BIOLOGICAL RELEVANCE OF JAZ PROTEINS IN THE INTERACTION BETWEEN *PSEUDOMONAS SYRINGAE* AND *ARABIDOPSIS THALIANA*

by

NISITA OBULAREDDY

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Abstract

BIOLOGICAL RELEVANCE OF JAZ PROTEINS IN THE INTERACTION BETWEEN *PSEUDOMONAS SYRINGAE* AND *ARABIDOPSIS THALIANA*

Nisita Obulareddy, PhD

The University of Texas at Arlington, 2013

Supervising Professor: Maeli Melotto

Stomata, micro-pores on the leaf surface, are formed by a pair of guard cells. In addition to controlling water loss and gas exchange between the plant and the environment, these cells act as immunity gates to prevent pathogen invasion of the plant apoplast. Some Plant pathogens produce virulence factors that enable them to reopen the stomatal immunity gates to favor bacterial entry. *Pseudomonas syringae pv. Tomato* strain DC3000 (*Pst* DC3000) produces a phytotoxin called coronatine that assists pathogen entry via re-opening the closed stomata. Earlier studies report that *Pst* DC3000 regulates a family of genes called *JASMONATE ZIM-DOMAIN* (*JAZ)* genes in COR dependant manner. Hypothesizing that COR regulation of *JAZ* genes is essential for bacterial entry, it is important to understand the molecular happenings at these initial stages of bacterial penetration. Laying emphasis on the above mentioned hypothesis this study provides 1) a brief procedure to obtain highly pure guard cell protoplasts (GCPs) using conditions that preserve the guard cell transcriptome as much as possible for a robust high-throughput RNA sequence analysis. 2) Direct effect of COR, on *JAZ* gene expression in whole leaves and guard cell protoplasts (GCPs). 3) Substantial genetic evidence that the N-terminus of JAZ9 is essential for plant's defense against *Pst* DC3000. This study will contribute to refining the current model of JAZ proteins in the plant cell during pathogen infection.

Preface

Chapter 1 was formatted and published in "*Brazilian journal of medical and biological research*" (2010) 43: 698-704.

Chapter 2 was published in scientific journal "*Molecular Plant-Microbe Interactions*" (2013) 26: 844-849.

Chapter 3 is in preparation for scientific journal "*Molecular Plant-Microbe Interactions*".

Chapter 4 is in preparation for scientific journal "*Plant journal*".

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Chapter1

Biological relevance of JAZ proteins in the interaction between *Pseudomonas syringae* and *Arabidopsis thaliana*

1.1 Introduction

Plants are the dominant sources of food for human beings and animals in the form of vegetables, fruits, cereals, and pulses. It is genuinely important that these economically propitious plants should be safeguarded from certain detrimental factors that can diminish their output. Some of the well-known factors that cause biotic stresses include bacteria, fungi, and pests and those that cause abiotic stresses include drought, temperature, and air pollutants. These factors are known to reduce crop yield in about 36.5% on average, out of which 14.5% decrement in yield is due to plant pathogens (Agrios 2005). With a goal of improving the production and quality of food, plant scientists cogitate over plant-pathogen interaction. In my research, I will focus on interaction between *Arabidopsis thaliana,* commonly called thale cress or Arabidopsis, and *Pseudomonas syringae* - a model pathosystem to understand basic mechanisms of plant pathogen interactions (Whalen et al. 1991).

Arabidopsis is a small flowering plant which is commonly used as a model organism in field of plant research (Rensink and Buell 2004). This plant has some characteristics that make it a better choice in comparison to others. First, Arabidopsis has a very small genome with 157 Mbp (Bennett et al. 2003). Second, its entire genome has been sequenced (The Arabidopsis Genome Initiative, 2000), and finally advanced research information and genetic and genomic resources about this model plant are available at The Arabidopsis Information Resource (TAIR, CA; www.arabidopsis.org, ARBC, OH).

P. syringae, a Gram-negative bacterium is capable of infecting a wide different pathovars (Cuppels, 1986). This was the first pathogen used in the laboratory environment to

infect Arabidopsis and successfully produce disease symptoms (Whalen et al. 1991). The most widely used strains to infect Arabidopsis are *P. syringae* pv*. tomato* strain DC3000 (*Pst* DC3000; Cuppels, 1986) and *P. syringae* pv. *maculicola* ES4326 (Davis et al. 1991). Infected leaves show water soaked patches and form necrotic lesions. These lesions are intermittently encompassed by chlorosis, bleaching/yellowing of plant tissues due to degradation of chlorophyll (Agrios, 1997). Further, the genome of *Pst* DC3000 has been sequenced (Buell et al. 2003) which helps in rapid advances of ongoing research.

P. syringae invades plant through wounds or natural openings called stomata. Stomata are predominantly present on the aerial parts of plant that first appeared at least 420 million years ago (Ruszala et al. 2011) and are possibly a major route for foliar pathogen entry. They have a pair of cells known as guard cells that encompass a pore called stoma which help in regulating the opening/closing of stoma (Swarthout 2008). Carbon dioxide enters the plant through the stomata and is used up for photosynthesis, at the same time oxygen that is released during this process exits through the same openings. Other than physiological functions of stomata, Melotto and others have discovered a novel groundbreaking function of stomata. Through an array of progressive experiments they found that stomata can close in response to live bacteria and/or bacterial motifs called pathogen/microbial associated molecular patterns (PAMPs/MAMPs, Melotto et al. 2006). By definition PAMPs are the molecular motifs of microbes that are recognized by receptors in host called pattern recognition receptors (PRRs; Boller and He, 2009). Some examples of PAMPs are lipopolysaccharide (LPS), bacterial flagellin, and lipoteichoic acid. When leaves or epidermal peels are treated with suspension of *Pst* DC3000/ *Escherichia coli* O157:H7 or PAMPs there is significant variety of plants. Based on the host range, specific strains are differentiated into 50

reduction in number of open stomata, which implies that stomata can restrict entry of foliar pathogens (Melotto et al. 2006). This evinces that plants have certain mechanisms to

restrain the entry of pathogens. Nonetheless, the plant pathogen *Pst* DC3000 is able to overcome this stomatal defense. Unlike *E. coli* O157:H7, *Pst* DC3000 could re-open the stomata indicating that this bacterium has certain virulence factors to overcome stomata based defense (Melotto et al. 2006) indicating an arms race at the initial interface.

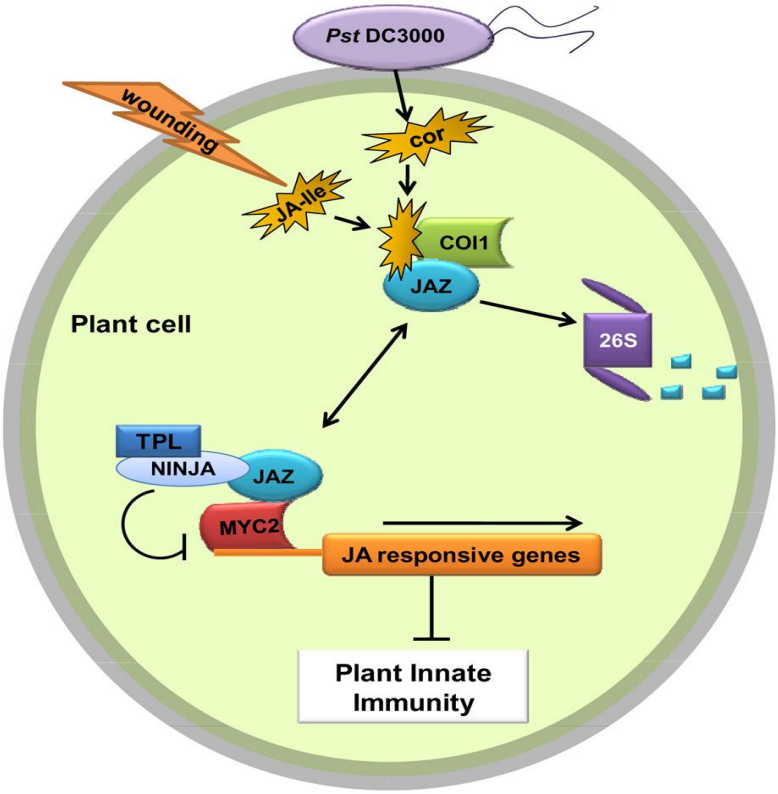
Two prominent virulence factors that are present in *Pst* DC3000 include: 1) the *hrc/hrp* gene encoded TTSS (type three secretion systems), which delivers numerous effector proteins into host cell (Alfano and Collmer 1997, He 1998, Preston 2000), and 2) phytotoxin coronatine (COR; Bender et al. 1999). Mutants of *Pst* DC3000 for both TTSS and COR were studied by Melotto et al. (2006). In their study they found that the *hrc*$^{-}$ mutant *Pst* DC3000, was able to re-open the stomata however a *cor*$^{-}$ mutant of *Pst* DC3000 could not. This fact clearly indicates that COR is the virulence factor responsible for re-opening of the stomata (Melotto et al. 2006).

COR is a phytotoxin produced by different pathovars of *P.syringae* (Bender et al. 1999). It induces modifications in the plant's physiology such as anthocyanin production, alkaloid accumulation, ethylene emission, tendril coiling, and root inhibition (Bender et al. 1999, Feys et al. 1994, Weiler et al. 1994 and Lauchli and Boland, 2003). This toxin acts as a virulence factor and helps in disease development (Bender et al. 1999).

COR requires COI1 to perform its virulence functions (Melotto et al. 2008-B). COI1 is a plant F-box protein (Xie et al. 1998) that interacts with Arabidopsis SKP1-like1, CULLIN1, and RING-BOX PROTEIN1 and forms E3 ubiquitin ligase called SCF^{COI1}complex (Xu et al. 2002, Ren et al. 2005). E3 ubiquitin ligases in plants are involved in ubiquitination, *i.e*. attachment of ubiquitin monomers to proteins that are targeted to the 26S proteosome pathway for degradation by proteolysis. COR consisting of coronafacic acid (CFA) and coronamic acid (CMA) is structurally and functionally similar to jasmonates (JA), especially jasmonyl isoleucine (JA-Ile) (Feys et al. 1994, Greulich et al. 1995, Koda et al. 1992, Weiler et al. 1994).

JA are phytohormones that regulate plant responses to abiotic and biotic stresses. JA are also involved in carbon partitioning, mechanotransduction, senescence, reproductive development, and defense (Devoto and Turner 2003, Farmer et al. 2003, Rojo et al. 2003, Lorenzo and Solano 2005). Given that the expression of defense genes is dependent on JA signaling (Kessler and Baldwin, 2002; Glazebrook, 2005; Browse and Howe, 2008), intensive studies to identify regulation of JA signaling in Arabidopsis has identified eight genes encoding proteins of unknown function that had a very similar sequence structure consisting of three domains; NT domain at the N-terminus, ZIM domain in the middle and Jas domain at the C-terminus. Bioinformatic analysis of the whole Arabidopsis genome revealed a total of 12 genes that encode at least 19 proteins of this family (Thines et al. 2007). As these proteins have a 28 amino acid ZIM domain, and therefore they were named jasmonate ZIM-domain (JAZ) proteins (Thines et al. 2007, Chini et al. 2007).

The existence of 12 different *JAZ* genes in Arabidopsis suggests a functional redundancy which makes it difficult to understand the implication of individual *JAZ* gene in the plant. Constitutive expression of individual *JAZ* genes also does not illustrate any JA associated phenotype (Thines et al. 2007, Chini et al. 2007); however expression of truncated JAZ proteins where the Jas domain is deleted, showed JA-insensitivity and dominant negative phenotype in Arabidopsis. This dominant negative phenotype can also occur as a result of alternative splicing which is evident in JAZ10 (Yan et al. 2007, Chung et al. 2010). These truncations in JAZ proteins have reduced COI1 binding potential, thereby causing dominant JA-insensitive phenotypes.



Baker CM, Chitrakar R, Obulareddy N et al., 2010

Fig1.1 Model for the role of JAZ proteins in plant cell. In absence of JA-Ile or COR, Jas domain of JAZ proteins binds to MYC2 and inhibits the JA signaling. In presence of JA-Ile and COR, the JAZ proteins bind to COI1, and get degraded through 26S proteasome pathway. Once JAZ proteins are degraded, MYC2 enhances the transcription of JA responsive genes, and JA signaling is therefore induced. Copyright for this figure as stated "The Brazilian Journal of Medical and Biological Research (BJMBR) applies the Creative Commons Attribution License (CCAL) to all works published (read the human-readable summary or the full license legal code). Under the CCAL, authors retain ownership of the copyright for their article and can allow anyone to download, reuse, reprint, modify, distribute, and/or copy articles published in the BJMBR, as long as the original authors and source are cited. No permission is required from the authors or the publishers."

The interaction potential of Jas domain of JAZ proteins is extensive across a wide range of proteins that have crucial role in diverse hormone defense pathways. Some of the key interactions include transcriptional factors like MYC2, 3, and 4 which bind to promoters of JA responsive genes and drive their expression (Cheng et al. 2011, Fernández-Calvo et al.

2011, Niu et al. 2011, Chini et al. 2007, Chico et al. 2008). The Jas domain also interacts with ethylene signaling transcription factors EIN3 and EIL (Zhu et al. 2011). In addition to the C-terminus interactions, the ZIM domain and N-terminus binding proteins have also been identified thus building an intricate network of JAZ proteins. The N-terminus of JAZ1, 3 and 9 are known to interact with HISTONE DEACETYLASE 6 (HDA6; Zhu et al. 2011). Moreover N-terminus along with C-terminus are known to interact with gibberellic acid signaling regulators like RGA – a well characterized DELLA protein that competes with MYC2 to bind to JAZs (Hou et al. 2011). Keeping the complex system of JAZ proteins in mind, I focus on integrating the defense related JAZ functions with COR-regulated stomatal innate immunity. Limited understanding about the mode of action of COR with respect to JAZ proteins and JA signaling is known in guard cells, which will be discussed in brief further.

In presence of COR, JAZ proteins that are bound to MYC2 release and bind to COI1 (Melotto et al. 2008-B) thereby causing degradation of JAZ proteins which in turn up-regulates JA signaling (Fig 1.1 Baker et al). The up-regulation of JA signaling pathway antagonizes two critical signaling components in plant defense called salicylic acid (SA) and abscicic acid (ABA) that induce stomatal closure (Melotto et al. 2006) and are induced in response to recognition of PAMPs (Peterson et al. 2000, Anderson et al. 2004, Brooks et al. 2004, Glazebrook 2005; Lorenzo and Solano 2005; Laurie-Berry et al. 2006, Li et al. 2006). One assumption could be that COR is indirectly responsible for the inability of a plant to recognize PAMPs by up-regulating the JA signaling pathway (Geng et al. 2012). Based on these facts, Melotto and associates hypothesized that *P. syringae* utilizes COR to exploit the antagonism between JA, SA, and ABA pathways or any one of them to inhibit stomatal closure (Melotto et al., 2008-A). Therefore, to better understand how COR induces re-opening of PAMP-closed stomata, it is important to identify the role of COR-regulated *JAZ* genes and COI1 in guard cells, forming the basis of this study.

1.2 Research Goal

1.2.1 Determine biological the relevance of JAZ proteins in stomatal immunity

To understand COR mediated *JAZ* gene regulation in guard cells, isolation of healthy guard cell protoplast (GCPs) is crucial. GCP isolations have been documented in studies done by Pandey et al. (2002) and Leonhardt et al. (2004). However, knowledge of the quality of RNA extracted from guard cells using both approaches is fragmented; therefore a better procedure for GCP isolation and RNA extraction was determined in my study. Subsequently, the biological relevance of the interaction between COI1 and JAZ proteins was assessed in guard cells to understand the mechanism of COR action in re-opening the stomata. For those *JAZ* genes expressed in guard cells, I characterized JAZ knock-out and over-expressing plant lines to study the stomatal immune response to *Pst* DC3000 infection.

1.3 Specific Objectives

1.3.1 Develop an approach for efficient RNA extraction from GCP.

1.3.2 Report unique *JAZ* gene regulation by COR in guard cells.

1.3.2 Structure and Function Analysis of JAZ proteins in Arabidopsis.

The above-mentioned first objective is explained in detail in chapter 2 under the title “Guard Cell Purification and RNA Isolation Suitable for High Throughput Transcriptional Analysis of Cell-Type Responses to Biotic Stresses” (Obulareddy et al., 2013). The second objective is explained in chapter 3 entitled “Regulation of *JAZ* Gene Expression in Guard Cells of Arabidopsis by the Phytotoxin Coronatine”. The third objective is discussed in chapter 4 entitled “Structure and Function Analysis of JAZ protiens in Arabidopsis”.

Chapter 2

Guard Cell Purification and RNA Isolation Suitable for High Throughput Transcriptional Analysis of Cell-Type Responses to Biotic Stresses

2.1 Abstract

Stomata, micro-pores on the leaf surface, are formed by a pair of guard cells. In addition to controlling water loss and gas exchange between the plant and the environment, these cells act as immunity gates to prevent pathogen invasion of the plant apoplast. Here, I report a brief procedure to obtain highly pure guard cell preparations using conditions that preserve the guard cell transcriptome as much as possible for a robust high-throughput RNA sequence analysis. The advantages of this procedure included i) substantial shortening of the time required for obtaining high yield of >97% pure guard cell protoplasts (GCP), ii) extraction of enough high quality RNA for direct sequencing, and iii) limited RNA decay during sample manipulation. Gene expression analysis by reverse transcription quantitative polymerase chain reaction revealed that wound-related genes were not induced during release of guard cells from leaves. The optimized GCP isolation and RNA extraction protocols are simple, reproducible, and fast, allowing the discovery of genes and regulatory networks inherent to the guard cells under various stresses.

2.2 Introduction

Guard cells are highly specialized type of cells that surround natural pores on the leaf epidermis forming structures called stomata. The primary function of the stomata is to control gas exchange (CO_2 and O_2) between the leaf interior and the environment and, at the same time, control leaf water loss through transpiration. Thus, the guard cell controls stomatal movement (opening and closure) in response to external (*e.g.* light, temperature, relative humidity) and internal (*e.g*. endogenous hormones) stimuli. More recently, another important

function of the guard cell was discovered; it can sense and respond to epiphytic microbes and protect the leaf against microbial invasion by closing the stomatal pore (Melotto et al. 2006; Gudesblat et al. 2009; Schellenberg et al. 2010). This phenomenon has been defined as stomatal immunity as it requires well known molecular components of the plant innate immune system including the flagellin receptor FLS2 (reviewed by Zeng et al. 2010).

Some of the downstream molecular processes in the guard cell after microbe recognition are somewhat overlapping with the ones associated with abiotic stress. For instance, synthesis and signaling of the plant hormone abscisic acid (ABA) are required for stomatal closure in response to drought stress (Schroeder et al. 2001) and are also linked to stomatal immunity (Melotto et al. 2006). Because the guard cells respond to several external factors that can simultaneously stimulate them, it is important to dissect the molecular mechanism(s) underlying these responses. The guard cell is autonomous making it a useful model to understand cell type responses to stresses.

Procedures to isolate guard cell protoplasts (GCPs) for western blotting, reverse transcription polymerase chain reaction (RT-PCR), microarray analysis, and electrophysiological studies have been previously reported (Pandey et al. 2002, Leonhardt et al. 2004). With the advent of novel high throughput methods such as direct RNA sequencing (RNA-seq), the quantity, quality, and differential decay of RNA molecules, as well as preservation of whole cell transcriptomes during protoplasting are critical to the success of functional studies. Two important modifications of the traditional protoplasting procedure (Pandey et al. 2002) have been devised. In one modification, transcription inhibitors were added during complete digestion of the cell wall to avoid induction of stress-related genes (Leonhardt et al. 2004). However, the long procedure (>5 h) to release guard cell protoplasts may lead to RNA decay. In another modification of the procedure, a partial cell wall digestion with 1h of incubation was performed, in which intact guard cells were still attached to the epidermal tissue (Pandey et al. 2010). Although this short procedure may alleviate extensive

RNA decay, stress-related genes such as wounding, can still be induced in a very short period of time (Chung et al. 2008). Wounding response can occur when leaves are blended to release the epidermis

Because the understanding of stomatal immunity involves the identification of transcripts associated with biotic stress and its regulatory processes, it is essential to demonstrate that guard cell protoplasting procedure does not alter the cell's transcriptome.

Therefore, I sought to develop a protocol for obtaining RNA from guard cell protoplasts that is useful for high throughput RNA sequencing. The newly devised method had the following advantages: (1) it shortened the overall procedure from 6 to 2 hours, while maintaining the purity and yield of isolated guard cell preparations; (2) it increased the amount of RNA extracted by two to three fold independent of the extraction method; and (3) it increased the recovery of short-lived transcripts that might be associated with early stages of biotic stress.

2.3 Material and Methods

2.3.1 Plant material and growth conditions.

Arabidopsis thaliana (L. Heyhn.) ecotype Columbia (Col-0, ABRC stock CS60000) seeds were sown in a 1:1:1 (v:v:v) mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro), fine vermiculite, and perlite (Hummert International, Earth City, MO) and grown in controlled environmental chambers at 22^0C, 65±5% relative humidity (RH), and a 12-h photoperiod under light intensity of 100 $\mu mol.m^{-2}.s^{-1}$. Four- to five-week old plants were used for all experiments.

2.3.2 Guard cell protoplast isolation.

Guard cell protoplasts (GCP) were isolated from the second and third layers of rosette leaves using the same solutions described by Leonhardt et al. (2004) in the presence or absence of the transcription inhibitors actinomycin D (Sigma, St. Louis, MO) and cordycepin (Sigma). The complete protocol, chemical concentrations, and variations in the

incubation times are depicted in the Fig. 2.2 Purity and yield of GCPs were determined by observing cells under Nikon Eclipse 80i fluorescent microscope (Nikon Corporations, Tokyo, Japan) equipped with a digital camera. Cells counts were obtained by using a Petroff Hausser counting chamber (Hausser Scientific, Horsham, PA) using the equation: Total cell number = number of cells counts X dilution factor X 50,000, where 50,000 corresponds to cell depth x cell volume. A minimum of 500 cells were counted for each sample. GCP suspensions with purity >97% were centrifuged at 1000 x g for 5 min at room temperature and flash frozen in liquid nitrogen for subsequent RNA extraction. A minimum of two biological replicates were performed for each variation of the method and all GCP isolations were performed at 2-3 hours after the lights were turned on in the morning.

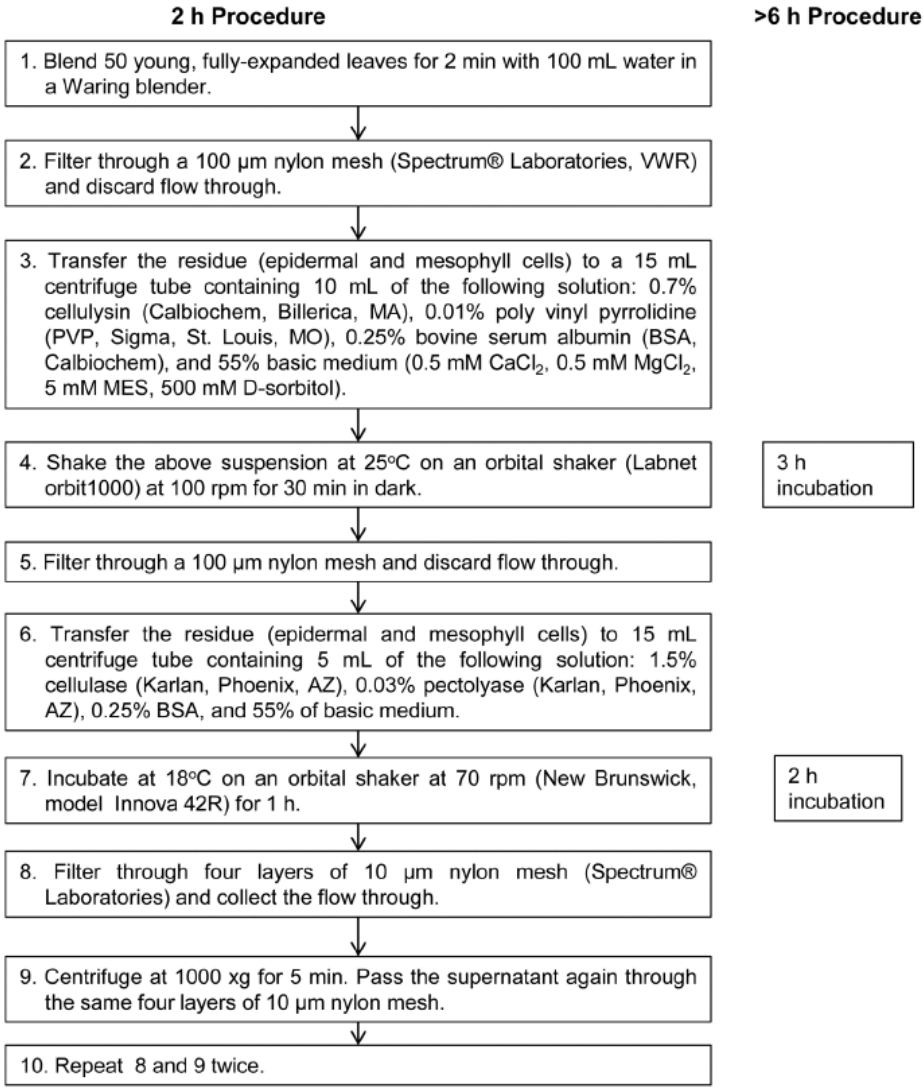


Fig 2.1 Flow chart of the GCP preparation procedure. The right column represents the long protocol (>6 h) and the left column indicates the steps where the protocol was shortened to be completed in 2 h. The two procedures were performed with (all steps) or without the transcription inhibitors cordycepin (0.01%) and actinomycin D (0.0033%).

2.3.3 Confocal microscopy imaging.

Green and red auto-fluorescence and differential interference contrast (DIC) images of the protoplasts were observed using a confocal laser scanning microscope (LSM 510

Meta, Carl Zeiss Inc., Thornwood, NY) with Argon laser at excitation of 488 nm and emission at 505-550 BP (green) and 560 LP (red). All channels were imaged simultaneously.

2.3.4 RNA extraction.

Frozen GCP preparations (~10^9 cells) were thawed using the lysis buffer supplied with each RNA extraction kit; RNeasy Plant Mini kit including the in-column DNA digestion option (Qiagen, Valencia, CA) or Trizol® reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. 450 µl or 1 ml of lysis buffer was used for the column-based or Trizol-based method, respectively. RNA yield and quality were determined using NanoDrop-1000 version 3.2 spectrophotometer (Thermo Scientific, Wilmington, DE) and the Agilent 2100 BioAnalyzer RNA 6000 Pico chip (Agilent Technologies, Inc. Wilmington, DE).

2.3.5 Gene expression analysis.

Total RNA was used to synthesize cDNA using 5 µg RNA template and 250 nM oligo dT in a 20 µl reaction using the Takara RNA PCR kit (AMV) (Clontech, Montain View, CA) according to manufacturer's recommendations. Reverse transcription reaction was carried out at 50°C for 30 min, 95°C for 5 min, and 4°C for 5 min. Quantitative PCR (qPCR) was performed in 20 µl reaction with iTaq Fast SYBR Green Supermix (BioRad, Hercules, CA) using 0.5 µl of the RT reaction described above, and 200 nM of reverse and forward gene-specific primers. Reactions were carried out with the Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA) using cycling conditions as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 58°C for 30 sec. A dissociation curve was determined for every reaction to confirm the presence of a single amplicon indicating that RNA samples were free of DNA contamination. Relative abundance of transcripts was calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001) using the housekeeping genes *ACT2 and TUB4* as internal controls. *ACT2* and *TUB4* have half-life of 6-12 and 12-24 h, respectively (Narsai et al. 2007) and their transcript levels show no difference among GCP

samples as described in the results. All gene-specific primers are described in the Table 2.1. A minimum of two biological replicates and three technical replicates were performed.

2.3.6 PCR efficiency.

Gene-specific primer sets that span an intron region were designed using the primer quest software from IDT-SciTools (http://www.idtdna.com/Primerquest/Home/Index) for qPCR analysis. To assess reaction efficiencies, standard curves were created using a five-fold serial dilution of cDNA pool. A linear regression between the amount of cDNA template and the cycle threshold (C_T) value was calculated to obtain a correlation coefficient (R^2) >0.97. The PCR efficiency was determined according to Schmittgen and Livak (2008).

2.3.7 Guard Cell Protoplasts And RNA Isolation From Pst DC3000 Treated Arabidopsis Leaves

50 young fully expanded leaves were collected from Arabidopsis (4-5 weeks old) one hour post dip inoculation with overnight grown bacterial inoculum having $O.D_{600}$: 0.8 -1.0. Leaves were blended immediately and proceeded to isolate GCPs in presence of transcription inhibitors as mentioned above. Plants dipped in 0.03% silwet were used as control. Two biological replicates of >99% pure GCP preparations were performed for RNA extraction using Qiagen columns according to the manufacturer's recommendation. The in-column DNase treatment with the RNase-free DNase set kit (Qiagen) was carried out for all samples. Total RNA was eluted using 30ul of RNase free water supplied with the kit. Total RNA was synthesized into cDNA in a 20-µl reaction containing approximately 3.5µg of RNA, 250 nM oligo dT, and reagents provided with the Takara RNA PCR kit (AMV) (Clontech, Montain View, CA, U.S.A.), according to manufacturer's recommendations. RT reaction was carried out at 50^{o}C for 30 min, 95^{o}C for 5 min, and 4^{o}C for 5 min.

2.4 Results and Discussion

2.4.1 Length of incubation for cell wall digestions does not affect GCP purity and yield.

High throughput sequencing for transcriptomic analysis requires that RNA samples are of excellent quality for assessing the level of gene expression accurately. GCP preparation, in particular, relies on extensive manipulation of the samples before RNA isolation and maintaining the integrity of the transcriptome during the procedure may be a challenge. Considering that the half-lives of some transcripts can be as short as 1 to 3 h (Narsai et al. 2007) and commonly used protocols take approximately 6 h to be completed (Leonhardt et al. 2004), it is likely that RNA decay will occur during GCP preparation. Thus, we determined the shortest incubation times to efficiently digest the plant cell wall and still yield pure and healthy GCPs.

Decrease in the incubation times in steps 4 and 7 of the protocol from 3 to 0.5 h and from 2 to 1 h, respectively (Fig. 2.1) does not affect the purity and yield of GCP preparations. GCPs are approximately ten times smaller than MCPs (Fig. 2.2A) and sample purity can be easily evaluated by observing cell preparation under light microscope and calculating the percentage of GCP present in the suspension. As these cells auto-fluoresce, cell viability can was also determined using a fluorescence microscope (Fig 2.2A). Both procedures yielded similar GCP purities with 98% and 97% for the short (2 h) and long protocol (>6 h), respectively (Fig. 2.2B). This purity is equivalent to other procedures (Pandey et al. 2002, Leonhardt et al. 2004). Likewise, very similar numbers of GCPs were recovered using either short or long protocol, an average of 4.8×10^9 and 5.3×10^9 cells per 50 leaves, respectively (Fig. 2.2C). This difference in GCP numbers is not statistically significant. The GCP yield was three orders of magnitude higher than the one reported by Pandey et al. (2002).

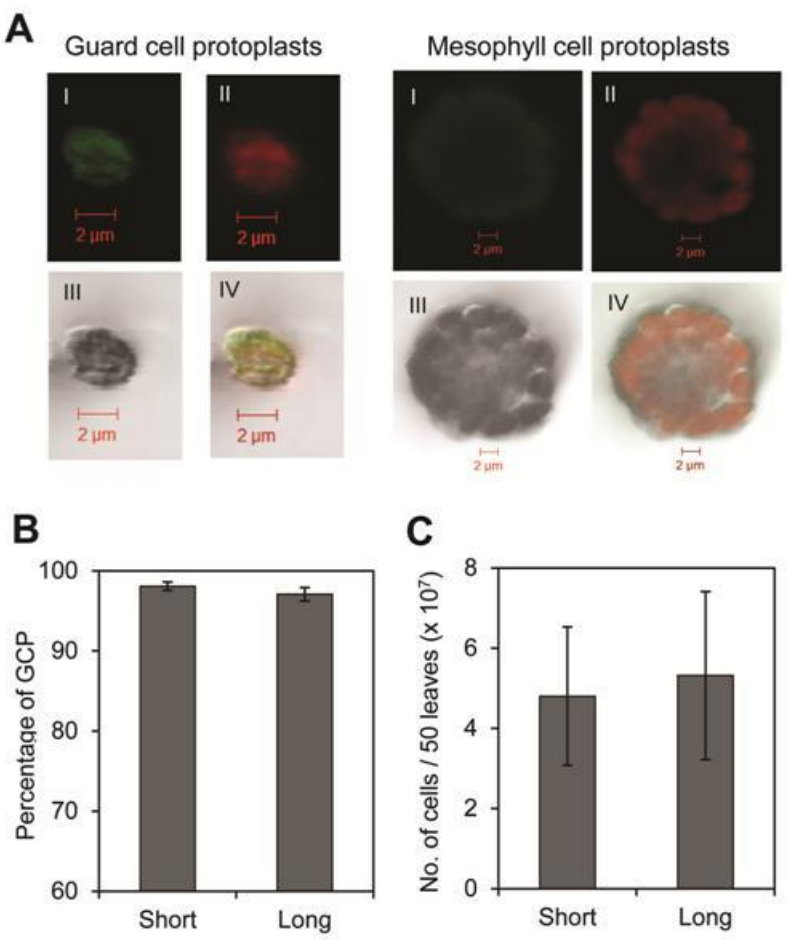


Fig. 2.2. Assessing the yield and purity of GCP preparations. A, Laser scanning confocal micrographs of guard cell and mesophyll cell protoplasts. Note the size difference. B, Number of GCPs isolated in long and short methods. C, Purity of GCPs extraction using long and short incubation protocols calculated as percentages of total protoplast extracted (MCP and GCP). Results are shown as means (n=3) ± standard error.

2.4.2 Amount of RNA extracted from GCPs is affected by digestion time, but not by the presence of transcription inhibitors.

To determine whether the length of the GCP preparation procedure could interfere with the amount of RNA extracted, I isolated GCPs from 50 leaves and divided the GCP suspension in two halves for RNA extraction using two different methods, Trizol® reagent or Qiagen column. Increasing incubation times to digest the plant cell wall negatively affected

($P < 0.05$) the RNA yield (µg) as determined by NanoDrop® spectroscopy, independent of the RNA extraction method of choice. Two- to three-fold more RNA could be extracted after short cell wall digestion (7-9 µg) as compared to after long digestion (3-3.5 µg) (Fig. 2.3A).

Next, I assessed the effect of the transcription inhibitors actinomycin D and cordycepin, on the amount of RNA extracted with Qiagen columns. In this experiment, RNA yields were also significantly decreased ($P < 0.001$) when GCPs were subjected to long digestion periods (Fig. 2.3B). However, similar RNA yields were obtained with or without the addition of transcription inhibitors during either long or short GCP preparation procedure (Fig. 2.3B). Taken together, these results suggest that lower RNA yield after longer GCP preparation may be due to RNA decay.

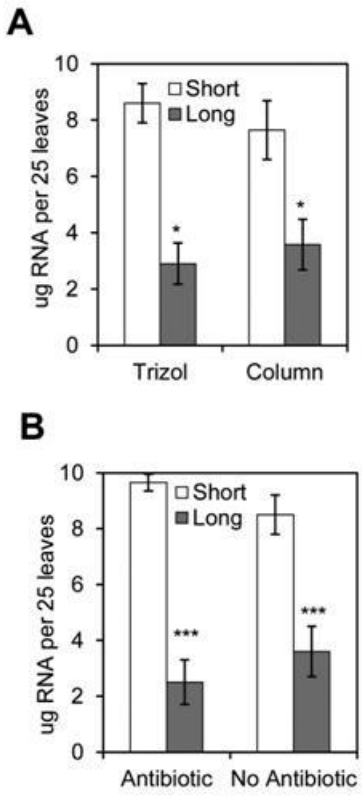


Fig. 2.3 Amount of RNA extracted from long and short protocols. A, GCP was isolated from 50 leaves and GCP suspension was equally divided for total RNA extraction using either the Qiagen column or Trizol reagent, thus yield is expressed in µg per 25 leaves. Transcription inhibitors were not added during guard cell protoplasting. B, Total RNA extracted from GCPs using Qiagen column in presence or absence of the transcription inhibitor antibiotics cordycepin (0.01%) and actinomycin D (0.0033%). Results are shown as means (n=3) ± standard error. Statistical significance between the means (short versus long) was detected with two-tailed Student's *t*-test (*** refers to $p<0.001$, * refers to $p<0.05$).

2.4.3 Quality of RNA is affected by extraction protocol, but not GCP preparation time.

To further determine the RNA quality for downstream application, total RNA extracted from GCPs was quantified using BioAnalyzer. I have not observed differences in the RNA amount extracted with either Trizol® reagent or Qiagen column (Fig. 2.4A) and the $A_{260:280}$

ratios of all RNA samples ranged from 2.0 to 2.2 based on NanoDrop® readouts. However, BioAnalyzer profiles indicated a significantly low overall quality of the RNA samples extracted with Trizol® reagent. The average RNA integrity number (RIN) for these samples was 4, ranging from 2.7 to 5.9 in four independent trials and the RIN number could not be determined in additional two biological replicates. These results highlight the importance of checking the RNA quantity and integrity using sensitive techniques such as BioAnalyzer profile. Therefore, I have not used Trizol®-extracted RNA for downstream application.

When RNA was extracted from GCPs with the Qiagen column, the RNA integrity based on RIN values averaged around 6 and were not significantly different between the GCP preparation protocols (short and long) or antibiotics addition (Fig. 2.4). Furthermore, the electropherograms and electronic gels for these RNA samples were very similar (Fig. 2.4B).

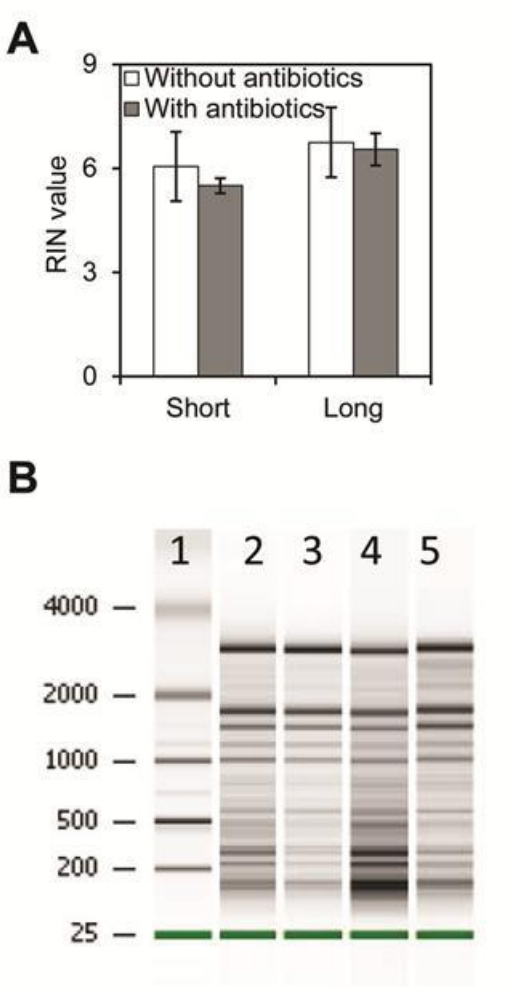


Fig 2.4 Quality of RNA extracted from GCPs using Qiagen columns. A, RIN values of RNA samples obtained from GCPs isolated following the short (2 h) or long (>6 h) methods with or without antibiotics. Results are shown as mean (n=4) • } standard error. B, Representative electronic gel derived from the BioAnalyzer profiles of RNA samples. Lanes were loaded as follows: 1 = RNA ladder, 2-5 = RNA extracted from GCPs isolated using short (2 and 4) or long incubations (3 and 5) in the absence (2 and 3) or presence (4 and 5) of antibiotics. The numbers on the left corresponds to the fragment size in bp. RIN values of these samples ranged from 4.7 to 6.6.

2.4.4 Actinomycin D and cordycepin prevent induction of wound-responsive genes during protoplasting.

Considering that protoplasting induces the expression of stress-associated genes, (Leonhardt et al. 2004; Wang et al. 2011), I tested whether the transcription inhibitors used

during protoplast isolation were efficient in preserving the expression levels of early wound-response genes. First, the quality of the cDNA synthesized with reverse transcriptase was assessed through agarose gel electrophoresis to ensure that only high quality cDNA was used for the gene expression analysis. cDNA smears ranging from 400 to >1000 base pairs were considered of good quality and used for qPCR analysis (Fig 2.5).

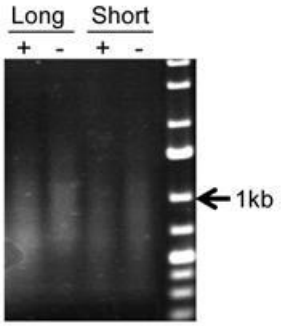


Fig 2.5 Agarose gel showing cDNA smears synthesized through reverse transcriptase reactions. Reactions were carried out with RNA samples extracted from GCP preparations in the presence or absence of antibiotics (+ and – symbols on top of the gel lanes) using long or short procedure.

Second, I evaluated the PCR efficiency according to Schmittgen and Livak (2008) and only reactions with efficiency within 15% of that observed for the reference gene were selected for assessing transcript abundance (Fig. 2.6).

Table 2.1. Gene-specific primers used in qpcr reactions and the expected amplicon sizes.

Gene	Primer	Sequence (5'-3')	Size (bp)
JAZ1	Forward	TGTAGTCGATTGAGTCAGTATCTAAAAGAGAACG	180
(At1g19180)	Reverse	CGGTTTAACATCTTGAACCATGGAATCCATGTTAG	
JAZ8	Forward	CAGCAAAATTGTGACTTGGAACTTCGTC	230
(AT1G30135)	Reverse	GTTATTCTTTGAGATTCTTCATTTGGTTGTGG	
DND1	Forward	GCAACACGCTGTATTGCGAGAACA	133
(At5g15410)	Reverse	AGAAGGATGCAGAAGGTCACTGGT	
S6K1	Forward	CTTCCAAGTCGCCTTTCTG	84
(At3g08730)	Reverse	CAAGCTTCCGCAGTTTCT	
LHY	Forward	GAGAGCCTGAAACGCTATAC	84
(AT1G01060)	Reverse	GAGACAACAACAGCAACAAC	
NINJA	Forward	CAACAGGTTGTTTGCCTTCGCCTT	91
(At4g28910)	Reverse	AGGAGGGATTGTCGCACTTTCTCA	

Table 2.1 continued…

Gene	Primer	Sequence (5'-3')	Size(bp)
TUB4	For	GCAGAGATGAGATGGTTAAGA	110
(At5g44340)	Rev	AACGCTGACGAGTGTATG	
JAZ2	For	CTTCTTCCTCTTCCTCTGGGACCAAAG	125
(At1g74950)	Rev	CATCAAACACCATAACTCGACCACCG	
PPC2	For	CTTCAGGAGTTACTCGCGGGTTTC	176
(At2g42600)	Rev	GGATGAGCTACTTCCATGAGACAATCTGG	
SKIP	For	ACAGTACCCAAGTCTCCCTCGTTT	145
(At1g77180)	Rev	ACTCTCCCTGTTACTGTCGATGCT	
ACT2	For	CACTTGCACCAAGCAGCATGAAGA	80
(At3g18780)	Rev	AATGGAACCACCGATCCAGACACT	

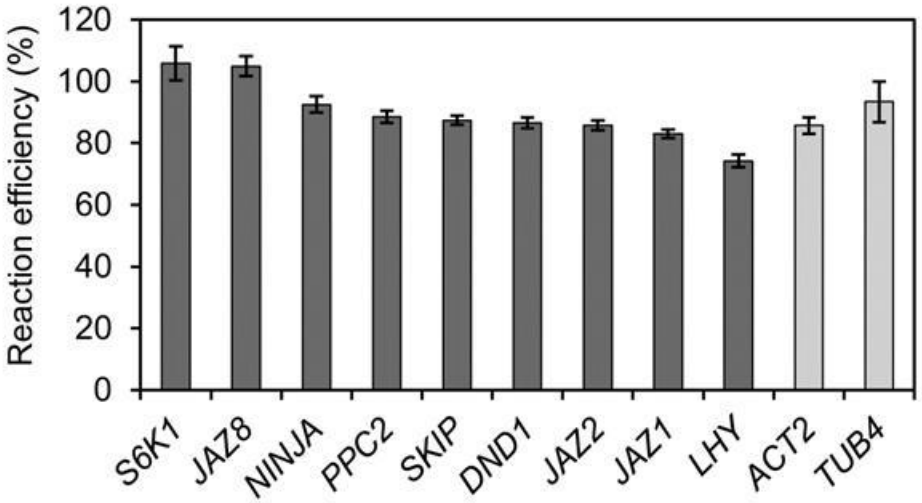


Fig 2.6 qPCR efficiency calculated based on the linear regression between the amount of cDNA template in the reaction and the cycle threshold (Ct). Results are shown as mean (n=3) ± standard error. *ACT2* and *TUB4* were used as reference gene for qPCR analysis.

Next, I selected two genes that are strongly induced by wounding as fast as 30 min, *JAZ1* and *JAZ8* (Chung et al. 2008) and determined their transcript abundances in RNA

samples extracted from GCPs isolated with short incubation times and in the presence or absence of transcription inhibitors. *JAZ1* and *JAZ8* transcripts were 23 and 3 times more abundant in samples without antibiotics as compared to samples with antibiotics, respectively (Fig. 2.7). Furthermore, besides *ACT2* that was used as internal control for qPCR, I assessed the expression of two other genes that have predicted half-lives higher than 6 h and are not known to be induced by stresses, *PPC2* and *TUB4.* No differences in transcript abundance were observed for these genes (Fig. 2.7). These results suggest that the addition of transcription inhibitors during protoplast in fact avoided the induction of genes in the guard cells, which is essential to evaluate global transcriptional changes in response to bacterial treatments.

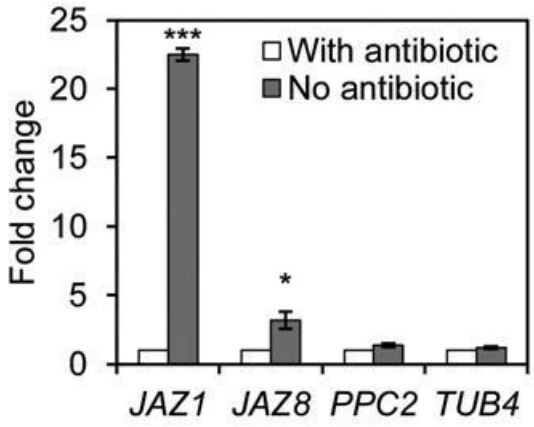


Fig 2.7 Effect of transcription inhibitor antibiotics (actinomycin and cordycepin) in wound-responsive gene transcription during GCP protoplasting. Relative transcript abundance of the indicated genes was determined by RT-qPCR analysis. Results are shown as mean (n=6) ± standard error. Statistical significance of the difference in the mean expression (with antibiotics versus without antibiotics) of was detected with two-tailed Student's *t*-test (*** = $p<0.001$, * = $p<0.05$).

2.4.5 mRNAs decay in guard cells.

To address the concern of RNA decay (Narsai et al. 2007) owing to lengthy procedures for protoplasting, I assessed transcript abundance of ten genes, two of which are commonly used as internal control for qPCR (*ACT2* and *TUB4*), after short and long

incubation procedures. These genes were selected based on their half-lives in Arabidopsis cell suspensions (Narsai et al., 2007) and were previously known to be expressed in guard cells (Leonhardt et al. 2004; Wang et al. 2011). I subjected all genes to the same qPCR controls described above (Fig. 2.6). Consistently, all four transcripts with predicted half-lives shorter than 3 h were three to five fold more abundant in GCP preparations using shorter incubations as compared to long incubation times (Fig. 2.8). Likewise, three gene transcripts with predicted half-lives between 3-6 h were all significantly more abundant in GCPs released with short incubations; however the fold changes were between 1.5 to 2.3 (Fig. 2.8). No changes were observed in the abundance of transcripts with half-life longer than 6 h, *PPC2*, *ACT2*, and *TUB4,* which was used as reference gene to create Fig. 2.8. Genes with shorter half-lives are mostly involved in regulatory functions (Narsai et al. 2007); therefore the time required for isolation of guard cells becomes crucial. These results indicate that an optimized GCP isolation protocol may yield RNA samples enriched with short-lived transcripts increasing the success to discover genes and regulatory networks of guard cells under biotic and abiotic stresses.

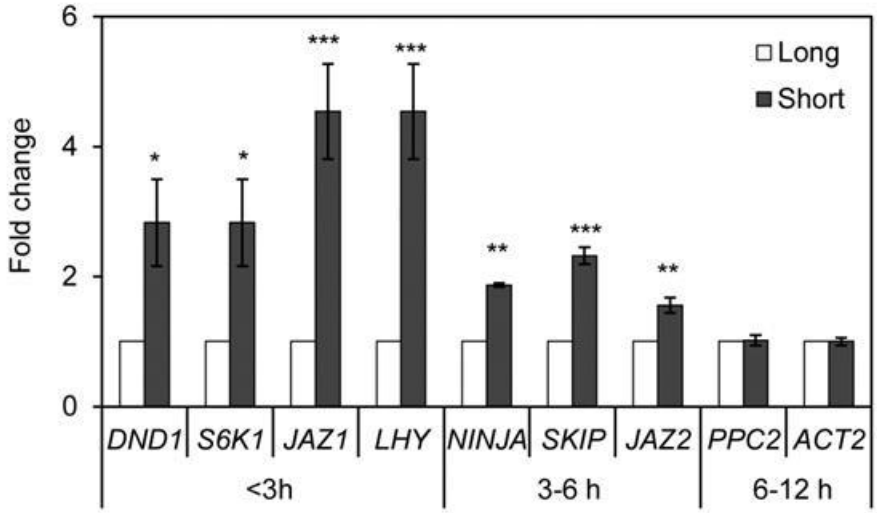


Fig 2.8 Effect of guard cell protoplast (GCP) preparation time on transcript abundance. Long procedure takes >6 h whereas the short procedure can be finished in about 2 h. Transcript abundance of the indicated genes relative to the >6 h procedure was determined by RT-qPCR analysis. Time periods in the X axis indicate the predicted half-life of the gene transcript. Results are shown as mean (n = 6) ± standard error. Statistical significance of the difference between means (short versus long procedure) was detected with two-tailed Student's t-test (*** = P<0.001, ** = P<0.01, * = P<0.05).

2.4.6 Guard Cell Protoplasts and RNA Isolation From Pst DC3000 Treated Arabidopsis Leaves

Previously, it has been determined that guard cells in intact leaves respond very quickly to the presence of bacteria by closing most of the stomatal pores within 2 h of exposure (Chitrakar and Melotto 2010) studying the molecular happenings in guard cells would be interesting. With this idea the effects of biotic stresses generated by *Pseudomonas syringae* pv. Tomato strain DC3000 (*Pst* DC3000) on the guard-cell transcription network through direct RNA-seq was assessed. Since a better approach to isolate guard cells is now available (Obulareddy et al. 2013), guard cell protoplasts were isolated from Arabidopsis one-hour post inoculation with *Pst* DC3000. Total RNA that was isolated from isolated GCPs using Qiagen column and RNA yield and quality were determined using NanoDrop-1000 version 3.2 spectrophotometer (Thermo Scientific, Wilmington, DE). Approximately a

minimum of 11ug and a maximum of 13ug of RNA yeild was achieved from the GCPs isolated with good 260/280 and 260/230 ratios as shown in Table1. Approximately 3.5ug of this RNA was used to generate cDNA and stored at -20^{o}C till further use.

Table 2.2 Total RNA yield from GCPs that was isolated from 50 Arabidopsis leaves

Sample	ng/ul	260/280	260/230
Control-1	441.25	2.16	2.44
Control-2	423	2.19	2.47
Pst DC3000-1	447.77	2.17	1.94
Pst DC3000-2	394.98	2.19	2.43

2.5 Conclusion

In this study, I demonstrate the feasibility of a robust, straight-forward, and fast procedure to obtain highly pure GCPs and enough high quality RNA to assess the transcriptome of guard cells using direct RNA sequencing. The number of detectable genes expressed in the guard cell was considerably extended providing a unique opportunity to infer the metabolic activities carried out by this special type of cells. The new procedure and protocol adjustments described here will provide new sequence data and increase the likelihood to detect short-lived RNA transcripts involved in the tight regulation of the signal transduction of guard cells under stress conditions, ultimately facilitating the mechanistic understanding of plant-pathogen interactions at the leaf surface.

Chapter 3

Regulation of *JAZ* Gene Expression in Guard Cells of Arabidopsis by Phytotoxin Coronatine

3.1 Abstract

Coronatine (COR) is a phytotoxin that facilitates the entry of *P. syringae* into the leaves of Arabidopsis by regulating stomatal movement. *P. syringae* is also known to regulate *JASMONATE ZIM-DOMAIN* (*JAZ)* genes in COR-dependent manner. To investigate direct effect of COR, I examined *JAZ* gene expression in whole leaves and guard cells. Young, fully expanded leaves were treated with 10, 60 and 100μM COR and *JAZ* gene expression was assessed in whole leaves and GCPs. Subsequently, comparative analysis of constitutive expression of *JAZ* genes in GCPs and whole leaves was also performed. My findings suggests that 1) COR induces the expression of all *JAZ* genes as early as 5 minutes in guard cells, however only JAZ1/3/5/6/7/8/9/10/11/and12 are induced in whole leaves, in addition induction of *JAZ* genes in dose dependant manner is more apparent in guard cells, 2) regulation of *JAZ4* and *JAZ2* genes by COR is restricted to guard cells, 3) Abundance of *JAZ* gene transcripts in guard cells is prominent as compared to whole leaves, 4) Expression of key proteins of the JAZ network are possibly different in guard cells as compared to mesophyll cell enriched whole leaves, and 5) JAZ4 might play a role in early stages of disease development. This study has shed light on the understanding of the role of JAZ proteins in disease progression in Arabidopsis.

3.2 Introduction

Plant hormones are not merely the growth regulators, they also respond to various stimuli like environmental stress, wounding, herbivory and pathogen infection (Browse 2009, McConn and Browse 1996, McConn et al. 1997, Staswick et al. 1998, Vijayan et al. 1998). In course of this response, plant hormones strategically control disease progression in plants

caused by various biotic agents. This process of preventing disease in plants is achieved by regulation of some defense genes that are dependent on plant hormones. Jasmonic acid (JA) is one such plant hormone that plays a central role in mounting defense in Arabidopsis (Howe et al. 1996, McConn et al. 1997, Feys et al. 1994). One gene family that is known to be responsive to JA signaling is *JAZ* gene family (Yan et al. 2007, Chung et al. 2010, Thines et al. 2007, Chini et al. 2007). Equivalently, pathogens have also evolved various virulence strategies to overcome these defense responses executed by plants. Phytotoxin, COR – a structural mimic of bioactive JA, is a virulence factor produced by *Pst* DC3000 that helps in disease development by overcoming stomatal immunity (Melotto et al. 2006). It has also been reported recently that induction of *JAZ* genes during *Pst* DC3000 infection is dependent on COR (Demianski et al. 2012).

From these earlier studies it evident that COR modulates stomatal immunity as well as *JAZ* gene expression. Stomatal response to pathogens is rapid and prompt, I therefore investigated direct regulation of *JAZ* gene expression by COR at early time points in young, fully expanded leaves of Arabidopsis. A similar study was also done in specialized cells called guard cells that form the stomatal pore and this analysis would help me to discover unique *JAZs* that are regulated by COR. Later comparative analysis of constitutive expression of *JAZ* genes in guard cells and whole leaves was also performed. My results indicate that 1) COR regulates *JAZ* gene expression as early as 5 minutes in whole leaves and in guard cells, 2) *JAZ4* and *JAZ2* genes are induced by COR in guard cells and not in whole leaves that are enriched by mesophyll cells, 3) *JAZ* gene transcripts are more abundant in guard cells in comparison to whole leaves, and 4) This study provides evidence of distinctive JA signaling network in guard cells as compared to whole leaves. 5) *JAZ4* might modulate key hormonal pathways to favor bacterial entry at early stages of infection. These findings not only extend the understanding of association of phytotoxin COR and *JAZ* genes, but also their collective role in disease progression in Arabidopsis. In any case, more studies

on phenotypic analysis of *jaz4*$^{-}$ mutants are needed to confirm the importance of *JAZ4* in bacterial penetration.

3.3 Methods

3.3.1 COR treatment.

COR stock (1mg /ml) was dissolved in methanol and was used to prepare 10, 60 and 100µM working solution. Three whole leaves were dipped in respective working concentrations for 5 and 15 minutes. I made sure the leaves were immersed in the solution throughout the treatment, and post treatment they were immediately flash frozen in liquid nitrogen to extract RNA. For GCP isolations, 50 young fully expanded leaves of Arabidopsis were also dipped for 5 minutes in all three working concentrations and proceeded for GCP isolations immediately. For control, leaves were dipped in water for 5 or15 minutes as needed.

3.3.2 Guard cell protoplast isolation.

Post COR treatment, GCPs were isolated according to the procedures described in Obulareddy et al. (2013) in the presence of the transcription inhibitors actinomycin D (Sigma, St. Louis, MO) and cordycepin (Sigma, St. Louis, MO). Purity and yield of GCPs were determined as mentioned in section 2.3.2 in chapter 2. GCP suspensions were centrifuged at 1000 x g for 5 min at room temperature and flash frozen in liquid nitrogen for subsequent RNA extraction. A minimum of two biological replicates were performed for each treatment and all GCP isolations were performed at 2-3 hours after the lights were turned on in the morning.

3.3.3 RNA extraction from whole leaves and guard cells.

From each set of leaves, RNA was extracted using RNeasy plant mini kit (Qiagen,Valencia - CA), quantified using Nanodrop-1000 Ver 3.2 (Thermo scientific, Wilmington-DE) and stored at -80^{o}C. After isolating GCPs from Col-0, intact cells were counted using hemocytometer under microscope. RNA was extracted from guard cells using

the approach described in section 2.3.4 of chapter 2, and stored at -80^{o}C as well. The stored RNA was used for Polymerase Chain Reactions (PCR).

3.3.4 RT-PCR procedure.

3.3.4.1 Primer design

Gene-specific primer sets that span an intron region were designed using the primer quest software from IDT-SciTools (http://www.idtdna.com/Primerquest/Home/Index).for qPCR analysis. To assess reaction efficiencies, standard curves were created using a five-fold serial dilution of the cDNA pool. A linear regression between the amount of cDNA template and the cycle threshold (C_T) value was calculated to obtain a correlation coefficient (R^2) >0.97. The PCR efficiency was determined according to Schmittgen and Livak (2008). Primers used for end-point PCR were designed to amplify the complete coding sequence using software from Oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/OligoCalc.html). The physical constants with 50% GC content, melting temperature of 60^{o}C, and primer length of about 24 nucleotides were maintained. Since the primers spanned the introns genomic DNA contamination was easily detected based on the size of amplicons

3.3.4.2 Quantitative RT-PCR

Complementary DNA (cDNA) was synthesized from 5µg RNA using the Takara kit (Takara. Bio. Inc. Ver.3.0, Shiga, Japan). Then using cDNA as a template, qPCR was performed using iTaq Fast SYBR Green Supermix (BioRad, Hercules, CA) using 0.25µl of cDNA template from the RT reaction described above and 250 nM of reverse and forward primers. Reactions were carried out in Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA). Gene expression levels relative to the water control were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). I used a housekeeping gene *ACT2* (AT3G18780) as an internal control, which has same expression pattern even

after COR treatment. PCR conditions were maintained according to manufacturer's instructions (Takara. Bio. Inc. Ver.3.0, Shiga, Japan).

3.3.4.3 End-point PCR

End-point PCR was performed with the Takara ExTaq HS (Clontech). The expression level of the housekeeping gene *ACT2* (At3g18780) was used as a control for the amount of RNA template used for PCR amplifications in different samples. The number of PCR cycles was optimized for each gene so that we could stop the reaction at the first cycle in which an amplicon could be observed by agarose gel electrophoresis and differences expression levels among treatments could be easily detected. For 1 step RT-PCR 100ng of RNA for each sample and carried out cDNA construction and PCR amplification in the same tube following manufacturer's instructions.

3.3.5 T-DNA lines genotyping

The presence of a T-DNA insertion within any given gene can be easily detected by a proper PCR strategy. Two *JAZ4* gene specific primers one forward and one reverse and a T-DNA left border primer-Lba1 primer (that is present in the T-DNA inserted) as shown in Fig 3.1 were used in a single reaction. If a Salk line is homozygous for the insertion, PCR product was formed with a combination of Lba1 primer and *JAZ4* forward primer visualized as single band in agarose gel, similarly if the line is homozygous for no insertion then PCR product was formed as a result of J*AZ4* forward and *JAZ4* reverse primers. However for heterozygous lines both PCR products will be formed visualized as double bands in agarose gel.

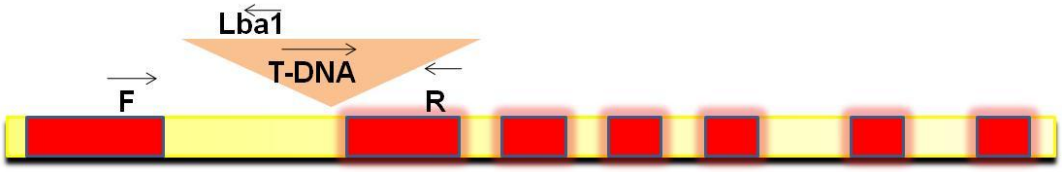


Fig 3.1 Schematic representation of T-DNA insertion and primers for screening *jaz4*-mutants. Yellow boxes determine the intron or untranslated regions, red boxes determine exons and orange triangle determines T-DNA insertion.

Later for those lines that are homozygous for T-DNA insertion are chosen and proceeded to RT-PCR. Using semiquantitative RT-PCR the homozygous lines were checked for complete absence of transcript.

3.3.6 Stomatal assay and bacterial growth assays

Post genotyping, homozygous T-DNA insertion were subjected to stomatal and bacterial growth assays to determine whether any *JAZ* gene mutation made stomata hypersensitive (due to removal of a JAZ repressor) to COR-mediated inhibition of PAMP/bacterium-induced stomatal closure. Stomatal assays were done by treating the whole leaves with bacterial suspensions and observing the width of stomatal aperture under microscope (Chitrakar and Melotto et al. 2010). Also, for bacterial pathogenesis assays *P. syringae* pv. *tomato* strains, *Pst* DC3000 (wild type) and mutant derivative, *Pst* DC3118, were cultured at 30^0C in low salt LB medium (10 $g.l^{-1}$ tryptone, 5 $g.l^{-1}$ yeast extract, 5 $g.l^{-1}$ NaCl, pH=7.0) supplemented with appropriate antibiotics until an OD_{600} of 0.8 was reached. Bacteria were collected by centrifugation and re-suspended in water to the final concentration of 10^7 $CFU.ml^{-1}$ containing 0.003% Silwet L-77 (Lehle seeds Co., Round Rock, TX) for dip-inoculated plants or 10^5 $CFU.ml^{-1}$ containing 0.008% Silwet L-77 for vacuum-infiltrated plants. Inoculated plants were immediately incubated under the following conditions: 25^0C, 65±5% relative humidity, and 12 h of daily light (100 $\mu mol.m^{-2}.sec^{-1}$). Bacterial population in the plant apoplast was determined as previously described (Katagiri et al. 2002). Statistical significance of the results was calculated using 2-tailed, paired wise Student's *t*-test. The experiment was repeated three times with similar results.

3.3.7 Photomorphogenesis

Arabidopsis seeds were stratified after sowing at 4°C for 2 days, the pots were then transferred to a growth chamber with 50µmol $m^{-2}\cdot s^{-1}$ continuous cool-white fluorescent light at 22 °C under 12-hour photoperiod. As a control some pots were transferred to a growth chamber with 100µmol $m^{-2}\cdot s^{-1}$ continuous cool-white fluorescent light at 22 °C under 12-hour

photoperiod Pictures were taken after 4 weeks and after 14 weeks. Plants were checked every week and number of days taken to flower was noted for graphical representation.

3.4 Results and Discussion

3.4.1 *JAZ* genes were induced in Arabidopsis leaves by COR in dose dependent manner

Previous studies have shown that *Pst* DC3000 induced *JAZ* genes in COR-dependent manner (Demianski et al. 2012) and also most *JAZ* genes are regulated by JA (Chini et al. 2007, Thines et al. 2007, and Chung et al. 2008). Having known this, to study the direct impact of COR on *JAZ* genes, using semi quantitative PCR, COR regulation was studied in young fully expanded leaves of Arabidopsis Gene specific primers (Table 3.1) were used to perform this experiment. Most of the *JAZ* genes were induced, however no clear bands were detected for *JAZ4, JAZ5* and *JAZ11* (Fig. 3.2.) Simultaneously, *JAZ* genes expression analysis was performed in guard cell protoplasts (GCPs) as well after 15 and 25 minutes of COR treatment. GCPs were extracted using long protocol as described in Chapter 2. Using RT-PCR, I could not detect any levels of transcripts for many *JAZ* genes. Only *JAZ1, JAZ2* and *JAZ9* showed clear transcript levels (Fig 3.3). For further confirmation, the experiment was repeated using the same procedure mentioned above. Since *JAZ* genes were induced at earlier time points, COR treatment was restricted till 15 minutes in the later experiments (Fig 3.4A). Some of the genes, *JAZ1, JAZ4, JAZ6, JAZ8, JAZ10* and *JAZ11* were induced as early as 5 minutes while others, *JAZ3, JAZ5, JAZ7,* and *JAZ9* were induced at 10 minutes by COR and no change in the expression pattern was observed in *JAZ2* and *JAZ12.*

To improve the technique for analysis of *JAZ* regulation in guard cells, I used a more sensitive technique; one-step RT-PCR that combines the cDNA synthesis (reverse

transcription) reaction and PCR reaction in the same tube, thereby reducing the possibility of contamination, and helps minimize carryover contamination between the samples. To check the robustness of this technique only 3 *JAZ* genes *JAZ3, JAZ4, and JAZ9* were studied (Fig 3.4B). As predicted this technique improved the detection of *JAZ* genes in guard cells and moreover COR regulation of *JAZ* genes was also clearly detected. This indicated the need of robust procedures for *JAZ* gene expression analysis.

For more robust results, I also analyzed *JAZ* gene regulation by COR at 10, 60 and 100μM dosages for 5 minutes by qPCR in Arabidopsis leaves. Gene-specific primers (Table 3.2) were used to perform this experiment. Fold change was calculated relative to the water treatment. At the minimal dosage of 10μM, I observed that *JAZ3, JAZ10 and JAZ12* were induced and *JAZ1, JAZ2, JAZ5, JAZ6, JAZ8* and *JAZ9* were repressed significantly as early as 5 minutes post inoculation (Fig. 3.4).

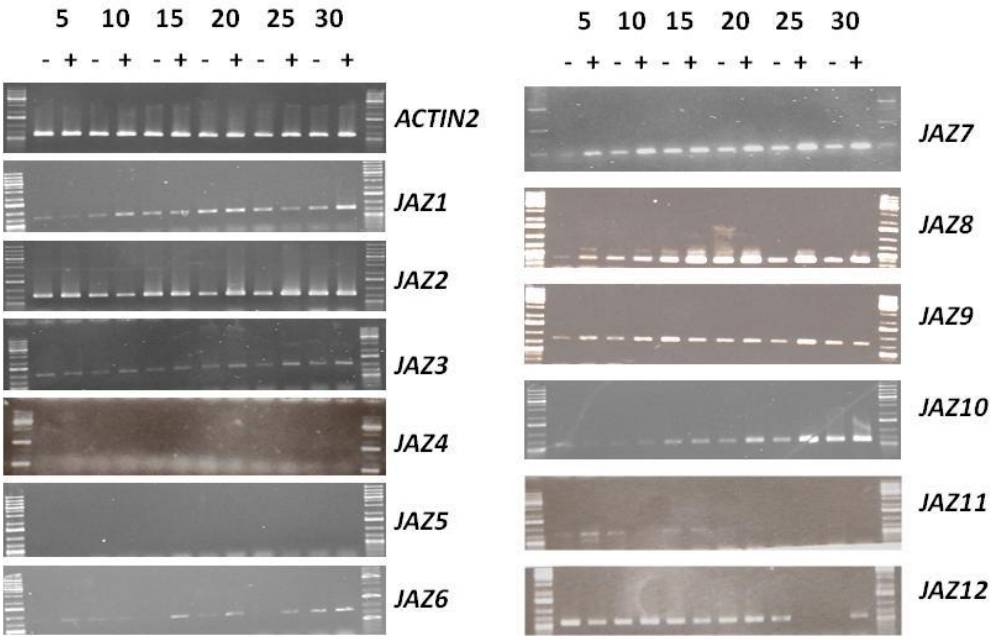


Fig 3.2 Effect of COR treatment on *JAZ* gene expression in leaves of Arabidopsis Col-0 plants until 30 minutes. Leaves from five-week-old plants were submerged in 60μM of COR solution (indicated as +) or water (indicated as -) as a control for the indicated times. Total RNA were extracted and for RT-PCR as described in methods. The *ACT2* gene was used as control for uniform RNA amount for all reactions.

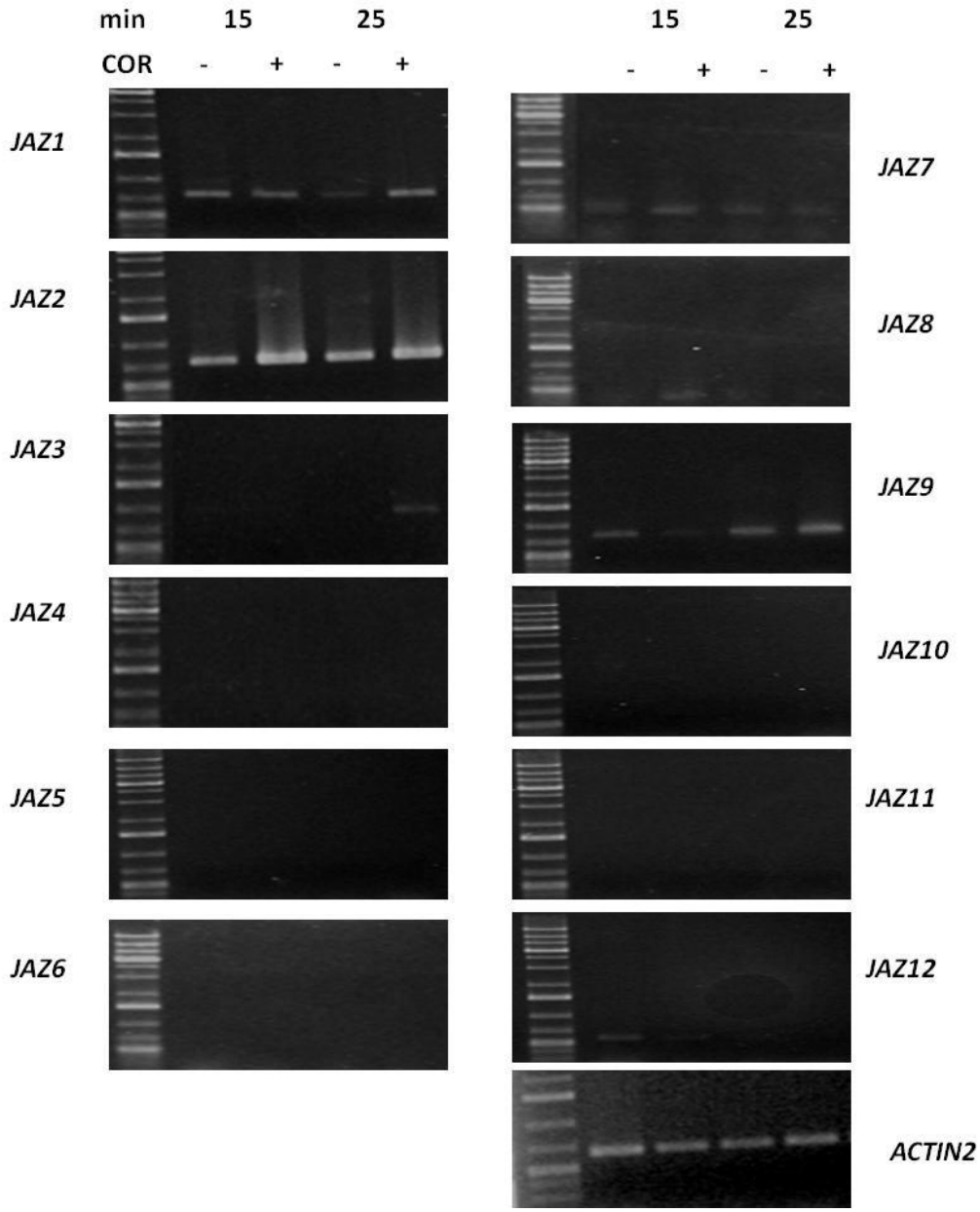


Fig 3.3 Regulation of *JAZ* genes by COR in guard cells isolated using long protocol. Leaves from five-week-old plants were submerged in 60μM of COR solution (indicated as +) or water (indicated as -) as control for the indicated times. GCPs were isolated immediately after treatment and total RNA was extracted for RT-PCR as described in methods. The *ACT2* gene was used as control for uniform RNA amount for all reactions.

Table 3.1 Gene-specific primers used in RT-PCR reactions and the expected amplicon sizes.

Gene	Primer	Sequence (5'-3')	Size (bp)
ACT2 (At3g18780)	For	GCCATCCAAGCTGTTCTCTC	629
	Rev	GAACCACCGATCCAGACACT	
JAZ1 (At1g19180)	For	CACCATGTCGAGTTCTATGGAATGTTC	761
	Rev	TCATATTTCAGCTGCTAAACCGAG	
JAZ2 (At1g74950)	For	CACCATGTCGAGTTTTTCTGCCGAGTGTTA	749
	Rev	CCGTGAACTGAGCCAAGCTGG	
JAZ3 (At3g17860)	For	CACCATGGAGAGAGATTTTCTCGGG	1058
	Rev	TTAGGTTGCAGAGCTGAGAGAAGAAC	
JAZ4 (At1g48500)	For	CACCATGGAGAGAGATTTTCTCGGGC	932
	Rev	TTAGTGCAGATGATGAGCTGGAGGACA	
JAZ5 (At1g17380)	For	CACCATGTCGTCGAGCAATGAAAATGCCTA	824
	Rev	TAGCCTTAGATCGAGATCTTTC	

Table 3.1 continued…

Gene	Primer	Sequence (5'-3')	Size (bp)
JAZ6 (At1g72450)	For	CACCATGTCAACGGGACAAGCGC	809
	Rev	CTAAAGCTTGAGTTCAAGGTTTTTGG	
JAZ7 (At2g34600)	For	CACCATGATCATCATCATCAAAAACT	443
	Rev	CTATCGGTAACGGTGGTAAGGGG	
JAZ8 (At1g30135)	For	CACCATGAAGCTACAGCAAAATTGTG	395
	Rev	TTATCGTCGTGAATGGTACGGTGAAG	
JAZ9 (At1g70700)	For	CACCATGGAAAGAGATTTTCTGGGTT	803
	Rev	TTATGTAGGAGAAGTAGAAGAGTAAT	
JAZ10 (At5g13220)	For	CACCATGTCGAAAGCTACCATAGA	593
	Rev	TTAGGCCGATGTCGGATAGTAAGG	
JAZ11 (At3g43440)	For	CACCATGGCTGAGGTAAACGGAGA	716
	Rev	TCATGTCACAATGGGGCTGGTTTC	
JAZ12 (At5g20900)	For	ACCATGACTAAGGTGAAAGATGAGCC	563
	Rev	CTAAGCAGTTGGAAATTCCTCCTTG	

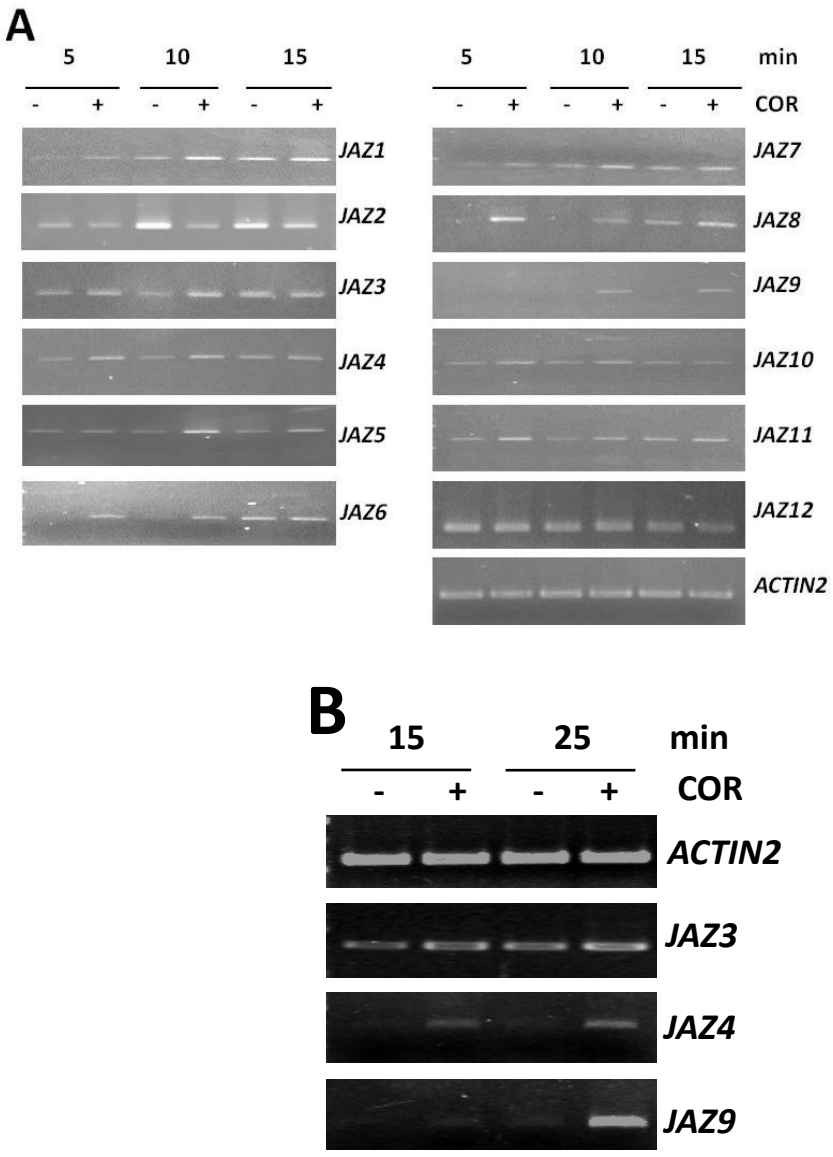


Fig 3.4 *JAZ* gene expression was assessed in leaves (A) and guard cells isolated using long protocol (B) of Arabidopsis Col-0 plants. Leaves from five-week-old plants were submerged in 60μM of COR solution(indicated as +) or water (indicated as -) as control for the indicated times. Total RNA were extracted and 2-step RT-PCR was performed for whole leaves (A) and 1-step RT-PCR for GCPs(B) as described in methods. *ACTIN2* gene was used as control for uniform RNA amount for all reactions.

At a dosage of 60μM COR; *JAZ1, JAZ5, JAZ6, JAZ7, JAZ8, JAZ11* and *JAZ12* were induced and this induction drive was maintained in *JAZ5, JAZ6, JAZ7, JAZ8, JAZ9* and

JAZ11 with further increased dosage of COR to as much as 100μM (Fig. 3.5). I did not observe any induction of *JAZ4* and *JAZ2* but significant repression was noted at 100μM dosages for *JAZ4* (Fig. 3.5). Taken together these results indicate that not all *JAZ* genes are regulated in similar mechanism by COR. Similar conclusions were derived from earlier studies where *JAZ* gene regulations were studied in response to wounding, methyl-JA and *P. syringae* treatment (Demianski et al. 2012, and Chung et al. 2008). Although most *JAZ* genes have similar response to the same treatment, they might have fine separation in functional aspects leading to the difference in the induction kinectics of *JAZ* genes by COR. It could be possible that *JAZ* genes that are induced as early as 5 minutes might be the key molecules of COR driven signaling cascade to override stomatal immunity in Arabidopsis. I further assessed regulation of *JAZ* genes at 15 minutes using the same dosages of COR with qPCR as mentioned above. Overall, the induction patterns were not evident at 15 minutes as compared to 5 minutes. Moreover, all *JAZ*s were either repressed or returned to their basal levels at higher COR concentrations (Fig. 3.6) suggesting that 15 minutes is already late to study the early *JAZ* gene regulation by COR. This result is in accordance with studies done by Chung et al., (2008) where rapid induction of *JAZ* genes was noted as early as 5 minutes due to JA. Earlier studies show that induction of *JAZ* genes due to methyl JA or bacterial treatment or due to wounding have always been transient (Yan et al. 2007, Chung et al. 2010, Thines et al. 2007, Chini et al. 2007, Demianski et al. 2012, and Chung et al. 2008). Similar to these findings *JAZ* genes that were induced as early as 5 minutes due to coronatine treatment relapsed at 15 minutes indicating COR mediated induction of JAZ genes is also short lived.

According to the current models and as mentioned earlier at lower concentrations of JA, the JAZ proteins repress JA responsive gene expression by binding to transcription factor MYC2 that regulates expression of JA responsive genes and however at higher concentrations of JA, JAZ proteins bind to COI1 and are degraded via 26S proteosome

pathways (Baker et al 2010., Chini et al., 2007). This suggests that *JAZ* genes might be distinctively regulated at higher and lower concentrations. In my gene expression analysis as well, I found that most *JAZ* genes are repressed due to COR at lower concentrations and are induced with increased dosages of COR. These results not only validate the functional mimicry of COR and JA, but also demonstrate that COR results in major reprogramming of JAZ expression, and that different JAZ genes exhibit distinct patterns of COR induced expression.

Table 3.2 Gene-specific primers used in qRT-PCR reactions and the expected amplicon sizes

Gene	Primer	Sequence (5'-3')	Size (bp)
TUB4 (At5g44340)	For	GCAGAGATGAGATGGTTAAGA	110
	Rev	AACGCTGACGAGTGTATG	
ACT2 (At3g18780)	For	CACTTGCACCAAGCAGCATGAAGA	80
	Rev	AATGGAACCACCGATCCAGACACT	
JAZ1 (At1g19180)	For	CGTGTAGTCGATTGAGTCAGTATCTAAA	180
	Rev	CGGTTTAACATCTTGAACCATGGAATCC	
JAZ2 (At1g74950)	For	CTTCTTCCTCTTCCTCTGGGACCAAAG	127
	Rev	CATCAAACACCATAACTCGACCACCG	
JAZ3 (At3g17860)	For	CGGTTCAGTTTGTGTTTACGATGA	97
	Rev	CGAAAAGACTTGAGGCATAGAGGA	

Table 3.2 continued…

JAZ4	For	GAGTTTAGCATCCACGCAACAA	110
(At1g48500)	Rev	TGCGTTTCTCTAAGAACCGAGCCA	
JAZ5	For	CAGGGCATTCCAAAGGCGAACC	115
(At1g17380)	Rev	CTTTCCCTCCGAAGAATATGGTCAGC	
JAZ6	For	CTATTGGTGAGGCCTCTACTTCTACCG	110
(At1g72450)	Rev	CCAAAGAATATGGTCAACTGTGAATTTCCAGA	
JAZ7	For	GATGCAAACAAAATGCGACTTGGAACTTCG	129
(At2g34600)	Rev	TGGTTAATATCTGAGATTCTTGCTTTGGTTGTG	
JAZ8	For	CAGCAAAATTGTGACTTGGAACTTCGTC	129
(At1g30135)	Rev	GTTATTCTTTGAGATTCTTCATTTGGTTGTGG	
JAZ9	For	TCATTCAATGCAGCTCCTCGT	64
(At1g70700)	Rev	TCCGAGCTTGAGGGATGAAG	
JAZ10	For	CGCTCCTAAGCCTAAGTTCCAGAAATTTCTC	119
(At5g13220)	Rev	GTTTCCAGTGGAAGCTAACAGCGATTTG	
JAZ11	For	GTTCTGTTTCCGCCGGACTTGAC	120
(At3g43440)	Rev	CCATTGAAGACTCTACAACTCCCACCAAAG	
JAZ12	For	CTATTGCAAGGAGGCATTCGCTTCAAC	110
(At5g20900)	Rev	GTTGGGACATCTGTCTTTTTGAAGTCTGAAG	

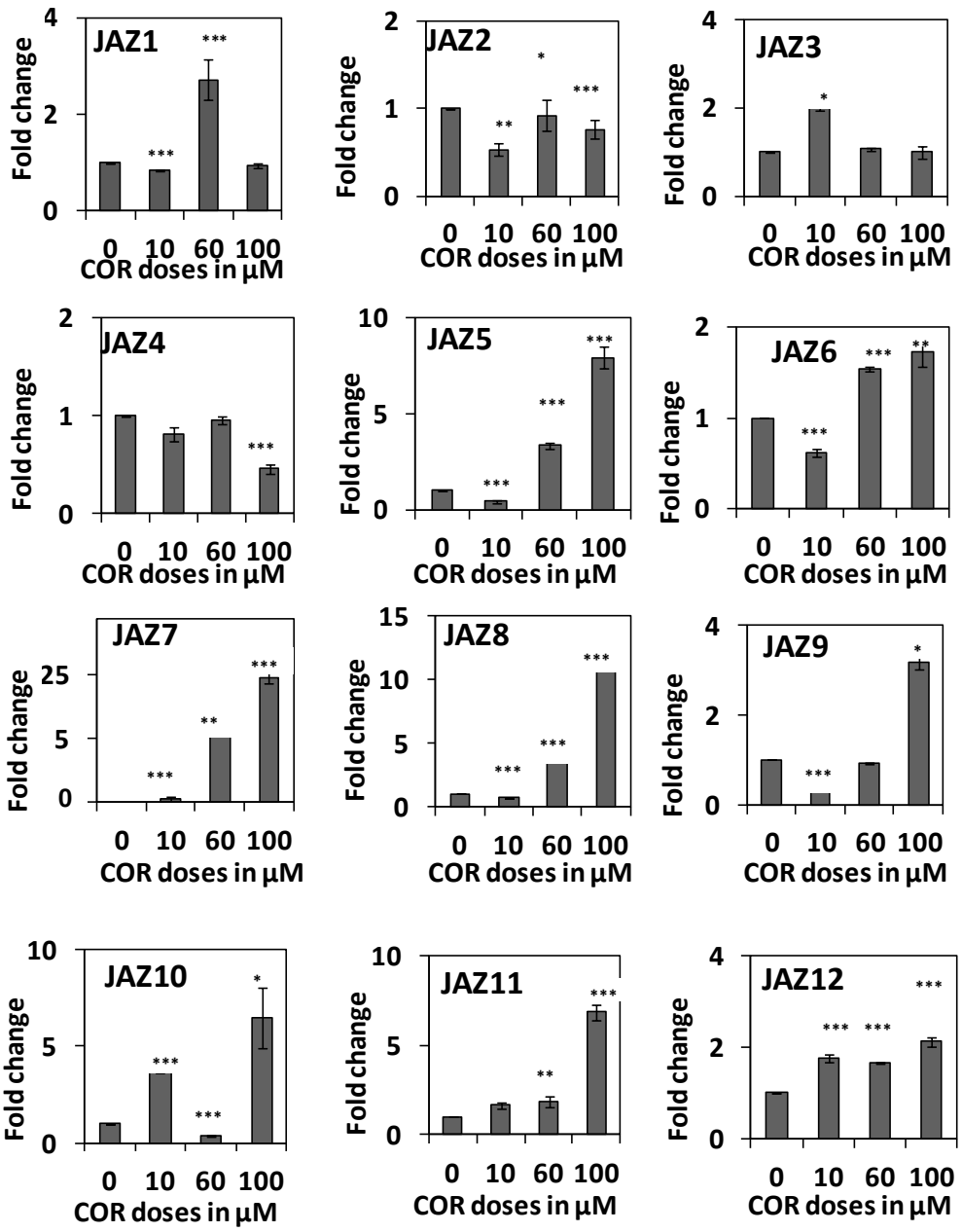


Fig 3.5 *JAZ* genes are regulated by COR as early as 5 min in whole leaves. Data points indicate dosages of COR in µM concentrations to study the relative expression of respective *JAZ* genes after placing Col-0 leaves in COR solution as compared to leaves dipped in water for 5 min. Results are shown as average (n=3 technical replicates) with standard error bars. The asterisks above the bars indicate statistical significance in comparison to water treated as calculated with two-tailed Student's *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001).

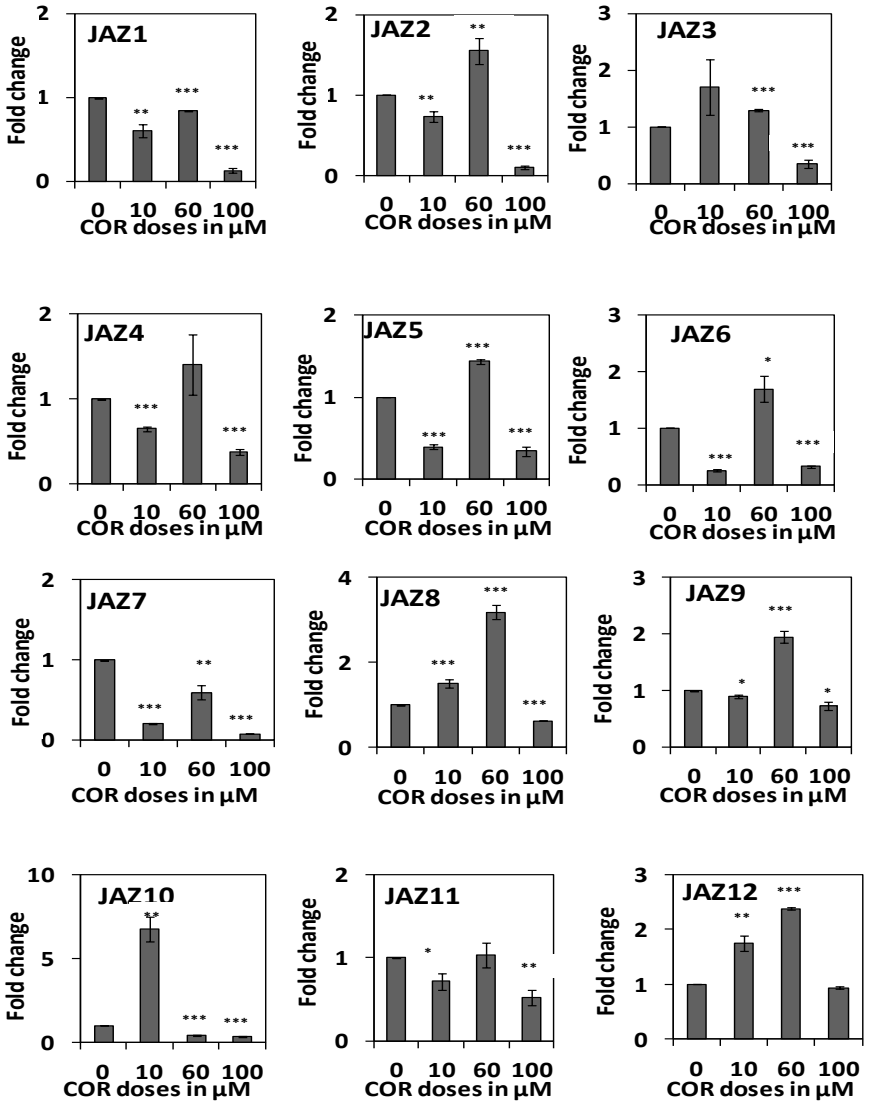


Fig 3.6 *JAZ* gene regulation by COR relapses at 15 minutes in whole leaves. Data points indicate dosages of COR in µM concentrations to study the relative expression of respective *JAZ* genes after placing Col-0 leaves in COR solution as compared to leaves dipped in water for 15 min. Results are shown as average (n=3 technical replicates) with standard error bars. The asterisks above the bars indicate statistical significance in comparison to water treated as calculated with two-tailed Student's *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001).

3.4.2 COR-mediated induction of JAZ genes is stronger in guard cells as compared to whole leaves

To understand whether *JAZ* genes are targets of COR to favor *Pst* DC3000 entry into the apoplast via stomata, it is important to study the regulation of *JAZ* genes by COR in guard cells that form stomata - the port of entry to *Pst* DC3000. To study this, guard cell protoplasts were isolated as described in the section 3.2.2. Considering the previous results, where I observed that most *JAZ* genes relapse from induced state in whole leaves at 15 minutes, COR mediated *JAZ* gene regulation study was restricted to 5min in GCPs. A better linear correlation of induction of *JAZ* genes with COR dosage in GCPs was observed. For example *JAZ*1, *JAZ2, JAZ3, JAZ5, JAZ6, JAZ7, JAZ8* and *JAZ12* followed the dosage pattern and *JAZ4, JAZ10* and *JAZ11* were induced only at 100µM dosage of COR (Fig 3.7). A minimal of 10µM COR was sufficient to induce *JAZ9* and increased dosages of COR did not induce this gene to any further extent. Two way Anova analysis to understand the effect of COR mediated regulation on *JAZ* genes in leaves and GCPs was performed. Anova analysis revealed the COR regulation of most *JAZ* genes is significantly different from GCPs and whole leaves (Table 3.4). Summary of the gene regulation of *JAZ* genes by COR can be found in Table 3.3. These findings also suggest that COR regulates *JAZ* genes distinctively in guard cells as compared to whole leaves, and further outcome of this analysis is also that COR mediated induction of *JAZ2* and *JAZ4* is unique to guard cells as compared to whole leaves.

Two very important functions of COR relevant to this study is that COR re-opens bacterium triggered stomatal closure (Melotto et al., 2006) and *Pst* DC3000 regulates *JAZ* gene expression analysis in COR dependant manner (Demianski et al., 2012). The result obtained above connects these two important functions of COR. The unique regulation of these *JAZs* in guard cells by COR might be of importance to aid or restrict entry of *Pst* DC3000. Not only the regulation of *JAZ* genes correlated to the dosages of COR, the

distinctive regulation at lower and higher COR dosages were not observed in GCPs indicating tissue specific differences of *JAZ* regulation. Significant repression has not been found at 10uM dosage for any *JAZ* gene in GCPs.

3.4.3 JAZ genes are more abundant in guard cells than whole leaves

Since COR mediated regulation of *JAZ* genes was striking in guard cells, it was important to study the basal levels of *JAZ* transcripts in guard cells. I therefore studied abundance of *JAZ*s in GCPs in relation to whole leaves using *TUB4* as housekeeping gene. This study revealed the profuse presence of most *JAZ* genes in guard cells as compared to whole leaves other than *JAZ3* and *JAZ6* (Fig. 3.8). This cell specific abundance of *JAZ* genes might explain the distinct and robust regulation of *JAZs* in guard cells and a better dosage response in other *JAZ* genes due to COR. It could be possible that *JAZ* abundance is the underlying reason that their regulation by coronatine is prominent and rapid in GCPs. Regulation of *JAZ* genes by a wide variety of environmental factors like humidity (Panchal et al. under review), touch (Sehr et al. 2010), light (Wang et al. 2012, Robson et al. 2010) and biotic factors like *Pst* DC3000 (Demianski et al. 2011), absicisic acid, jasmonic acid strengthens my result that these genes are abundant in the cells that are a major part of the dermal tissue system that constantly endures changes in environment and encounters different biotic and abiotic stresses.

Abundance of *JAZ* genes in guard cells as compared to whole leaves suggests the importance of restricting *JAZ* studies to guard cells. However it might also be possible that *JAZ* genes are also present in the mesophyll cells and their distinctive regulation in these two different kinds of cells suggests that *JAZ* genes perform distinctive functions.

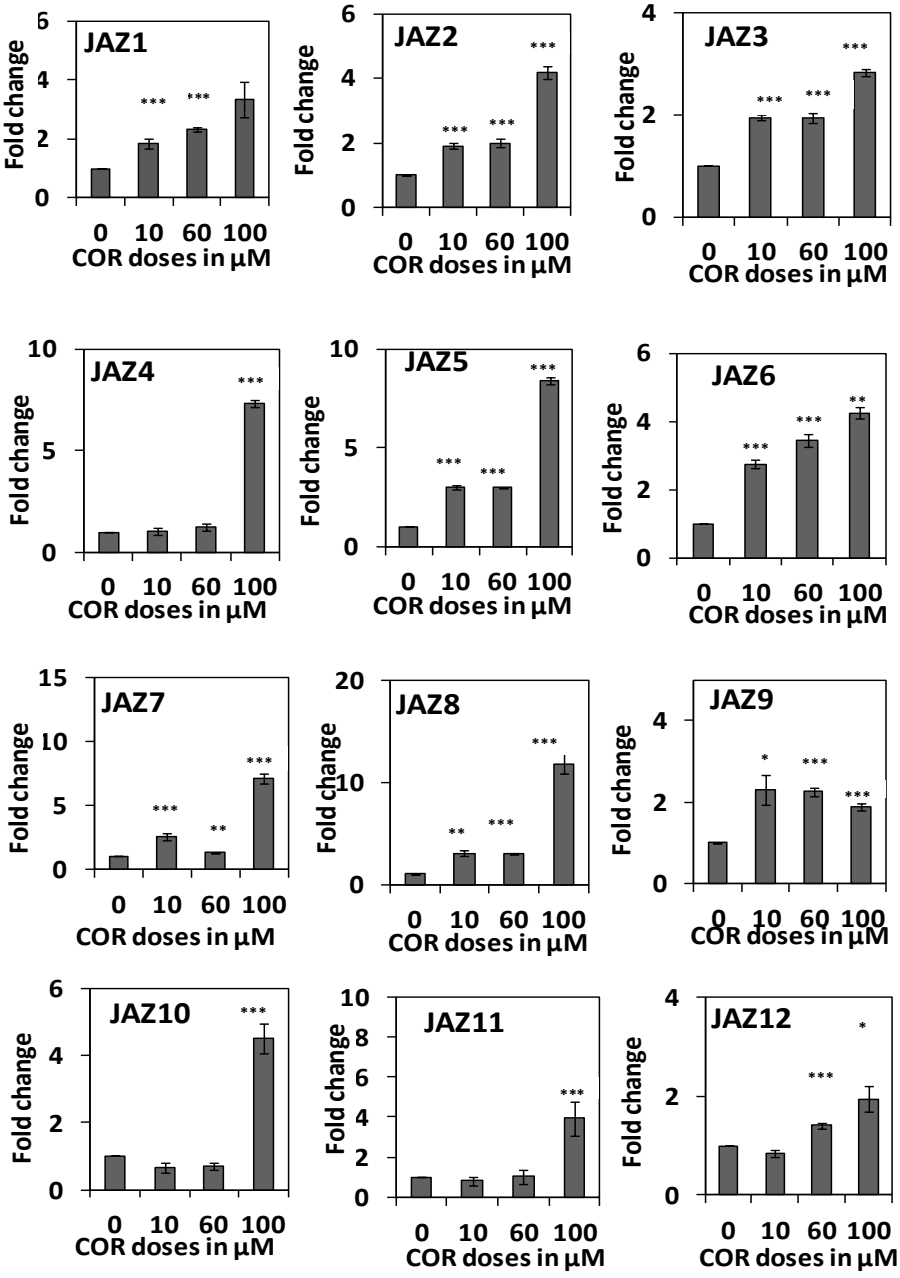


Fig. 3.7 Robust regulation of *JAZ* genes by COR in guard cells isolated using short protocol. Data points indicate the relative expression of *JAZ* genes after COR treatment of Col-0 leaves and GCP isolations, water treatment for 5 min was used as control. Results are shown as average (n=3 technical replicates) with standard error bars. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point as calculated with two-tailed Student's *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001). Two biological replicates were performed for each gene.

Table 3.3 Expression analysis of *JAZ* genes 5 minutes post COR treatment in whole leaves and GCPs

	LEAF			GCP		
	10µMCOR	60µMCOR	100µMCOR	10µMCOR	60µMCOR	100µMCOR
JAZ1	---	+++	None	+++	+++	None
JAZ2	--	-	---	+++	+++	+++
JAZ3	+	None	None	+++	+++	+++
JAZ4	None	None	---	None	None	+++
JAZ5	---	+++	+++	+++	+++	+++
JAZ6	---	+++	++	+++	+++	++
JAZ7	+++	++	+++	+++	++	+++
JAZ8	---	+++	+++	++	+++	+++
JAZ9	---	None	+	+	+++	+++
JAZ10	+++	---	+	None	None	+++
JAZ11	None	++	+++	None	None	+++
JAZ12	+++	+++	+++	None	+++	+

+ indicates induction, - repression, and none denotes, no significant difference was noted. Numbers of +/- signs indicate degree of induction and repression

Table 3.4 Two way Anova analysis of *JAZ* regulation due to coronatine treatment in tissue specific manner

ANOVA for JAZ1

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	4.23	2	2.12	7.21	8.8E-03	3.89
Tissue type	4.53	1	4.53	15.43	2.0E-03	4.75
Tissue type and COR	5.72	2	2.86	9.74	3.1E-03	3.89

ANOVA for JAZ2

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	5.24	2	2.62	49.00	1.7E-06	3.89
Tissue type	17.29	1	17.29	323.21	4.8E-10	4.75
Tissue type and COR	4.82	2	2.41	45.09	2.6E-06	3.89

ANOVA for JAZ3

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	1.08	2	0.54	9.62	0.003218	3.89
Tissue type	2.92	1	2.92	51.82	1.09E-05	4.75
Tissue type and COR	3.31	2	1.65	29.31	2.41E-05	3.89

ANOVA for JAZ4

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	33.49	2	16.74	316.73	4.13E-11	3.89
Tissue type	27.13	1	27.13	513.24	3.24E-11	4.75
Tissue type and COR	43.90	2	21.95	415.23	8.35E-12	3.89

ANOVA for JAZ5

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	137.24	2	68.62	324.64	3.6E-11	3.89
Tissue type	3.48	1	3.48	16.44	1.6E-03	4.75
Tissue type and COR	7.12	2	3.56	16.84	3.3E-04	3.89

Table 3.4 continued…

ANOVA for JAZ6

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	5.20	2	2.60	48.57	1.8E-06	3.89
Tissue type	21.39	1	21.39	399.30	1.4E-10	4.75
Tissue type and COR	0.29	2	0.15	2.71	1.1E-01	3.89

ANOVA for JAZ7

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	580.74	2	290.37	62.65	4.5E-07	3.89
Tissue type	221.35	1	221.35	47.76	1.6E-05	4.75
Tissue type and COR	227.90	2	113.95	24.59	5.7E-05	3.89

ANOVA for JAZ8

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	120.23	2	60.12	107.42	2.2E-08	3.89
Tissue type	93.70	1	93.70	167.43	2.1E-08	4.75
Tissue type and COR	44.72	2	22.36	39.96	5.0E-06	3.89

ANOVA for JAZ9

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	4.37	2	2.18	24.60	5.7E-05	3.89
Tissue type	1.71	1	1.71	19.21	8.9E-04	4.75
Tissue type and COR	8.35	2	4.18	47.04	2.1E-06	3.89

ANOVA for JAZ10

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	76.06	2	38.03	28.83	2.6E-05	3.89
Tissue type	10.51	1	10.51	7.97	1.5E-02	4.75
Tissue type and COR	8.75	2	4.38	3.32	7.1E-02	3.89

Table 3.4 continued…

ANOVA for JAZ11

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	14.011	2	7.006	11.51	1.6E-03	3.89
Tissue type	0.003	1	0.003	0.01	9.4E-01	4.75
Tissue type and COR	9.123	2	4.561	7.49	7.7E-03	3.89

ANOVA for JAZ12

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
COR dosages	1.65	2	0.82	17.06	3.1E-04	3.89
Tissue type	0.93	1	0.93	19.17	9.0E-04	4.75
Tissue type and COR	0.51	2	0.26	5.30	2.2E-02	3.89

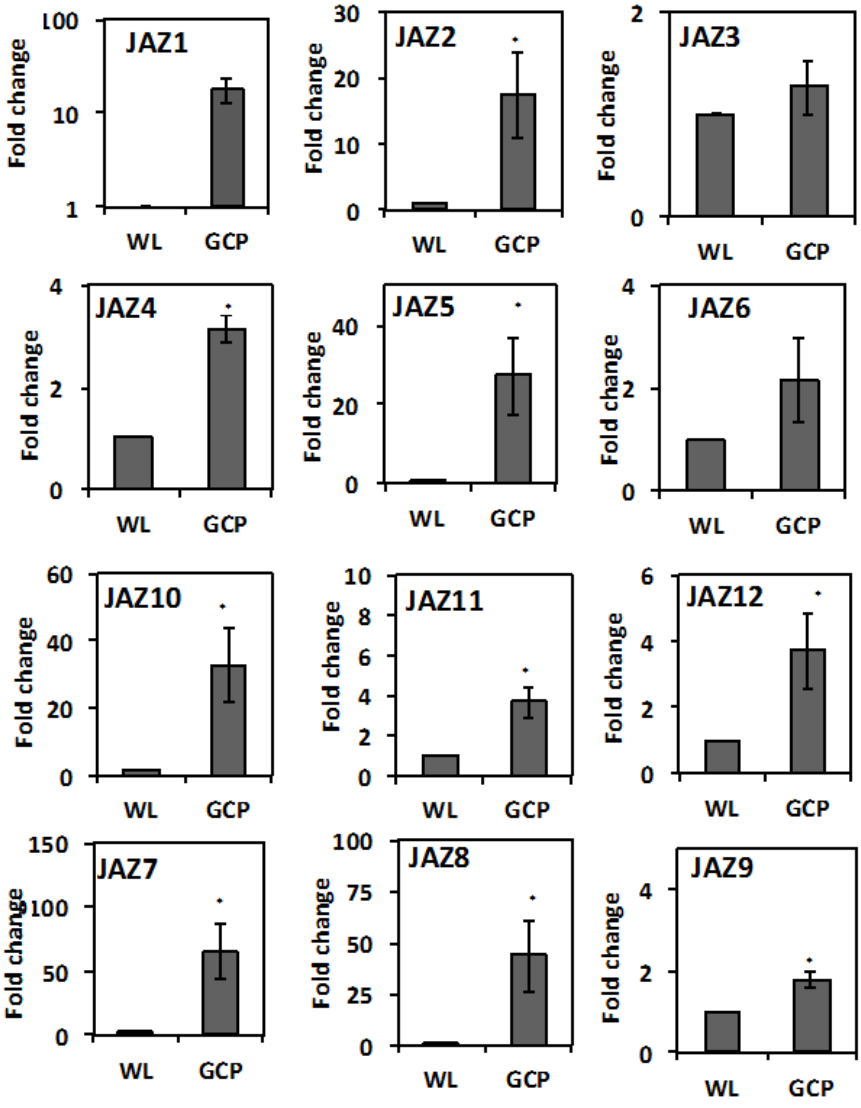


Fig 3.8 *JAZ* gene expression is profuse in guard cells. Transcript abundance of the indicated genes relative to the cell type was determined by RT-qPCR analysis. Results are shown as mean (n=6) ± standard error. Statistical significance of the difference in the mean abundance of each gene (whole leaves versus guard cells) was detected with two-tailed Student's *t*-test (* = $P<0.05$).

3.4.4 Jasmonic acid network might be different in guard cells

From the above results it is known that *JAZ* genes are abundant in guard cells as compared to whole leaves and it also understood that *JAZ* genes are key signaling molecules of JA network in plants. I therefore performed gene expression analysis (qPCR) of some key signaling molecules of JA signaling like *NINJA, COI1*, and *LOX3* in guard cells using the primers shown in Table 3.3.

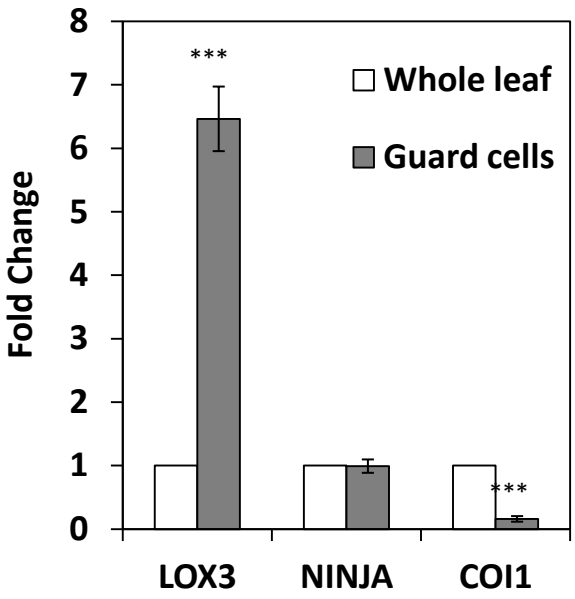


Fig 3.9 Key *JAZ* regulators are differentially expressed in guard cells. Transcript abundance of the indicated genes relative to the cell type was determined by RT-qPCR analysis using *UBC 21* as housekeeping gene. Results are shown as mean (n=6) ± standard error. Statistical significance of the difference in the mean abundance of each gene (whole leaves versus guard cells) was detected with two-tailed Student's *t*-test (*** = *P*<0.001).

My results indicate that *LOX3* transcripts are also constitutively profuse in guard cells but COI1, which is a central regulator of signaling in JA network is found to be more in whole leaves as shown in Fig 3.9. Another important protein in *JAZ* signaling is the NINJA that is a repressor of *JAZ* transcripts. qRT-PCR analysis reveals no significant difference in the magnitude of mRNA quantity of *NINJA* transcript in guard cells and whole leaves (Fig 3.9). These results suggest that JA signaling might not be the same in guard cells and mesophyll

cells. Moreover, the specialized *JAZ* regulation in these cells might aid or restrict pathogen entry by fine tuning the JA network in favor of pathogens. The functional prediction of this result needs further exploration; it could be possible that COR has other targets in guard cells as COI1 has very low basal levels in these specialized cells. A recent study reports that three NAC transcription factors are necessary for coronatine to re-open stomata (Zheng. et al. 2012). It could be possible that NAC transcription factors have a major role in COR – JAZ network thereby modulating stomatal immunity eventually. However the role of COI1 in guard cells cannot be completely ruled out because, it might be possible that COI1 will be induced in guard cells at the time of infection to carryout ubiquitination processes and therefore gene expression analysis of COI1 post COR treatment in guard cells can also be done to exclude this possibility.

Table 3.5 Gene-specific primers used in qRT-PCR reactions and the expected amplicon sizes.

Gene	Primer	Sequence (5'-3')	Size (bp)
LOX3 (At1g17420)	Forward	GGATGCTTTTGCTGATAAAATTGGTCGA	125
	Reverse	CGATTTCTTTGACCAATCCTTTAAAACT	
NINJA (At4g28910)	Forward	CAACAGGTTGTTTGCCTTCGCCTT	91
	Reverse	AGGAGGGATTGTCGCACTTTCTCA	
COI1 (AT3G57260)	Forward	GAAGCCACTTACCATCCTTT	165
	Reverse	TCTTGAGACTTTGAAGCTAGAC	

3.4.5 Genotype and phenotype analysis of jaz4^{-} mutants

Very little is known about the function of the individual JAZ proteins. Individual mutants of *JAZ* genes were studied earlier which did not show any obvious phenotypes (Chini et al. 2007, Thines et al. 2007, Yan et al. 2007). It has long been considered that these proteins have overlapping functions but despite this consideration specialized function of each JAZ protein cannot be ruled out. It may well be possible that most JAZ related phenotypes are restricted to guard cells and are going undetected.

Since *JAZ4* was regulated by COR only in guard cells and not in whole leaves, it was important to investigate whether *jaz4*$^{-}$ mutants exhibited any phenotype related to stomatal immunity. Therefore, two T-DNA insertion lines, SALK_001245 and SALK_141628C that have T-DNA insertions in first intron and fourth exon of the *JAZ4* gene respectively (Fig 3.10A), were screened for absence of transcript (Fig 3.10). *JAZ4* gene structure reveals that this gene has four splice variants (Fig 3.10A) which make it necessary to check the absence of all these transcripts in the mutant lines. The detailed information of the splice variants will

be discussed in Chapter 4. RT-PCR analysis revealed the absence of all the transcripts of *JAZ4* in SALK_001245 line and presence of *JAZ4.1* and *JAZ4.2* in wild type Col-0 plants (Fig 3.10B). Even after repeated analysis *JAZ4.3* and *JAZ4.4* transcripts were not detected in Col-0. It could be possible that these two splice variants were very low abundant that they cannot be detected with RT-PCR. Therefore genotypying of SALK_141628C and SALK_001245 will be completed in the near future with qPCR by designing primers that span all the splice variants.

I later studied the phenotypic analysis of SALK_001245 and SALK_141628C by performing dip inoculations with the bacterial strains *Pst* DC3000 (Fig 3.11) and *Pst* DC3118 (Fig 3.12). Bacterial dip inoculations with *Pst* DC3000 showed increased bacterial growth in the apoplast on day1 in both T-DNA lines SALK_001245 (Fig 3.11B) and SALK_141628C (Fig 3.11A) as compared to wild type plants. However when the same experiment was repeated with the line SALK_001245 (Fig 3.11C), I did not observe the same trend, also no visible difference in the symptoms was observed between Col-0 and SALK_001245. I am in the process of repeating this experiment for a robust conclusion.

When *jaz4*$^{-}$ plants, SALK_001245 and SALK_141628C were dip-inoculated with *Pst* DC3118 no striking difference was observed as compared to wild type Col-0 plants (Fig 3.12C-D), These results indicate, in absence of COR, stomatal defense is not altered in both these mutant lines, however, SALK_001245 plants showed significant stomatal closure in response to *Pst* DC3118 two hours post-inoculation as compared to Col-0 (Fig 3.13). It could be possible that absence of *JAZ4* results in weaker phenotype that is evident in stomatal assays but not in dip inoculations by *Pst* DC3118. When plants were vacuum infiltrated or syringe infiltrated with the same bacteria bypassing stomatal immunity, significant increased bacterial population was noted on day1 (Fig 3.11A-B) this result suggests that *JAZ4* is required for early apoplastic immunity as well.

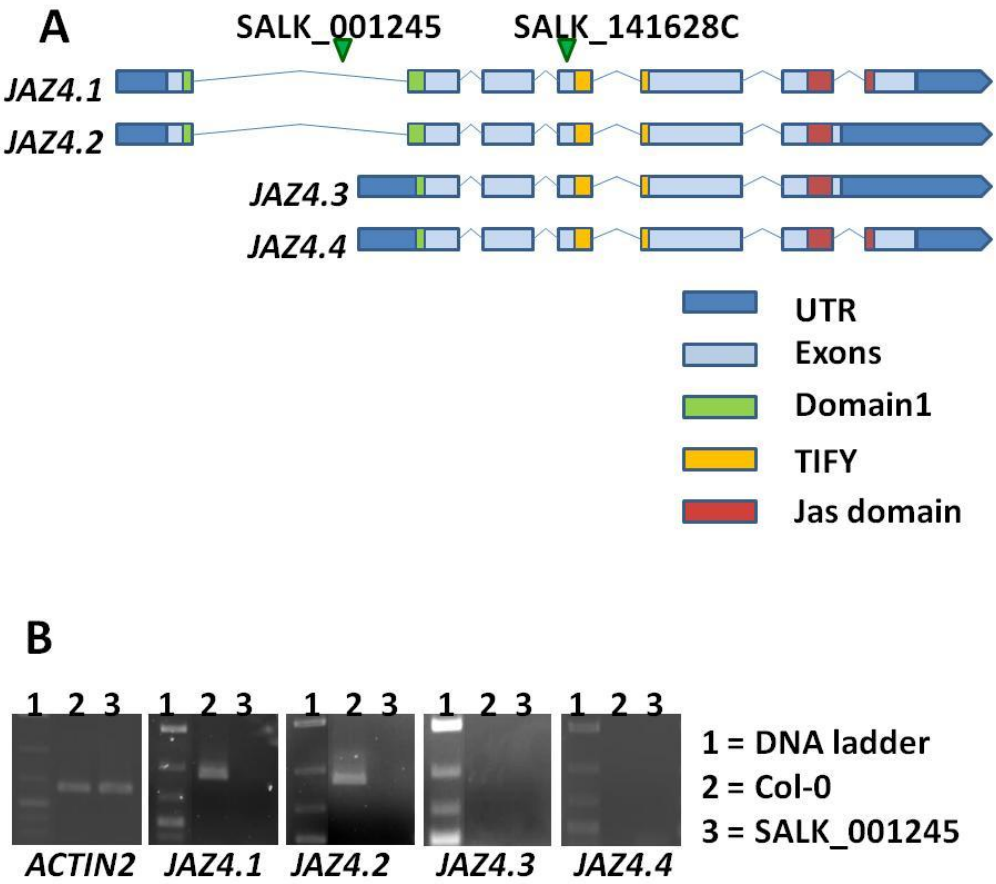


Fig 3.10 Genotyping of *jaz4*$^{-}$ mutant. Schematic diagram of the *JAZ4* gene with all four splice variants. (A) The position of a T-DNA insertion is indicated as green arrow heads for both the SALK_001245 and SALK_141628C. (B) Constitutive expression levels of *JAZ4* splice variants in the T-DNA insertion mutant SALK_001245 and Col-0 plants by RT-PCR analysis.

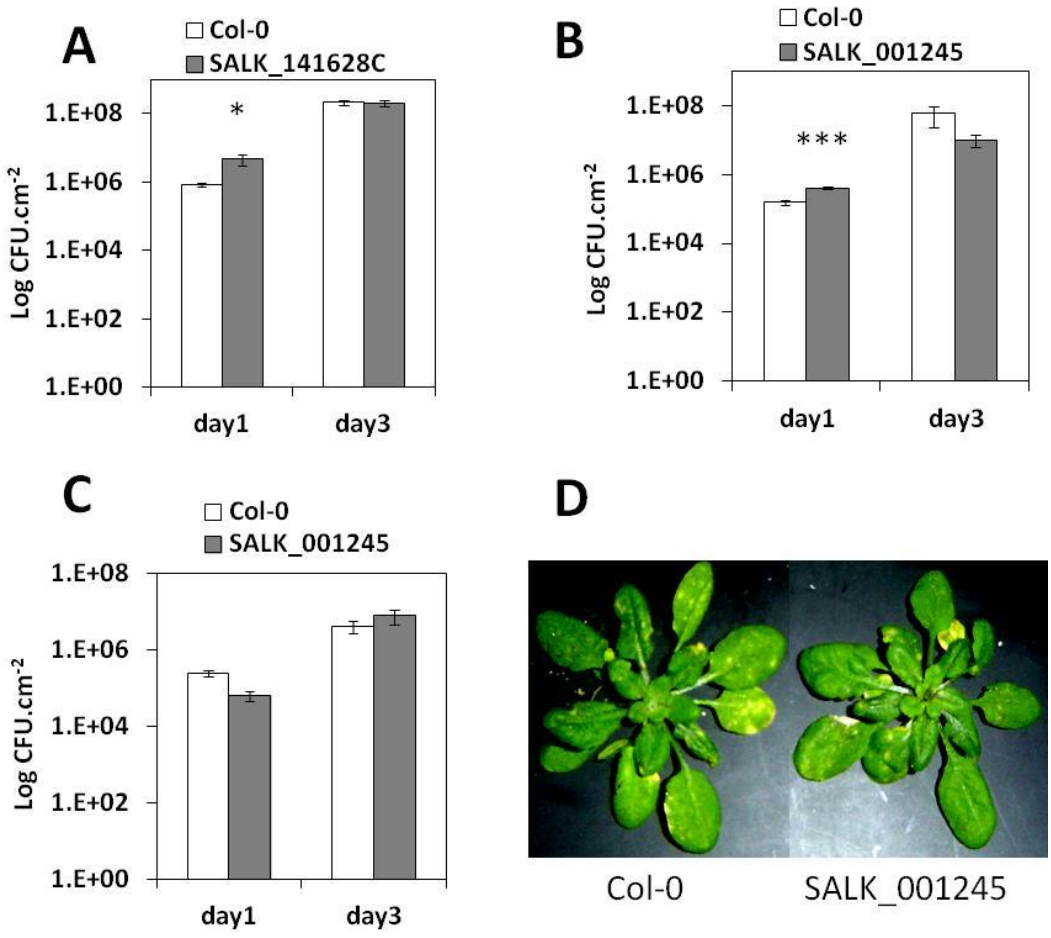


Fig 3.11 Bacterial inoculations of *jaz4*⁻ mutants with *Pst* DC3000 population in the apoplast of plants inoculated by dipping (A) SALK_141628C or (B) and (C) SALK_001245. Picture at the right in panel (D) was taken 3 day post inoculation. Results are shown as the mean (n=6) ± standard error (SE). Statistical significance was detected with two-tailed Student's t-test (* = $p<0.05$).

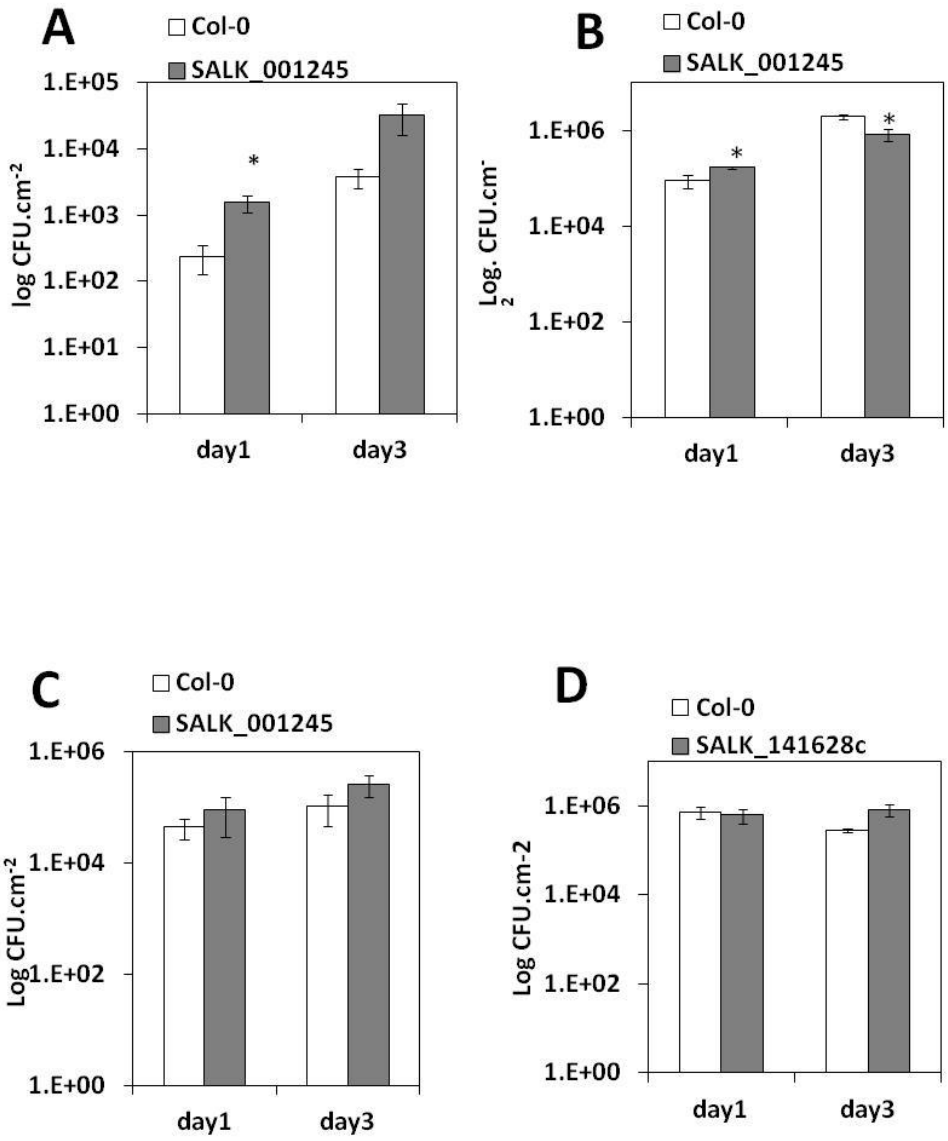


Fig 3.12 Bacterial inoculations of *jaz4*- mutants with *Pst* DC3118. Panels (A) and (B) are the inoculations done with vacuum and syringe infiltration respectively in SALK_001245. below are bacterial populations of *Pst* DC3118 inoculated by dipping in (C) SALK_001245 and (D) SALK_141628C

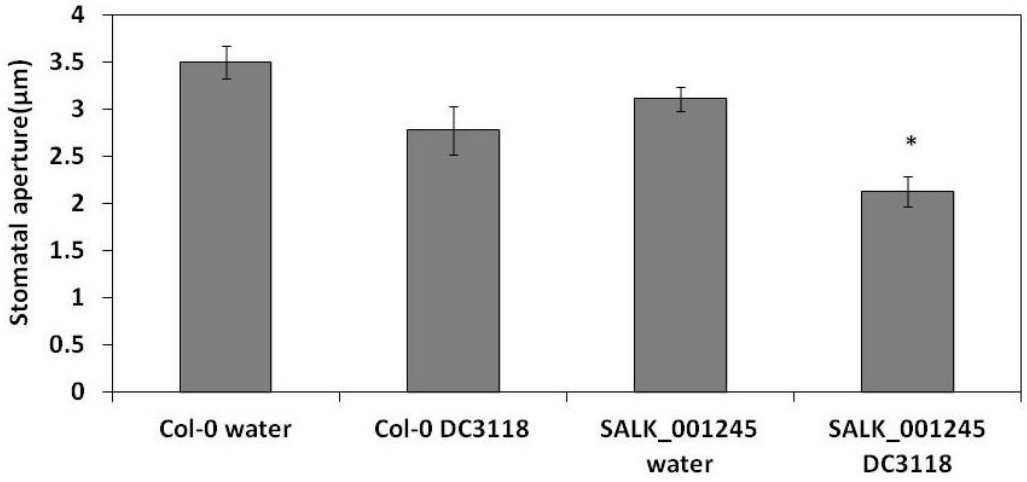


Fig 3.13 Wild type (Col-0) and *jaz4*- mutant line (Salk_001245) were dipped in bacterial suspension and the stomatal apertures were measured after 2 hours. Results are shown as means (n=60) ± standard errors and Statistical significance was detected with two-tailed Student's t-test (*** = p<0.01).

3.4.6 JAZ4 modulates key marker genes of jasmonic acid and salicylic acid signaling pathways

To understand whether JAZ4 can modulate jasmonic acid or salicylic acid hormonal pathways, expressions of key marker genes were studied using RT-PCR. To check the JA regulation in *jaz4*$^{-}$ (SALK_001245), *OPR3 and LOX3* - the JA biosynthetic genes, *MYC2* - the key transcription factor in JA signaling and primary JA responsive genes (Chung et al. 2008), and *PDF1.2* - a plant defensin gene necessary for JA related defense mechanisms were selected (Brown et al. 2003). Up-regulation of *OPR3* and *MYC2* indicates the induced JA signaling in *jaz4*$^{-}$ (SALK_001245) as compared to wild-type. Simultaneously other JA signaling genes like *LOX3* showed repression and no difference was noted in *PDF1.2* transcripts between *jaz4*$^{-}$ (SALK_001245) and Col-0 (Fig 3.14). Similarly to check the SA regulation in *jaz4*$^{-}$ (SALK_001245); *PR-1* and *PR-5* gene expressions were studied as these genes were known to be dependent on SA pathway (Kojima et al. 2012). These genes were constitutively strongly expressed in this line as compared to wild-type (Fig 3.14). These

results indicate that JAZ4 might be playing a dual role by regulating both the hormonal pathways simultaneously. However similar experiments must be repeated in other *jaz4*$^{-}$ (SALK_141628C) to draw stronger conclusions.

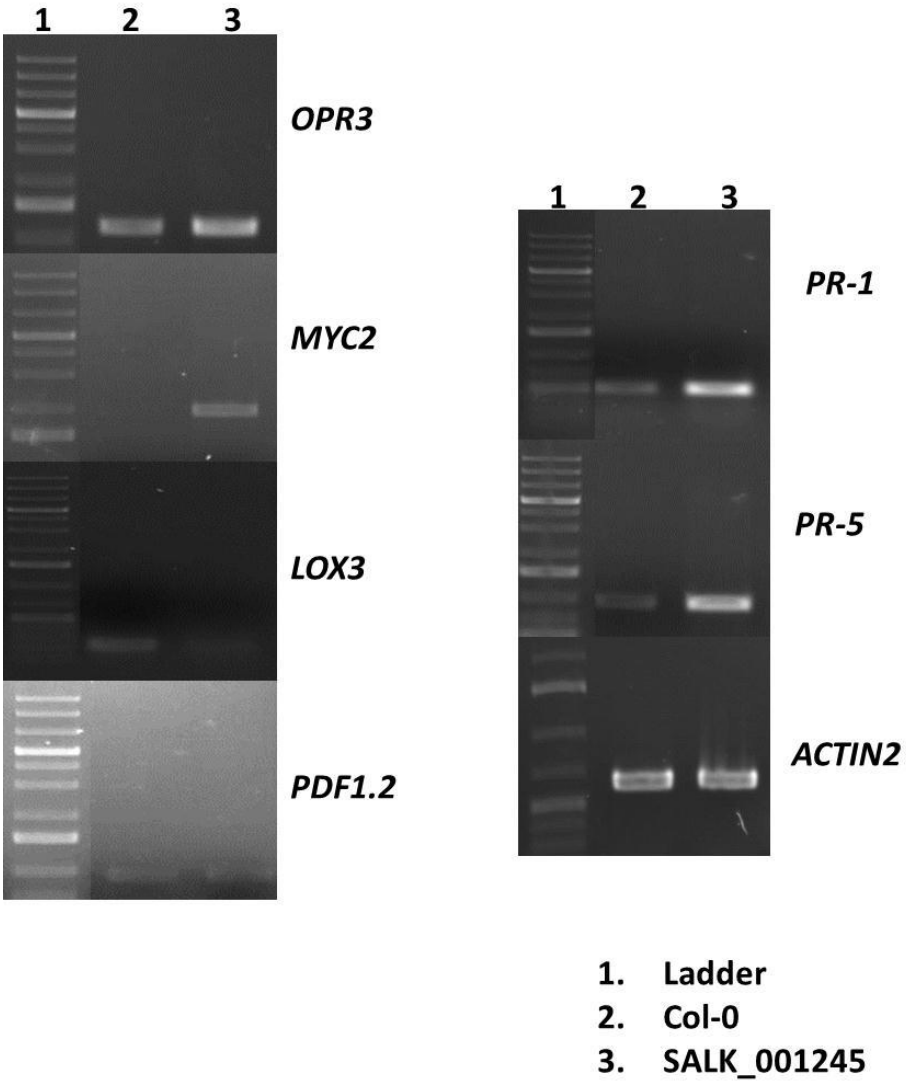


Fig 3.14 RT-PCR analysis of JA and SA responsive genes in *jaz4*$^{-}$ mutant (SALK_001245). Constitutive expression analysis of JA responsive genes (*OPR3, MYC2, LOX3, PDF1.2*) and SA responsive genes (*PR-1*, and *PR-5*) with respect to Col-0 using *ACT2* as a housekeeping gene was performed.

In addition to key marker genes, gene expression analysis of other *JAZ* genes was also done. Results indicate that absence of *JAZ4* modulates *JAZ1, JAZ7* and *JAZ8* indicating

the role of *JAZ4* on expression of other *JAZ* genes (Fig 3.15). This experiment was done by Debanjana Roy and has been included here with her permission. Earlier studies have discovered the potential of JAZ proteins to form homo and heterodimers. Therefore absence of JAZ4 might be regulating other JAZs. It could also be possible that since JA signaling has been upregulated which is evident from Fig 3.12 the upregulated JA has an effect on other *JAZ* genes.

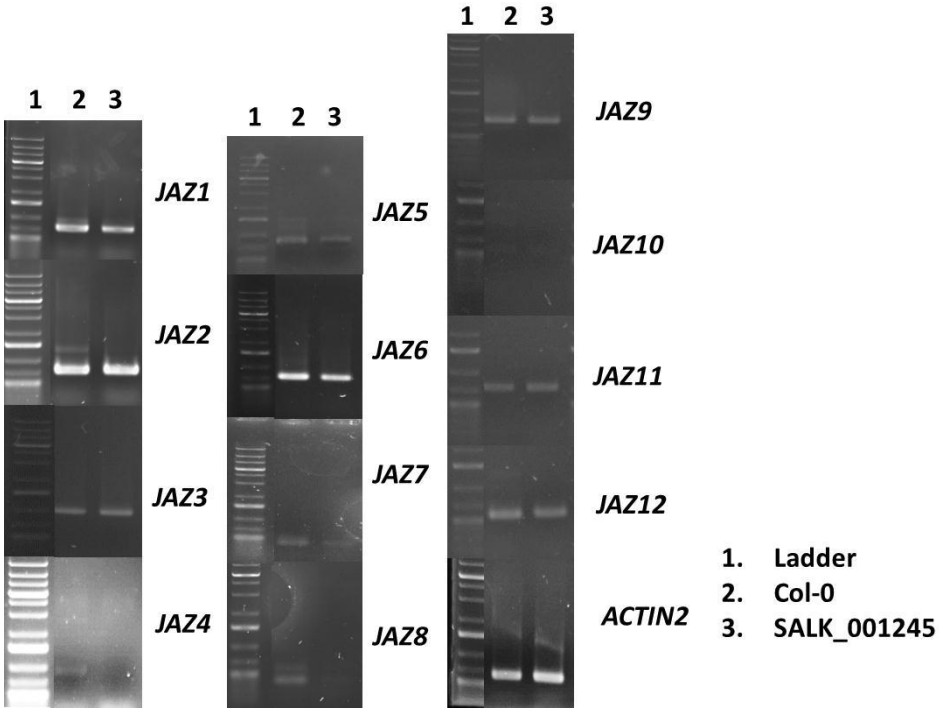


Fig 3.15 RT-PCR analysis of other *JAZ* genes in *jaz4*$^{-}$ mutant. Constitutive expression analysis of all *JAZ* genes with respect to Col-0 using *Actin2* as a housekeeping gene.This experiment was performed by Debanjana Roy and I thank her for giving me permission to include this figure here

Table 3.6 Gene-specific primers used in RT-PCR reactions and the expected amplicon sizes.

Gene	Primer	Sequence (5'-3')	Size (bp)
MYC2 (At1g32640)	Forward	AATGACTGATTACCGGCTACA	1535
	Reverse	TTAACCGATTTTTGAAATCAA	
OPR3 (At2g06050)	Forward	ATGGCCTTAGCTAAAGAGTTAATG	1176
	Reverse	TCAGAGGCGGGAAAAAGGAGCCAA	
PR-1 *(At2g14610)*	Forward	ATGAATTTTACTGGCTATTCTCG	486
	Reverse	TTAGTATGGCTTCTCGTTCACA	
PR-5 *(AT1g75040)*	Forward	ATCATCATCACCCACAGCAC	981
	Reverse	AAGTATCCGTAGATCAATCATTTTGT	
LOX3 (At1g74950)	Forward	ATGGCCTTAGCTAAAGAGTTAATG	749
	Reverse	TATCAGAAACTGTTGATTGGCGAC	

3.4.7 JAZ4 has no role in photomorphogenesis

Recent studies have reported that *JAZ* genes respond to environmental cues like light (Yang et al. 2012), and JAZ9 over-expressor plants flower early in long day conditions (16 h 120 µmol. $m^{-2}\cdot s^{-1}$ light/8 h dark, 22 °C/18 °C) and produce long hypocotyls at low light conditions (10 µmol. $m^{-2}\cdot s^{-1}$ continuous white light at 22 °C for 6 d; Yang et al. 2012). To check the possibility of JAZ4 response to different light conditions JAZ4 plants were grown for 14 weeks under low light conditions (50 µmol.m−2·s−1 white light at 22 °C, 12h photoperiod for 14 weeks). Growth was monitored intermittently. At the end of 4 weeks both plants (mutant and wild type) grown under low light showed slow growth as compared to normal light. No difference was noted between Col-0 and *jaz4*$^{-}$ (Salk_001245) in their growth rate under the same light condition (Fig 3.16), and number of days taken to flower (Fig 3.17 A-B).

However *jaz4*- plants showed increased anthocyanin production at low light conditions (Fig 3.17C) which might be due to constitutively up-regulation of jasmonic acid in this line (Kazan et al. 2011). Upregulation of JA defense signaling is identified to strictly restrict plant development; this is a prominent example of growth–defense tradeoff in plants. In *jaz4*$^{-}$ mutants although JA upregulation is noted through gene expression analysis and increased anthocyanin production no growth retardation was observed. This indicates that the JA upregulation in these plants might not strong enough to exhibit development phenotypes.

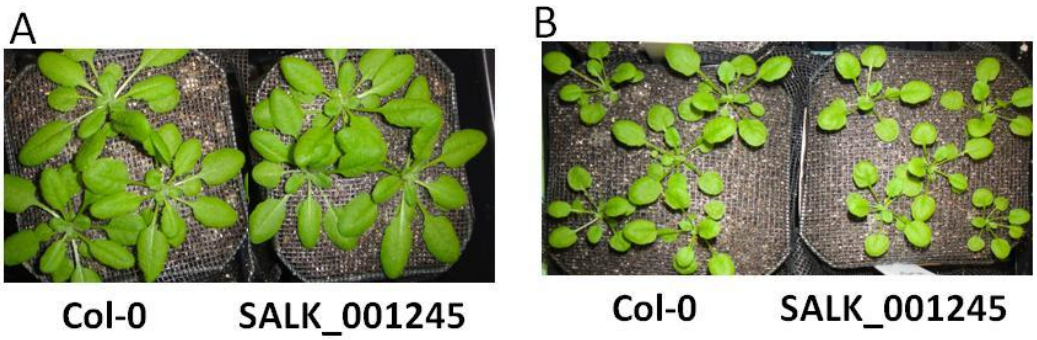


Fig 3.16 Photomorphogenetic analysis of *jaz4*$^{-}$ mutant for 4 weeks. (A) Plants when grown under 100µmol. m−2·s−1 or (B) 50µmol. m−2·s−1white light at 22 °C, 12h photoperiod for 4 weeks. Pictures were taken at the end of 4 weeks. Note the growth retardation of plants under low light conditions

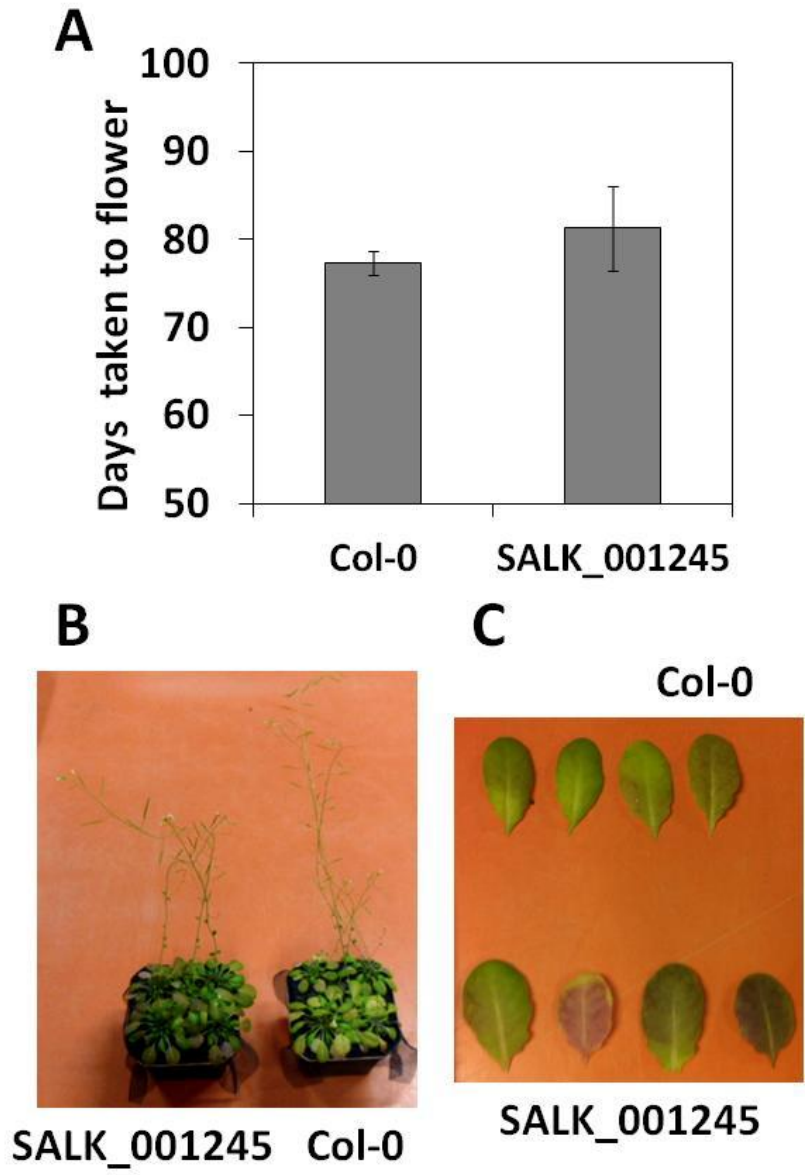


Fig 3.17 Photomorphogenetic analysis of *jaz4*⁻ mutant for 14 weeks. Plants were grown under 50µmol.m−2·s−1white light at 22 °C, 12h photoperiod for 14 weeks. (A) Days taken for the plants to flower were noted and represented as graph (B) Pictures were taken at the end of 14 weeks. (C) Note the increased anthocyanin production in the mutant plants under low light conditions as compared to the wild type Col-0 plants.

3.5 Conclusion

This chapter is more focused on studying the direct regulation of COR on *JAZ* genes. Gene expression analysis after treating leaves with different dosages of COR was performed in guard cell and whole leaves. My results indicate that 1) COR regulates *JAZ* gene expression as early as 5 minutes in whole leaves and guard cells, 2) *JAZ2* gene is induced by COR in guard cells and not in whole leaves that are enriched by mesophyll cells, 3) *JAZ4* gene is regulated at higher dosages of COR in only guard cells at 5 minutes, 4) *JAZ* gene transcripts are more abundant in guard cells in comparison to whole leaves, and 5) This study provides evidence of distinctive JA signaling network in guard cells as compared to whole leaves. 5) *JAZ4* might favor *Pst* DC3000 entry at early stages of infection, however more studies on phenotypic analysis of *jaz4*$^{-}$ mutants are needed to confirm the importance of *JAZ4* in bacterial penetration. 6) *jaz4*$^{-}$ (SALK_001245) mutants exhibit increased *Pst* DC3118 multiplication when Arabidopsis plants were inoculated with infiltration procedures.7) *jaz4*$^{-}$ (SALK_001245) mutants showed increased anthocyanin production when grown at low light conditions.

Chapter 4

Structure and Function Analysis of JAZ Proteins in Arabidopsis

4.1 Abstract

The mode of action of coronatine in plant cells has beginning to be elucidated. Two components of the coronatine receptor complex have been identified, namely COI1 (the F-box subunit of and E3 ligase complex) and JAZ (a transcriptional repressor of jasmonic acid pathway) proteins, suggesting that coronatine acts in the plant by inducing the degradation of proteins and hijacking the jasmonic acid (JA) signaling pathway. This study is focused on determining the function of the JAZ4 protein in mediating plant immunity at early stages of bacterial infection. Using a combination of approaches, including gene expression analysis, ectopic expression of truncated proteins, and gene knock-outs I determined that JAZ4 occurs in four natural isoforms in Arabidopsis, allowing for additional levels of regulation of plant innate immunity. Furthermore, I have substantial genetic evidence that the N-terminus of JAZ9 is essential for plant's defense against *Pst* DC3000 and it also restricts ubiquitin-dependent degradation of JAZ9. The N- terminus of JAZ4 and JAZ9 is also required for normal photomorphogenesis. This study will contribute to refining the current model of JAZ proteins in the plant cell during pathogen infection.

4.2 Introduction

Coronatine (COR) is a phytotoxin produced by several pathovars of the bacterial pathogen *P. syringae* that plays a central role in suppressing plant defenses and thereby enhancing tissue necrosis and chlorosis (Bender et al. 1999, Block et al. 2005, Brooks et al. 2005, Cui et al. 2005, Melotto et al. 2006, 2008, Mittal and Davis, 1995, Underwood et al. 2007). This virulence of COR is achieved by facilitating the binding of COI1 and a family of proteins called JAZ repressors. The structure of JAZ proteins revealed the presence of three domains in them; a weakly conserved N-terminus domain also called domain1, a central ZIM

domain otherwise called domain2 and a highly conserved C-terminus Jas domain or domain3 as shown in Fig 4.1 (Thines et al. 2007, Chini et al. 2007). Each of these conserved motifs are known to have an important role in recruiting other repressors or interacting with key proteins of several hormonal pathways such as MYC2, MYC3, MYC4; COI1; DELLA; NINJA; and other JAZ proteins (Cheng et al. 2011, Fernández-Calvo et al. 2011, Niu et al. 2011, Chini et al. 2007, Chico et al. 2008 Zhu et al. 2011 Hou et al. 2011).

A lot of emphasis has been laid on the Jas domain and ZIM domain eventually leading to the discovery of various JA related phenotypes associated with Jas domain and novel protein-protein interactions through the ZIM domain. However, not until recently the importance of N-terminus of JAZ proteins has been studied. Hou et al (2011) have shown that the N-terminus and C-terminus of JAZ proteins are essential for some critical interactions with the DELLA proteins RGA, GAI, and RGL1 that regulate gibberellic acid (GA) signaling. Having known that GA is a growth promoting plant hormone and JA a defense related hormone, Yang et al. (2012) carried out experiments and determined a molecular cascade by which JA antagonizes GA signaling, and showed how plants prioritize defense over growth.

As the mentioned above, researchers have identified novel interactions of JAZ proteins via N-terminus, however their function in disease pathogenicity is not yet determined. Here, I report that 1) the N-terminus of JAZ4 and JAZ9 is required for resistance against *Pst* DC3000 infection in Arabidopsis, 2) JAZ4 exists in four natural isoforms; *JAZ4.1, JAZ4.2, JAZ4.3,* and *JAZ4.4,* 3) the N-terminus of JAZ4 and JAZ9 is required for stomatal immunity, 4) plants expressing truncated JAZ4 protein lacking the N-terminus exhibit photo-morphogenetic phenotypes and increased anthocyanin production when grown under low light intensity, and 5) the N-terminus of JAZ9 is required to prevent degradation of JAZ9 via 26s proteasome independent pathway.

4.3 Methods

4.3.1 T-DNA lines genotyping

T-DNA insertion lines that have kanamycin resistant gene were screened for *JAZ* knock-outs following the method described in Chapter 3.

4.3.2 Stomatal assay and bacterial pathogenesis assays

T-DNA insertion lines that were homozygous for insertion were proceeded to bacterial growth and stomatal assays as mentioned in chapter3

4.3.3 RT-PCR procedure

RNA was extracted from young, fully expanded leaves using the RNeasy plant mini kit (Qiagen,Valencia - CA), , quantified using Nanodrop-1000 Ver 3.2 (Thermo scientific, Wilmington-DE) and stored at -80^{o}C. Complementary DNA (cDNA) was constructed from 5µg total RNA and olido-dT using Takara kit (Takara. Bio. Inc. Ver.3.0, Shiga, Japan). Then using cDNA as template end-point PCR was performed as mentioned in chapter3

4.3.4 Photomorphogenesis observations

Arabidopsis seeds were stratified after sowing at 4 °C for 2 days the pots were then transferred to a growth chamber with 50 µmol $m^{-2}{\cdot}s^{-1}$ continuous cool-white fluorescent light at 22 °C under 12-hour photoperiod. As a control some pots were transferred to a growth chamber with 100 µmol.$m^{-2}{\cdot}s^{-1}$ continuous cool-white fluorescent light at 22 °C under 12-hour photoperiod Pictures were taken after 4 weeks and after 14 weeks. Plants were checked every week and number of days taken to flower was noted.

4.3.5 Generating transgenic lines

Arabidopsis plants ectopically-expressing truncated JAZ9 proteins (N-terminus) fused with green fluorescent protein (GFP) were created. Arabidopsis transformations were done using floral dip method as explained by Clough and Bent (1998) using the plant

transformation vector pB7WGF2 (Karimi et al. 2002). Plants that expressed fluorescent protein and that were Glufosinate herbicide resistant (Nelson et al. 2007) were used for further experiments.

4.3.6 Methyl JA (MeJA)-induced degradation assay

To assess the effect of MeJA on JAZ9, transgenic leaves expressing GFP::JAZ9 driven by the CaMV 35S promoter were floated on water, 50µM MeJA, or 50µM MejA + 5µM MG132 with the abaxial surface in contact with the solution. The leaf surface in contact with solution was imaged at Green and red auto-fluorescence and differential interference contrast (DIC) using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY) with Argon laser at excitation of 488 nm and emission at 505-550 BP (green) and 560 LP (red) after 30 minutes of treatment. All channels were imaged simultaneously.

4.4 Results and Discussion

4.4.1 Structure and functional analysis of JAZ proteins

Individual genes in JAZ gene family did not reveal any JA related phenotypes. It was only very recently that a full length JAZ protein i.e., the JAZ9 photomorphogenetic phenotype was determined (Fig 4.1). The three domains present in JAZ proteins interact with wide variety of proteins magnifying their functional complexity .Inspite of an intricate network most of the disease and JA related phenotypes have been restricted to Jas domain of JAZ proteins. To better understand the role of N-terminus of JAZ proteins JAZ3/4/9 were chosen. Alignment of the N-terminus of JAZ3/4/9 revealed that this amino acid sequence is highly conserved among these genes (Fig 4.2).

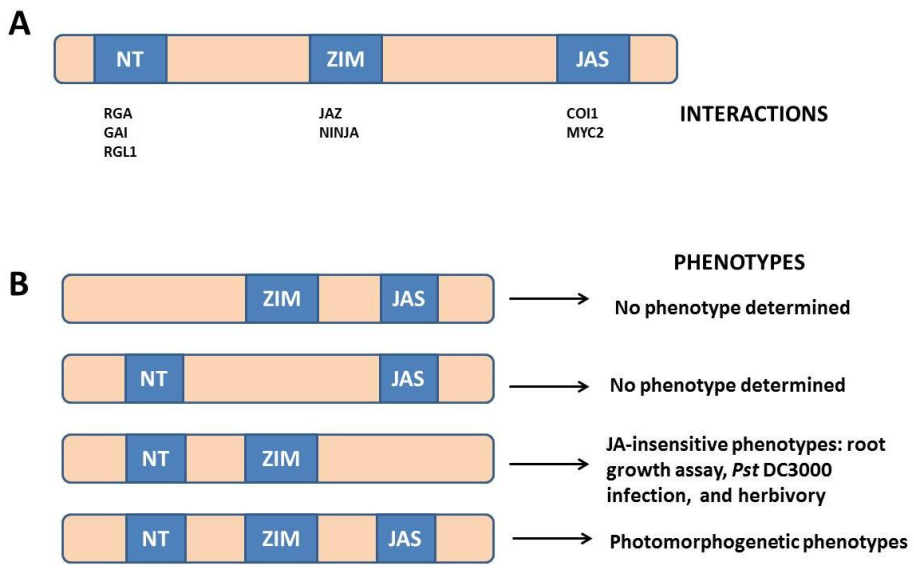


Fig 4.1 Schematic representation of JAZ proteins with associated phenotypes. (A) JAZ proteins with three domains, blue colored boxes indicate conserved domains. Proteins that bind to each domain are listed below (B)Upon deletion of some domains JAZ proteins either exhibit the respective phenotypes or go undetected

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JAZ3.1.NT       MERDFLGLGSK-NSPITVKEETSESSRDSAPNRG-MNWSFSNK- 40
JAZ4.1.NT       MERDFLGLGSK-LSPITVKEETNE---DSAPSRGMMDWSFSSKV 40
JAZ9.1.NT       MERDFLGLSDKQYLSNNVKHEVND---DAVEERG-LSTKAAREW 40
                ********..*   . .**.*..:   *:. .** :. . : :
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Fig 4.2 Amino acid alignment of JAZ3/4/9 N-terminus. Domain 1 is highlighted.

4.4.2 Domain1 of JAZ9 has an important role in innate immunity of Arabidopsis

JAZ9 over-expressers and gene – knockout plant lines did not show any increased or decreased bacterial multiplication as compared to wild -type Arabidopsis plants when inoculated with *Pst* DC3000 (M. Melotto, unpublished). However, truncation of domain1 of JAZ9 which is the JAZ9Δ1 showed significant hyper-susceptibility to *Pst* DC3000 in dip inoculation (Fig. 4.3A) and syringe infiltration (Fig. 4.3C) inoculation. These results suggest domain 1 of JAZ9 has a crucial role in disease development.

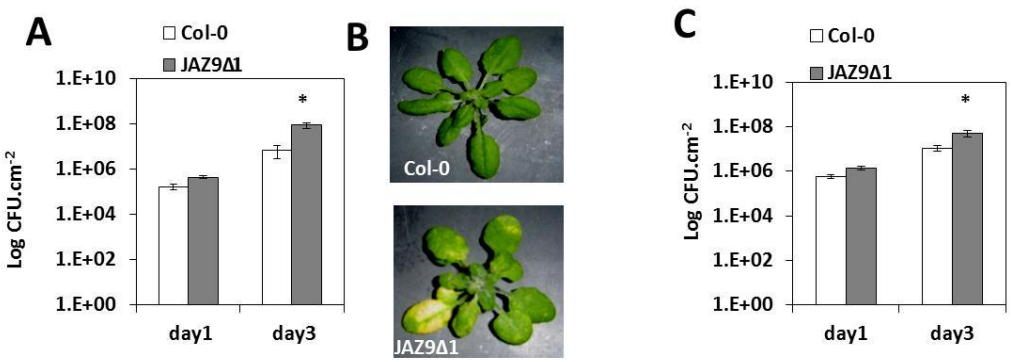

Fig 4.3 Susceptibility of Arabidopsis plants to bacterial infection. (A) Col-0 (WT), and Col-0 plants transformed with JAZ9Δ1 (the entire domain 1 deleted) were dip-inoculated with *Pst* DC3000 bacterial suspension ($1x10^7$ CFU.ml^{-1}), (B) followed by disease symptoms (C) Alternatively, these plants were syringe-infiltrated with $1x10^5$ CFU.ml^{-1} *Pst* DC3000 inoculum. Results are shown as means (n=6) ± standard errors. The bacterial growth experiment was repeated three times.

4.4.3 Role of N-terminus of JAZ4 in innate immunity of Arabidopsis

To understand the role of *JAZ4* in disease development, it was important to assess *jaz4*$^-$ mutant response to pathogen infection. Therefore T-DNA insertion line SALK_051205 was used for this assay. This line is known to have a T-DNA insertion in the first intron of *JAZ4* as shown be the green triangles in Figure 4.3A. Homozygous lines for the insertion were screened and analyzed for the lack of transcripts.

JAZ4 was predicted to exist in three isoforms through bioinformatic analysis available at The Arabidopsis Information Resource (TAIR, CA; www.arabidopsis.org, ARBC, OH). In

the experiments directed towards detecting these isoforms in Arabidopsis, I found that *JAZ4* exists in four natural isoforms as a result of post-transcriptional modifications. These include *JAZ4.1* that forms a full length protein*; JAZ4.2* that retains the Jas intron and results in a premature stop codon within the retained intron losing a part of the Jas domain; *JAZ4.3* as a result of loss of N-terminus in addition to retaining the Jas intron similar to *JAZ4.2;* and *JAZ4.4* that has part of the N-terminus deleted. Illustration of all the *JAZ4* splice variants is shown in Fig 4.3A. Since the difference in the splice variants lies in either N-terminus or C-terminus, primers spanning the unique regions in both the termini are needed to amplify each splice variant. Therefore the resulting PCR amplicon will be the size of full length spliced transcript and thus qPCR analysis to detect splice variants cannot be performed.

RT-PCR analysis has revealed the presence of *JAZ4.1* and *JAZ4.2* in wild type Col-0 but not in SALK_051205, whereas *JAZ4.3* and *JAZ4.4* were present in this line and not in Col-0. Since this line does not have *JAZ4.1* and *JAZ4.2,* it was named *JAZ4ΔNT*. Infection assays with this line revealed the importance of N-terminus of JAZ4 in disease progression in Arabidopsis. Dip inoculation (Fig. 4.5A) as well as syringe infiltration (Fig. 4.5C) with *Pst* DC3000 showed enhanced bacterial growth accompanied by more severe disease symptoms as compared to Col-0 (Fig. 4.5B and Fig. 4.5D). Although it is clear enough that N-terminus of JAZ4 is required for plants to resist the pathogenic bacterium *P. syringae*, it is important to note the presence of more than one splice variants in JAZ4ΔNT line used, therefore transgenic lines over-expressing each splice variants of *JAZ4* will reveal their individual role in disease phenotype against *Pst* DC3000. it could be possible that truncations in the N-terminus of JAZ4 and JAZ9 leads loss of function mutations which could result in formation of a weaker repressor complex that leads to up-regulation of JA signaling thereby eventually leading to compromised innate immune responses. Therefore to check if JA signaling is upregulated I studied gene expression analysis of JA responsive genes in the JAZ4ΔNT plants.

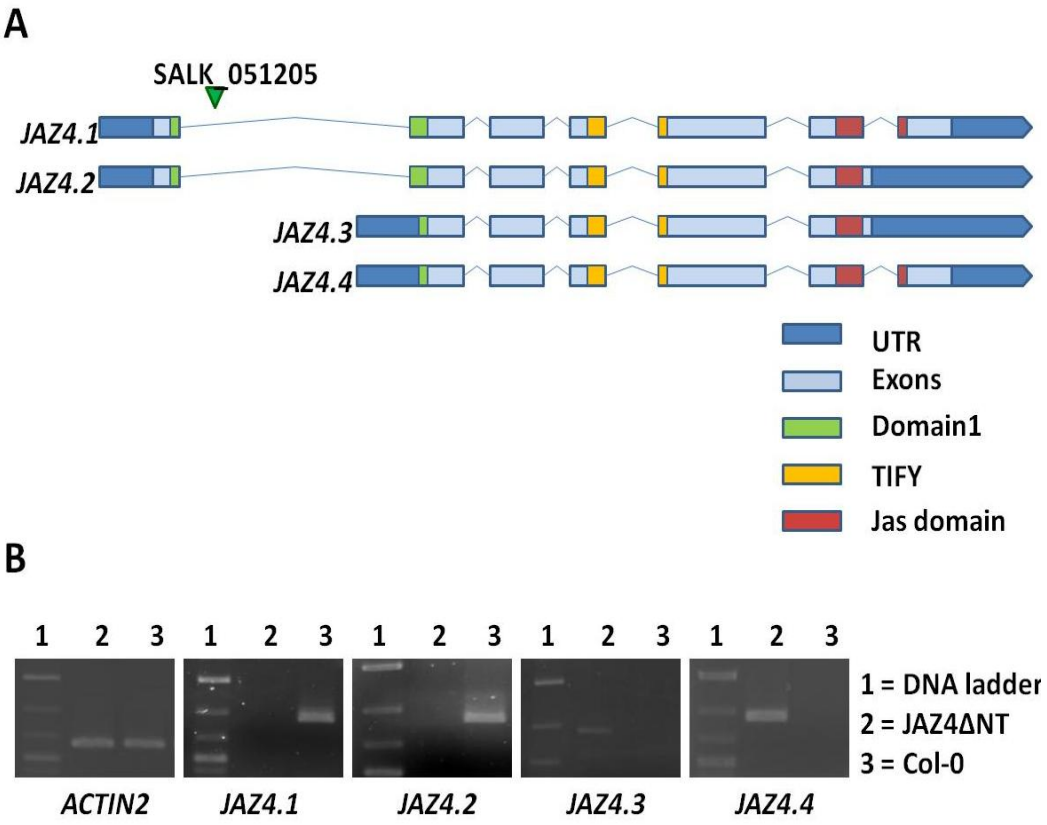

Fig 4.4. Genotype JAZ4ΔNT plants (A) Diagram depicting the genomic structure of splice variants of the *JAZ4* gene and approximate T-DNA insertion point (green triangle). Rectangles in dark blue represent UTR and light blue represent exons containing domain 1 (green), TIFY domain (orange), and Jas domain (red), (B) Constitutive expression levels of *JAZ4* splice variants in the T-DNA insertion mutant JAZ4ΔNT (SALK_051205) and Col-0 plants.

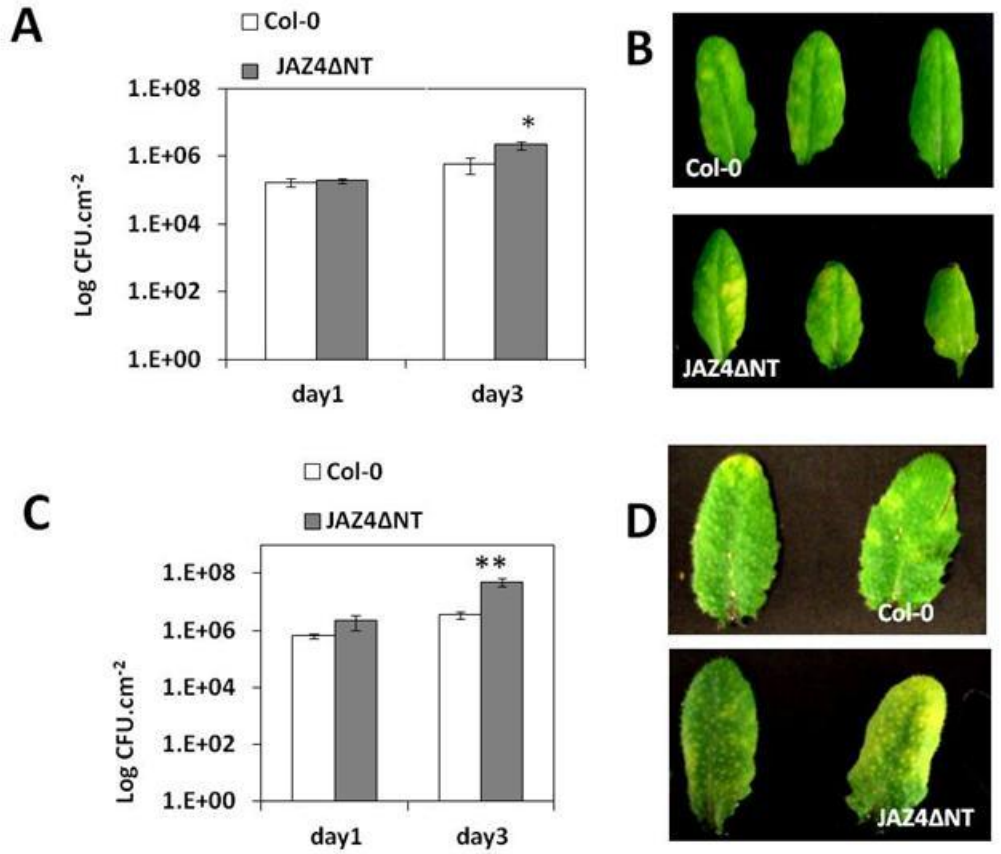

Fig 4.5 Disease phenotype of JAZ4ΔNT plants. (A) *Pst* DC3000 population in the apoplast of plants inoculated by dipping, (B) followed by disease symptoms or (C) by syringe infiltration and (D) followed by disease symptoms. Pictures were taken 3 day post inoculation. Results are shown as the mean (n=6) ± standard error (SE). Statistical significance was detected with two-tailed Student's t-test (* = $p<0.05$ and ** = $p<0.01$).

4.4.4 Gene expression analysis in JAZ4ΔNT plants

To check the JA regulation in JAZ4ΔNT (SALK_051205), same JA responsive genes studied in chapter3 were studied in this line as well. Up-regulation of *OPR3, LOX3,* and *MYC2* indicates the induced JA signaling in JAZ4ΔNT (SALK_051205) as compared to wild-type (Fig 4.6). No difference was noted in *PDF1.2* transcript levels between JAZ4ΔNT (SALK_051205) and Col-0 (Fig 4.6). Similarly to check the SA regulation in JAZ4ΔNT (SALK_051205); *PR-1* and *PR-5* gene expressions were studied as these genes were known

to be dependent on SA pathway (Kojima et al. 2012). No difference in the transcript levels was noted in JAZ4ΔNT as compared to wild-type (Fig 4.6). These results suggest that the hyper susceptibility of JAZ4NT to *Pst* DC3000 infection might be due to up-regulation of JA signaling and not suppression of SA signaling

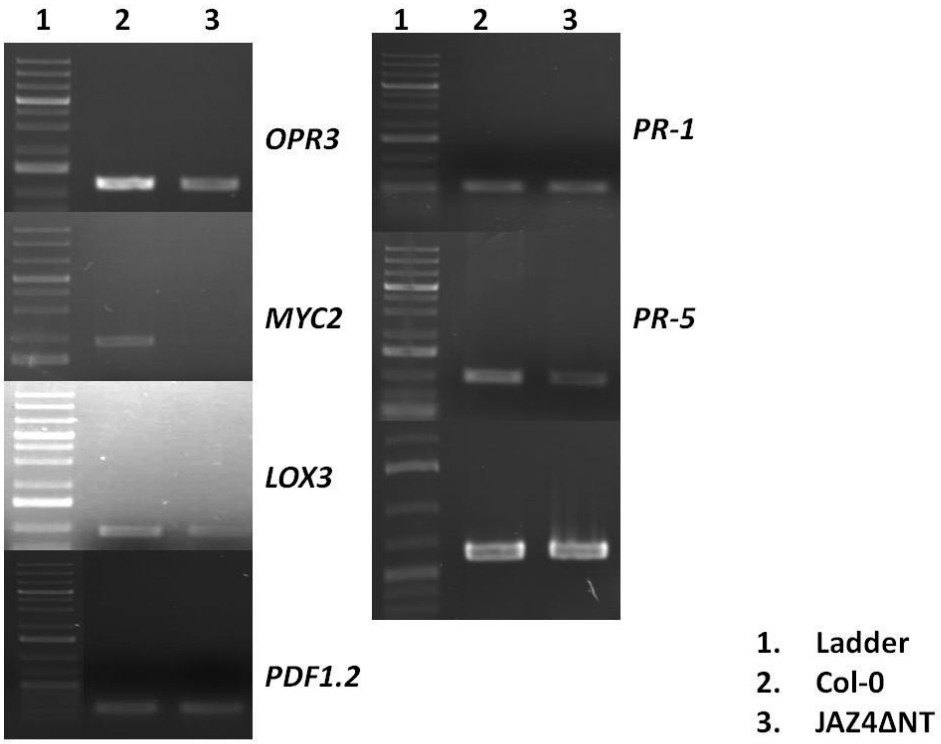

Fig 4.6 RT-PCR analysis of JA and SA responsive genes in JAZ4ΔNT mutant. Constitutive expression analysis of key marker genes with respect to Col-0 using *ACT2* as a housekeeping gene.

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In addition to SA and JA responsive genes, expression analysis of all *JAZ* genes in JAZ4ΔNT was also performed to investigate whether the absence of N-terminus of JAZ4 has effect on other *JAZ* genes. Some *JAZ* genes; *JAZ7,* and *JAZ8* were repressed in JAZ4ΔNT as compared to Col-0 (Fig 4.7)

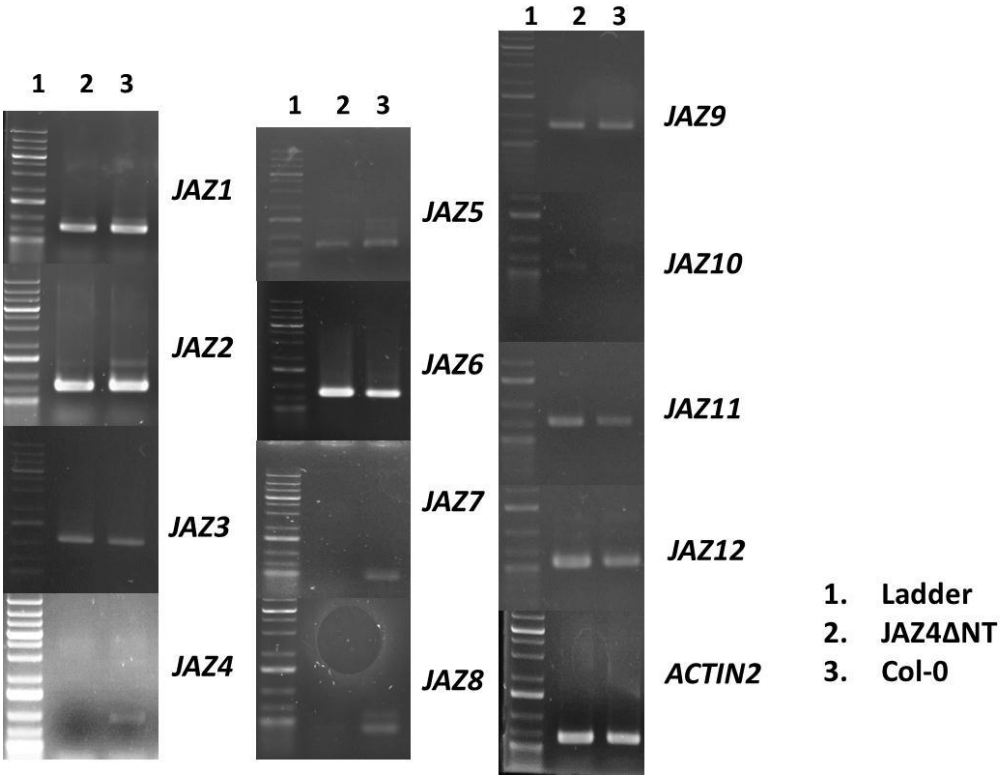

Fig 4.7 RT-PCR analysis of all *JAZ* genes in JAZ4ΔNT mutant. Constitutive expression analysis of key marker genes with respect to Col-0 using *ACT2* as a housekeeping gene. This experiment was performed by Debanjana Roy.

4.4.3 Role of N-terminus of JAZ proteins in stomatal immunity

Since both JAZ9Δ1 transgenic and JAZ4ΔNT mutant plants showed enhanced susceptibility to pathogen infection and both JAZ4 and JAZ9 are expressed and induced by coronatine in guard cells , it was important to investigate the stomatal immune responses in these lines. Therefore, inoculation of JAZ9Δ1 plants with *Pst* DC3118 using dip (Fig. 4.9A)

and syringe infiltration (Fig. 4.9B) procedures were performed. as compared to when JAZ9Δ1 and wild-type Col-0 plants were dipped in *Pst* DC3118 inoculum more bacterial growth was observed in JAZ9Δ1 plants increased bacterial growth was not observed when plants were inoculated with syringe infiltration (a procedure of inoculation that bypasses stomatal immunity) (Fig. 4.9A-B). This result hints towards impaired stomatal defense in this line and therefore increased bacterial penetration. I have not observed the same trend in JAZ4ΔNT plants when inoculated with *Pst* DC3118 (Fig 4.8), when plants were inoculated with *Pst* DC3118 significant growth was not observed either with infiltration (Fig 4.8A), or with dip inoculation procedures (Fig 4.8 B-C), However, stomatal assays done with the same bacteria showed that these plants were not capable of triggering stomatal closure as compared to wild type (Fig. 4.9 C). This could be a possible reason for enhanced disease susceptibility in these plants.

These results suggest that transgenic plants expressing JAZ9 Δ1 constructs exhibit stronger phenotype of compromised stomatal immunity than JAZ4ΔNT plants. Assessment of stomatal immunity by a more sensitive technique, measuring the stomatal aperture revealed the impaired stomatal defense in this line. The stronger phenotype in JAZ9^1 plants could be due to construct where JAZ9Δ1 was expressed under the 35s promoter which expresses more protein than the JAZ4ΔNT which is under the influence of native promoter. In either case these results suggest that absence of N-terminus of JAZ proteins leads to suppression of both stomatal and apoplastic innate immune responses. The suppression of both the immune responses clearly indicates the importance of N-terminus of JAZ proteins in controlling infection against *P.syringae* infection.

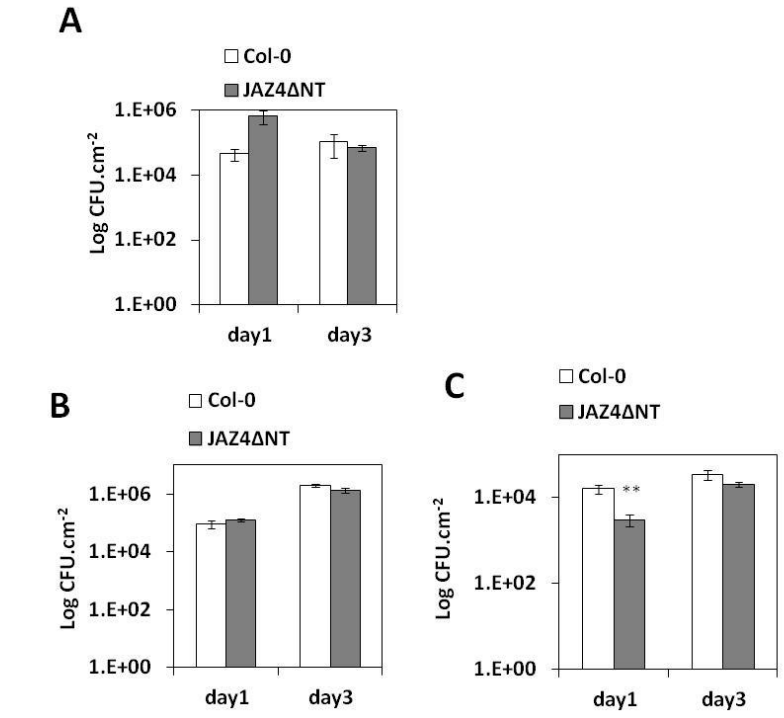

Fig 4.8 Bacterial inoculations of JAZ4ΔNT with *Pst* DC3118. Panels (A) inoculations done with vacuum infiltration and below are bacterial populations of *Pst* DC3118 inoculated by dipping (B-C). Results are shown as means (n=6) ± standard errors. The bacterial growth experiment was repeated two times for panel A. Statistical significance was detected with two-tailed Student's t-test (*** = $p<0.01$).

Table 4.1 Gene-specific primers used in RT-PCR reactions and the expected amplicon sizes.

Gene	Primer	Sequence (5'-3')	Size (bp)
ACT2 (At3g18780)	For	GCCATCCAAGCTGTTCTCTC	629
	Rev	GAACCACCGATCCAGACACT	
JAZ4 (At1g48500)	For	CACCATGGAGAGAGATTTTCTCGGG	761
	Rev	TTAGTGCAGATGATGAGCTGGAGGA	
JAZ4.2 (At1g48500.2)	For	CACCATGGAGAGAGATTTTCTCGGGC	749
	Rev	CGAAGCCAATATACAGCAAACCTGTG	
JAZ4.3 (At1g48500.3)	For	GTCGTCCACTTTAGCATAGCTAGATCTGAG	1058
	Rev	CGAAGCCAATATACAGCAAACCTGTG	
JAZ4.4 (At1g48500.4)	For	GTCGTCCACTTTAGCATAGCTAGATCTGAG	932
	Rev	TTAGTGCAGATGATGAGCTGGAGGACA	

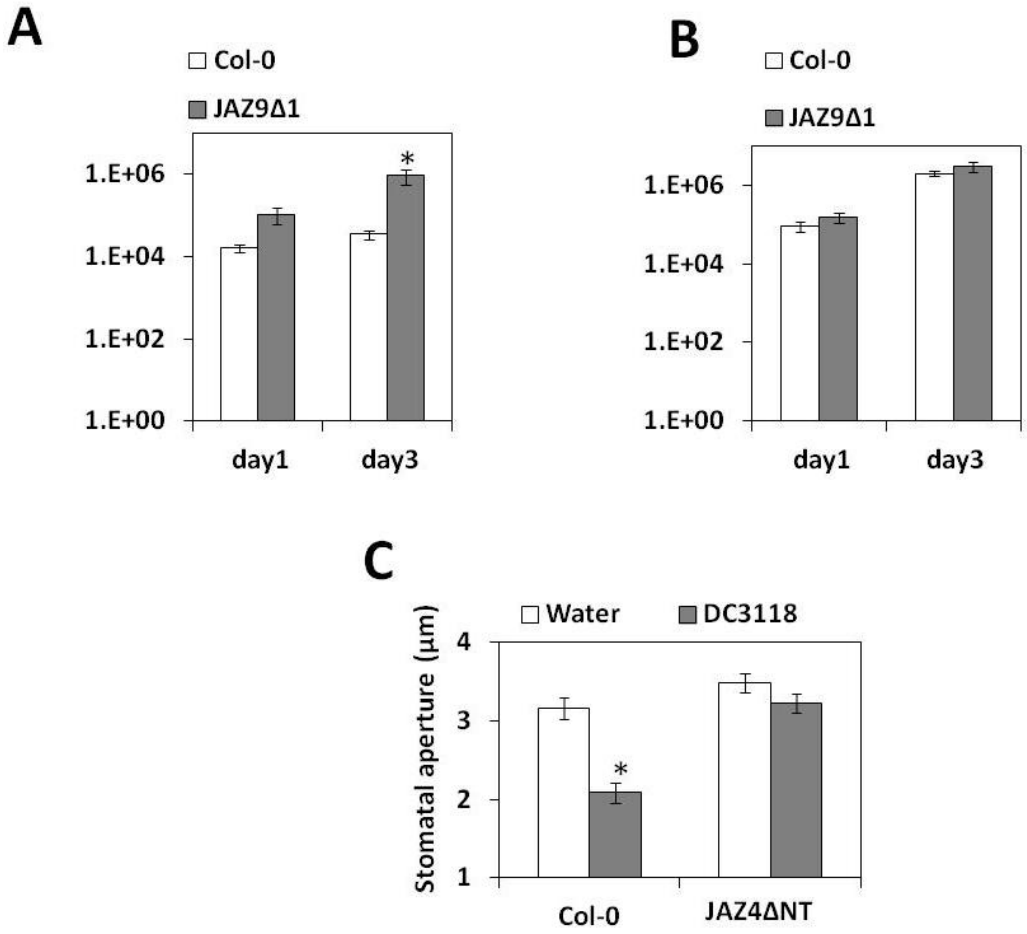

Fig 4.9 Key role of stomata in disease susceptibility of plants lacking N-terminus of JAZ proteins. (A). Col-0 (WT), and Col-0 plants transformed with JAZ9Δ1 (JAZ9 with the entire domain 1 deleted) were dip-inoculated into *Pst* DC3118 bacterial suspension ($1x10^7$ $CFU.ml^{-1}$) (B) Alternatively these plants were syringe-infiltrated with $1x10^5$ $CFU.ml^{-}$ *Pst* DC3118 inocula. Results are shown as means (n=6) ± standard errors. The bacterial growth experiment was repeated three times. (C) Stomatal response of JAZ4ΔNT to the COR-deficient bacterium *Pst* DC3118 2h post inoculation. Results are shown as means (n=60) ± standard errors and Statistical significance was detected with two-tailed Student's t-test (*** = $p<0.01$).

4.4.5 N-terminus of JAZ proteins might have a role in photomorphogenesis

Recent studies have reported that N-terminus of JAZ proteins especially JAZ9

interact with RGA - a DELLA protein that regulates gibberellic acid (GA) signaling. They have

also reported that these genes respond to environmental cues such as light (Yang et al. 2012), JAZ9 over-expressors flower early in long day conditions (16 h 120 µmol.m−2.s−1 light/8 h dark, 22 °C/18 °C), and produce long hypocotyls at low light conditions (10 $\mu mol.m^{-2}\cdot s^{-1}$ continuous white light at 22 °C for 6 d; Yang et al. 2012). For that reason, these JAZ9Δ1 and JAZ4ΔNT plants were subjected to low light intensity (50 $\mu mol.m^{-2}\cdot s^{-1}$ white light at 22 °C, 12h photoperiod for 14 weeks). At the end of 4 weeks, plants grown under low light showed visible delayed growth than plants that grew under normal light conditions (Fig 4.10 - 4.11). However in low light conditions, JAZ4ΔNT plants grew even slower than Col-0 plants, which was visible at the end of 4 weeks (Fig 4.10). Slow growth was not noted in JAZ9Δ1 plants (Fig 4.11). Both JAZ9Δ1 and JAZ4ΔNT plants showed delayed flowering with a more pronounced phenotype in JAZ4ΔNT plants (Fig 4.12C-E). Moreover, their leaves also showed increased anthocyanin production (Fig 4.12D) which is a determined response in plants having induced JA signaling as a result of over expression of MYC transcription factors (Dombrecht et al 2007). Earlier studies have shown that under long day conditions JAZ9 over-expressers exhibit early flowering (Yang et al 2012). The authors have also proved that, at these conditions the *JAZ*s interact with DELLA proteins like RGA which is responsible for the phenotype. Since the JAZ4ΔNT and JAZ9Δ1 transgenic plants lack the N-terminus of their respective JAZ proteins they might lack the interaction with DELLAS leading to visible photomorphogenetic phenotype in these plants. To better understand the role of JAZ4ΔNT and JAZ9Δ1 in photo-morphogenesis, it would be feasible to monitor the level of DELLA proteins in these lines or study the interaction of JAZ4 with DELLA proteins.

Inspite of intense research on JA signaling it is still not very clear how plants prioritize growth over defense on vice versa. It is well known that JA signaling restricts plants development thereby up-regulating defense mechanisms. This study reveals that JAZ4ΔNT plants are known to have induced JA signaling, and also show signs of growth restrictions

under low light indicating the significance of N-terminus of JAZ proteins in two important mechanisms - plant growth and defense.

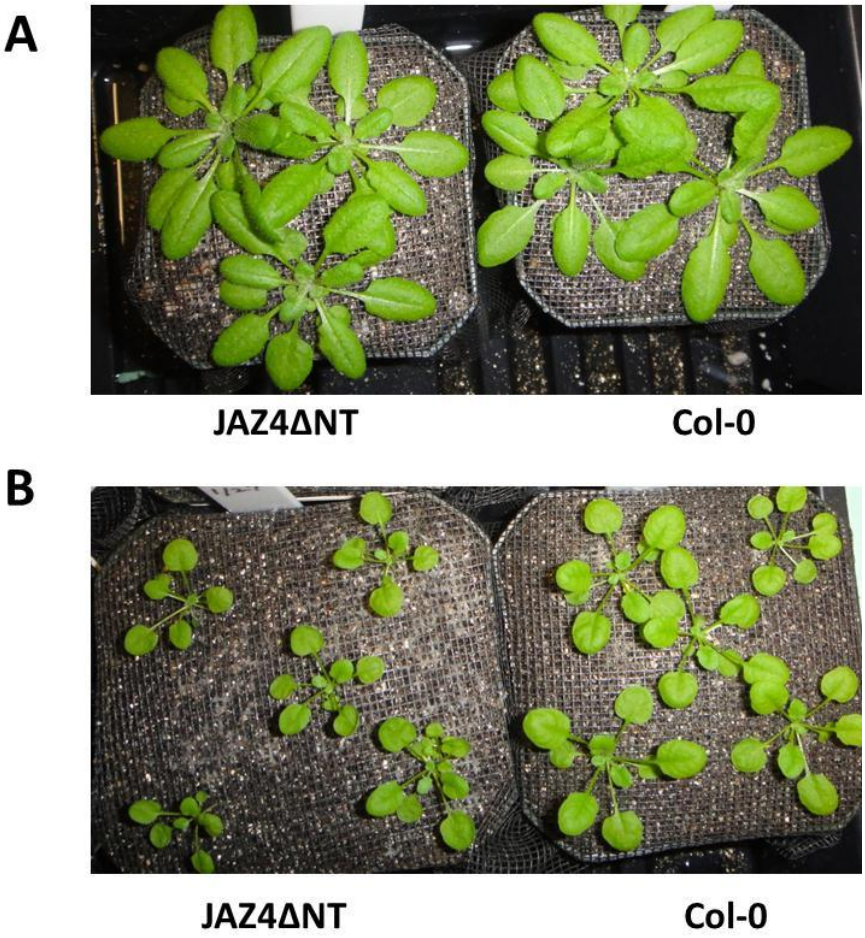

Fig 4.10 Photomorphogenetic analysis of JAZ4ΔNT plants for 4 weeks. (A)Plants when grown under 100μmol.m^{-2}·s^{-1} or (B) 50μmol.m−2.s−1 white light at 22 °C, 12h photoperiod for 4 weeks. Pictures were taken at the end of 4 weeks. Note the growth retardation of plants under low light conditions. Also JAZ4ΔNT plants showed even more slow growth under low light as compared to Col-0

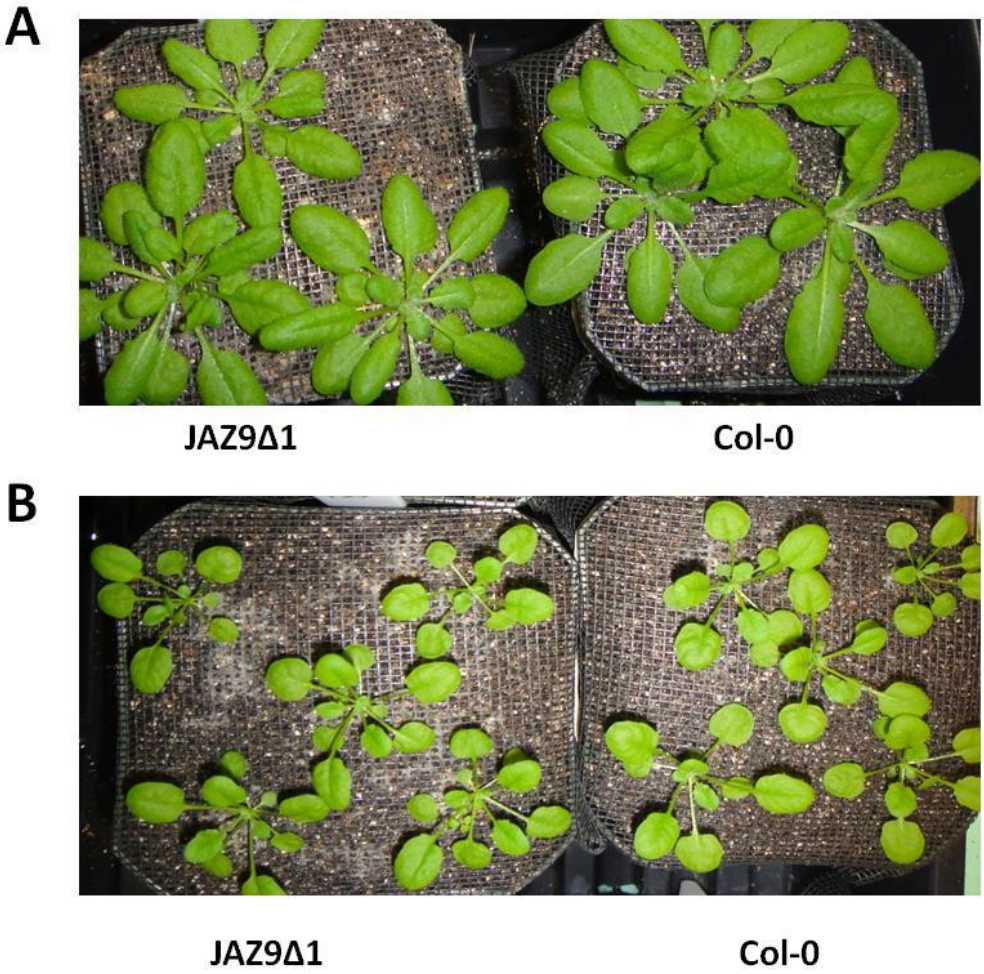

Fig 4.11 Photomorphogenetic analysis of JAZ9Δ1 transgenic plants for 4 weeks. (A) Plants when grown under 100µmol.m−2·s−1 or (B) 50µmol.m^{-2}·s^{-1}white light at 22 °C, 12h photoperiod for 4 weeks. Pictures were taken at the end of 4 weeks. Note the growth retardation of plants under low light conditions. No difference has been noted as compared to wild-type plants under both light conditions

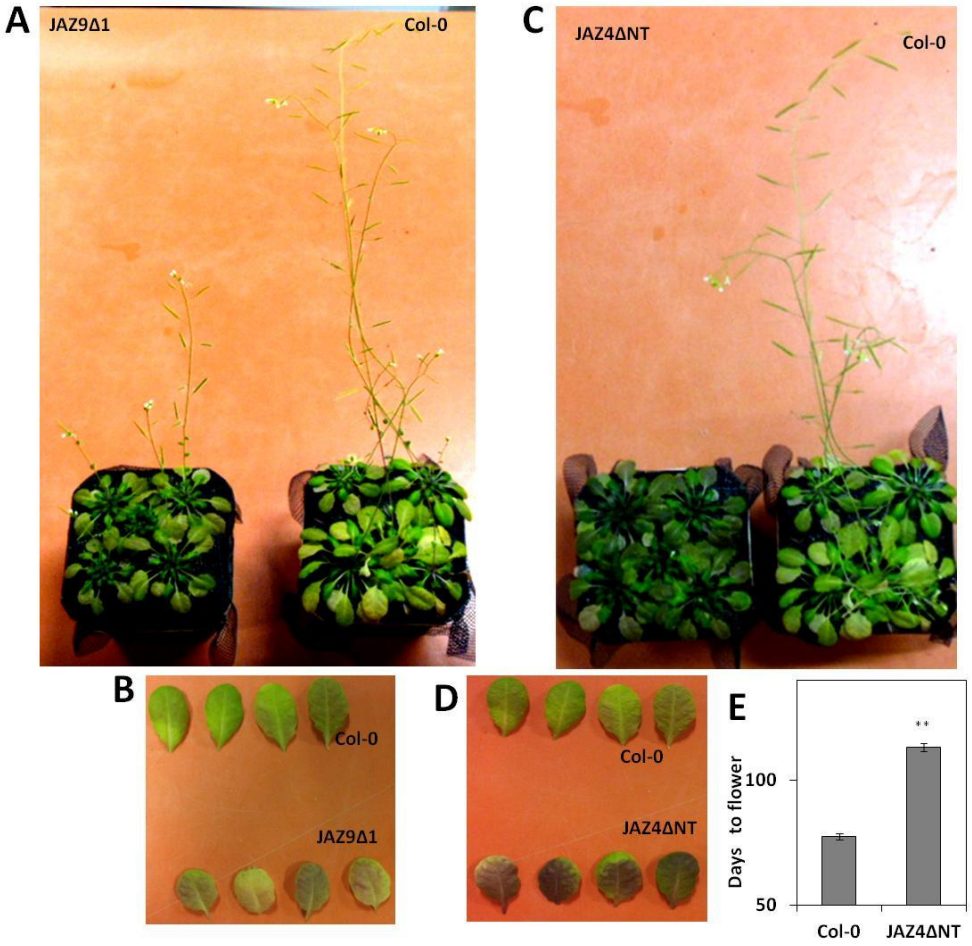

Fig 4.12 Photomorphogenetic analysis of JAZ4ΔNT and JAZ9Δ1 plants for 14 weeks. (A) Plants when grown under 50µmol.m^{-2}·s^{-1} white light at 22 °C, 12h photoperiod for 14 weeks resulted in delayed flowering (B) followed by increased anthocyanin production of JAZ9Δ1. (C) However in JAZ4ΔNT delayed flowering and (D) increased anthocyanin production was more prominent. (E) Statistical representation of days taken to flower can also be noted and significance was detected with two-tailed Student's t-test (** = $p<0.01$).

4.4.6 MeJA jasmonate degrades JAZ9Δ1 in an ubiquitin-independent manner in guard cells of Arabidopsis.

Transgenic JAZ9 plants were generated expressing GFP::JAZ9 constructs under the control of CaMV 35S promoter. JAZ9 protein was localized in guard cells reconfirming the findings that JAZ9 is abundant in guard cells in Chapter 3, it might be possible that JAZ

proteins have some internal signal to localize in guard cells. JAZ proteins bind to COI1 in presence of COR and JA-Ile and are eventually degraded via ubiquitination-26S proteasome pathway (Melotto et al 2008). Here similar studies were done using JAZ9 transgenic plants. Consistent with the previous results, I found that JAZ9 protein degraded in presence of 50μM MeJA (Fig 4.12). In presence of 50μM MeJA and 5μM MG132 (a proteasome inhibitor), JAZ9 protein was found to be stabilized (Fig 4.12D) indicating that MeJA induced degradation of JAZ9 is dependent on proteasome pathway. However in transgenic plants expressing GFP::JAZ9Δ1, 50μM MeJA induced degradation of this protein even in presence of 5μM MG132 (a proteasome inhibitor - Fig 4.13). These results indicate that N-terminus of JAZ9 prevents MeJA induced protein degradation that is not proteasome dependent by some unknown mechanism. My results also suggest proteasome independent degradation of JAZ proteins in absence of their N-terminus (Fig 4.13). It might be even possible that N-terminus in JAZ4 and JAZ9 is required to form a stable repressor complex which would be dispersed if absent and thereby causing induced JA signaling making the plant susceptible to *Pst* DC3000. Earlier studies in chapter3 have shown that COI1 is least expressed in guard cells which drove a speculation that COR might be having other targets that are expressed in guard cells. This 26s proteosome independent degradation of GFP::JAZ9Δ1, also gets associated to the idea of COR having targets in guard cells other than COI1. In any case more experiments directed towards interaction and localization studies are needed to unravel the function of N-terminus in these proteins.

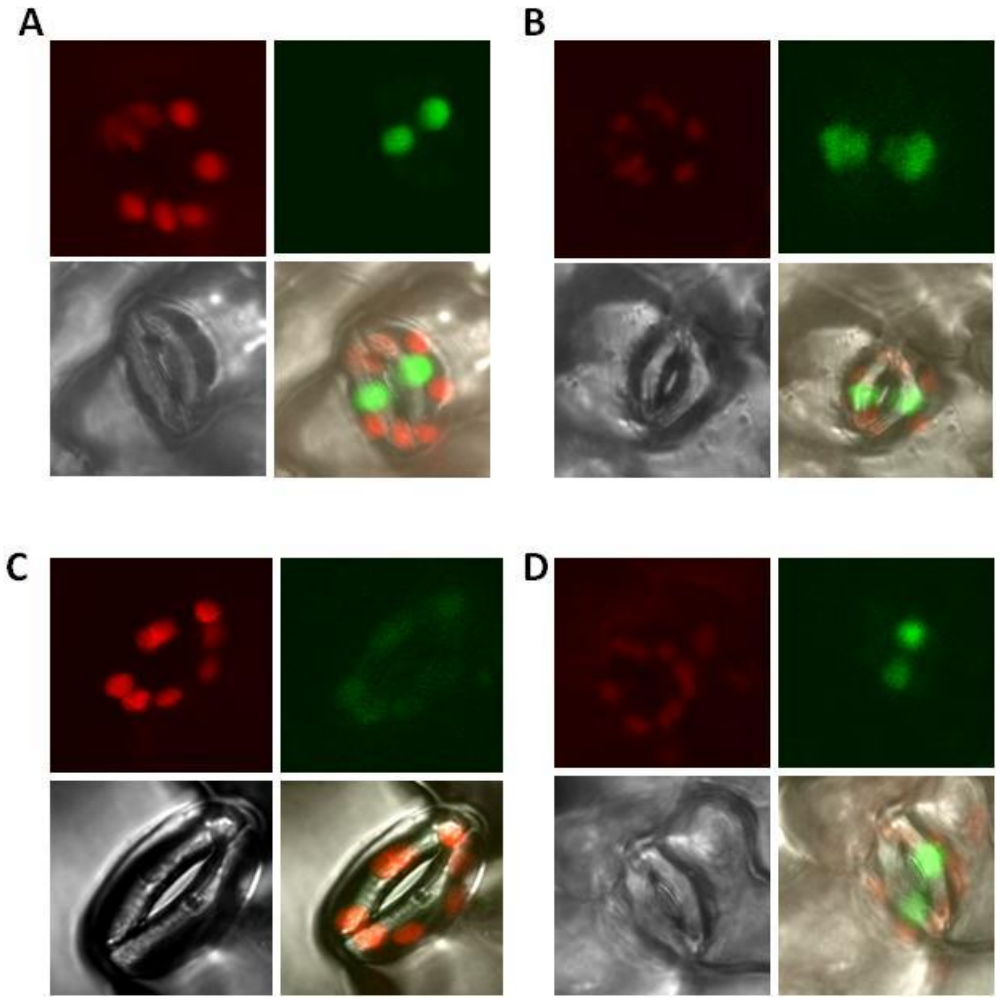

Fig 4.13 MeJA induced degradation of JAZ9 protein localized in guard cells is dependent on 26s proteasome pathway. (A) Confocal images of Arabidopsis leaves reveal JAZ9 localized in guard cells at time 0 (B) This localization was also noted when leaves were incubated in water. (C) MeJA induced JAZ9 degradation after treating leaves for 30 minutes and (D) this degradation was not observed in MeJA + MG132 treated leaves.

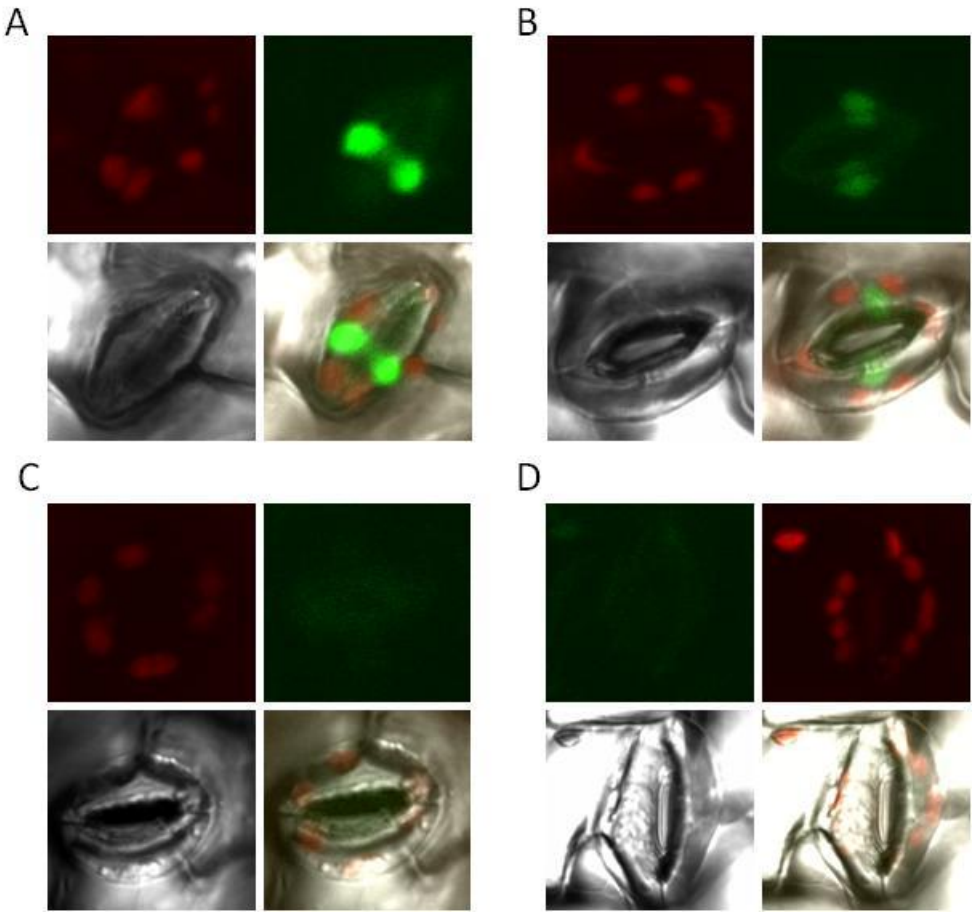

Fig 4.14 MeJA induced degradation of JAZ9Δ1 protein localized in guard cells is not dependent on 26s proteasome pathway. (A)Confocal images of JAZ9Δ1 localized in guard cells of Arabidopsis leaves at time 0 and (B) when leaves were incubated in water for 30 minutes. (C) However MeJA and (D) MeJA + MG132 induced JAZ9Δ1 degradation after treating leaves for 30 minutes indicating proteasome-independent degradation of JAZ9Δ1.

4.5 Conclusion

Recent studies have discovered the important interactions of N-terminus of JAZ proteins; inspite of several studies the role of N-terminus of JAZ proteins in biotic stresses encountered by Arabidopsis have not been studied yet. With a goal to identify the importance of N-terminus of JAZ proteins in disease progression in Arabidopsis structure and functional analysis of JAZ4 and JAZ9 was carried out in this chapter. My findings in this chapter suggest that 1) the N-terminus of JAZ4 and JAZ9 is required for resistance against *Pst* DC3000 infection in Arabidopsis, 2) JAZ4 exists in four natural isoforms; *JAZ4.1, JAZ4.2, JAZ4.3,* and *JAZ4.4,* and absence of N-terminus in JAZ4 exhibits constitutive upregulation of jasmonic acid signaling 3) the N-terminus of JAZ4 and JAZ9 is required for stomatal immunity in addition to apoplastic immunity, 4) plants expressing truncated JAZ4 protein lacking the N-terminus exhibit photo-morphogenetic phenotypes and increased anthocyanin production when grown under low light intensity, and 5) the N-terminus of JAZ9 is required to prevent degradation of JAZ9 via 26s proteasome independent pathway

Appendix A Generation of Double knock-out of *JAZ3* and *JAZ9*

Introduction

As mentioned earlier *JAZs* are a family of 12 genes and single knock-out of these genes did not reveal any pathogenecity related phenotype (Thines et al. 2007, Chini et al. 2007). Therefore to identify the functions of multiple JAZs simultaneously, double knock-outs of *JAZ9* and *JAZ3* were generated and their phenotypic studies were assessed.

Methods

Endpoint PCR

RT-PCR was done as described in chapter3 using gene specific primers. Same *Actin2, JAZ3* and *JAZ9* primers described in Table 3.1 were used.

Bacterial inoculation

P. syringae pv. *tomato* strains, *Pst* DC3000 (wild type) and mutant derivative, *Pst* DC3118, were cultured at 30^0C in low salt LB medium (10 $g.l^{-1}$ tryptone, 5 $g.l^{-1}$ yeast extract, 5 $g.l^{-1}$ NaCl, pH=7.0) supplemented with appropriate antibiotics until an OD_{600} of 0.8 was reached. Bacteria were collected by centrifugation and re-suspended in water to the final concentration of 10^7 $CFU.ml^{-1}$ containing 0.003% Silwet L-77 (Lehle seeds Co., Round Rock, TX) for dip-inoculated plants or 10^5 $CFU.ml^{-1}$ containing 0.008% Silwet L-77 for vacuum-infiltrated plants. Inoculated plants were immediately incubated under the following conditions: 25^0C, 65±5% relative humidity, and 12 h of daily light (100 $\mu mol.m^{-2}.sec^{-1}$). Bacterial population in the plant apoplast was determined as previously described (Katagiri et al. 2002).

Results

Using RT-PCR analysis, genetic screening for lines that lack both JAZ9 and JAZ3 transcripts was performed. Single mutants of *jaz9* (salk_046563) and *jaz3* (salk_139337) that were crossed previously by (Melotto M Unpublished) were screened. This genetic study generated a line that did not express either *JAZ3* or *JAZ9*. Figure1 below demonstrates that the double knock-out did not express any of these two transcripts.

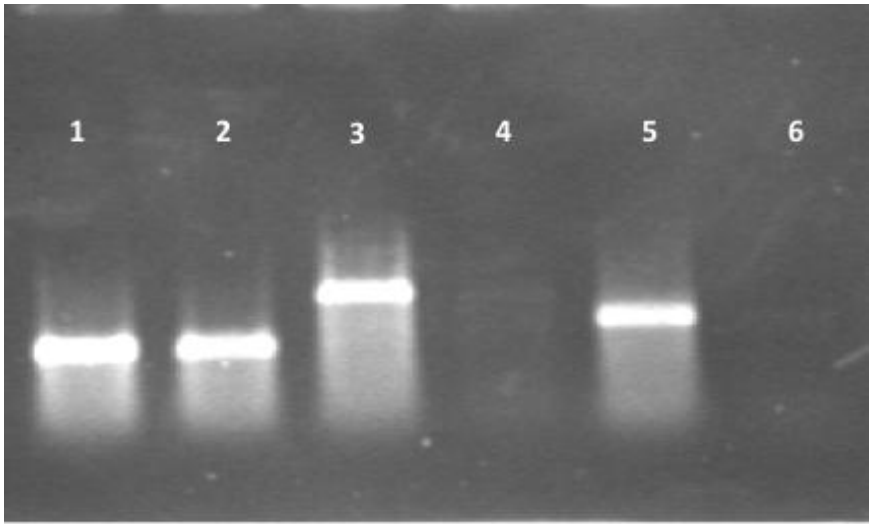

Fig1 Generating double knock-out of *JAZ3* and *JAZ9* using RT-PCR. Single mutants of *jaz9* (salk_046563) and *jaz3* (salk_139337) were crossed and screened for the loss of both the transcripts using end-point PCR using *ACT2* as housekeeping gene (Lane1 and lane2). Please note the absence of JAZ3 and JAZ9 transcripts in the double mutant line (Lanes 4 and 6) as compared to Col-0 (Lane3 and 5) respectively.

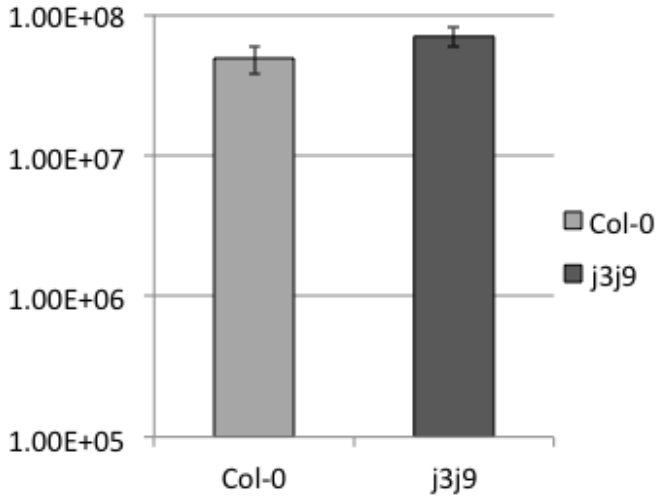

Fig2 4-5 weeks old plants were dip inoculated with 10^8 CFU/ml *Pst* DC3000 bacterial inoculum. Bacterial plating was done 3 days post inoculation. No statistical significance was observed between wildtype (Col-0) and double knock-out plants.

Seeds collected from the above mentioned double knock-out were grown and 4-5 weeks old plants were dip inoculated with *Pst* DC3000 and bacterial growth was assessed after 3dpi. As shown in Fig 2 plants did not show any phenotype. However COR mutant bacterial growth and other inoculation procedures were not performed on this line which might provide a clear vision of disease phenotypes in this line.

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Biographical Information

Nisita Obulareddy began her career as a Plant pathologist in the year 2005. She completed her Masters in Biotechnology in Bharathidasan University, India under supervision of Dr. Subbu Rathinam. As a part of her Masters studies she worked on a project titled "*Development if immunodiagnostic kit for rapid detection of Staphylococcus aureus in food samples"* where she developed interest in microbiology. Later she worked in a research lab for a year as project assistant in a plant pathology lab on an assignment named "*Development of grain mold disease resistance in sorghum using antifungal proteins"* under supervision of Dr. K. Ulaganathan.

Moving further in her area of interest which is Plant pathology, she later began her doctoral studies at University of Texas, Arlington – TX in 2008. During her graduate studies, Nisita Obulareddy excelled in various aspects of plant pathological research like experimental designing, scientific writing, and analyzing results that would help her in future. As a graduate student she also gained immense experience of teaching and training undergraduate students in different scientific procedures.

Amassing all the previous knowledge Nisita's imminent plan is to continue in the same field of science that is geared towards crop protection and is keenly looking for her next research challenge.