar. I autoclaved the media solution under high temperature pressure conditions.

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Top Agar

I made 1L of top agar by adding 1.5g of bacto agar to 1L of distilled, deionized water. I then autoclaved the solution under high temperature pressure conditions

Growth of *Arabidopsis* Plants

I grown the *Arabidopsis* plants in Metro-Mix 360 soil (Scotts Company in Marysville, Ohio, USA). All growth chambers were set at 23 to 25°C, 60 to 70% relative humidity, and photosynthetic photon flux density (PPFD) of 100-150 µmol m^2 sec^{-1} with a 10h photoperiod.

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Sterilizing Arabidopsis seeds

I mixed the seeds with 10% bleach with SDS added (one drop of 20% SDS per 10ml of bleach). I vortexed the seeds in solution for 30 to 45 minutes. The bleach solution was removed by pipette or by vacuum suction, while working

in a sterile laminar flow hood. I often repeated this step with shorter vortex time to improve sterilization.

I rinsed the seeds with ddH_2O three times. During rinsing cycle the same volume of water as bleach solution was added to each tube containing the seeds. I vortex the tube each time and allow the seeds to settle to the bottom of the tube before removing water. I usually suspended sterilized seeds in top agar medium.

Rapid isolation of Genomic DNA

The tissue samples were collected using the lids of sterile Eppendorf tubes to pinch out uniform sized disks and left in the tube. The issue samples were then macerated using either individual plastic hand Eppendorf tube grinders or an electrical drill fitted with Eppendorf compatible drill bit. The grindings were done in room temperature with the samples immersed in 400µl of extraction buffer (200mM Tris HCl pH7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS). The samples were processed through grindings until there were no visible large chucks of debris, after which the samples were vortexed briefly and left at room temperature until all other samples were processed with the same manner. The extractions were later performed by centrifuging the sample at 13000 rpm for 1 minute. 300µl of the clean supernatant was taken out and transferred to a fresh Eppendorf tube and mixed with 300µl of isopropanol and left at room temperature for 2 minutes. Following centrifugation at 13000 rpm for 5 minutes, the supernatants were decanted and the pellets were left for air drying and dissolved in 50µl of ddH_2O.

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Infiltration for bacterial growth curve and hypersensitive response

The mutants and the wild type plants were grown in the short day growth chamber (8 hours of light followed by 16 hours of dark) till 4-5 weeks. Infiltrate of leaves of 4-5 weeks old plants (total of 5-10 plants/line) were done using 5×10^4 cfu ml^{-1} of virulent strain (*Pst* DC3000 low virulence) and 1×10^5 cfu ml^{-1} for avirulent (*Pst* DC3000 expressing *avrRpm1*). After infiltration, plants were grown in short day growth chamber until bacterial count in these plants was determined.

To extract bacteria from infected leaves, I used a lacerator [what is this???] cut leave disks with diameter of about 5 mm and place them in 1.5ml Eppendorf tubes containing 500µl of 10mM $MgCl_2$ or $MgSO_4$. Using a compatible hand grinder or electric grinder bit, I hand grind the sample briefly then initiated full scale grinding either by continued with hand grinding or used an electric grinder. The grinding is complete once no large chunks of leave tissue were visibly blocking the actions of pipetting. Serial dilution was prepared using the same 10mM $MgCl_2$ or $MgSO_4$.

I did serial dilution by preparing Eppendorf tubes with 900 of 10mM $MgCl_2$ or $MgSO_4$ mixed with 100µl of the dilatants. 100µl of each serial dilution were used for plating. The plates were prepared with LB media and appropriate antibiotics (rifampicin (50 µg/ml) and kanamycin (50 µg/ml)). I used glass beads to evenly spread the bacterial and I incubated the plates at 28°C for 2 days. I counted the number of colonies for the plates that were visually countable.

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Salt stress assay

The seeds were sterilized as described above and approximately 100 seeds (about 2.5mg) were plated on half MS media containing 50mM of NaCl. Plates were incubated in short day growth incubator (8 hours of light and 16 hours of dark) at constant temperature (28°C). Plated were observed regularly for any visible phenotypes.

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Heat stress assay **Keluo: Fix all sections as I did for "Salt stress assay"**

I plated approximately 100 seeds (about 2.5mg) on half MS media. The seeds are sterilized using our method and their growth is conducted in short day growth incubator (8 hours of light and 16 hours of dark) at constant temperature (28°C). I heat treated the seedlings by placing them in dark incubator for 30 minutes at specified temperatures after they were grown for a week and half. I recorded observations for any visible phenotypes at specified days after treatment.

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Oxidative stress assay with PQ

I plated approximately 100 seeds (about 2.5mg) on half MS media containing 0.50µM of PQ. The seeds were sterilized using our method and their growth was conducted in short day growth incubator (8 hours of light and 16 hours of dark) at constant temperature (28°C). I recorded observations for any visible phenotypes periodically.

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PCR analysis and amplifications

The PCR amplifications were carried out using *Red Taq*™ polymerase (Sigma, St Louis, MO, USA). PCR reaction solutions were usually 15 to 20 µl with pre-manufactured PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$), 200µM dNTPs, 2µM of primers, 2U of polymerases, and 1 to 10ng of template DNA. PTC-225 thermocycler (MJ Research, Waltham, MA, USA) were used to program and carry out the PCR cycles. Most PCR programs included 5 minutes of initial denaturation for 5 minutes at 95°C, followed by 3 steps of cycling that were composed of 30 seconds of denaturing at 94°C, 30 seconds of annealing at 50 to 70°C, and 30 seconds to 2 minutes of extension at 72°C. The 3 steps of cycling were used in range from 25 to 35 cycles. The PCR program was terminated by doing a final extension for 10 minutes at 72°C and then held all samples at 10°C until further intervention.

Identification of homozygous T-DNA insertion lines

For a particular T-DNA insertion line, the DNA sequence flanking the T-DNA insertion site was obtained from "SALK Insertion Sequence Database" (http://signal.salk.edu/). Based on this sequence, a set of primers flanking the insertion site that would amplify a 500 to 1000bp sequence were designed. At the same time, I have grown at least 16 plants for each heterozygous line and collected leave tissues at 4 to 5 weeks. I isolated DNA using rapid isolation method and then ran the PCR. I identified homozygous lines by looking for samples that failed to amplify because

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T-DNA insertions made the process impossible. I identified as least two homozygous plants per T-DNA insertion lines and used them to produce more future homozygous lines.

Results and Discussion

T-DNA insertion knockout lines 20-1, 18-1, 14-1, 11-1, 8-1, 7-1, and 4-1, were infiltrated with *Pst* DC3000 and *Pst* DC3000 expressing *avrRpm1* (Table 2). Growth of pathogens was not altered by knocking out AT4G33010, AT3G14210, AT2G45180, AT2G29350, and AT1G51800 genes, suggesting that these genes do not play a critical role in resistance against these pathogens. However, line 8-1 at the forth day post Pst DC3000 infiltration shown significantly decrease in bacterial growth in the plant tissue. These results suggest that gene corresponding

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to line 8-1 is a negative regulator of defense in Arabidopsis. In a separate growth curve analysis, it was confirmed that line 18-1 and 11-1 do not exhibit abnormal Pst DC3000 resistance. Recall that in arabidopsis, due to R-gene-mediated resistance, *P. syringae* can induce HR and stop the growth and spread of the bacterial. Even though according to Genevestigator AT1G67360's expression is not significantly regulated (table 1b) under *P. Syraingae* biotic stress, in this case it is flagged as a gene that might play a role in R-gene-resistance and HR pathway and should be characterized further for its potential monopolistic role. Though Line 4-1 and 11-1's silenced genes are significantly up regulated under *P. Syraingae* biotic stress in normal plants, knocking them out did not appear to have any visible affects on pathogen resistance. [Keluo: This paragraph is very confusing. Please state clearly which lines (and therefore which corresponding genes) showed altered resistance to pathogens and accordingly draw conclusions – critical for resistance, positively regulate defense or negatively regulate defense. If the results wer not clear say so and suggest that these experiments need to be repeated].

Through the quantitative analysis of the growth curve did not provide enough evident to indicate majorities of the lines involved in the experiment behave abnormally during the course of pathogen invasion, the subjective evaluation of the leave tissues told another story. Figure 2 shows the leaves of lines 18-1, 14-1, and 11-1 after infiltration with Pst DC3000 developed significant amount of lesions that could be the result of reduced pathogen resistance. [What does this paragraph mean????].

T-DNA insertion lines 7-1, 8-1, 13-1, 15-1, and 18-1 underwent paraquat (PQ) stress treatment to test the viability of those plants with suppressed expression of AT1G51800, AT1G67360, AT2G40000, AT3G01290, and AT3G14210. AT2G40000's expression is moderately up regulated during oxidative stress conditions (table 1b). It was found when its expression was suppressed as in the case of line 13-1; the plant's growth was significantly retarded by the presence of paraquat compared to the wild type. AT1G67360, AT1G51800, and AT3G01290 are not significantly up regulated for wild type plants under oxidative stress conditions. Their corresponding T-DNA insertion lines 8-1, 7-1, and 15-1 were shown with moderate growth retardation compared with wild type. Not enough concrete evidence is there to support how line 18-1 is affected by the absence of AT3G14210's expression. Figure 3 shows that the growth retardation of line 7-1 and 8-1 were dominated by smaller sized seedlings and lower rate of germination. Those seedlings also appeared less healthy in terms of their color than the wild type that underwent the same treatment. Figure 4 shows more severe states of growth retardation of lines 13-1 and 15-1. In addition to have smaller sized seedlings, lower rate of germination, the germinated seedlings were also dying. Figure 5 shows that line 18-1 did not have significant different growth pattern due to paraquat induced oxidative stress compared to wild type.

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Genes AT2G29350 and AT2G40000 are significantly up regulated during salt stress (table 1b). Their corresponding T-DNA insertions lines 11-1 and 13-1 were tested for NaCl stress tolerance. Table 4 shows that with the absence of

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AT2G29350 and AT2G40000's expressions, neither line was significantly affected in terms of their ability to tolerate NaCl stress compared to wild type (figure 4).

Gene AT1G67360 is significantly up regulated during heat stress (table 1b). Gene AT3G01290, on the other hand, is not significantly regulated (table 1b). Line 8-1 and 15-1 are T-DNA insertion lines for these two genes and they were heat stressed to determine their role in regulating heat stress. These experiments have shown that the absence of these genes can actually increase the heat stress tolerance of both of these lines. The tolerance is more prominent at the 4th day post treatment than at the 7th day post treatment.

The series of biotic and abiotic stress treatment concluded AT1G67360, AT2G40000, AT1G67360, AT1G51800, and AT3G01290 these genes are critical for abiotic stress tolerance in *Arabidopsis*. Their phenotypes strongly support prioritizing these genes for detailed characterization of their role in regulating abiotic stresses in *Arabidopsis*.

My results demonstrate that AT1G67360 plays a critical role in regulating pathogen defense and heat stress. These results should be further tested and experiments should be designed to characterize this gene in more detail to better understand the mechanism of regulating of pathogen and heat stress by this gene.

PQ treatment experiments identified AT2G40000, AT1G67360, and AT1G51800's roles in oxidative stress tolerance. None of these genes were significantly up regulated in response to PQ (table 1b). The absence of AT2G40000's expression was especially prominent as line 13-1's growth was

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significantly retarded under such stress condition compared to the wild type. As a stress related gene, more characterization is required to deduct its roles in HR as it is directly involved with wounding and abscisic acid regulation (Guan and Nothnagel, 2004; Leonhardt et al, 2004). AT1G67360 has been documented for electron carrier activity and it is probably involved in electron transport system (Armengaud et al, 2004). It is more likely that this gene codes a mitochondria membrane protein and is needed for the oxidative reduction process for producing energy (Taji et al, 2004). Based on the predicted function, it is likely that this gene could be involved in the regulating PCD associated with HR. Detailed characterization of this gene should help better understand its role in HR-associated cell death. AT1G51800 is currently only known as a gene coding for a receptor like kinase, its moderate importance in PQ induced oxidative stress requires more study before it can even be reasoned.

Lastly, loss-of-function of AT3G01290indices heat stress tolerance yet lower than normal PQ induced oxidative stress. The expression profile of this gene does not show it is significantly regulated for neither stress. My findings are not very informative in terms of the direction on future research on this gene because at this point SA's relation with HSP gene transcription in plants is not clear. Maybe its role in hydrogen peroxide-mediated stress can be further explored.

In summary, my results have identified some candidate genes that are likely to be key regulators of defense against biotic and abiotic stresses. Additional knockout lines of these need to be analysed to make sure that the

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phenotypes of these knockout lines is not the result of mutation of an unrelated genes. If the results of new knockout line are consistent with my results, these genes can be studied in detail to determine the molecular mechanism of regulation of their activity.

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Appendix

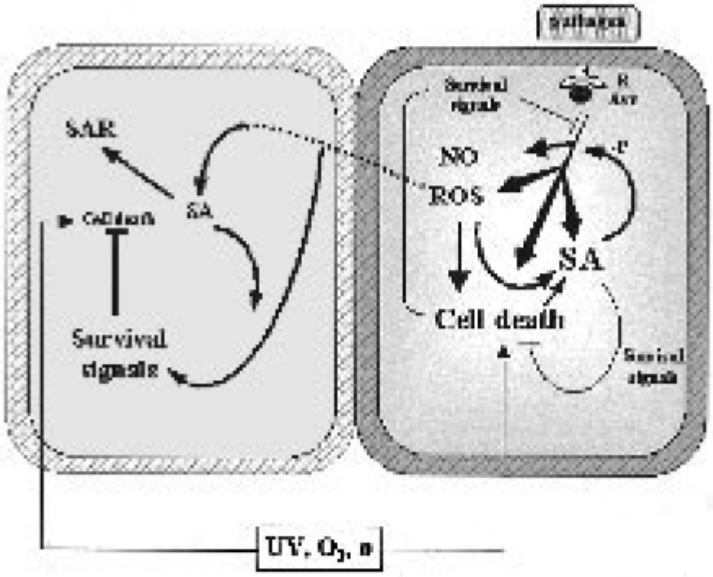

Figure 1. Model of SA function in the HR. The scheme represents two cells at the end stage of HR lesion. The right cell is infected with pathogen and the left cell is uninfected. Suppressor of PCD counteracts the effect of biotic and abiotic injuries promoting cell death. Diffusible signals generated at infected cells induce a direct or indirect increase of SA levels in the area. At this stage SA and reactive oxygen intermediates are at sub-optimal for PCD induction but the cell is pushed to a ready state. Once those cells in ready state perceive the right levels of Avr factor, the R-gene-dependent specific defenses are activated. Accumulating SA up regulates phosphorylation dependent processes that lead to either oxidative burst or phenylpropanoid biosynthesis. It is in this way the SA-dependent feedback loop promotes lesion formation. The uninfected cells are also put into ready state but their PCD signals are maintained below threshold (Chen et al, 2004).

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Table 1a. List of Genes and Their Available T-DNA Insertions Lines Used for Stress Treatment

Line(s)	Locus Name	Line IDs
8-1	AT1G67360	SALK_148081
7-1	AT1G51800	SALK_137388
4-1	AT1G26390	SALK_083228

20-1	AT4G33010	SALK_110091
18-1	AT3G14210	SALK_150833
14-1	AT2G45180	SALK_017818
13-1	AT2G40000	SALK_016065
11-1	AT2G29350	SALK_021619
15-1	AT3G01290	SALK_124393

The T-DNA insertion knock line shown here have been verified to be homozygous and their gene expressions should be completely blocked. These lines were used in the biotic and abiotic stress treatments.

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Table 1b. List of Genes and Their Expression Profile Under Various Stress Treatments

Line(s)	Locus Name	Heat Stress 1	Heat Stress 2	Oxidative Stress	Salt Stress	P. Syringae
8-1	AT1G67360	2.12	2.74	0.06	1.67	0.86
7-1	AT1G51800	-0.38	-3.51	0.06	-0.68	0.61
4-1	AT1G26390	-1.52	-0.79	0.38	0.63	2.78
20-1	AT4G33010	-0.80	0.02	0.06	-0.32	-0.89
18-1	AT3G14210	-0.11	-0.99	0.06	-0.08	-0.88
14-1	AT2G45180	0.15	-2.25	-0.31	-1.09	-0.88
13-1	AT2G40000	0.97	1.23	1.00	2.23	0.45
11-1	AT2G29350	-0.28	-0.16	2.67	3.78	3.58
15-1	AT3G01290	0.15	-0.56	0.53	0.08	-0.16

The expression profiles were retrieved from Genevestigator. The numerical values are the quantitative changes in the levels of expression on log scales compared to normal conditions. Values greater than 2 are highlighted in red and values less than –2 are highlighted in blue.

Deleted: of the T-DNA insertions lines' silenced genes under normal conditions and stress treatments are presented here using tools

Table 2. Pathogen Treatment and Growth of Various T-DNA Insertion Lines

Keluo: Convert these dilutions to one value and report as cfu/leaf disc.

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Pathogen	RPM						
Day after infiltration	0						
Lines	cs	20-1	18-1	14-1	11-1	8-1	7-1	4-1

No dilution	98	121	127	74	76	84	131	159
10 times dilution	0	0	0	0	0	0	0	0
100 times dilution	0	0	0	0	0	0	0	0
1000 times dilution	0	0	0	0	0	0	0	0
10000 times dilution	0	0	0	0	0	0	0	0

Pathogen	DC							
Day after infiltration	0							
Lines	cs	20-1	18-1	14-1	11-1	8-1	7-1	4-1
No dilution	71	120	118	116	123	151	93	89
10 times dilution	0	0	0	0	0	0	0	0
100 times dilution	0	0	0	0	0	0	0	0
1000 times dilution	0	0	0	0	0	0	0	0
10000 times dilution	0	0	0	0	0	0	0	0

Pathogen	RPM							
Day after infiltration	4							
Lines	cs	20-1	18-1	14-1	11-1	8-1	7-1	4-1
No dilution	n/a	n/a	1560	n/a	1564	n/a	n/a	n/a
10 times dilution	2044	1324	166	1908	226	152	1272	864
100 times dilution	168	35	3	4	4	5	13	1
1000 times dilution	6	2	0	0	0	0	0	0
10000 times dilution	0	0	0	0	0	0	0	0

Pathogen	DC							
Day after infiltration	4							
Lines	cs	20-1	18-1	14-1	11-1	8-1	7-1	4-1
No dilution	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
10 times dilution	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
100 times dilution	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
1000 times dilution	2076	1348	N/a	N/a	1236	460	1140	676
10000 times dilution	274	386	783	867	214	52	203	154

T-DNA insertion knockout lines 20-1, 18-1, 14-1, 11-1, 8-1, 7-1, and 4-1, each were subjected to Pst DC3000 low virulence and Pst DC3000 expressing avrRPM1. The colony count is an indicator of bacterial growth. Fields were not available because the colonies were too numerous to count.

Table 2 Continued

Pathogen	RPM							
Day after infiltration	4							
Lines	cs	20-1	18-1	14-1	11-1	8-1	7-1	4-1

No dilution	336	688	167	N/a	444	364	116	386
10 times dilution	43	82	8	956	51	20	17	29
100 times dilution	1	8	1	111	3	2	0	1
1000 times dilution	2	1	0	4	0	0	0	0
10000 times dilution	0	0	0	0	0	0	0	0

Pathogen	DC							
Day after infiltration	4							
Lines	cs	20-1	18-1	14-1	11-1	8-1	7-1	4-1
No dilution	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
10 times dilution	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
100 times dilution	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
1000 times dilution	N/a	N/a	1116	N/a	N/a	564	1512	N/a
10000 times dilution	1776	1228	194	1024	736	49	24	1452

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Table 3. Response of various T-DNA insertion lines to paraquat (oxidative stress)

Line	trail 1	trail 2	trail 3

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7-1	0	+	+
8-1	+	+	+
13-1	+	++	++
15-1	0	+	+
18-1	++	n/a	0

The comparisons are made subjectively based on appearance. Lines were rated sensitive for having smaller, fewer and less healthy looking seedlings, and rated resistant for having bigger, higher level of germination, or healthier looking seedlings compared to wild-type Col-0. +, sensitive: -, resistant; 0, no difference.

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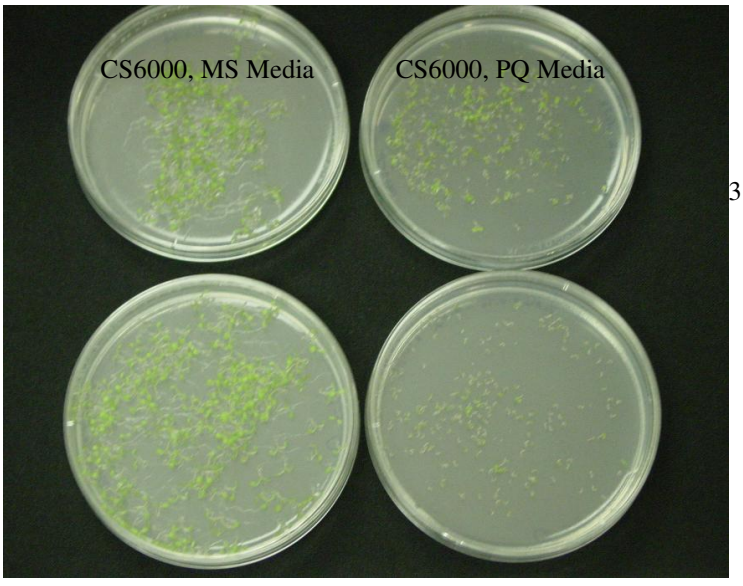

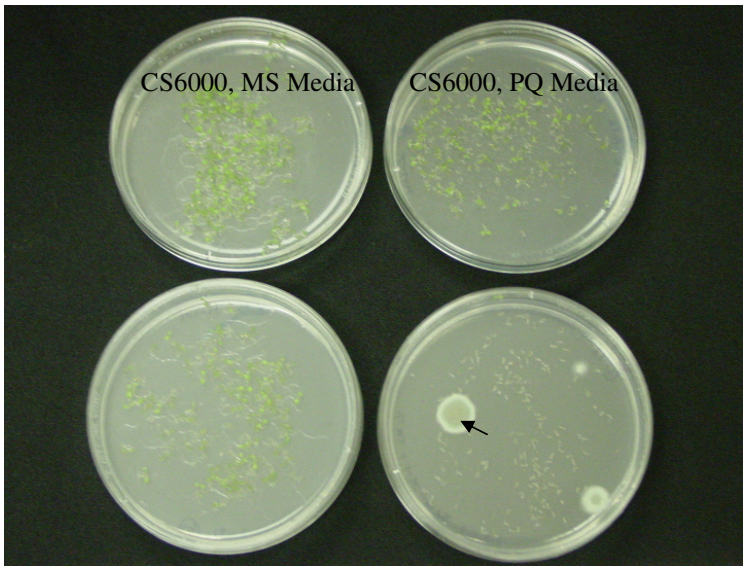

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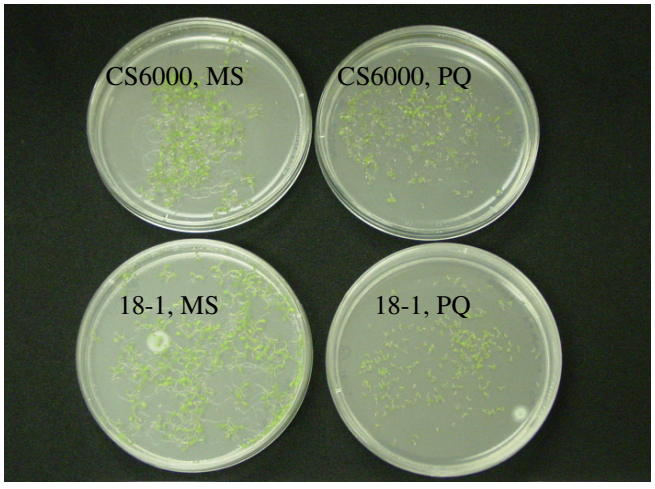

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Table 4. Response of various T-DNA insertion lines to NaCl stress.

Line	Trail 1	Trail 2	Trail 3
11-1	0	0	-
13-1	0	0	0

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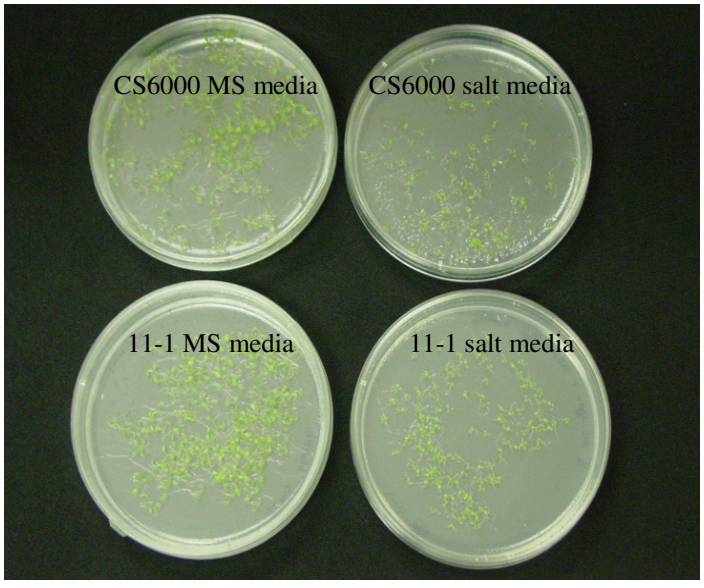
The comparisons are made subjectively based on appearance. Being sensitive means smaller, less number of, or less healthier looking seedlings. Being resistant means bigger, higher level of germination, or healthier looking seedlings. Degree of sensitivity compared to wild type (+ sensitive, - resistant, 0 no difference)



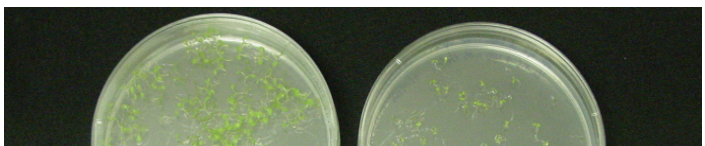


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Table 5. Heat stress Treatment of T-DNA insertion line 8-1 and 15-1

Trial 1			
40℃		45℃	
4 DPHST	7 DPHST	4 DPHST	7 DPHST

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8-1	N/a	--	--	0
15-1	N/a	--	--	0

Trail 2

	45℃	
	4 DPHST	7 DPHST
8-1	--	-
15-1	--	-

Seeding were grown of about 10 days and were heat treated in dark incubator for 30 minutes at indicated temperature. Seedlings were scored after indicated days post heat stress treatment (DPHST).

Lines were rated sensitive for having smaller, fewer and less healthy looking seedlings, and rated resistant for having bigger, higher level of germination, or healthier looking seedlings compared to wild-type Col-0. +, sensitive: -, resistant; 0, no difference.

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ic knockout lines for existing and unstudied genes

	rimer
	CAG ACT CAG CAA TCC AAC CAC C
	CAG ACT CAG CAA TCC AAC CAC C
	AGGAAGAGCTCCTAAAGGAGTTAAAACC
	aa atttaaat ca TTC TCT TCA ACC AAT TTG ATT GGT TC
	TCCGTCACCA ATCTGACTCA
	aaggcgcgccgcaactgt ATGAACAAAA CAAACCC
	TAG TGT GGA TTG TGG TTT GGC ACC
	aaggcgccacaATGGCTGCTAAGAGATCTATCTAC
	aaggcgccacaATGGCTGCTAAGAGATCTATCTAC
	CAG CGA GTC AAC TCG GAA TCA ACC
	CGC TAT ATA TTT TAC GAT TAG TCACAATCA AAA CGG
	CGC ATT TCT CAA TAA TCC GAA ACT GTG TGG
	GTG AAG CAA CCT CTC CAC CTC C
	GTG AAG CAA CCT CTC CAC CTC C
	ACC CCA CAC ACC CTC AGA TC
	aggcgccATGGCATCGGCGGTTCAG
	TGTTAGTGAAAAATACCATGCACGG
	ATG CCA GCA CCA CAT TCG GT
	GGATTGCGGTTCGCCTAGAG

dopsis Biological Resource Center, Ohio. Primers wre designed based on the equence of the T-DNA specific primer was 5'tggttcacgtagtgggccatcg3'. One gene- r was used to determine the site of insertion of T-DNA and genotype of the plant.

tccgatctagtaacatagatg.

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Deleted: Some lines had homozygous lines in the first generation while a few minor ones required us to go to the next generation. The DNA for PCR analysis were taken directly from the leaves and extracted using our rapid extraction

	Annealing Temperature used °C	Homozygous lines obtained	Number of generations grown before Homozygous line is obtained
GTT GGC C	62	TRUE	2
GTT GGC C	62	TRUE	2
CATTGTACATGGC	62	TRUE	2
AGT AGT TGA GAA G	62	TRUE	1
GTTT	62	TRUE	2
GCTTTGATGATC	62	TRUE	1
AAG TAC GGG	62	TRUE	2
ATTGTTGAAGAGAAG	62	TRUE	1
ATTGTTGAAGAGAAG	62	TRUE	1
GAT GTC C	62	TRUE	2
TGA GTA GGA ATT GAA CC	62	TRUE	2
GA CAA TCC C	62	TRUE	2
AAA ATC G	62	FALSE	n/a
AAA ATC G	62	TRUE	1
AA TCA GAC G	62	TRUE	2
TGG	60	TRUE	1
CCTG	60	TRUE	1
GC CAT G	60	FALSE	n/a
G	60	FALSE	n/a