Analysis of Transcriptional Responses in Plants Related with Induced Systemic Resistance by Plant Growth Promoting Fungi

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(植物生育促進菌類による全身抵抗性誘導に関わる植物の転写応答解析)

2013

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CHAPTER 1

GENERAL INTRODUCTION

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Food production is affected by a myriad of factors including, but not limited to, decreasing area of arable land, pestilence, climate change, underdeveloped infrastructures, and political factors. Research continues to increase agricultural yields and improve practices, particularly in developing countries. The reliance on fertilizers and pesticides, which are inappropriately managed, has significantly compromised human health and the integrity of natural resources that support life itself, such as soil and water. This has led to the development of the concepts of sustainability. Sustainability can be defined as the "successful management of resources to satisfy changing human needs while maintaining or enhancing the quality of the environment and conserving resources" (13). Specially, sustainability in agriculture can be characterized by, for example, the maintenance of soil fertility and structure over a long period of time such that the economic yields from crops can be achieved through minimum inputs. However, it is not easy to develop any form of agriculture that could be truly sustainable. As an alternative, the modification of strategies or practices is required such that chemical fertilizer and pesticide inputs are reduced but not eliminated, and that there is maximum use of the soil microbiota like the beneficial microorganisms which have innate roles in nutrient capture and cycling nutrients to the plant root system.

Currently, beneficial micro-organisms are increasingly used as inoculants for biofertilization, phytostimulation and biocontrol, because *r*educed use of fertilizers and fungicides in agricultural production is necessary to help maintain the ecosystems and to develop sustainable agriculture. The use of both bio-fertilizers and biocontrol systems can have minimal affect on environment and such strategies have been widely researched. Plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) are naturally occurring soil microorganisms that colonize roots and stimulate plant growth. Such bacteria and fungi have been applied to a wide

range of agricultural species for the purpose of growth enhancement, including increase seed emergence, plant weight, crop yields and disease control (47, 60). The mechanisms of plant growth promotion by PGPR and PGPF have been reported, including plant hormones production (70, 72, 118) substrate degradation (mineralization) and suppression of deleterious microorganisms (48, 73).

Plant growth is influenced by an abundance of abiotic and biotic factors. Plant growth hormones dominatingly affect plant growth, whereas the photosynthetic rate is dominated by temperature, irradiance and gaseous atmosphere (42). These physiological functions have been utilized as classical plant growth regulators. However, along with the composition of the nutrient medium, the composition of the gaseous atmosphere is another important factor for proper growth and development of plants (*12).* Several gaseous components are present in the atmosphere especially nitrogen, oxygen, carbon dioxide and different types of volatile compounds produced by surrounding organisms including the plant itself (*16, 103*). Changes in these components during different physiological functions in vitro largely affect the photosynthesis and other biological functions of the plant (*16*).

Recently, it has been demonstrated that plants have evolved the capacity to release and detect volatile organic compounds (VOCs) in their environment, and plant growth is promoted by VOCs from beneficial microorganisms (95, 124). VOCs, the major source of secondary metabolites and important components in ecosystems (10), are intensively studied due to their access as a biocontrol resource. VOCs characterized by low molecular weight and high vapor pressure are produced by all organisms as part of their normal metabolism, and play important roles in communication within and between organisms (98). VOCs mediated interactions among plant-plant, plant-insect and bacteria–plant have been frequently documented (24, 26, 52, 95, 99).

Plants also perceive the presence of pathogenic microbes via metabolites derived from the pathogen and activate defensive responses against the pathogens (2). Though the details of the molecular interactions are unknown as of now, low-molecular-weight plant volatiles such as terpenes, jasmonates and green leaf components have been identified as potential signal molecules for the plant (33). Koitabashi (63, 64) reported that a filamentous fungus isolated from the wheat leaf produces volatile materials that could suppress diseases and promote growth of different plants. Subsequently, volatile- producing fungus *Muscodor albus* was reported to have the capacity of growth enhancement and biological control of soil-borne diseases (80). Although the signaling network between plants and microbes has been extensively studied for the past 20 years, little is known on the role of microbial VOCs in regulating plant growth and development.

Many reports have focused on the effects of volatiles produced by rhizobacteria or plant growth promoting rhizobacteria on plant disease control. Several volatiles produced by rhizobacteria have exhibited antibacterial or antifungal activities (51). Two volatiles, 2,3-butanediol and acetoin (3-hydroxy-2 butanone), produced by *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been identified as important factors in inducing systemic resistance and promoting plant growth (96, 32). Volatiles produced by a few strains of *Streptomyces* are also reported to have potential for biocontrol (122, 69).

While most studies have focused on the interaction between rhizobacteria and plant pathogens, little is known about the plant response to VOC emitted by PGPF and the resistance that is conferred.

Therefore, in the present study, we aimed to establish whether the PGPF-released VOC can induce systemic resistance in plants, and if they can, to determine what types of signaling pathways are involved in this ISR.

Plants respond to adverse environmental stress and pathogen attack by expressing specific genes and synthesizing a large number of stress proteins that have putative roles in stress adaptation and plant defense (110, 92). The signals that mediate systemic responses must be transmitted rapidly throughout the plant and may involve cell-to-cell signaling. Putative systemic signals include ethylene (29), salicylic acid (27), jasmonic acid (35), and abscisic acid (133). Communication between these plant hormones might modulate the expression of abiotic and biotic stress–responsive genes in plants. However, the interactions between these hormone-mediated signal pathways and molecular mechanisms governing their cross-regulation have remained generally unresolved.

An example of a PGPF is *Penicillium simplicissimum* GP17-2, which was found to control soil-borne diseases effectively (47). Examination of local and systemic gene expression revealed that culture filtrate of GP17-2 modulate the expression of genes involved in both the SA and JA/ET signaling pathways. Phytohormones are acting on this signal transduction alone or interact each other in a cooperative, competitive or interdependent way. This relationship between phytohormones is a part of the transcriptional network for complex phytohormones responses. These transcriptional networks are biologically important for plants to respond against any kind of environmental stress. Promoter regions of stress-inducible genes contain *cis*-acting elements involved in stress-responsive gene expression. Precise analysis of *cis*-acting elements and their transcription factors can give us an accurate understanding of regulatory systems in stress-responsive gene expression. The DNA microarray has recently emerged as a powerful tool in

molecular biology research, offering high throughput analysis of gene expression on a genomic scale. Microarrays have already been used to characterize genes involved in the regulation of circadian rhythms, plant defense mechanisms, oxidative stress responses, and phytohormone signaling. Microarray data can serve a long list of up-regulated as well as genes with no response to stresses, and thus has a potential to identify corresponding *cis*-regulatory elements. In *Arabidopsis* plant, thousands of genes have been found as up-regulated and down-regulated from microarray analysis of the stress-inducible genes (Kubota et al. unpublished). In order to identify *cis*-regulatory elements without using microarray there are some other methods have also been established. A large number of *Arabidopsis cis*-regulatory elements have been identified by a recently developed bioinformatics methodology named LDSS (Local Distribution of Short Sequences) (127). There are 308 octamers have successfully been detected that belong to a group of putative *cis*-regulatory elements, Regulatory Element Group (REG), in addition to novel core promoter elements (131) by applying LDSS method in *Arabidopsis* genome. Biological role of most of the REG is still not very clear. In order to give biological annotation to *cis*-regulatory elements, one of the best methods is to analyze the microarray data and to predict *cis*-elements from the genes response to environmental stress.

In my laboratory, microarray analysis to see transcriptional response of Arabidopsis treated with GP17-2 in roots has been performed. Taking advantage of the in house data, I analyzed the microarray data in detail, by comparing selected public microarray data of pathogen, phytohormones, hydrogen peroxide (H_2O_2), and wound responses. Utilizing the microarray data, I achieved *in silico* promoter analysis in order to reveal participating *cis*-regulatory elements involved in the GP17-2-mediated ISR. An octamer-based frequency comparison method that has been developed in our laboratory was used for the prediction.

Some promoters are known to be activated by osmotic stress, high salt, drought, or ABA treatment (125, 123). Moreover, different *cis*-acting elements in these promoters are involved in stress-responsive gene expression (126). ABRE (ABA-responsive element) and DRE/CRT (dehydration-responsive element/C repeat) are major cis-acting elements in abiotic stress-inducible gene expression. DRE/CRT elements with the core sequence C/DRE (GCCGAC) play an important role in regulating gene expression in ABA-independent regulatory systems and can be found in promoter regions of many dehydration-, high-salt-, and cold-stress inducible genes in Arabidopsis, such as rd29A, kin1, and cor15a (6,123, 54). Various types of ABRE-like sequences have been reported, including the G-box sequence (CACGTG), which is present in a large number of environmentally regulated genes (79). Other cis-regulatory elements, such as MYB (C/TAACNA/G), MYC (CANNTG), LTRE (CCGAC) play key roles in activating gene expression in response to osmotic stress and/or ABA (6, 1, 87).

Applications in plant genetic engineering with transcription factors driven by stress-induced promoters provide an opportunity to improve the stress tolerance of crops (121). However, the activities of native promoters identified so far have certain limitations, such as low expression activity and low specificity. A series of synthetic promoters for higher-level expression of foreign genes has been reported in the literature (82, 94, 102, 61,11). With the information currently available on the regulatory mechanisms of abiotic stress tolerance in plants, it is now feasible to construct strong inducible promoters artificially. Thus, in the current study, I have selected *cis*-regualtory elements derived from stress-induced promoters (e.g. PGPF, phytohormone) in Arabidopsis, to construct artificial promoters. The pattern of inducibility driven by these artificial synthetic promoters was characterized in stable transgenic Arabidopsis by monitoring expression of the luciferase (LUC) reporter gene, upon exposure of these plants to

various stress conditions. In addition, promoter activity was assessed through luminescence estimation of LUC expression in transgenic plants under various stress conditions (biotic and phytohormone) as compared to the wild type Col-0 and /or vector control.

Therefore, this study was conducted to explore the molecular characterization and transcriptional responses during ISR by plant growth promoting fungi (PGPF). To achieve the goal, at first the volatile organic compounds were isolated from PGPF and analyzed for growth promotion and disease suppression effect in the first two chapters. Then microarray data of PGPF treated gene expression were analyzed and compared with phytohormone responses to find out the involvement of phytohormones during ISR induced by PGPF. Analyzing the microarray data with the help of bioinformatics, I have extracted some putative *cis*-regulatory elements, prepared synthetic vectors by inserting them in luciferase reporter gene based vector. Finally, the synthetic vectors were subjected to *in vivo* analysis to examine the biological response against different biotic and abiotic stress.

CHAPTER 2

Analysis of volatile organic compounds emitted by plant growth- promoting fungus *Phoma* sp. GS8-3 for growth promotion effects in tobacco

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2.1 INTRODUCTION

Plant growth is influenced by an abundance of abiotic and biotic factors. Plant growth hormones dominatingly affect plant growth, whereas the photosynthetic rate is dominated by temperature, irradiance and gaseous atmosphere (42). These physiological functions have been utilized as classical plant growth regulators. However, along with the composition of the nutrient medium, the composition of the gaseous atmosphere is another important factor for proper growth and development of plants (12). Several gaseous components are present in the atmosphere especially nitrogen, oxygen, carbon dioxide and different types of volatile compounds produced by surrounding organisms including the plant itself (16, 103). Changes in these components during different physiological functions in vitro largely affect the photosynthesis and other biological functions of the plant (16).

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In the past few years the role of volatile emissions from rhizobacteria in plant development has been widely studied. Ryu et al. (95) first reported a blend of airborne chemicals released from specific strains of PGPR, *Bacillius subtillis* GB03 and *Bacillius amyloliquefaciens* IN937a, which promoted growth of *Arabidopsis thaliana* seedlings. Gutiérrez-Luna et al. (41) also reported that VOCs from some strains of *Bacillius* sp. has growth promotion effect . While most studies have focused on the effect of VOCs released from PGPR and plant pathogens, little is known about the molecular mechanisms of response and resistance offered by PGPF- released VOCs.

Previously, different PGPF isolates like *Phoma* sp. (GS8-3, GS8-1) and *Penicillium simplicissimum* (GP17-2) have been reported for their growth promotion effect (77,78,107,108, 115). However, VOCs from these have not been analyzed. The first report regarding the growth promotion effect of VOCs produced by PGPF was by Yamagiwa et al. (124) where they introduced a new PGPF, *Talaromyces wortmannii* having growth promotion effect on several plant species such as *Brassica campestris*, *Arabidopsis thaliana*, *Phaseolus vulgaries*, *Nicotiana benthamiana* and *Cucumis sativas.* The major volatile component isolated from that PGPF was a terpenoid-like volatile *β*-caryophyllene which significantly promoted plant growth and induce resistance of turnip (124).

Considering that the fungi produce a wide range of VOCs (32) and VOCs produced from microorganisms play important role in plant growth, we aimed to analyze plant growth promotion effect of VOCs released from previously reported plant growth promoting fungus Phoma sp. GS8-3.

2.2 MATERIALS AND METHODS

2.2.1 Fungal cultures

One hundred fungal isolates were used in this experiment. All of the isolates were obtained from the plant pathology laboratory of Gifu University. Air borne fungi were isolated from leaves of turf grass around Gifu city and the soil borne fungi were isolated from the rhizosphere of cucumber, tomato and leaf mustard. Most of the isolates were identified by sequence comparison in the ITS regions of the rRNA gene including 5 of the selected fungi: *Cladosporium* sp. (D-c-4), *Ampelomyces* sp. (D-b-7, F-a-3), *Mortierella* sp. (U-c-1) and *Phoma* sp. (GS8-3) (data not shown). The fungal isolates were cultured on potato dextrose agar (PDA), and the periphery of actively growing cultures were cut with a cork borer of 5 mm diameter and used in the experiment. The fungal cultures were maintained on PDA slants and stored at 5^0 C.

2.2.2 Preparation of Plant materials

Seeds of *Nicotiana tabacum* L. cv. Xanthi-nc were surface-sterilized (70% ethanol soaking for 2 minutes, followed by 5% sodium hypochlorite soaking for 2 minutes), rinsed (five times) in sterile distilled water, and placed on petri dishes containing Murashige and Skoog salt (MS) medium (Wako) containing 0.8% agar and P^H was adjusted to 5.7. The seeds were incubated in growth cabinets (Nihon ika kikai seisakusho, LH-100S) set to a 12-h-light/12-h-dark cycle at 25 ^oC .

2.2.3 Screening of fungal isolates showing plant growth promotion

Plastic petri dishes (90×15 mm) containing a center partition (I plates; Atekuto) were prepared with 5 ml MS solid medium on one side, and 5 ml PDA on the other side. Fourteen days old

tobacco seedlings (10 seedlings per plate) were transferred to the MS solid medium side of the I plates. Treatments were done by inoculating the I plates with a disk of fungal isolate on the center of PDA medium. Control was maintained by using PDA medium without fungal disk. The plates were sealed with parafilm and arranged in a randomized design within the growth cabinets and incubated at 25 ^{o}C with a 12-h-light/12-h-dark photoperiod.

2.2.4 Design of screening of fungal isolates

Test fungal isolates were selected randomly considering the origin of isolates and pattern of growth promotion. Among the 7 test fungi, 4 were selected from the air-borne fungal group: *Cladosporium* sp. (D-c-4), *Ampelomyces* sp (D-b-7and F-a-3) and C-b-9 (unidentified); whereas the other 3: *Phoma* sp. (GS8-3), E-a-2 (unidentified) and *Mortierella* sp. (U-c-1) were from the soil borne fungal group. Considering the pattern of growth promotion effects, D-c-4 (*Cladosporium* sp.), GS8-3 (*Phoma* sp.), and D-b-7 (*Ampelomyces* sp.) were selected from the group of fungi that have higher growth promotion effect; whereas F-a-3 (*Ampelomyces* sp.) was selected from the medium group and unidentified E-a-2 and C-b-9 and U-c-1 (*Mortierella* sp.) were selected from the fungi having lower growth promotion effect.

2.2.5 Measurement of CO_2 regulation by the test fungus

The test fungal isolates were inoculated in a 300 ml Erlenmeyer flask containing 100 ml PDA and cultured in an incubator set to 12-h-light / 12-h-dark cycle for 7 days at 25^0 C. Three, 5, 7, 9, 12 and 14 days after inoculations, CO_2 concentration in the jar was measured by a CO_2 detector.

2.2.6 Analysis of volatiles produced from a selected fungal isolates GS8-3 for plant growth promotion effect

The assay was performed in two Erlenmeyer flasks that were tied in a glass tube with adapters for air inlet and outlet. The first Erlenmeyer flask was prepared with 100 ml PDA medium and the second flask was prepared with 100 ml MS solid medium. The tobacco seedlings incubated for 14 days (20 seedlings per a flask) were transferred to the MS solid medium containing flask. PGPF isolate GS8-3 was used as test fungus and incubated on the PDA medium of the Erlenmeyer flask. Air was passed over the fungal culture to the plant culture one-way at 10 ml/min. In another set, a charcoal and silica-gel tube (SIBATA) was used as an absorbent of the volatile compounds produced by the test fungus to compare the effect of the compounds on plant growth. The absorbent was connected at the middle part of the glass tube which was tied to the fungal culture flask connected to the plant culture. Control was maintained by using PDA medium without fungal disk. The whole set up was incubated were incubated at 25^0 C with a 12 h-light/12 h-dark photoperiod for 14 days. The tube was moved every third day and new one was set.

2.2.7 Measurement of atmospheric CO_2 in vitro and analysis of its effect on plant growth

Three sets of I plates were used in this experiment. The I plates were prepared with 5 ml MS solid medium on one side, and 5 ml PDA on the other side. In the first design, 14 days old tobacco seedlings were transferred to the MS solid medium side (10 seedlings per plate) and the PDA side of the I plates were inoculated with a disk containing GS8-3. In the second design, the PDA side of I plates contained only PDA without fungus. And in the third design, the PDA side of the I plates were inoculated with a disk containing GS8-3 but the MS solid medium were without plants. Then the I plates were placed in the AnaeroPack rectangular jar (2.5 liters) (Mitsubishi Gas Chemical, Tokyo, Japan) that contained an AnaeroPack MicroAero (Mitsubishi Gas Chemical, Tokyo, Japan). The AnaeroPack MicroAero is a non disposable oxygen-

absorbing and carbon dioxide-generating agent for use in anaerobic jar. The experiment was performed under 7 % (vol) preliminary CO_2 concentration with an AnaeroPack MicroAero in the jar. The jar was placed in growth cabinets set to a 12-h-light / 12-h-dark cycle for 7 days at 25 ^oC. Tobacco plants with and without fungus were also grown for comparing plant growth in the jar without Anaeropack MicroAero. The CO_2 concentration and plant growth was compared between the treatments with or without the Anaeropack MicroAero. There were five replicates for each treatment and the CO_2 concentration in the jar was measured by CO_2 detector (New Cosmos Electric Co., Osaka, Japan) at 1, 3, 5 and 7 days after treatment.

2.2.8 Extraction and Analysis volatile metabolites

GS8-3 was cultured in 10 ml solid phase micro extraction (SPME) vials (Supelco, Sigma-Aldrich Co. US) for 3, 5, 7 and 9 days. The volatile metabolites were extracted by headspace SPME during 30 min at 25 ^oC. Polydimethylsiloxane / Divinylbenzene (PDMS/DVB) (65µm) fibers were used for volatiles profiling. Fibers were obtained from Supelco, and conditioned prior to analyses according to the manufacturer's recommendations.

GC-MS: A Hewlett-Packard 5890 gas chromatograph equipped with a split injector HP-5 MS capillary column (30 m length, 0.25 mm i.d.) was combined by direct coupling to a Hewlett-Packard 5972 A mass spectrometer. Working conditions were: injector 250 ^oC, transfer line to MS system 250 ^oC, oven temperature-start 40 ^oC, hold 2 min, programmed from 40 to 200 ^oC at 10 ^oC min-1, from 200 to 250 ^oC at 15 ^oC min-1, hold 5 min; carrier gas (He) 1.0 ml min-1; injection of the analytes was done in split mode (1/10); electron impact ionization 70 eV. Peak areas (of total ion current) were used for comparison of volatile compound fractions. Compounds were identified using the US National Institute of Standards and Technology (NIST) Mass

spectral Library or by comparison of retention times and spectra with those of authentic standards and Kovats retention indices with literature data.

2.2.9 Analysis of plant growth promoting effect of volatile organic compounds produced by PGPF isolate GS8-3

I plates were prepared with 5 ml MS solid medium on one side. Fourteen-day old pre-germinated tobacco seedlings were transferred to the side of I plates. The compounds identified through GS-MS analysis were purchased (synthetic chemicals) to carry out plant growth promotion test. The compounds were diluted in CH_2Cl_2, or the solvent alone was mixed with 0.1 lanolin, and 20 µl of the resulting suspension was applied to a sterile paper disk (d=1cm). Each of the compounds was tested for plant growth promoting effect by placing 1.8×10^{-4} and 1.8×10^{-2} µg singly and in combination with the compounds, on sterile filter paper discs placed on the blank side of I plates. The plates were sealed with parafilm and arranged in a randomized design within the growth cabinets and incubated at 25 ^{o}C with a 12-h-light/12-h-dark photoperiod. There were four replications for each treatment and the experiments were repeated three times.

2.2.10 Statistical Analysis

Data of growth promotion was analyzed by the analysis of variance (ANOVA). The significance of effect of fungal treatments was determined by the magnitude of the *F* value ($P = 0.05$). When a significant *F* test was obtained for treatments, separation of means was accomplished by Fisher's protected least significant difference (LSD) test.

2.3 RESULTS

2.3.1 Screening of fungal isolates showing plant growth promotion

One hundred fungal isolates were screened for the growth promotion effect in tobacco plant. Almost all of the fungal isolates were found to promote plant growth except the isolate U-c-1. Among them, 70 isolates were found to promote plant growth almost double compared to control treatment after 7 days of transplanting (Fig. 2.1). Seven isolates such as D-c-4 (*Cladosporium* sp.), D-b-7 (*Ampelomyces* sp.), F-a-3 (*Ampelomyces* sp.), GS8-3 (*Phoma* sp.), C-b-9 (unidentified air borne fungus), U-c-1 (*Mortierella* sp.) and E-a-2 (unidentified soil borne fungus) were randomly selected for rescreening for their growth promotion effect in tobacco maintaining time course as 3, 5, 7, 10 and 14 days after treatment. All isolates showed significantly higher growth at 14 days comparing to control triggering gradual growth promotion until 7 days and then with a sharp increase of plant fresh weight (Fig. 2.2). U-c-1 was found to have comparatively poor growth promotion effect while D-c-4 has the highest that validated the preliminary result in which this isolate belonged to the top group isolates. In this experiment, I plates (Atekuto) were used which have a central partition that avoids physical contact between the fungus and the plant seedlings and allowing only airborne signal transmission.

2.3.2 Measurement of CO_2 production by the test fungus and analysis of its effect on plant growth

Since CO_2 plays an important role in plant growth it is necessary to measure CO_2 regulation by the test fungal isolates and their role on plant growth. Test isolates showed variable trend in CO_2 production. D-b-7 and D-c-4 showed highest production of CO_2 at 14 days after inoculation that indicates a positive correlation between the increase of CO_2 regulation and growth promotion of

tobacco though both the patterns are different (Fig. 2.3 and Fig. 2.2). However, F-a-3 showed higher rate of CO_2 production for the first 9 days but subsequently it gradually decreased. In the case of GS8-3, CO_2 concentration showed an increase for the first 7 days but after that marginally decreased. In the case of U-c-1 and E-a-2, slowly increasing CO_2 concentration pattern was noticed whereas C-b-9 was notable in showing an exceptionally slow increase in CO_2 production. These results suggest that F-a-3, C-b-9 and GS8-3 could promote growth of tobacco at 14 days after inoculation despite of the decrease in CO_2 production. Among the seven fungi, GS8-3 was selected for further analysis. Because *Phoma* sp. GS8-3 has previously been reported as a PGPF, as well as a biocontrol agent (Meera et al. 1995; Meera et al. 1994; Shivanna et al. 1995; Shivanna et al. 2005; Sultana et al. 2009).

2.3.3 Analysis of volatile substances produced from selected fungal isolate for plant growth promotion effect

To confirm the growth promotion effect of the volatile chemicals released from the test fungal isolate GS8-3, another experiment was done by using absorbent of volatile substances. GS8-3 inoculated seedlings in which absorbent was not used showed more than 7 times growth promotion whereas fungus inoculated plants where absorbent was used showed 1.5 times growth promotion over control (Fig. 2.4). This result confirms the positive effect of airborne chemical signaling produced by GS8-3 on plant growth.

2.3.4 Measurement of atmospheric CO_2 in vitro and analysis its effect on plant growth

Atmospheric CO_2 was measured *in vitro* by using the AnaeroPack MicroAero to identify the relation of plant growth with CO_2 level *in vitro*. The experiment was performed with 7 % (vol) CO_2 concentration preliminarily kept in the jar with an AnaeroPack MicroAero. In the case of

GS8-3 only, the CO_2 concentration gradually increased and reached 7 % (vol) to 9 % (vol) in the jar at seven days of cultivation whereas in the case of tobacco plant only, the CO_2 concentration rapidly decreased after three days and was detected at 1 % (vol) in the jar at seven days after planting (Fig. 2.5). When tobacco plants were cultivated in the same jar with GS8-3 under MicroAero, the CO_2 concentration gradually decreased to 5 % (vol) after seven days of cultivation.

The growth of tobacco seedlings were compared between different jars with or without the fungus and MicroAero condition (Fig. 2.6). Fresh weight (g) of tobacco plants was significantly increased when cultivated under MicroAero condition compared with the jar without MicroAero. The highest plant growth was found in the tobacco plants treated with the fungus only which is similar with the plants cultivated with MicroAero only. Contrastingly, plant growth was found to be very poor and leaves had become slightly bleached when tobacco plants were treated with GS8-3 and cultivated under MicroAero condition. Furthermore, the growth of GS8-3 in the jar with tobacco plants under MicroAero condition seemed poor comparing to that in the jar without an AnaeroPack MicroAero (Fig. 2. 6).

2.3.5 Extraction and Analysis of volatile metabolites regulated from test fungus

A total of 15 volatile organic compounds were extracted from the PGPF GS8-3 using SPME coating PDMS/DVB fibers. Among these, 14 were identified as C4-C8 hydrocarbons including alcohols (2-methyl-propanol, 3-methyl-butanol, 1-hexanol, 2-heptanol, 4-methyl-phenol, phenyl ethyl alcohol), carboxylic acids (acetic acid, methacrylic acid and tiglic acid), ketones (2-Hexanone, 2-heptanone, 3-hydroxy-2-butanone/acetoin) and their ester (isobutyl acetate) (Table-2.1). To investigate the relationship between mould growth and release of fungal volatile

substances with time, the volatiles were extracted from different sets of fibers at 3, 5, 7 and 9 days of growth. GS8-3 produced 2-methyl-propanol and 3-methyl-butanol as main volatile organic components during the culture periods. However, the number and concentration of the volatiles produced by GS8-3differed with increasing age of the fungus.

2.3.6 Effect of synthetic VOCs on plant growth

Synthetic VOCs that were identified from GS8-3 in 3 and 5 days aged culture individually and two of their mixtures were tested for their growth promotion effects at four concentrations. In addition, other two VOCs (2,3-butanediol and 1-octen-3-o1) that were previously identified having growth promotion effect on *Arabidopsis* by other researchers have also been chosen to compare their effects on tobacco. Mixture-1 that included the VOCs : 2-methyl-propanol: 3-methyl-butanol: methacrylic acid: isobutyl acetate (30:60:7:3) extracted from GS8-3 at 3 days showed 1.4 times significant increase in fresh weight of tobacco over solvent control at 1.8 $x10^{-2}$ µg concentration (Table-2.2). Besides these, mixture-2 (at 1.8×10^{-2} µg) that included acetic acid: 2-methyl-porpanol:acetoin: 3-methyl-butanol: methacrylic acid: isobutyl acetate: tiglic acid: phenylethyl alcohol (14:20:6:46:9:2:2:3) and methacrylic acid (at 1.8×10^{-4} µg), acetic acid (at 1.8×10^{-4} µg)and tiglic acid (at 1.8×10^{-4} µg) individually showed noticeable good effects on growth promotion though they are not significant. Fresh weight of tobacco was varied in different concentrations of synthetic VOCs. At high concentration such as at 1.8 and 1.8×10^{2} µg, most of the compounds caused bleaching of the cotyledon leaves (data not shown).

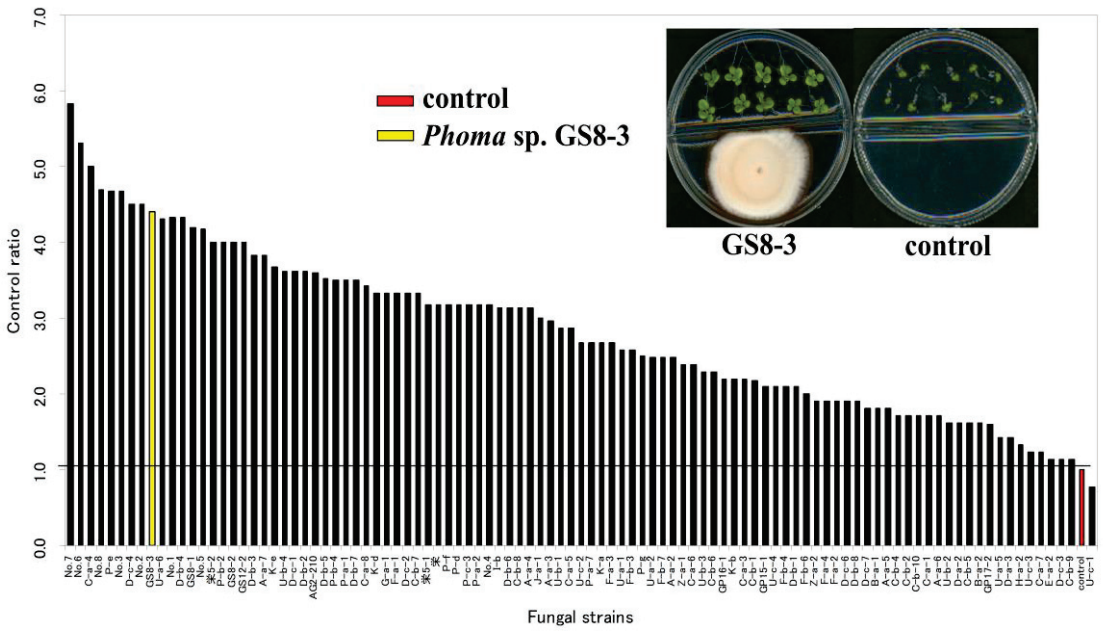


Fig. 2.1. Analysis of growth promotion in tobacco with exposure to airborne chemicals released from 100 fungal isolates compared with control (PDA only). Representative example of 7 day-old tobacco seedlings grown on I plates with exposure to airborne fungal isolate (GS8-3) and PDA only are shown in *Inset*. I- plates were prepared as gnotobiotic system to avoid contamination. Figure is showing the fresh weight of tobacco under different treatments with control ratio as fresh weight of control is 1. Data are the mean of three independent experiments.

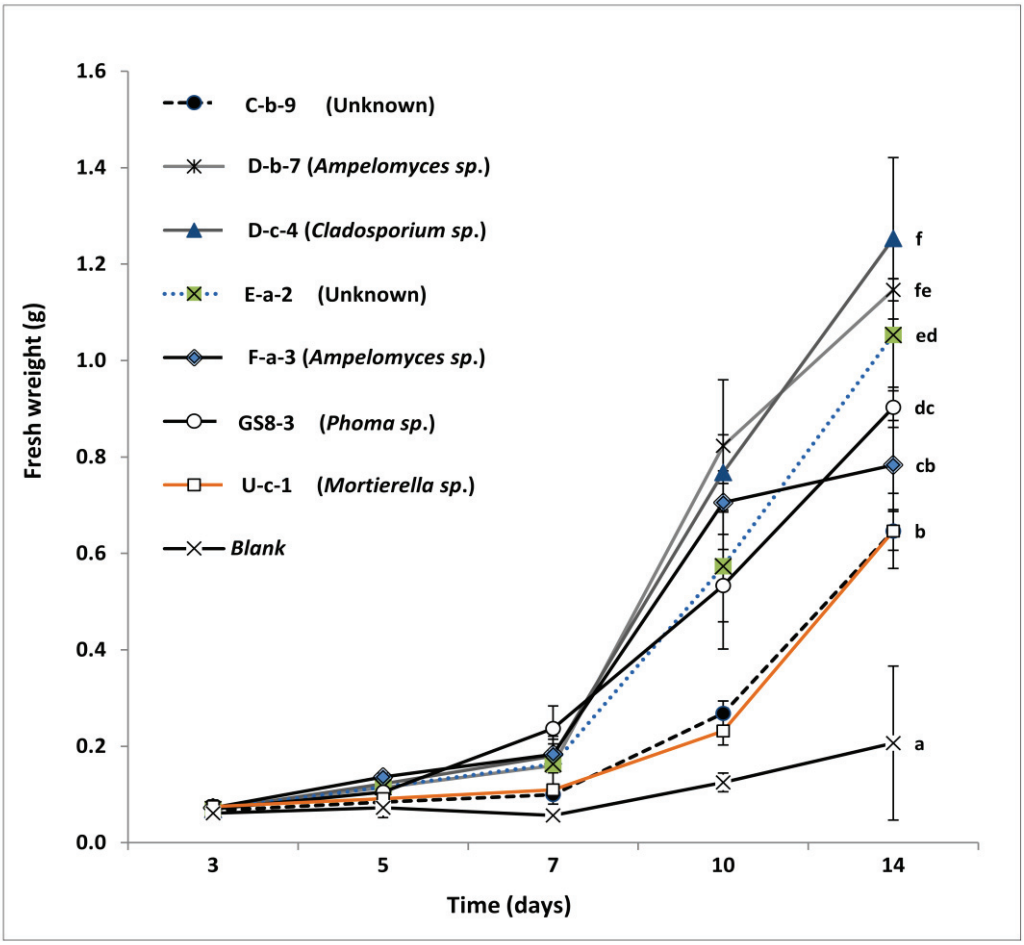


Fig. 2.2. Growth of tobacco seedlings during 14 days with exposure to airborne chemicals released form selected fungal isolates compared with PDA alone (blank). There were four replicates for each treatment and the experiments were repeated three times. Data are the mean of three independent experiments. Different letters indicate significant differences between treatments according to Fisher's LSD at P=0.05

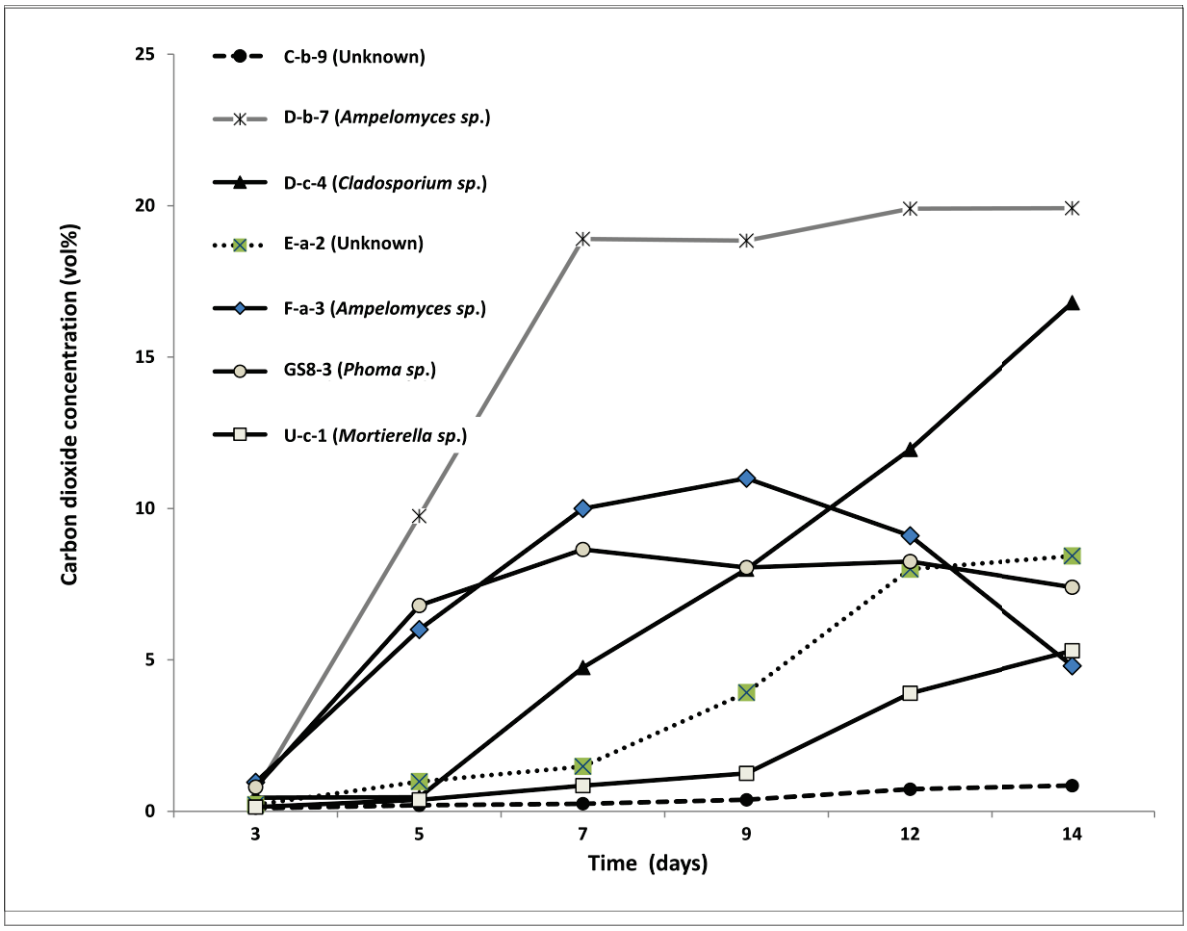


Fig. 2.3. Production of CO_2 by the selected fungal isolates during 14 days of growth period.

The test fungal isolates were inoculated in a 300 ml Erlenmeyer flask containing 100 ml PDA and cultured in an incubator set to 12-h-light / 12-h-dark cycle for 7 days at 25^0 C. Three, 5, 7, 9, 12 and 14 days after inoculations, CO_2 concentration was measured by a CO_2 detector. Data are the mean of three independent experiments.

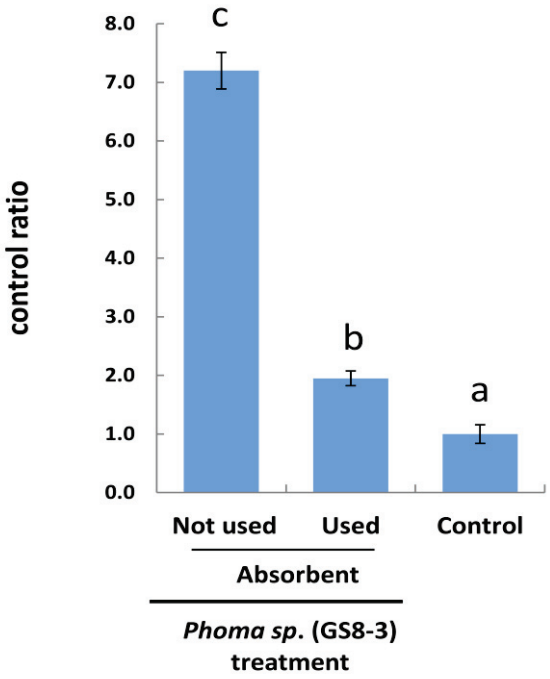


Fig. 2. 4. Growth promotion effect of volatile substances of *Phoma sp.* (GS8-3) in tobacco.

PGPF *Phoma sp.* (GS8-3) was used as test fungus. Charcoal and silica-gel tube that absorbs volatiles as soon as they are produced was used to block the flow of volatile compounds toward the plant flasks were used for comparison. Control treatment was maintained using PDA only inside the flask without any fungal isolate. Data show fresh weight of tobacco under different treatments with control ratio as fresh weight of control is 1.Values are means of 3 independent trails. Different letters on the bars indicate significant differences between treatments according to Fisher's LSD at P=0.05.

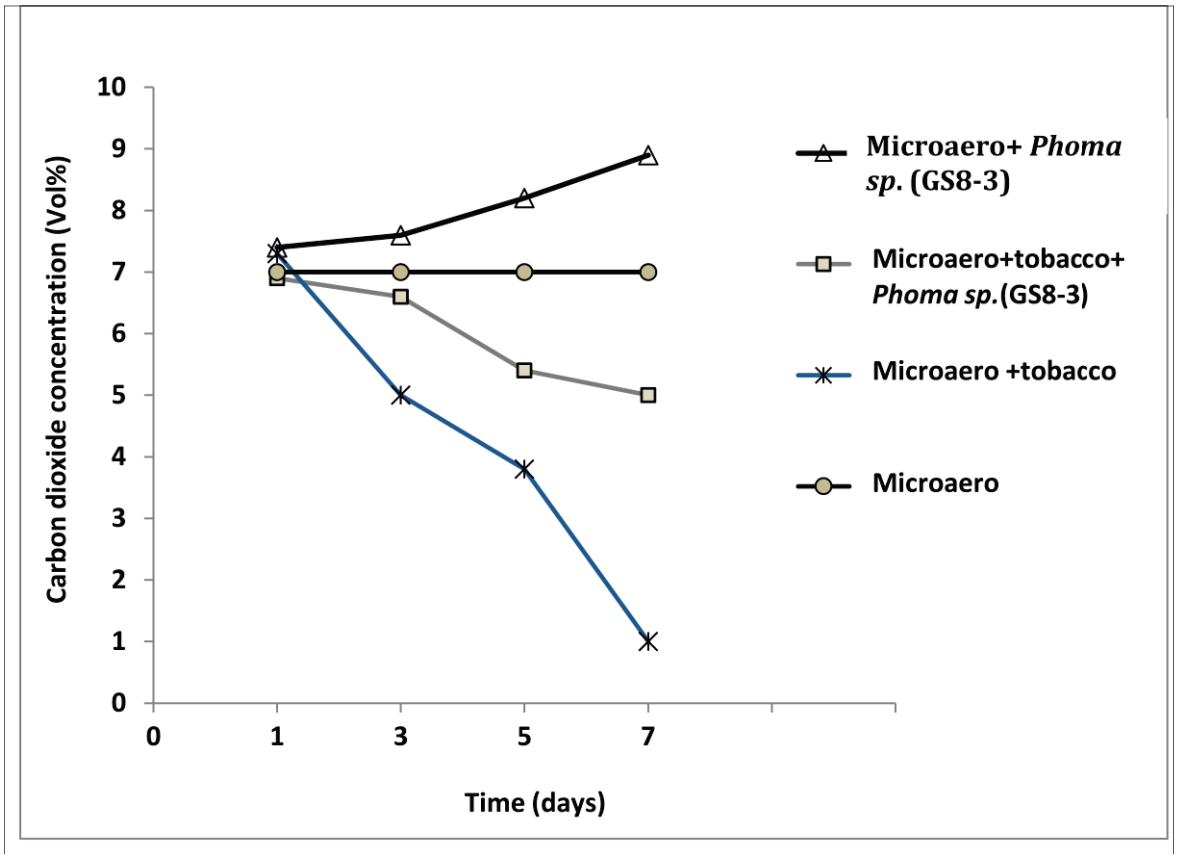


Fig. 2.5. Concentrations of CO_2 *in vitro* under MicroAero condition with or without tobacco plants and/or *Phoma sp.* (GS8-3). Three sets of I plates were used in this experiment. In the first set, 14 days old tobacco seedlings were transferred to MS media and PDA media on the other side inoculated with *Phoma sp.* (GS8-3). Second and third sets were prepared with fungus or plants only. The I plates were placed in the AnaeroPack rectangular jar with 7 % (vol) preliminary CO_2 concentration by an AnaeroPack MicroAero. The jar was placed in growth cabinets set to a 12-h-light / 12-h-dark cycle for 7 days at 25 ^oC. There were five replicates for each treatment and CO_2 concentration in the jar was measured by CO_2 detector at 1, 3, 5 and 7 days after treatment.

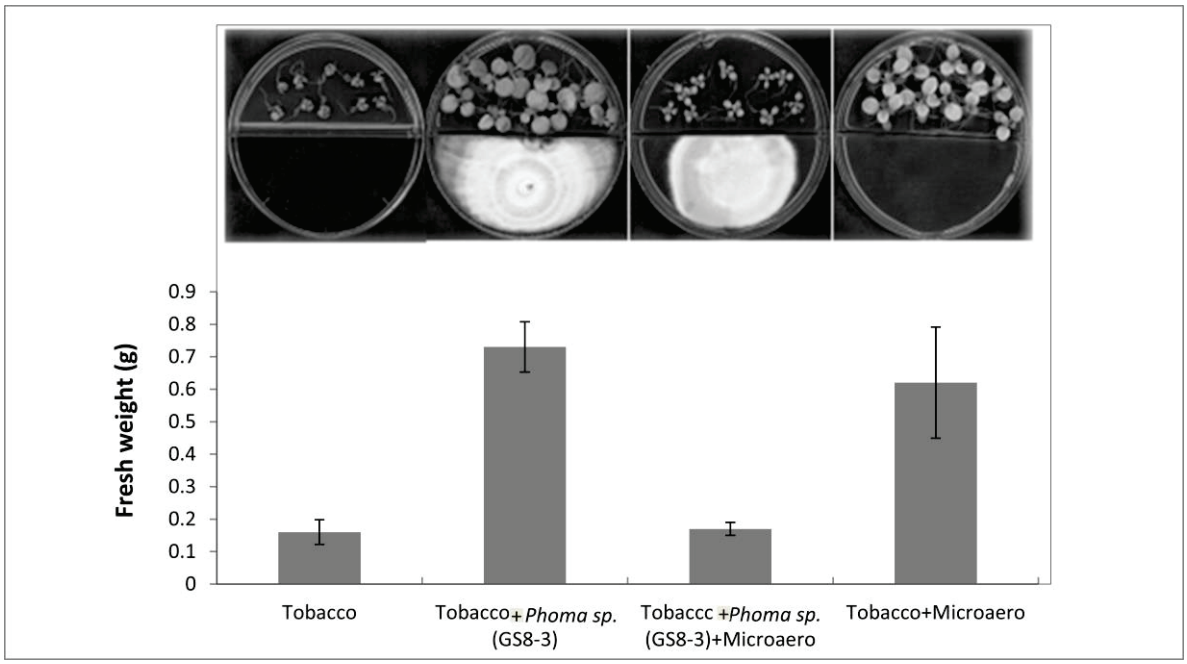


Fig. 2.6. Growth promotion of tobacco seedlings with MicroAero condition and/or *Phoma sp.* (GS8-3). Tobacco seedlings were grown for 14 days after treatment: from left, the seedlings grew alone (blank), with *Phoma sp.* (GS8-3), with *Phoma sp.* (GS8-3) under microaero condition, or under microaero condition without *Phoma sp.* (GS8-3). There were four replicates for each treatment and the experiments were repeated three times. The data are means of three independent experiments. Bars marked with same letters are not significantly different according to Fisher's LSD at $P = 0.05$.

Table 2.1. VOCs extracted from the PGPF isolate *Phoma sp.* (GS8-3) after 3, 5, 7 and 9 days.

Compounds	RI	Peak area (%)			
		3 days	5 days	7 days	9 days
Acetic acid		0	13.7	0	0
2-Methyl-propanol	621	28.9	19.8	9.4	17.5
3-Hydroxy-2-butanone/ Acetoin	710	0	6.0	0	0
Unknown	713	0	0	0	3.2
3-Methyl-butanol	740	62.1	45.9	83.5	59.6
Methacrylic acid	761	7.0	8.8	0	7.1
Isobutyl acetate	789	2.0	1.5	0	0
2-Hexanone	811	0	0	0	2.1
Octane	801	0	0	0	1.9
Tiglic acid	849	0	1.6	0.4	1.0
1-Hexanol	870	0	0	0	3.6
2-Heptanone	894	0	0	0.4	2.3
2-Heptanol	902	0	0	0.4	0
4-Methyl-phenol	1080	0	0	3.2	0
Phenyl ethyl alcohol	1126	0	2.7	2.7	0
Total		100	100	100	100

RI = Retention index. Compounds identified base on the comparison of retention index and mass spectra with NIST database.

Table 2.2. Plant growth promotion effect with exposure to volatile organic compounds (VOCs) released from PGPF isolate *Phoma sp.* (GS8-3) on tobacco.

VOCs	Concentration (μg)	
	1.8×10^{-4}	1.8×10^{-2}
2-Methyl-propanol	1.0	0.9
3-Methyl-butanol	0.9	1.1
Phenyl ethyl alcohol	1.1	1.0
3-Hydroxy-2-butanone	1.0	0.8
2,3- Butanediol[d]	0.9	0.9
1-Octen-3-ol[e]	1.0	1.0
Methacrylic acid	1.2	1.1
Isobutyl acetate	1.0	1.0
Acetic acid	1.2	1.0
Tiglic acid	1.3	1.0
Mixture 1[b]	1.0	1.4[a]
Mixture 2[c]	0.9	1.2

Tobacco seedlings were treated for 14 days with VOCs. Table showed the fresh weight of treated plant with control ratio as the fresh weight of control is 1. [a] indicted significant different at P <0.05 (LSD).

[b] is the mixture that duplicated volatiles produced by GS8-3 at 3 day ; 2-methyl-propanol: 3-methyl-butanol: methacrylic acid: isobutyl acetate = 30:60:7:3.

[c] Mixture -2 is the mixture that duplicated volatiles produced by GS8-3 at 5 day ; acetic acid: 2-methyl-porpanol: 3-hydroxy-2-butanone: 3-methyl-butanol: methacrylic acid: isobutyl acetate: tiglic acid: phenyl ethyl alcohol = 14:20:6:46:9:2:2:3.

[d] & [e], VOCs that were previously reported by other researchers were used to compare the growth promotion effects.

2.4 DISCUSSION

We investigated a total of 100 airborne and the soil borne fungal isolates for their growth promotion effect in tobacco plant and 70 isolates were found to promote plant growth almost double compared to the control treatment (Fig. 2.1). Among these, randomly selected seven isolates were rescreened maintaining time course and were found to have significantly higher growth promotion effect. In this study, we maintained air-tight cultivation using I plates that restricts physical contact between the fungus and the plant seedlings and allowed only gaseous exchange. This result suggests that the volatile or gaseous compounds released from the fungal strains have growth promotion effect on tobacco plants and our result supports the data of Ryu et al. (2003). These fungi included *Phoma* sp. GS8-3 which has previously been reported as a PGPF, as well as a biocontrol agent (Meera et al. 1995; Meera et al. 1994; Shivanna et al. 1995; Shivanna et al. 2005; Sultana et al. 2009), and was used as a test fungus in the next experiments. In another test, plant growth was found more than double in the case of GS8-3 treated seedling without using absorbents comparing to control treatment or the GS8-3 treated plants where charcoal and silica- gel tube absorbents were used (Fig. 2.4). These materials adsorbed the volatiles as soon they were produced by the organism and block the transfer of volatiles to the seedlings. Our method supports the method of Fernando et al. (2005). In this experiment, air-tight cultivation plates suggesting that the condition of gaseous atmosphere was normalized or CO_2 concentration was elevated through the fungal colony or culture. Thus, CO_2 produced by the fungus inside the chamber might have the possibility of affecting plant growth. Because many reviews have been published as well on the increased growth of plant species by improved CO_2 supply (Buddendrof-Joosten and Woltering, 1994; Chu et al. 1995; Desjardins, 1995; Pospíšilová et al. 1992; Sionit et al. 1982). Consequently, in this study we also considered the effect of the amount of CO_2 *in vitro* during the analysis of the growth promotion effects of PGPF- released

volatile metabolites in tobacco. Although previous researchers (Ryu et al. 2003; Yamagiwa et al. 2011) who worked on plant growth promotion effect of VOCs from microorganisms have not mentioned the involvement of CO_2, we located a report (Farag et al. 2006) where considerable amounts of CO_2 were recovered along with the VOCs during profiling of some PGPR. Thus, we checked the CO_2 production by the previously mentioned 7 test fungi until 14 days of culture. Data showed that among the isolates, D-b-7 and D-c-4 were gradually increasing CO_2 production that indicates positive correlation between the increase of CO_2 regulation and growth promotion of tobacco (Fig. 2.2 and Fig. 2.3). Thus, we assumed that CO_2 might play important role of plant growth promotion effect in case of D-b-7 and D-c-4. But in other isolates including GS8-3, no such correlation was found as GS8-3 still could increase plant growth significantly in spite of the decrease in CO_2 production after 7 days. Thus, we could distinguish the effect of VOCs in case *Phoma* sp. GS8-3 rather than the effect of CO_2. Moreover, among the isolates, only the *Phoma* spp. have been reported as effective PGPF for many crop species from a detailed study in our laboratory over years (Hyakumachi and Kubota, 2004) . Therefore, in this work we have chosen *Phoma* sp. GS8-3 as a test fungus to analyze the effects VOCs released from this fungus for better understanding of the growth promotion mechanisms of that PGPF. For further inspection, we measured the atmospheric CO_2 concentration in the presence or absence of GS8-3 and its effect on plant growth *in vitro*. We used AnaeroPack MicroAero for CO_2 supplement *in vitro*, i.e., a non disposable oxygen-absorbing and carbon dioxide-generating agent for use in anaerobic jar. Result showed that level of CO_2 inside the jar was increased when inoculated with GS8-3 with or without plants until 7 days of inoculation (Fig. 2.5). Another set of experiment was done without using MicroAero and fresh weight of tobacco plants was measured and compared in both situations at 14 days of planting. Fresh weight (g) of tobacco plants was significantly increased

when cultivated under MicroAero condition compared with the jar without MicroAero (Fig. 2.6). This result supports the findings of Haisel et al. (1999) as they reported that tobacco plantlets better supplied with CO_2 had high net photosynthetic rate, and low transpiration rate and stomatal conductance. But the highest plant growth was found in the case of tobacco plants treated with GS8-3 alone in the absence of MicroAero though it was statistically similar with the plants cultivated with MicroAero only. From the previous experiment (Fig. 2.3) we found that GS8-3 decreases CO_2 production after 7 days of inoculation. This result indicates that aside from CO_2, GS8-3 produce some VOCs that could promote plant growth. Plant growth was notably poor and leaves became minimally bleached when tobacco plants were treated with GS8-3 and cultivated under MicroAero condition (Fig. 2.6). In addition, the growth of GS8-3 in that jar seemed poor compared to that in the jar without an AnaeroPack MicroAero (Fig. 2.6 Inset). It may be the cause that excess CO_2 inhibited the growth of the fungi and changed the gaseous content inside that chamber by reacting with the VOCs. Previous reports (Burges and Fenton, 1953; Stotzky and Goos, 1965) indicate that higher concentrations (more than 5% increases in concentration) of CO_2 inhibit the growth of microorganisms, especially soil borne fungi. The altered gaseous atmosphere might be the cause behind growth retardation and bleaching symptoms of tobacco seedlings. However, the effect of growth promotion on tobacco by GS8-3 alone was higher than that by CO_2 supply using MicroAero.

In the next step, we separated the volatile components emitted from GS8-3 at different culture periods by gas chromatography, and identified by mass spectrometry. Identified VOCs belonged mostly to four classes of C4-C8 hydrocarbons where 2-methayl-propanol and 3-methayl-butanol were mostly found in considerable concentrations for all the fungal age (Table-2.1). Compounds of these characteristic metabolites were detected as indicator substances for mould growth

(Börjessonet al. 1992). These two components were previously extracted from some PGPR (Farag et al. 2006). Volatiles were found variable in number and amount by the age of fungus. Among the identified VOCs, acetoin (3-hydroxy-2-butanone) was discussed in many reports (Farag et al. 2006; Ryu et al. 2003; Ryu et al. 2004) for their growth promoting and ISR triggering ability in *Arabidopsis* when released from PGPR. We opted to analyze all the VOCs extracted at 3 days and 5 days of GS8-3 culture for the growth promotion effect, as the rest of the compounds have been found in trace amounts. Aside from these, 2,3-butanediol (Ryu et al. 2003) , and 1-octen-3-o1 (Kishimoto et al. 2007; Meruva et al. 2004; Schnurer et al. 1999) have also been checked in tobacco as these two metabolites were previously reported to promote growth and to induce defense response in *Arabidopsis*. Synthetic VOCs and their mixtures were performed at four concentrations. Mixture -1 (2-methyl-propanol: 3-methyl-butanol: methacrylic acid: isobutyl acetate in 30:60:7:3 ratio respectively) showed greatest level of growth promotion (1.4 times) compared to control (Table-2.2). Mixture -2 (acetic acid: 2-methyl-porpanol: acetoin: 3-methyl-butanol: methacrylic acid: isobutyl acetate: tiglic acid: phenylethyl alcohol in 14:20:6:46:9:2:2:3 ratio respectively) also showed better result than control. This supports the findings of Ryu et al. (2003), that better growth promotion effect is seen from all VOC blends. Though the VOCs did not show individual growth promotion effect significantly, few of them like methacrylic acid, acetic acid and tiglic acid still show good control ratio. Yamagiwa et al. (2011) also reported similar level of growth promotion effect of the volatile β-caryophyllene in turnip. However, we failed to notice a positive effect of 2, 3-butanediol and 1-octen-3-o1 in tobacco. Probably, the growth stimulating ability of VOCs differ according to plant species. As the fresh weight of tobacco plants varied at different concentrations of synthetic VOCs, from our observations, VOCs at lower concentrations showed better growth promotion than at higher concentrations.

Previously, we have mentioned that plant growth promoting microorganisms promote plant growth by producing growth regulating hormones (Loper and Schroth, 1986; MacDonald et al.1986; Timmusk et al. 1999), mineralizing nutrient substrates (Hyakumachi and Kubota, 2004.) and suppressing deleterious microorganisms (Farag et al. 2006; Kishimoto et al. 2007; Ryu et al. 2004). Ryu et al. (2003) revealed the possible involvement of PGPR regulated VOCs in the growth regulatory signaling pathways by using different mutant plants. They also speculated the possibility of using PGPR VOCs in other cultivation methods other than air-tight cultivation. We have also analyzed the growth promotion effects of PGPF produced VOCs in open air-cultivation system. But in our case, VOCs were not found as effective growth inducer in open-air system (data not shown).In this report, we have tried to find out the potential role of PGPF regulated VOCs in the orchestra of growth regulatory mechanisms. We found that *Phoma* sp. GS8-3 could induce growth promotion in tobacco in airtight cultivation system that suggests it's contemporary participation in the growth promotion effect of plant growth promoting fungi. However, the involvement of PGPF released VOCs in the growth regulatory signaling pathways remains to be determined.

CHAPTER 3

Systemic resistance induced by volatile organic compounds emitted by plant growth-promoting fungi in *Arabidopsis thaliana*

Systemic resistance induced by volatile organic compounds emitted by plant growth-promoting fungi in *Arabidopsis thaliana*

3.1 INTRODUCTION

Non-pathogenic, filamentous, saprophytic rhizosphere fungi that significantly enhance the growth of plants are known as plant growth-promoting fungi (PGPF) (Hyakumachi, 1994; Shivanna et al. 1994). In the search for alternate disease control strategies to minimize the use of chemical pesticides, the discovery of PGPF brought new expectations to researchers worldwide. In the past few years, PGPF from the genera of *Fusarium*, *Penicillium*, *Phoma*, and *Trichoderma* have been frequently studied and evaluated for their high suppressive abilities against a variety of plant diseases as a result of direct antagonism against soil-borne pathogens or by inducing systemic resistance in the plant (Ahmad and Baker, 1988; Shivanna et al. 1996; Shivanna et al. 2005; Hossain et al. 2007; Yoshioka et al. 2012). PGPF have been extensively studied to elucidate the mechanisms underlying the disease suppressiveness using different forms of inocula such as barley grain inocula or cell free culture filtrates (Hossain et al. 2007; Yoshioka et al. 2012; Koike et al. 2011; Meera et al. 1994). Molecular characterizations of the mechanism of the disease suppressive effects of PGPF or its culture filtrate proved that multiple signaling pathways are involved in ISR by PGPF and are mainly mediated by SA/JA-ET signals (Hossain et al. 2007; Yoshioka et al. 2012; Sultana et al. 2008).

Recent studies have also revealed that volatile organic compounds (VOC) released from some PGPF strains can effectively promote plant growth and enhance disease resistance (Yamagiwa et al. 2011; Naznin et al. 2013). In our previous study, we screened about 100 fungal strains by growing them in sealed I-plates (containing a center partition) with tobacco seedlings but without

physical contact between the strain and seedling; most plants increased growth when exposed to the volatile substances of the fungi. The volatile blends isolated from *Phoma* sp. GS8-3 significantly increased plant growth at low concentrations (Naznin et al. 2013). Yamagiwa et al. (2011) reported that the volatile compound β-caryophyllene emitted from the PGPF *Talaromyces wortmannii* FS2 significantly enhanced the growth of komatsuna (*Brassica campestris* L. var. *perviridis*) seedlings and their resistance to *Colletotrichum higginsianum*. Although reports on VOC from PGPF are relatively recent and few in number, the role of volatiles emitted from plants and other microorganisms on plant development have been studied extensively (Farmer, 2001; Ryu et al. 2004).

Many reports have focused on the effects of volatiles produced by rhizobacteria or plant growth promoting rhizobacteria on plant disease control. Several volatiles produced by rhizobacteria have exhibited antibacterial or antifungal activities (Kai et al. 2009). Two volatiles, 2,3-butanediol and acetoin (3-hydroxy-2 butanone), produced by *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been identified as important factors in inducing systemic resistance and promoting plant growth (Ryu et al. 2004; Farag et al. 2006). Volatiles produced by a few strains of *Streptomyces* are also reported to have potential for biocontrol (Wan et al. 2008; Li et al. 2010).

While most studies have focused on the interaction between rhizobacteria and plant pathogens, little is known about the plant response to VOC emitted by PGPF and the resistance that is conferred. Therefore, in the present study, we aimed to establish whether the PGPF-released VOC can induce systemic resistance in plants, and if they can, to determine what types of signaling pathways are involved in this ISR. We isolated the VOC from different PGPF and examined the disease suppression efficacy of VOC in a hydroponic culture system using the

model plant *Arabidopsis thaliana* (Arabidopsis) and bacterial leaf speck pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) and explicated the molecular basis of VOC-induced ISR in Arabidopsis.

3.2 MATERIALS AND METHODS

3.2.1 PGPF isolates

Fungal isolates *Cladosporium* sp. (D-c-4), *Ampelomyces* sp. (D-b-7, F-a-3) and *Phoma* sp. (GS8-3) used for VOC analysis were collected and identified at the laboratory of Plant Pathology, Gifu Univerisity.

3.2.2 Test plants and pathogen

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were provided by Dr. K.S. Park (NIAST, Suwon, Korea). Mutants *ein3* (Chao et al. 1997), *npr1* (Cao et al. 1994) and *jar1* (Staswick et al. 1992) were obtained from NASC (The Nottingham *Arabidopsis* Stock Centre) and transgenic line NahG was a personal gift (Lawton et al. 1995). All the mutants and transgenic *Arabidopsis* lines were developed against the background of the Col-0 ecotype. Virulent pathogen *Pseudomonas syringae* pv. *tomato* (*pst*) DC3000 was provided by Y. Ichinose (Okayama University, Okayama, Japan).

3.2.3 Extraction and analysis of volatile metabolites from PGPF isolates

Three PGPF isolates were cultured in 10 mL solid phase micro extraction (SPME) vials (Supelco, Sigma-Aldrich Co. US), and the volatile metabolites were extracted by headspace SPME during 30 min at 25°C. Polydimethylsiloxane/divinylbenzene (PDMS/DVB) (65 μm) fibers were used for volatile profiling. Fibers were obtained from Supelco and conditioned before analyses according to the manufacturer's recommendations. The composition of VOC 1, VOC 2 and VOC 3, isolated from *Phoma* sp. (GS8-3), *Ampelomyces* sp. (F-a-3) and *Cladosporium* sp. (D-c-4), respectively, were identified using GC-MS analysis as described by Miyazawa et al. (2008). Compounds were identified using the U.S. National Institute of Standards and

Technology (NIST) Mass Spectral Library or by comparing the retention times and spectra with those of authentic standards and Kovats retention indices with literature data.

3.2.4 Hydroponic culture of plants

Arabidopsis plants were grown in a hydroponic culture system developed by Toda et al. (1999). In this system, seeds were sown on nylon mesh (50 holes per inch) and were placed in a plastic photo-slide mount (50 x 50 mm; Fuji film, Japan). These mesh mounts were floated in a plastic case with the help of small pieces of styrofoam on 5 L of 1:10 MGRL nutrient solution (pH 5.6) and kept in a growth chamber at 24^{o}C with a 12 h day/12 h night cycle (Fujiwara et al. 1992). The nutrient solution was renewed every 7 days, and the culture was continued for 2 weeks.

3.2.5 Application of Volatile organic compounds (VOC)

The volatile compounds, VOC 1, VOC 2, and VOC 3 (Table 3.1) that were identified through GC-MS analysis and commercial methacrylic acid and isobutyl acetate (synthetic chemicals) were dissolved in CH_2Cl_2 and diluted to a 0.1 M solution. VOC were mixed with 0.1 g of lanolin before use and then 50 µL of one of the VOC was applied to a sterile paper disk and kept on a glass petri dish (3 cm). A dilution series (1 µM to 100 mM) of *m*-cresol and MeBA was also prepared and used to analyze dose-specific effects on disease severity. Hydroponically grown, 13-d-old *Arabidopsis* plants were transferred to a medium-sized (13 x 32 x 18.5 cm) plastic case containing 1/10 MGRL and kept in a large plastic case with the VOC in the glass petri dish. The whole system was then covered quickly and held for 24 h before inoculation with the pathogen.

3.2.6 Inoculation

The virulent bacterium *Pst* DC3000 was cultured in Kings' B broth containing rifampicin (50 mg/L) for 2 days at 28°C. The bacterial cells were collected by centrifugation, washed twice with sterilized distilled water (SDW) and resuspended in SDW to a final concentration of 7.0 x 10^7–8.0 x 10^7 colony forming units (cfu)/mL (OD_{600} = 0.070–0.080). The surfactant Silwet L-77 (0.01% v/v; Nihon Unica, Tokyo, Japan) was added as a spreading agent during inoculation. One day after the VOC treatment, 2-wk-old plants were sprayed with 200 mL of bacterial suspension. The inoculated plants were then kept at 100% relative humidity in the dark for 2 days to induce disease development. Plants were then transferred to the growth chamber with 12 h day/12 h night cycle and held for 3 more days.

3.2.7 Assessment of disease severity

Five days after the pathogen challenge, disease severity was scored, and the number of colony forming units of *Pst* (cfu)/g of leaves was determined for 10 randomly selected plants. Severity was scored for each plant as the percentage of total leaf surface with symptoms, from 0 = no symptoms to 100 = most severe with necrotic symptoms, and calculated using the formula described by Hossain et al. (2007). To determine the number of *Pst* DC3000 cells in inoculated leaves, we collected and weighed all leaves from the samples, rinsed them thoroughly in sterile water, then homogenized them in sterilized distilled water. Leaf suspensions were plated on KB agar supplemented with rifampicin (50 mg/L), and after 48 h incubation at 28°C, the number of cfu of *Pst* per gram of leaves was calculated. The experiment was repeated 3 times.

3.2.8 RT-PCR analysis

After the 24-h VOC treatment, aerial parts from 15 randomly selected plants were sampled in 1.5 mL Eppendorf tubes, ground in liquid nitrogen and homogenized with 600 µL of the extraction

buffer (20 g of guanidine thiocyanate, 0.2 g of *N*-lauroylsarcosine sodium salt and 0.2 g of trisodium citrate dihydrate dissolved in 40 mL of RNase free water) and 10 μL of 2-mercaptoethanol. The aqueous phase resulting from centrifugation at room temperature was re-extracted with a phenol : chloroform : isoamyl alcohol (PCI) (25 : 24 : 1; v/v) mixture. The upper aqueous phase was precipitated with isopropanol followed by a 75% ethanol rinse. The precipitated RNA was collected, air-dried briefly and dissolved in RNase-free water. After treatment with RNase-free DNase and inactivation of the DNase according to the instructions of the supplier (Takara Bio, Shiga, Japan), approximately 1 μg of total RNA was reverse transcribed to single-strand cDNA, and a sample of the obtained cDNA was amplified by RT-PCR, as described by Suzuki et al. (2004) to analyze the expression of a set of well-characterized defense-related genes. The expression of candidate priming gene was analyzed using the following primers: F-5′-GTAGGTGCTCTTGTTCTTCC-3′, R- 5′-TTCACATAATTCCCACGAGG-3′ (*PR-1;*At2G14610, product size 421 bp) and F-5′-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3′), R-5′-AATCCATGGAATACACACGATTTAGCACC-3′ (*PDF*1.2a; At5G44420, product size 281 bp). Expression of defense-related genes was determined by semi-quantitative RT-PCR. PCR products were separated on a 1.5% agarose gel, and intensities of bands were scanned with Typhoon 9400 Variable Mode Imager (GE Healthcare UK, Amersham, UK). The signal strength of each band was expressed numerically with the program image Quant 5.2 (GE Healthcare), and the relative expression level of each gene was calculated. β-tublin (*TUB8*; AT5G23860) was used as an internal standard using primers Forward-5′-CGTGGATCACAGCAATACA-3′ and Reverse-5′-CCTCCTGCACTTCCACTT-3′.

3.2.9 Real-time quantitative RT-PCR analysis

Real-time RT-PCR assay was performed using real-time PCR, ABI PRISM 7000 system (Applied Biosystems, Tokyo, Japan) using the default thermocycler program for all genes. Approximately 1 μg of total RNA was reverse transcribed to single-strand cDNA as described by Suzuki et al. (2004) after inactivation of DNase I according to the manufacturer's instructions (Takara Bio, Shiga, Japan). A sample of the obtained cDNA was amplified to monitor the expression of a set of selected genes. Power SYBR Green Master Mix was used according to the manufacturer's instruction; 1 μL of cDNA to 10 μL of SYBR Green Master mix: 0.8 μL of 5 μM primer F&R: 7.4 μL SDW. Primers used for real-time PCR are listed in Table 3.2. The relative signal intensity compared with control plants was calculated using $2^{-\Delta\Delta Ct}$ from the threshold cycle (Ct) values according to the manufacturer's software. Relative RNA levels were calibrated and normalized against expression levels of the internal control genes *UBQ5* and *ACT2*.

3.2.10 Statistical analysis

The experimental design was completely randomized, consisting of three replications for all treatments. The experiment was repeated at least twice. Data were subjected to analysis of variance (ANOVA), and a Student's *t*-test was used to determine statistically significant differences between treated samples and untreated control.

3.3 RESULTS

3.3.1 Extraction and identification of volatile metabolites from PGPF isolates

When the volatile metabolites were extracted from 2-wk-old cultures of three PGPF isolates using headspace SPME and identified using gas chromatography–mass spectrometry (GC-MS), most of the VOC from *Phoma* sp. (isolate GS8-3, VOC 1) and *Ampelomyces* sp. (isolate F-a-3, VOC 2) were C4–C8 hydrocarbons (Table 3.1). VOC 1 comprised 2-methyl-propanol (9.4%), 3-methyl-butanol (83.8 %), 2-heptanone (0.4%), 2-heptanol (0.4%), 4-methyl-phenol (3.3%) and phenylethyl alcohol (2.8%). VOC 2 comprised 2-methyl-propanol (3%), 3-methyl-butanol (22.6 %), 4-heptanone (2.5%), 3-octanone (1.1%), *m*-methyl-anisole (1.9%), *m*-cresol (59.8%), phenylethyl alcohol (8.6%) and cubenene (0.6%). Only one volatile component, methyl benzoate (MeBA) (100%), was identified from *Cladosporium* sp. (isolate D-c-4, VOC 3).

3.3.2 VOC emitted from PGPFs suppress disease severity

Arabidopsis plants were treated with one of the volatile organic compounds (VOC 1, VOC 2 or VOC 3) isolated from the 3 PGPFs in hydroponic culture (Table 3.1). After 24 h of treatment, plants were inoculated with bacterial leaf speck pathogen *P. syringae* pv. tomato (*Pst*) DC3000, and disease symptoms and number of bacteria were evaluated 5 days after inoculation. As shown in Fig. 3.1 (A, B), Arabidopsis Col-0 plants treated with VOC 2 (*Ampelomyces* sp. F-a-3) and VOC 3 (*Cladosporium* sp. D-c-4) resulted in a significant reduction in disease severity compared with the control. Disease severity, based on an index for percentage of total leaf surface with symptoms then calculated as the percentage protection compared with the control, in Arabidopsis plants was 39% after treatment with VOC 2 and 34% with VOC 3 (MeBA). On the other hand, disease severity in plants treated with VOC 1 isolated from *Phoma* sp. (GS8-3) was higher than

in the control. Results in Fig. 3. 1(C) present the number of colony-forming units (cfu g^{-1}) of *P. syringae* pv. tomato (*Pst*) DC 3000 in challenged leaves and reveal that the plants treated with VOC 2 and VOC 3 caused an approximately 2.4- and 3.8-fold decrease in cfu g^{-1}, respectively, compared with the control.

3.3.3 VOC induced high expression of defense-related genes

To evaluate the roles of SA and JA in the VOC-induced defense responses in Arabdiposis, the expression of SA- and JA-dependent marker genes was analysed by semi-quantitative PCR (Fig. 3.2. A and B). The expression level of the SA-inducible gene *PR*-1 and of JA-inducible gene *PDF* 1.2 was significantly higher in aerial parts of Arabidopsis treated with VOC 2 and VOC 3 (MeBA) than in the control. On the other hand, VOC 1-treated plants did not express defense-responsive genes. Expression of *PR*-1 was 2 and 2.5 times higher than in the control in VOC 2- and VOC 3 (MeBA)-treated plants, respectively. *PDF* 1.2 was expressed 3.9 and 2.6 times higher in VOC 2- and VOC 3 (MeBA)-treated plants, respectively, over the control. Thus, both SA- and JA-signalling are involved in the VOC-induced defence in Arabidopsis.

Because MeBA was identified as the major (100%) volatile compound in VOC 3 emitted by *Cladosporium* sp. D-c-4 that elicits ISR (Figs. 3.1, 3.2), while VOC 2 was extracted as a blend of volatiles (Table 3.1), we further analyzed VOC 2 to identify the major active volatile compound emitted by *Ampelomyces* sp. F-a-3.

3.3.4 *m*-Cresol is a major component with an important role in disease supression by Ampelomyces sp.

As we see in Figs. 3.1 and 3.2, VOC 2 (blend of volatiles) from *Ampelomyces* sp. and VOC 3 (MeBA) from *Cladosporium* sp. significantly suppressed disease against *Pst* DC3000. In the

blend of volatiles produced by *Ampelomyces* sp. F-a-3, *m*-cresol occupied the leading position (59.8%). Therefore, in the next step, we analysed all the components extracted from *Ampelomyces* sp. for their ability to reduce disease and the pathogen population. Together with the F-a-3 volatiles, 2 of the VOC, methacrylic acid and isobutyl acetate, found to be common components in 3- and 5-d-old cultures of *Phoma* sp. GS8-3 in our previous study (Naznin et al. 2013), were also included in the ISR test. Fig.3.3 shows that 3 of the VOC from *Ampelomyces* sp. (F-a-3), 3-octanone, *m*-cresol, phenyl ethyl alcohol, and the test volatiles methacrylic acid and isobutyl acetate induced systemic resistance in Arabidopsis against *Pst* DC3000 by 5 days after inoculation. Among the VOC, 3-octanone was highly effective in disease supression, and the bacterial population was reduced the most by treatment with *m*-cresol. From 14-d cultures, 3-octanone was identified as a trace component (1.1%), whereas *m*-cresol was greatest (59.8%) in the total volatile blend. We thus considered this compound to be the major active volatile component involved in the ISR by *Ampelomyces* sp. F-a-3.

3.3.5 Dose-specific effects of *m*-cresol and MeBA on ISR

To observe the effects of *m*-creosl and MeBA on ISR at different concentrations, we pretreated plants with a dilution series of the compounds (1 μM to 100 mM) before pathogen inoculation, then scored the percentage disease severity and the pathogen population. As we see in Fig.3.4, both *m*-cresol and MeBA induced ISR at all concentrations. Although the effect varied at different concentrations of *m*-cresol and MeBA, they both reduced disease severity more at the higher concentrations in the case of both VOC, and the pathogen population was decreased the

most at 100 mM. However, *m*-cresol and MeBA both induced ISR significantly over the control even at low concentrations.

3.3.6 Systemic resistance induced by methyl benzoate is compromised in Arabidopsis genotypes defective in JA-dependent signalling pathway

Previously, we checked the induction of defense-related genes *PR*-1 and *PDF*-1.2 in Arabidopsis plants treated with VOC blends (Fig. 3.2). To elucidate the signalling pathways leading to the ISR mediated by the major VOC, we exposed different Arabidopsis mutants or transgenic plants that are impaired in a specific regulatory pathway to the major VOC that triggered ISR: SA-deficient mutant *npr1*, impaired in NPR1 activity or nonexpressor of PR genes; Arabidopsis transgenic plant NahG, defective in SA-dependent signalling; an ethylene-insensitive3 (*ein*3) mutant and a JA-deficient mutant *jar1*. Application of methyl benzoate (MeBA) extracted from *Cladosporium* sp. D-c-4 significantly decreased development of leaf specks caused by *Pst* DC3000 in the *npr1* mutant, impaired in NPR1 activity and in the *ein3* mutant, impaired in ET-dependent signalling (Fig. 3.5 A). Bacterial growth also followed a trend similar to lesion development in *npr1* and *ein3* (Fig. 3.5 B), indicating that ISR mediated by MeBA is independent of SA and ET signalling. On the other hand, disease severity and the pathogen population were higher in the JA-signalling defective *jar1* mutant implicating the involvement of JA-signalling pathways in ISR by MeBA. Remarkably, disease severity in Arabidopsis transgenic plant NahG was not signficantly reduced by treatment with MeBA, albeit the bacterial population was significantly lower than in the control. This result indicates a partial recriutment of the signal transduction molecule SA in MeBA-mediated ISR.

3.3.7 *m*-Cresol failed to induce systemic resistance in Arabidopsis mutants impaired in SA-/JA-dependent signalling pathways

m-Cresol was also tested to determine the molecular patterns of induced systemic resistance in Arabidopsis plants using the same set of genotypes as those used in the MeBA treatment. Results showed that the percentage protection and the reduction of bacterial population were compromised in the SA-signalling-defective transgenic plant NahG, the NPR1-activity-impaired mutant *npr1*, and the JA- signalling-impaired mutants *jar1* plants treated with *m*-cresol (Fig. 3.5. C&D). On the other hand, lesion development and proliferation of bacterial pathogens in ET-signalling-impaired Arabidopsis mutant plants were significantly reduced in contrast to the control. These results indicate that the SA-signalling pathway is essential for *m*-cresol-induced systemic resistance in Arabidopsis plants, including partial JA-signalling.

3.3.8 Induction of Arabidopsis defense-related genes in plants treated with major VOC, MeBA and *m*-cresol

To define more clearly the role of SA-, JA- and ET-signal transduction pathways in the induction of systemic resistance by VOC, we further studied the induction pattern of marker genes for these pathways in plants exposed to the major VOC (Table 3.2). Plants were treated with VOC for 24 h, and transcription of SA-inducible gene *PR1, PR2, PR5* and ET-inducible gene *PR4*, JA-/ET-inducible gene *PR3, PDF1.2* and JA-inducible *AtVSP2* and *MYC2* was analysed by real-time quantitative RT-PCR. Result showed that relative expression of SA-inducible gene *PR1* and *PR2* was significantly higher (more than 6-fold and 2.5-fold, respectively) in *m*-cresol-treated plants (Fig. 3.6). *PR1* also showed high expression after the MeBA treatment (>2 fold), supporting the previous data on SA involvement (Fig. 3.2). On the other hand, JA/ET-inducible

marker gene *PDF1.2*, JA-inducible gene *MYC2*, and *VSP2* showed significantly higher relative expression in case of MeBA-treated plants. *m*-Cresol also significantly induced the JA/ET-inducible gene *PDF1.2* (>2 fold), strengthening support for the involvement of JA based on the previous data (Figs. 3.2, 3.5). The expression of ET-inducible marker gene *PR4* was also higher (>1.6 fold) after *m*-cresol treatment, differing from the data in Fig. 3.5, but not after the MeBA treatment. The JA/ET-inducible gene *PR3* and SA-inducible marker gene *PR5* were not noticeably expressed in our experiments.

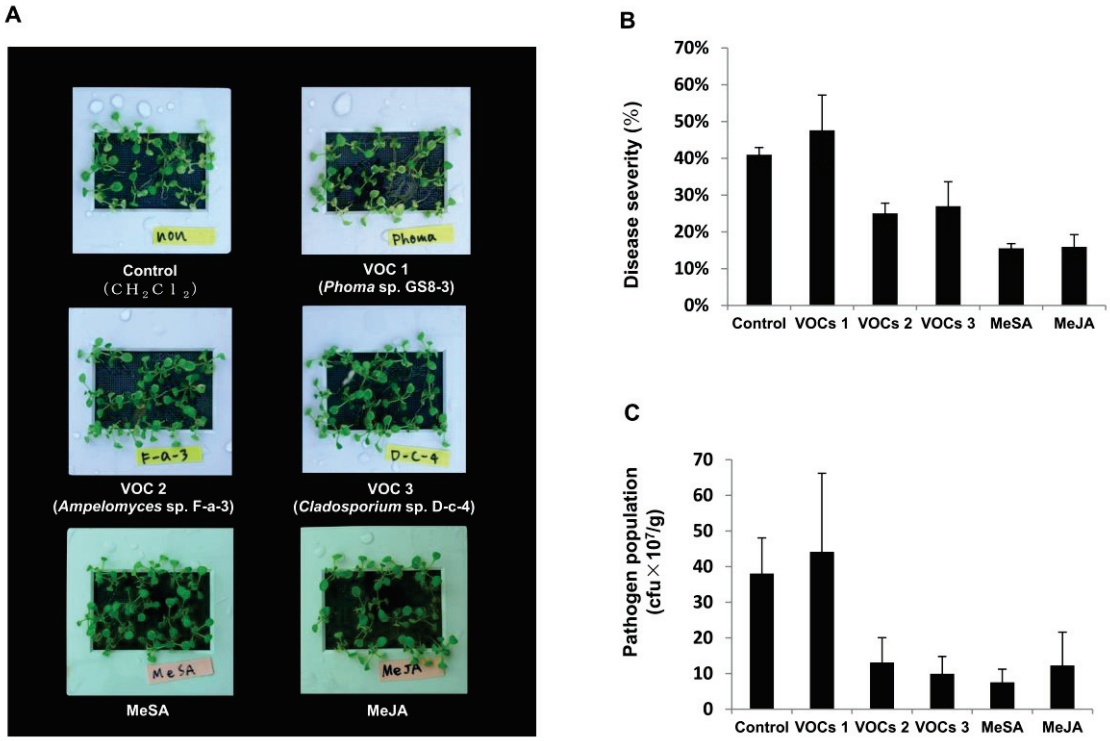


Fig. 3.1. Suppression of disease symptoms and numbers of *Pst* DC3000 after VOC pretreatment in *Arabidopsis thaliana*. A. Plants (17-d-old) on mesh screen in a slide mount 5 days after challenge inoculation with *Pst* DC3000. Plants were treated with VOC1 (*Phoma* sp. GS8-3), VOC2 (*Ampelomyces* sp. F-a-3) and VOC 3 (*Cladosporium* sp. D-c-4) for 24 h then inoculated with *Pst*. VOC1 and VOC2 were used as blend of volatiles; VOC3 was methyl benzoate (MeBA) only. Control was treated with CH_2Cl_2 only; MeSA and MeJA were used as positive controls. B. VOC-induced reduction of disease severity. Severity was scored for each plant as the percentage of total leaf surface with symptoms, from 0 = no symptoms to 100 = most severe with necrotic symptoms. C. Growth of *Pst* DC3000 (cfu g^{-1} fresh mass) in leaves. Asterisks indicate values differ significantly (Student's *t*-test, $P = 0.01$) from the control. Data are from representative experiments that were repeated at least 3 times with similar results.

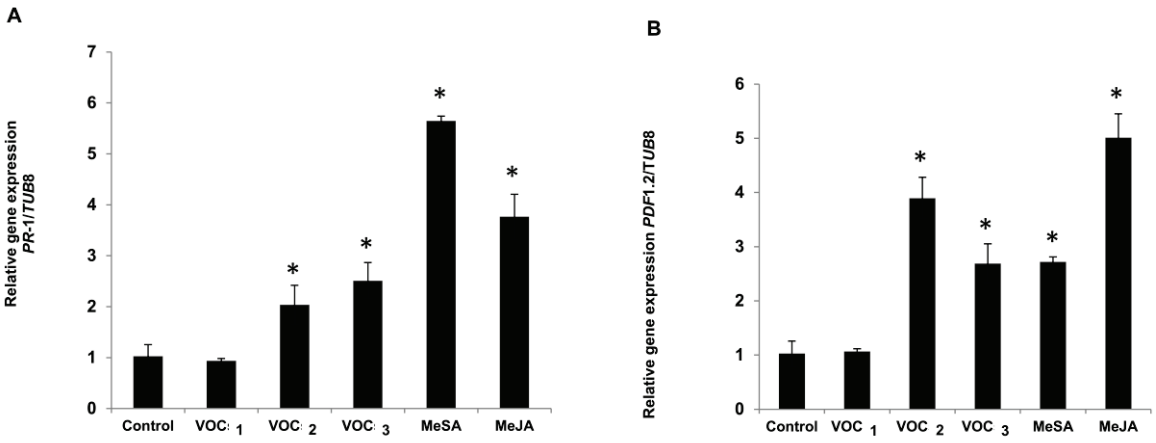


Fig. 3.2. Expression of of defense-related genes. A. SA-responsive gene *PR*-1 and B. JA-responsive gene *PDF* 1.2 in leaves of *Arabidopsis thaliana* treated with VOC 1, VOC 2 or VOC 3 (MeBA) of PGPFs in semi-quantitative RT-PCR analysis. Asterisks indicate statistically significant differences (Student's *t*-test, $P = 0.01$) compared with the control. Data are from representative experiments that were repeated at least 3 times with similar results.

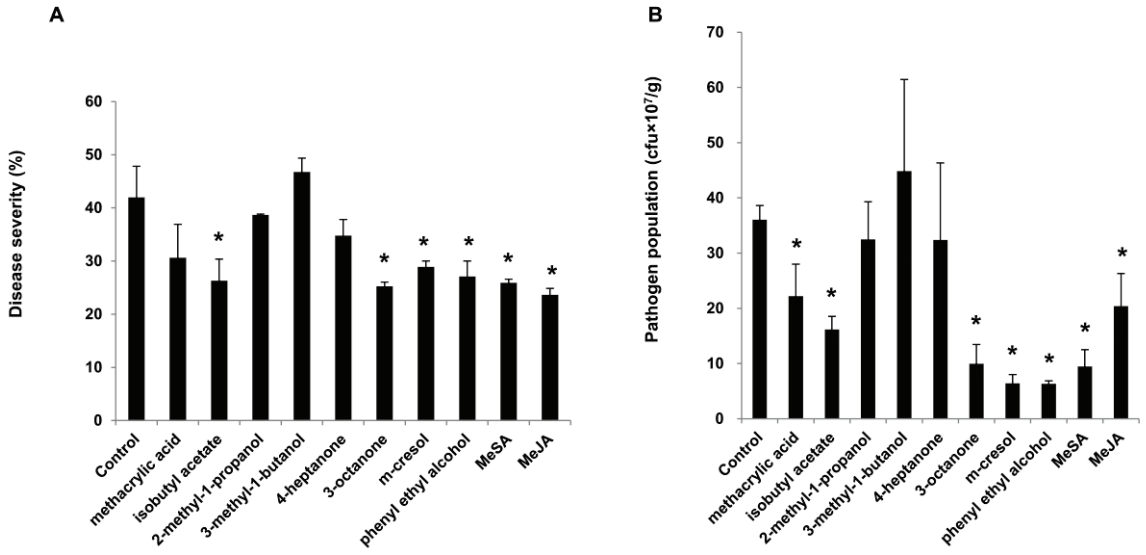


Fig. 3.3. Supression of disease symptoms and pathogen population by VOC isolated from *Ampelomyces* sp. A. VOC-induced reduction of disease severity caused by *Pst* DC3000 in Arabidopsis. Severity was scored for each plant as the percentage of total leaf surface with symptoms, from 0 = no symptoms to 100 = most severe with necrotic symptoms. B. Growth of *Pst* DC3000 (cfu g^{-1} fresh mass) in leaves. Plants were pretreated with 50 µL of one of the volatile components (0.1 M) for 24 h before inoculation. Methacrylic acid and isobutyl acetate were also tested as volatiles. Controls received only CH_2Cl_2; MeSA and MeJA were used as positive control treatments. Asterisks indicate statistically significant differences (Student's *t*-test, $P = 0.01$) compared with the control. Data are from representative experiments that were repeated at least 3 times with similar results.

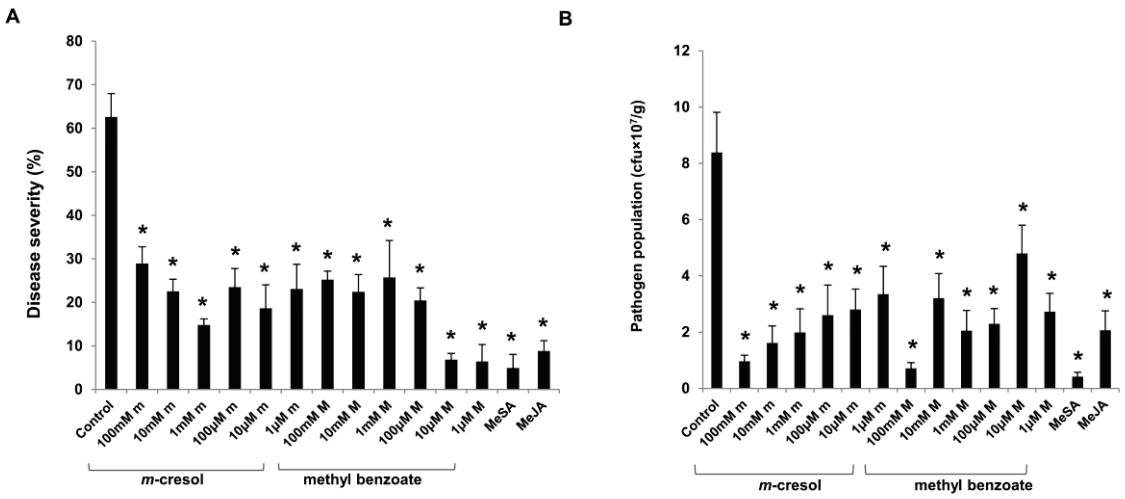


Fig. 3.4. Systemic resistance induced by *m*-cresol and methyl benzoate (MeBA) at different concentrations. A. Reduction in disease severity and B. Growth of *Pst* DC3000 in leaves after pretreatment of plants with *m*-cresol and MeBA at different concentrations followed by challenge inoculation with *Pst* DC3000. Disease severity was scored for each plant as the percentage of total leaf surface with symptoms, from 0 = no symptoms to 100 = most severe, with necrotic symptoms. Asterisks indicate statistically significant differences (Student's *t*-test, *P* = 0.01) compared with the control. Data are from representative experiments that were repeated at least 3 times with similar results.

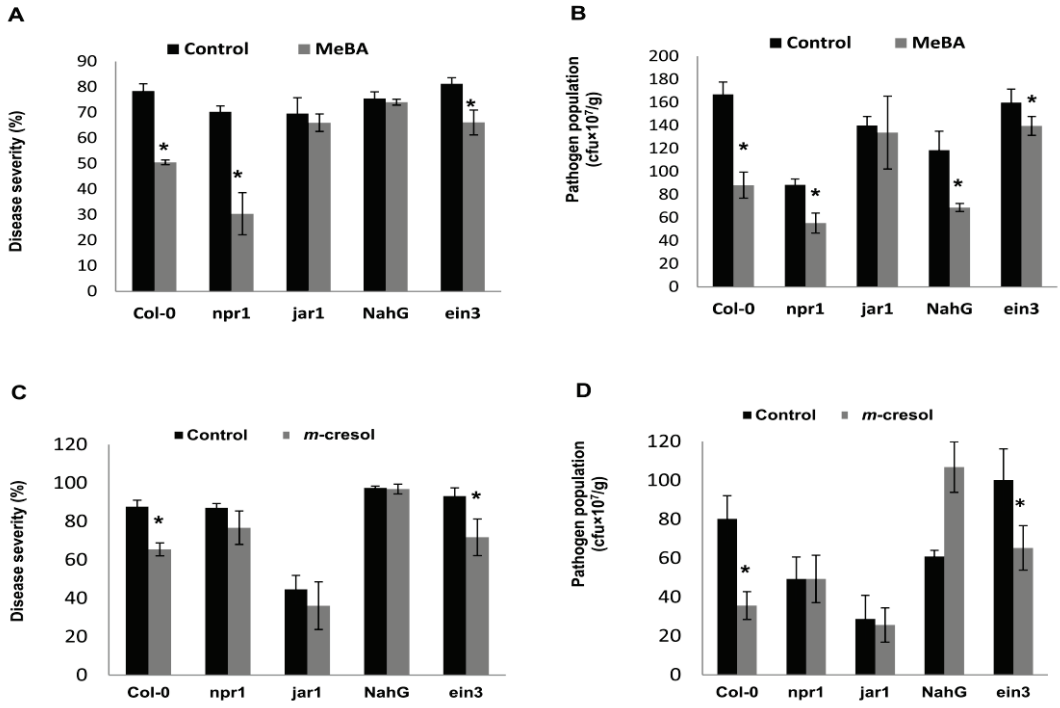


Fig. 3.5. Suppression of disease symptoms and *Pst* DC3000 population by VOC methyl benzoate (MeBA) and *m*-cresol. Arabidopsis transgenic plants and mutants impaired in defense signalling pathways and wild-type (Col-0) plants were used. A. Reduction in disease severity and B. Growth of *Pst* DC3000 in leaves after MeBA pretreatment followed by challenge inoculation with *Pst* DC3000. C. Reduction in disease severity and D. Growth of *Pst* DC3000 in leaves after *m*-cresol pretreatment followed by challenge inoculation with *Pst* DC3000. Data are percentage of disease severity (scored for each plant as the percentage of total leaf surface with symptoms, from 0 = no symptoms to 100 = most severe with necrotic symptoms) or number of cfu g^{-1} fresh mass 5 days after challenge inoculation. Asterisks indicate statistically significant differences (Student's *t*-test, $P = 0.01$) compared with the control. Data are from representative experiments that were repeated at least 3 times with similar results.

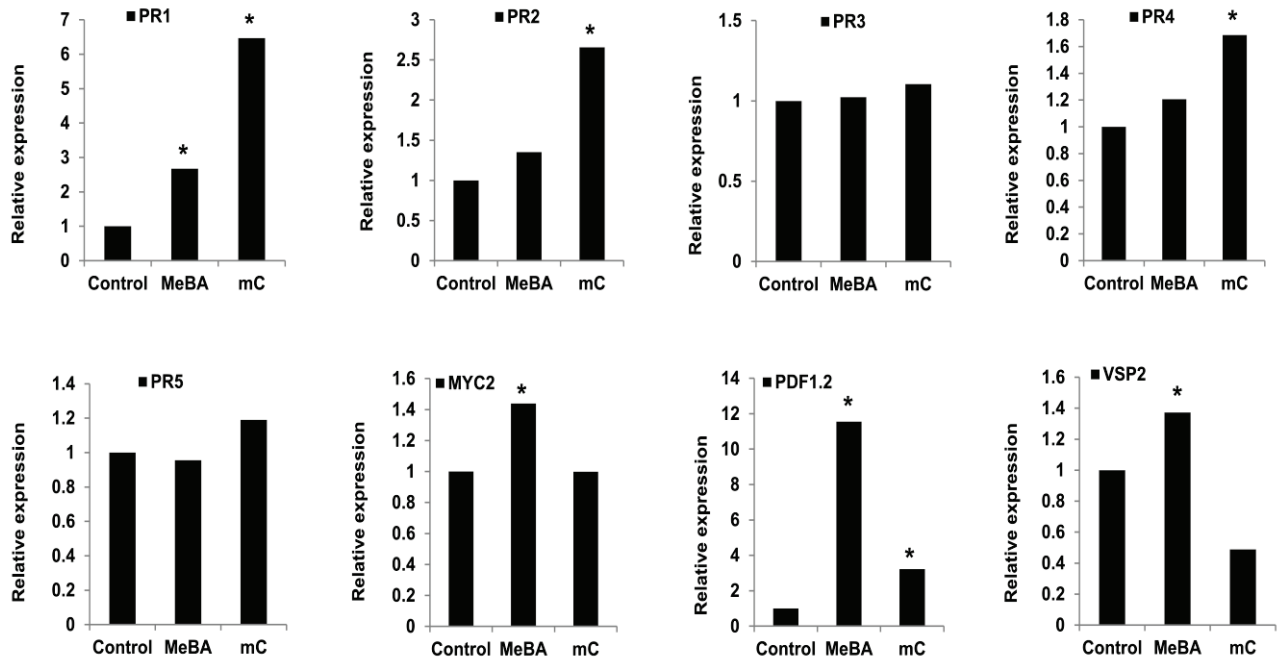


Fig. 3.6. Relative expression of defense-related genes on leaves of *A. thaliana* treated with *m*-cresol and MeBA. Amplification of JA-/ET-responsive genes *PR3* and *PDF1.2*, JA-responsive genes *AtVSP2* and *MYC2*, ET-responsive gene *PR4*, and SA-inducible genes *PR1*, *PR2* and *PR5* were analyzed with real-time qRT-PCR. Leaves from 15 representative plants were sampled 5 days after inoculation. Asterisks indicate statistically significant differences (Student's *t*-test, $P = 0.01$) compared with the control treatment.

Table 3.1: Retention index (RI) and peak areas for volatile organic compounds (VOC) extracted from 14-d-old cultures of the plant-growth-promoting fungi *Phoma* sp. (GS8-3), *Ampelomyces* sp. (F-a-3) and *Cladosporium* sp. (D-c-4) using SPME-based GC-MS analysis.

		Peak areas (%)		
Compounds	**RI**	**VOC 1** (*Phoma* sp. GS8-3)	**VOC 2** (*Ampelomyces sp*. F-a-3)	**VOC 3** (*Cladosporium* sp. D-c-4)
2-Methyl-propanol		9.4	3.0	-
3-Methyl-butanol		83.8	22.6	-
4-Heptanone		-	2.5	-
2-Heptanone		0.4	-	-
2-Heptanol		0.4	-	-
3-Octanone	986	-	1.1	-
m-Methyl-anisole	1022	-	1.9	-
4-Methyl-phenol	1080	3.3	-	
m-Cresol	1081	-	59.8	-
Methyl benzoate	1095	-	-	100.0
Phenylethyl alcohol	1116	2.8	86	-
Cubenene	1376	-	0.6	-
Total		100.0	100.0	100.0

Note: Compounds were identified by comparing the RI and mass spectra with data in the NIST database.

Table 3. 2: Gene-specific primers used in real-time qRT-PCR analysis

AGI code	Target gene	Primer sequences	Product size (bp)
Salicylic acid regulated gene			
At2g14610	PR-1	F5′-TTCTTCCCTCGAAAGCTCAA-3′	174
		R 5′-AAGGCCCACCAGAGTGTATG-3′	
At3g57260	PR-2	F 5′-AGCTTAGCCTCACCACCAATGT-3′	83
		R 5′-CCGATTTGTCCAGCTGTGTG-3′	
At1g75040	PR-5	F 5′- TGTTCATCACAAGCGGCATT-3′	99
		R5′GTCCTTGACCGGCGAGAGTTAATGCCGC-3′	
Jasmonic acid / Ethylene regulated gene			
At3g12500	PR-3	F 5′-GGCCAGACTTCCCATGAAAC-3′	113
		R 5′-CTTGAAACAGTAGCCCCATGAA-3′	
At3g04720	PR-4	F 5′-GCAAGTGTTTAAGGGTGAAGAACA-3′	104
		R 5′-GAACATTGCTACATCCAAATCCAAG-3′	
At5g44420	PDF1.2	F 5′-TTTGCTGCTTTCGACGCAC-3′	80
		F 5′-CGCAAACCCCTGACCATG-3′	
At5g24770	AtVSP2	F 5′-TCAGTGACCGTTGGAAGTTGTG-3′	104
		R 5′-GTTCGAACCATTAGGCTTCAATATG-3′	
At1g32460	MYC2	F 5′-AGCAACGTTTACAAGCTTTGATTG-3′	76
		R 5′-TCATACGACGGTTGCCAGAA-3′	
Housekeeping gene / internal control			
At3g62250	UBQ5	F 5′-GACGCTTCATCTCGTCC-3′	256
		R 5′-GTAAACGTAGGTGAGTCCA-3′	
At2g37620	ACT2	F 5′-AGTGGTCGTACAACCGGTATTGT-3′	92
		R 5′-GATGGCATGAGGAAGAGAGAAAC-3′	

3.4 DISCUSSION

In our previous study, we validated that chemical signals were being emitted into the air from the fungi *Phoma* sp. (GS8-3), *Ampelomyces* sp. (F-a-3) and *Cladosporium* sp. (D-c-4) and contributed to promoting the growth of tobacco seedlings (Naznin et al. 2013). Here, we isolated the VOC from these PGPF and analyzed their potential for plant protection by pretreating Arabidopsis plants and challenging them with the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). Protection of the plant was manifested by both a reduction in disease severity and a decrease in pathogen proliferation in the leaves. The VOC emitted from the PGPF suppressed *Pst* infection via induced systemic resistance since there was less disease without direct contact between the VOC and the pathogen. *Phoma* sp. (GS8-3) and *Ampelomyces* sp. (F-a-3) emitted a blend of volatile components, whereas only one volatile (MeBA) was produced by *Cladosporium* sp. (D-c-4) after 14 days of culture (Table 3.1). Different strains of *Phoma* sp. including GS8-3 have previously been reported to promote growth and induce systemic resistance in plants (Chandanie et al. 2006; Sultana et al. 2009). In addition, we found that a volatile blend emitted by GS8-3 was able to increase plant growth (Naznin et al. 2013). But unexpectedly, in the present study, the VOC isolated from plants treated with *Phoma* sp. (GS8-3) did not suppress disease or reduce the pathogen population after inoculation with the pathogen (Fig. 3.1). On the other hand, volatile components isolated from *Ampelomyces* sp. (F-a-3) and *Cladosporium* sp. (D-c-4) did reduce disease symptoms and pathogen population significantly, as did the positive control treated with MeJA and MeSA. The mycoparasite *Ampelomyces quisqualis*, a well-known biocontrol agent, is widely used for controlling powdery mildew of different plants and is known to act by hyperparasitism (Elad et al. 1998; Gilardi et al. 2008), but our finding that *Ampelomyces* sp. emits VOC that can induce systemic resistance is undisputedly

the first report for this antagonist. Likewise, *Cladosporium* spp. is also a mycoparasite of powdery mildew fungi (Kiss, 2003), parasitizing the surface of the penicillate cells of the cleistothecia and causing plasmolysis of the conidia (Kiss, 2003; Mathur and Mukerji, 1981). Antifungal compounds were presumed to play role in this inhibitory effect or antibiosis, but the mode of action had not been studied in detail.

In the present study, we isolated a volatile compound from *Cladosporium sp*. (D-c-4) that could induce systemic resistance in plants. In addition, to determine the mode of action underlying the ISR by the VOC extracted from the PGPF strains, we checked two Arabidopsis defense-related genes *PR*-1 (SA) and *PDF*1.2 (JA/ET) for post-inoculation amplification. Our results showed that disease suppression by the VOC isolated from both F-a-3 and D-c-4 involved the SA and JA/ET pathways (Fig. 3.1 B), with methyl benzoate ($C_6H_5CO_2CH_3$) the only compound (100%) emitted by *Cladosporium* sp. (D-c-4). In the mixture of VOC emitted by *Ampelomyces* sp. (F-a-3), *m*-cresol ($CH_3C_6H_4OH$) significantly induced systemic resistance and was the most abundant of all the VOC, confirming it as the major active volatile compound in ISR (Fig. 3.3). Methacrylic acid and isobutyl acetate, were isolated as common components from *Phoma* sp. GS8-3 after 3 and 5 days of culture in our previous study (Naznin et al. 2013), so we included them in ISR tests. Because the volatiles varied in number and quantity over time during culture (Naznin et al. 2013), we isolated VOC from a 14-d-old fungal culture, when methacrylic acid and isobutyl acetate are absent from the VOC profile of *Phoma* sp. GS8-3. In Fig. 3.3, we see the major volatiles emitted from GS8-3; 2-methyl-1-propanol and 3-methyl-1-butanol failed to reduce disease and pathogen population in Arabidopsis. On the contrary, methacrylic acid and isobutyl acetate reduced disease severity and the pathogen population, leaving little doubt that the age of the fungal culture is the likely reason behind the negative effects of VOC emitted by

Phoma sp.GS8-3 in Arabidopsis; however, we did not test this further. From our results, the volatile compounds, methacrylic acid, isobutyl acetate, 3-octanone, *m*-cresol and phenyl ethyl alcohol, were found to reduce disease severity, and are potential candidates for biological control agents.

When we used these two major volatile organic components and well-characterized mutants and transgenic plants to clarify the signaling pathways involved in this VOC-mediated ISR, our data revealed that plant protection was completely arrested in mutant *jar1* after treatment with MeBA, a paradigm of JA-dependency. Although JA and ET are thought to be the signal transduction molecules for induced systemic resistance (ISR) by biological control agents and JA and ET share a common pathway in ISR (Pieterse et al. 1998), in our case, disease in an ethylene-impaired mutant plants (*ein3*) was significantly suppressed, similar to the wild-type plants, indicating that an independent JA-signalling pathway is involved in MeBA-accelareted ISR. Disease severity in Arabidopsis transgenic NahG, defective in SA-dependent signaling, was higher than in the control although the pathogen population was significantly reduced compared with the control. But NPR1-activity-impaired mutant plants did not differ from wild-type plants in being protected by the volatile-induced resistance. Previously, PGPF or PGPR (rhizobacteria)-mediated ISR in Arabidopsis was reported to involve a novel signaling pathway based on JA/ET signals and regulated by NPR1 (Hossain et al. 2007; Yoshioka et al. 2012; Pieterse et al. 1998). But in our case, PGPF-regulated MeBA-triggered ISR signalling pathways appear to be involved, mainly via JA as a signal molecule with the partial recruitment of SA, but the ISR signaled via JA/ET differs by requiring NPR1.

Similar to MeBA, *m*-cresol also induced ISR without involving an ET-signal molecule but involved a JA-signaling pathway. Our results showed that *m*-cresol used a complete SA-

dependent signalling pathway to trigger ISR that requires NPR1. The signal transduction pathway through SA accumulation is found in the systemic acquired resistance (SAR) induced by pathogen attack (Durrant and Dong, 2004), while it is thought that JA and ET are the signal-transducing molecules for induced systemic resistance (ISR) by biocontrol agents (BCAs) (Pieterse et al. 1998). However, there are some reports that SA can also work as an inducement factor of ISR by BCAs (Hossain et al. 2007; Yoshioka et al. 2012). Our results also proved the involvement of both SA- and JA-signal transduction in ISR.

For more confirmation of the molecular mechanisms behind the VOC-mediated ISR, we assessed transcription levels of Arabidopsis defense-related markers, SA-, JA/ET-inducible genes (Table 3.2) by real-time qRT-PCR analysis. Like the results of the mutant screening, *m*-cresol significantly induced the SA-inducible marker genes *PR1* and *PR2*, confirming that the volatile lowered disease severity by inducing systemic resistance mainly through the SA-signal transduction pathway. In addition, JA-inducible gene *PDF1.2* was expressed significantly by treating plants with *m*-cresol, strengthening our idea of a partial engagement of JA-regulation. On the other hand, of all the genes examined, expression of the JA/ET-signal gene *PDF1.2* was the highest in MeBA-treated plants, more than 11-fold higher than in the control. Moreover, the JA-inducible marker genes *MYC2* and *VSP2* were also amplified significantly by the MeBA treatment. Thus, we are more confident that the JA-signaling pathway is activated in MeBA-mediated ISR in Arabidopsis. MeBA also induced transcription of the SA-responsive *PR1* gene, supporting our mutant-screening data. Generally, regulation of *PDF1.2* after pathogen infection requires concomitant activation of JA- and ET-signaling pathways. However, our results provide substantial evidence that the PGPF-emitted VOC *m*-cresol and MeBA induce *PDF1.2* using the JA-signal independently of ET-signaling. Although, we cannot explain the reason, the ET-

responsive gene *PR*4 was significantly expressed by *m*-cresol treatment compared with the control, whereas ET-impaired mutants (*ein2*) showed no involvement of ethylene in the resistance elicited by MeBA or *m*-cresol. However, studies on other volatile components from different sources indicated various modes of action can be involved. For instance, Ryu et al. (2004) revealed that the rhizobacterial volatile 2-3-butanediol and acetoin employed an ET-signaling pathway independent of the SA- and JA-signals, completely opposite of the mechanism induced by our volatiles. Lee et al. (2012) also found ISR by a long-chain volatile isolated from *Paenibacillus polymyxa* E681 that primed expression of SA-, JA- and ET-signaling marker genes. From another study, C6-aldehyde volatiles from green leaves of Arabidosis induced resistance involving the JA-signaling pathway in Arabidopsis against a necrotrophic pathogen (Kishimoto et al. 2006). However, the response of Arabidopsis to different volatile compounds differed because the amount and type of the elicitors varied, depending on the source of the volatiles; each of the multiple pathogen-associated molecular patterns (PAMPs) used by microorganisms are recognized by different receptors, and they activate different pathways (Hossain et al. 2007).

In conclusion, the present observations highlight the use of volatile organic components emitted from beneficial fungi as a new strategy for biocontrol. Although a volatile compound is difficult to apply in the field due to its evaporative nature and its efficacy is low compared with other chemical pesticides, some volatile compounds have been used successfully in the field to control plant disease (Song and Ryu, 2013). On the other hand, chemical inducers of resistance are hampered by their own hazards including negative effects on plant growth (Heil et al. 2001). MeBA and *m*-cresol have been used as antimicrobial compounds (Morris et al. 1979), and according to the material safety data sheet of Science lab.com, both (especially *m*-cresol

according to Roberts et al. (1977) is corrosive to human skin and eyes at high concentrations. But in our observation, *m*-cresol was nontoxic to Arabidopsis plants even at high concentration (100 mM). Considering that point, both of these volatiles were able to prime systemic resistance even at very low concentrations, and perhaps only very low concentrations (1 μM) need to be applied (Fig.3.4). However, further experiments in the greenhouse or open field using different crop plants are needed before these compounds can be recommended for commercial use.

CHAPTER 4

Analysis of microarray data and prediction of transcriptional regulatory elements related with Disease resistance

Analysis of microarray data and prediction of transcriptional regulatory elements related with Disease resistance

4.1 INTRODUCTION

Plants utilize diverse and sophisticated signaling cascades for recognizing and responding to a wide range of biotic and abiotic stresses. Stress recognition and signaling is translated into biochemical reactions, metabolic adjustments and an altered physiological state. Thus, plants have evolved defense mechanisms by which they can increase their tolerance against such stresses. Consequently, a complex signaling network underlies plant adaptation to these adverse environmental conditions (Zhu 2001). There has been rapid progress in our understanding of these signaling pathways over recent years. From these studies, it has become apparent that these pathways rely on endogenous regulators, such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA), to induce defense reactions (Glazebrook 2001). In the past few decades, an increasing amount of research was devoted to the study of Induced Systemic Resistance (ISR) mechanisms. Salicylic acid (SA) is a key regulator of plant defenses, both in the enhancement of local defense responses and the establishment of the broad-based systemic acquired resistance (Mauch-Mani and Métraux, 1998). Its production at the site of infection has been linked with the induction of defense-related gene expression, the enhanced generation of reactive oxygen species (ROS) and programmed cell death (Mur et al., 1996; Shirasu et al., 1997). The role of reactive oxygen species, especially H_2O_2, in plant response to stresses has been the focus of much attention. Hydrogen peroxide has been postulated to play multiple functions in plant defense against pathogens. H_2O_2 may possess direct microbicidal activity at the sites of pathogen invasion. It is used for cell-wall reinforcing processes: lignification and oxidative cross-linking of hydroxyproline-rich proteins and other cell-wall polymers. It was found to be necessary for

phytoalexin synthesis. H_2O_2 may trigger programmed plant cell death during the hypersensitive response that restricts the spread of infection. H_2O_2 has been suggested to act as a signal in the induction of systemic acquired resistance by inducing defense genes. Recently H_2O_2 has been proposed to be involved in the signal transduction pathways leading to acclimation and protection from abiotic stresses (Elżbieta and Henryk, 2000). The connection between H_2O_2 and SA in the signaling networks has been extensively documented for a number of stress responses, including to pathogen elicitors, insect feeding, wounding, high temperature and ABA associated stomatal closure (Larkindale and Knight 2002; Apel and Hirt 2004; Peng et al. 2004; Mateo et al.).

Some non-pathogenic soil inhabiting saprophytes that significantly promote plant growth are called plant growth promoting fungi (PGPF). Colonization of root with PGPF can also lead to systemic resistance in distal parts of the plant (Meera et al. 1994, Meera et al. 1995). An example of a PGPF is *Penicillium simplicissimum* GP17-2, which was found to control soil-borne diseases effectively (Hyakumachi, 1994). Examination of local and systemic gene expression revealed that culture filtrate of GP17-2 modulate the expression of genes involved in both the SA and JA/ET signaling pathways. Phytohormones are acting on this signal transduction alone or interact each other in a cooperative, competitive or interdependent way. This relationship between phytohormones is a part of the transcriptional network for complex phytohormones responses. These transcriptional networks are biologically important for plants to respond against any kind of environmental stress. Promoter regions of stress-inducible genes contain *cis*-acting elements involved in stress-responsive gene expression. Precise analysis of *cis*-acting elements and their transcription factors can give us an accurate understanding of regulatory systems in stress-responsive gene expression. The DNA microarray has recently emerged as a powerful tool in molecular biology research, offering high throughput analysis of gene expression on a

genomic scale. Microarrays have already been used to characterize genes involved in the regulation of circadian rhythms, plant defense mechanisms, oxidative stress responses, and phytohormone signaling (Aharoni and Vorst, 2002). Microarray data can serve a long list of up-regulated as well as genes with no response to stresses, and thus has a potential to identify corresponding *cis*-regulatory elements. In *Arabidopsis* plant, thousands of genes have been found as up-regulated and down-regulated from microarray analysis of the stress-inducible genes (Kubota et al. unpublished). In order to identify *cis*-regulatory elements without using microarray there are some other methods have also been established. A large number of *Arabidopsis cis*-regulatory elements have been identified by a recently developed bioinformatics methodology named LDSS (Local Distribution of Short Sequences) (Yamamoto et al., 2007). There are 308 octamers have successfully been detected that belong to a group of putative *cis*-regulatory elements, Regulatory Element Group (REG), in addition to novel core promoter elements (Yamamoto et al. 2009) by applying LDSS method in *Arabidopsis* genome. Biological role of most of the REG is still not very clear. In order to give biological annotation to *cis*-regulatory elements, one of the best methods is to analyze the microarray data and to predict cis-elements from the genes response to environmental stress.

In my laboratory, microarray analysis to see transcriptional response of Arabidopsis treated with GP17-2 in roots has been performed. Taking advantage of the in house data, I analyzed the microarray data in detail, by comparing selected public microarray data of pathogen, phytohormones, hydrogen peroxide (H_2O_2), and wound responses. Utilizing the microarray data, I achieved *in silico* promoter analysis in order to reveal participating *cis*-regulatory elements involved in the GP17-2-mediated ISR. An octamer-based frequency comparison method that has

been developed in our laboratory was used for the prediction. Special care was taken for cross-detection by prediction of the SA/H_2O_2 response.

4.2 MATERIALS AND METHODS

4.2.1 Promoter analysis of genes related with Induced Systemic Resistance (ISR)

Microarray data of the PGPF *Penicillium simplicissimum* GP17-2 treatment (Kubota et al. unpublished) were subjected to comparative analysis with microarray public data of different phytohormones treatments. Raw data of 6 and 24 hrs post GP17-2 treatment and SA, JA, ET, ABA and hydrogen peroxide treatment (obtained from the database TAIR,6,) were analyzed to get fold change data of gene expression compared to control treatment using the software Excel (Microsoft Japan, Tokyo).

4.2.2 Analysis of microarray data and Prediction of *cis*-regulatory elements:

4.2.2.1 Promoter sequence:

Promoter sequence from -1,000 to -1 relative to the major TSS (Transcription start site) was prepared for 14,960 *Arabidopsis* genes. Major TSS was determined with large scale TSS tag sequencing (Yamamoto et al., 2009) or 5' end information of RAFL cDNA clones (Seki et al., 2002; Yamamoto and Obokata, 2008). Identification of core promoter elements in a position-sensitive manner was achieved as described previously (Yamamoto et al., 2009). Arabidopsis genome and its gene models were obtained from TAIR (TAIR 6).

4.2.2.2 Preparation of RAR tables and promoter scanning:

Microarray data (Table 4.1) was used to prepare gene lists that show expression with more than 3 fold over the control. RAR (Relative Appearance Rate) for each octamer was calculated as the following formula.

RAR= (count in an activated promoter set/number of promoters in the set)/ (count in total promoters/ number of total promoters)

For each octamer-RAR combination, *P* value was calculated by Fisher's Exact Test. *P* values were once transformed into LOD scored, and RAR values with the LOD score less than 1.3 (*P*= 0.05) were filtered out to set as 0. The masked RAR are referred to as RARf in this report. RAR and RARf values for REG annotation (Table 4.2) were calculated in a direction-insensitive manner, where information of the complementary octamers was merged. Promoter scanning with RAR, RARf and LOD tables were achieved using a home made Perl script and Excel (Microsoft Japan, Tokyo). Promoters used for scanning showed over 5 fold-activation by hormones and culture filtrate of PGPF treatment.

4.2.2.3 Scanning of promoter to select *cis*-regulatory elements:

To predict elements, all the promoters were scanned for the octamers of high RAR or RARf value. For scanning of the promoters, a newly developed method by Yamamoto et al. (unpublished) was followed. Phytohormone responsive promoters were scanned by analyzing the RAR and RARf (Fig. 4.2). In some cases, multiple octamers with high RAR/RARf make a cluster by overlap (Fig. 4.1). In that case, *cis*- elements were extracted as the overlapped region.

Example of promoter: **ACGTCCCTTCAAACTAGCT**

Octamer	RAR value
ACGTCCCT	3.8
CGTCCCTT	5.3
GTCCCTTC	4.2
TCCCTTCA	1.2

Predicted sequence = ACGTCCCTTC

Fig. 4.1. RAR value of the octamers in a promoter. In this case, the selection was ACGTCCCTTC

4.3 RESULTS

4.3.1 Comparative analysis of transcriptome related with Induced Systemic Resistance (ISR)

Microarray data of GP17-2 and phytohormone treatment were subjected to comparative analysis. Results showed that there is a peak of SA/H2O2 response at 6 hours post GP17-2 treatment, and another peak of abscisic acid (ABA) response at 24 hours post GP17-2 treatment (Fig. 4.2). These results indicate that the GP17-2 treatment causes a sequence of responses from SA/H_2O_2 to ABA. Therefore, GP17-2 response is partially overlapped salicylic acid, hydrogen peroxide and abscisic acid signaling during ISR caused by it.

4.3.2 *Cis* –element prediction for Phytohormones and CF of PGPF responses:

Prediction of *cis*-regulatory elements for PGPF and plant hormone responses were achieved based on the microarray data shown in Table 4.1. Because ISR of *Arabidopsis thaliana* by a PGPF, *Penicillium simplicissimum* GP17-2, is known to be activated by the treatment of roots by culture filtrate (CF) of culture medium of PGPF, this experimental scheme was selected as a model of ISR by PGPF. CF response was monitored at aerial parts excluding roots with application of CF in the presence or absence of pathogen (*Pseudomonas syringae* pv *tomato* DC3000) infection (Table 4.1). Microarray data was used to identify up-regulated genes by the stimuli, and the corresponding promoter sequences were subjected to statistical analysis to calculate.

4.3.2 Selection of Phytohormone Salicylic acid (SA) responsive *cis*-element from over-represented genes:

Salicylic acid responsive *cis*-elements were selected from the microarray data of Goda et al (2008).Total 197 up-regulated genes were analyzed for SA responsive *cis*-element selection.

Total six octamers have found with high RAR value against SA response. Among them, SA-1, SA-2 and SA-3 have been selected depending on the level of expression (RAR value >3.0) against Salicylic acid treatment. On the other hand, SA+CF-1, SA+CF-2 and SA+CF-3 were selected upon the expression level of the octamer to SA and culture filtrate of the PGPF (GP17-2) treatment. SA-1, SA-2 and SA+CF-2 were selected from the same promoter that has been reported as P-loop containing nucleoside triphosphate hydrolases superfamily protein; functions in nucleoside-triphosphatase activity, ATPase activity, nucleotide binding and ATP binding. In the same way, SA+CF-1 and SA+CF-3 were selected from same the promoter, that is a member of WRKY Transcription Factor; Group III, having sequence-specific DNA binding transcription factor activity and biologically involved in defense response to bacterium, regulation of transcription, DNA-dependent, salicylic acid mediated signaling pathway. SA-3 was selected from the promoter encodes a UDP-glucosyltransferase, UGT74E2, which acts on IBA (indole-3-butyric acid) and affects auxin homeostasis. The transcript and protein levels of this enzyme are strongly induced by H_2O_2 and may allow integration of ROS (reactive oxygen species) and auxin signaling (http://arabidopsis.org/servlets/TairObject?id=39846&type=locus). No REG was found among the selections. But, the octamer of SA-1 has 5 bases overlapping with one of the REG elements. Octamer of SA+CF-3 was also found having 7 bases overlapped with another REG element. RAR (RARf) value of the octamer of SA-1, SA-2, SA-3 was 4.36, 4.23 and 5.12 respectively against SA treatment where as octamers of SA+CF-1, SA+CF-2 and SA+CF-3 having RAR value of 3.5, 4.4 and 6.1 respectively for SA response and 3.3, 3.2 and 3.5 respectively for CF response (Table 4.2, Fig. 4.3).

4.3.3 Selection of CF responsive *cis*-element from over-represented genes:

Following the same scanning procedure, *cis*-regulatory elements were extracted for CF (CF-P) responsive and CF with Pathogen (CF+P) responsive elements from over expressed genes. Total four *cis*-elements were selected. Among them, CF+P1, A CGCG box containing sequence was found in the CF followed by Pathogen infection treatment responsive promoter known as biologically involved in carbohydrate biosynthetic process, response to water deprivation and having transferase activity, transferring glycosyl and hexosyl groups. CGCG box is well known as a member of the Calmodulin-binding protein family which is involved in multiple signaling pathways in plants (Yang and Poovaiah, 2002). CF+P2 was extracted from the promoter encodes protein phosphatase 2C (PP2C) and also reported as negative regulator of ABA signaling. Abscisic acid interacts antagonistically with salicylic acid signaling pathway (Chang-Jie Jiang et. al. 2010). This gene has also been reported responsive to abscisic acid stimulus, cold and water deprivation. The mRNA up-regulated by drought and ABA. The octamer of CF+P2 have 5 bases overlapped with a REG element. In case of CF (no pathogen) responsive element CF-P1, a REG element containing T/G box- AACGTG was extracted which is reported for MYC protein binding TF. Boter at el. (2004) revealed that JAMYC/AtMYC2 transcription factors recognizing a T/G-box AACGTG motif in this promoter fragment play key role in JA-induced defense gene activation. This REG element is positioned at -183 bp from TSS in the promoter that encodes the large subunit of ADP-glucose pyrophosphorylase, the enzyme which catalyzes the first and limiting step in starch biosynthesis. CF-P2 was extracted from the promoter which Encodes a chloroplast/cytosol localized serine O-acetyltransferase involved in sulfur assimilation and cysteine biosynthesis having cellular response to sulfate starvation and cold. RAR (or RARf) value of the octamers of CF+P1, CF+P2, CF-P1 and CF-P2 was 7.5, 10.33, 3.05 and 18.11 respectively against CF (+/-) pathogen treatment (Table 4.2).

4.3.4 Selection of H_2O_2 responsive *cis*-element from over-represented genes:

For H_2O_2 responsive genes, total 6 *cis*-regulatory elements were selected. H_2O_2-1 was extracted from the promoter which encodes a UDP-glucosyltransferase, UGT74E2, which acts on IBA (indole-3-butyric acid) and affects auxin homeostasis. The transcript and protein levels of this enzyme are strongly induced by H2O2 and may allow integration of ROS (reactive oxygen species) and auxin signaling. H_2O_2-2 was selected from the gene involved in carbohydrate metabolic process having endo-1, 4-beta-xylanase activity, hydrolase activity and hydrolyzing O-glycosyl compounds. H_2O_2-3 is extracted from the gene is noticed for cell differentiation, cell proliferation and organ morphogenesis. That octamer has 7 bases overlapped with one REG element. H_2O_2+ CF-1 was extracted from the promoter involved in response to Heat, high light intensity and hydrogen peroxide. H_2O_2+CF-2 was selected form the gene which has alcohol dehydrogenase (NAD) activity and catalyzes the reduction of acetaldehyde using NADH as reductant, biologically involved in cellular respiration, and response to cadmium ion, hypoxia, osmotic stress and salt stress. H_2O_2+CF-3 was extracted from the gene involved in toxin catabolic process and has glutathione transferase activity at molecular level. No REG element was matched with the H_2O_2 responsive *cis*-regulatory element selections. RAR (RARf) value of the octamer of H_2O_2-1, H_2O_2-2, H_2O_2-3 was 9.3, 8.6 and 10.46 respectively against H_2O_2 treatment where as octamers of H_2O_2+CF-1, H_2O_2+CF-2 and H_2O_2+CF-3 having RAR value of 6.9, 5.7 and 8.2 respectively for H_2O_2 response and 3.3, 5.1 and 4.4 respectively for CF response (Table. 4.2).

Cross-detection of the same elements by SA, H_2O_2, or CF suggests their possible crosstalk (Table 4.2) in the transcriptional network of ISR (Fig: 4.5). For instance, the octamer of H2O2-1, H_2O_2-2 and H_2O_2+CF-1 showed over-expression (high RAR value) against SA treatment. Likely,

CF-P2 also showed high expression value in case of H_2O_2 treatment (Table 4.2). This result indicates multiple cross-talking of the *cis*-regulatory elements in the transcriptional network of ISR by PGPF (Fig. 4.5).

4.3.5 Construction of synthetic promoter by the predicted *cis*-elements:

Synthetic promoters contain three copies of each element with spacer sequence (AAAA) (Fig. 4.4). Restriction enzyme sites were included at right and left end respectively.

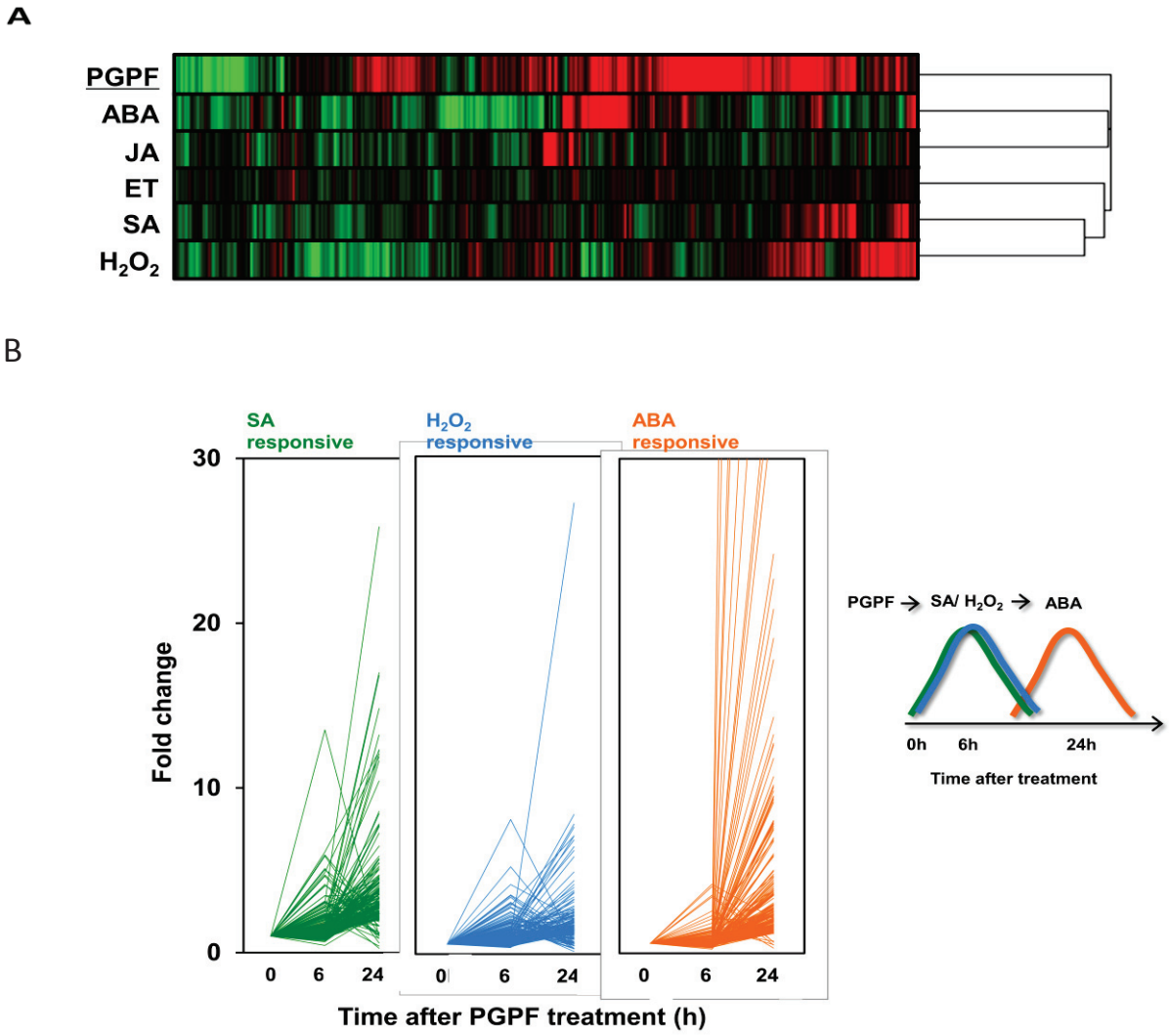


Fig. 4.2 Comparative analysis of microarray data of PGPF and phytohormone treatment.

A. Hierarchical luster comparing gene expression profiles of Arabidopsis plants treated by cell-free culture filtrate of *P. Simplicissimum* GP-17-2 (PGPF) with various phytohormone treatments.

B. Time-course expression pattern of GP17-2 responsive genes with more than 3 fold induction by SA, H_2O_2 and ABA

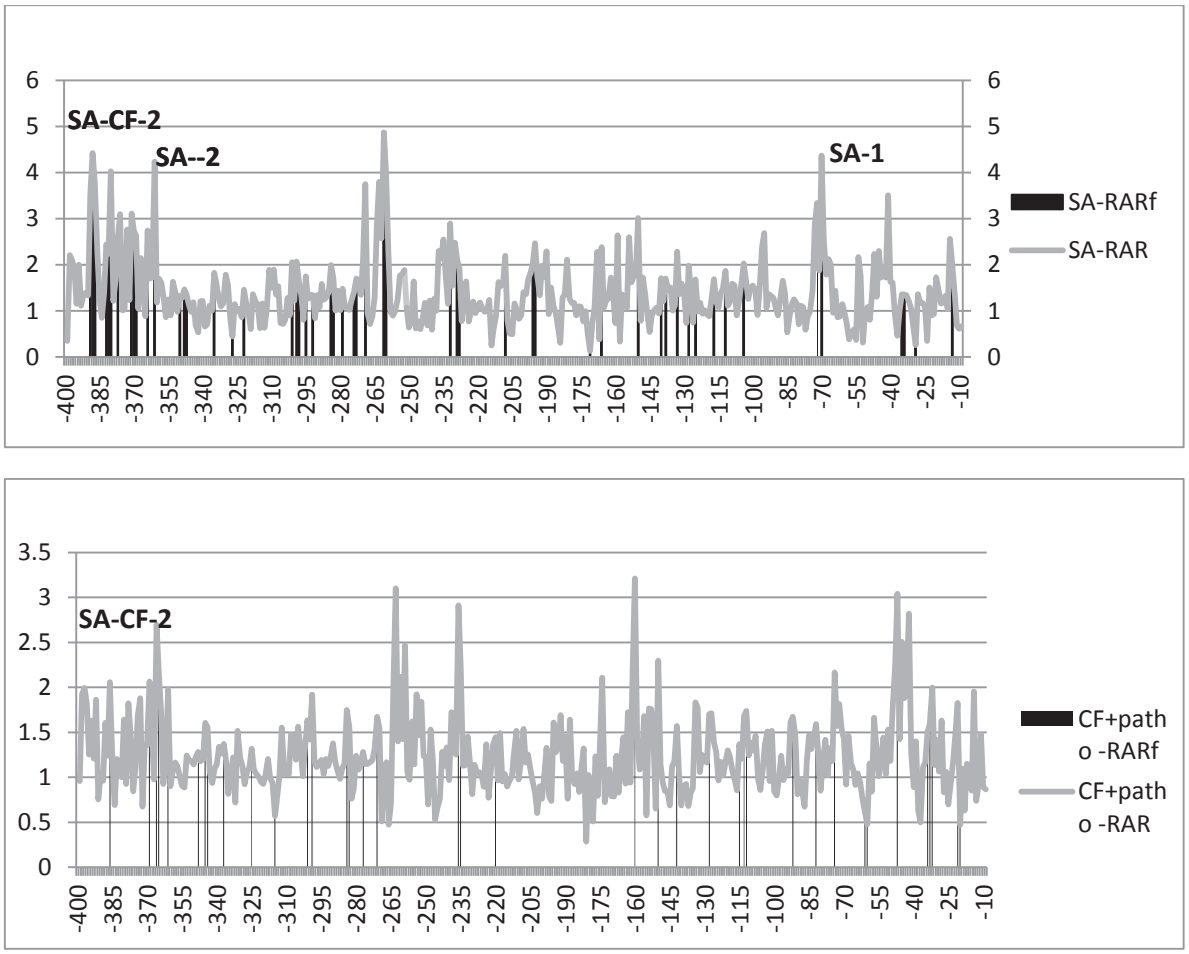


Fig. 4.3. Showing the fold of expression level of the octamers of SA and CF + Pathogen responsive promoter (-400--10). Line graph showing RAR and bar graph showing RARf value of the octamers**.**

SA-1: CAAAA **SA-1** AAAA **SA-1** AAAA **SA-1** AAAAG

SA-2: CAAAA **SA-2** AAAA **SA-2** AAAA **SA-2** AAAAG

SA-3: CAAAA **SA-3** AAAA **SA-3** AAAA **SA-3** AAAAG

SA+CF-1: CAAAA **SA+CF-1** AAAA **SA+CF-1** AAAA **SA+CF-1** AAAAG

SA+CF-2: CAAAA **SA+CF-2** AAAA **SA+CF-2** AAAA **SA+CF-2** AAAAG

SA+CF-3: CAAAA **SA+CF-3** AAAA **SA+CF-3** AAAA **SA+CF-3** AAAAG

CF+P-1: CAAAA **CF+P-1** AAAA **CF+P-1** AAAA **CF+P-1** AAAAG

CF+P2 : AGCTTAAAA **CF+P2** AAAA **CF+P2**AAAA **CF+P2** AAAAG

CF-P1: AGCTTAAAA **CF-P1** AAAA **CF-P1** AAAA **CF-P1** AAAAG

CF-P-2: AGCTTAAAA **CF-P-2** AAAA **CF-P-2** AAAA **CF-P-2** AAAAG

H_2O_2-1: CAAAA **H_2O_2-1** AAAA **H_2O_2-1** AAAA **H_2O_2-1** AAAAG

H_2O_2-2: CAAAA **H_2O_2-2** AAAA **H_2O_2-2** AAAA **H_2O_2-2** AAAAG

H_2O_2-3: CAAAA **H_2O_2-3** AAAA **H_2O_2-3** AAAA **H_2O_2-3** AAAAG

H_2O_2+CF-1: CAAAA **H_2O_2+CF-1** AAAA **H_2O_2+CF-1** AAAA **H_2O_2+CF-1** AAAAG

H_2O_2+CF-2: CAAAA **H_2O_2+CF-2** AAAA **H_2O_2+CF-2** AAAA **H_2O_2+CF-2** AAAAG

H2O2+CF-3: AGCTTAAAA **H2O2+CF-3** AAAA **H2O2+CF-3** AAAA **H2O2+CF-3** AAAAG

Fig. 4.4: Structures of synthetic promoter. Each of the elements was inserted with three times replication along with four spacer AAAA.

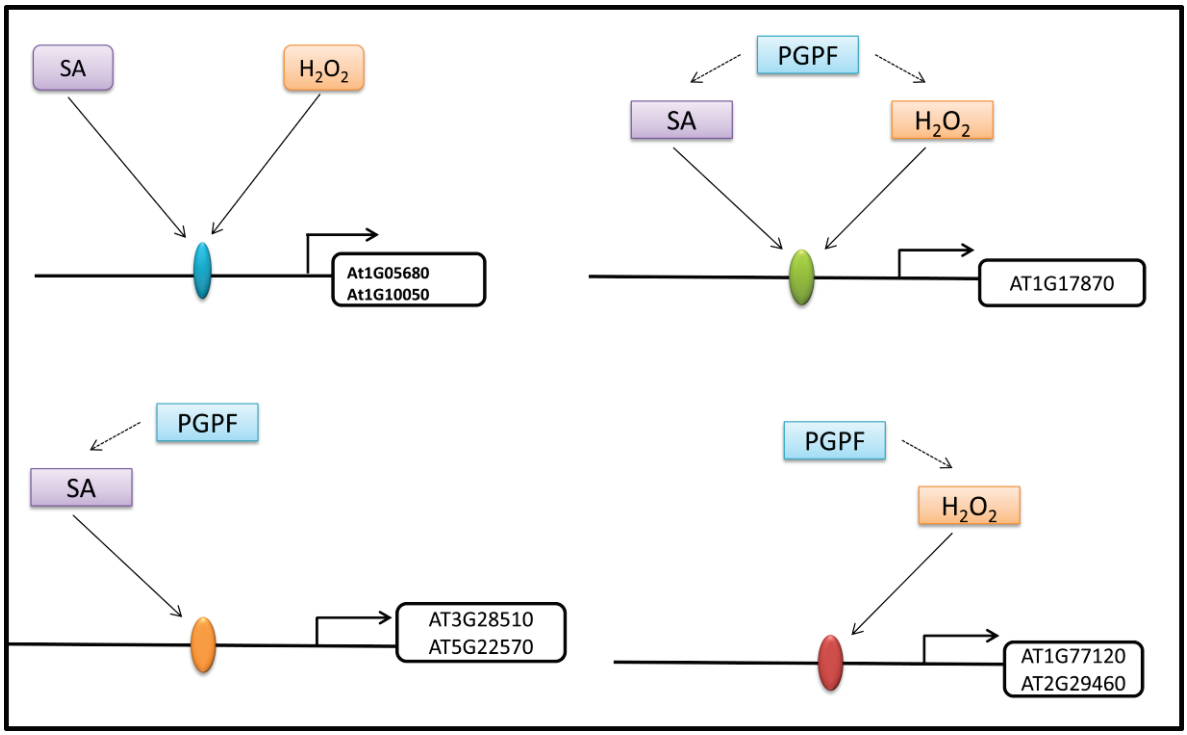


Fig. 4.5 Possible cross- talk between PGPF and phytohormone signaling pathways

Table 1. Extraction of overrepresented octamers in promoters with hormone and CF response.

Microarray	Ref	Number of up-regulated genes
CF response (6 h)	Kubota et al. unpublished	127
CF response (24 h)	Kubota et al. unpublished	361
CF response during pathogen infection (*Pseudomonas syringae* pv *tomato* DC3000, 24 h)	Kubota et al. unpublished	362
Pathogen infection (*Pseudomonas syringae* pv *tomato* DC3000, 24 h)	Kubota et al. unpublished	965
SA	Goda et al, 2008 (TAIR_ME00364)	197
H2O2	Yamamoto et al, 2004	260

Table 2: RAR (RARf) value of the selected octamers in response to different treatments

Element	Length of element (bp)	CF 6h	CF 24h	CF+P 24h	Path 24h	H2O2	SA
SA-1	12	0	0	0	0	0	4.36
SA-2	12	0	0	1.97	0	0	4.23
SA-3	12	0	0	0	0	0	5.12
SA+CF-1	14	0	3.34	0	1.63	0	3.52
SA+CF-2	12	0	3.2	0	0	0	4.4
SA+CF-3	14	0	3.5	0	0	0	6.15
H2O2-1	8	0	0	0	0	9.329	6.16
H2O2-2	11	0	0	0	0	8.629	3.8
H2O2-3	9	0	0	0	0	10.46	0
H2O2+CF-1	8	0	3.31	0	0	6.9	3.03
H2O2+CF-2	8	0	5.17	0	0	5.75	1.89
H2O2+CF-3	12	0	4.43	0	0	8.218	2.71
CF+P-1	15	0	0	7.5	0	0	0
CF+P-2	12	0	0	10.33	0	0	0
CF-P-1	14	0	3.05	2.28	2.47	0	0
CF-P-2	12	18.119	0	0	0	8.85	0

4.4 DISCUSSION

Plant development and environmental adaptation is organized by phytohormones through cell-to cell signal transduction. Multiple cross talking of different phytohormone activities are involved in the transcriptional regulation of this signal transduction. Phytohormones are acting on this signal transduction alone or interact each other in a cooperative, competitive or interdependent way. This relationship between phytohormones is a part of the transcriptional network for complex phytohormones responses. These transcriptional networks are biologically important for plants to respond against any kind of environmental stress.

Previous researches showed that some phytohormones are involved in activation of systemic immunity by pathogen infection. Specially, the salicylic acid content and expression of salicylic acid, jasmonic acid and ethylene inducible genes has increased significantly in the plants inoculated with PGPF and become resistant to pathogen infection. This suggests the involvement of several phytohormones in systemic resistance induced by PGPF.

There has been rapid progress in our understanding of these signaling pathways over recent years. In the past few decades, an increasing amount of research was devoted to the study of ISR mechanisms. Salicylic acid (SA) is a key regulator of plant defenses, both in the enhancement of local defense responses and the establishment of the broad-based systemic acquired resistance (Mauch-Mani and Métraux, 1998). Its production at the site of infection has been linked with the induction of defense-related gene expression, the enhanced generation of reactive oxygen species (ROS) and programmed cell death (Mur et al., 1996; Shirasu et al., 1997). The role of reactive oxygen species, especially H_2O_2, in plant response to stresses has been the focus of much attention. Hydrogen peroxide has been postulated to play multiple functions in plant defense against pathogens. H_2O_2 may trigger programmed plant cell death during the hypersensitive

response that restricts the spread of infection. H_2O_2 has been suggested to act as a signal in the induction of systemic acquired resistance by inducing defense genes. The connection between H_2O_2 and SA in the signaling networks has been extensively documented for a number of stress responses, including to pathogen elicitors, insect feeding, wounding, high temperature and ABA associated stomatal closure (Larkindale and Knight 2002; Apel and Hirt 2004; Peng et al. 2004; Mateo et al. 2006). Considering the above mentioned circumstances, my present study was aimed to identify the phytohormones and/or PGPF responsive element in the promoter of stress inducible genes. My long term goal was to understand characteristics of promoter that support the huge transcriptional network where multiple hormones are supposed to be committed to the PGPF induction of disease resistance.

Promoter sites that are over-representing in the microarray-positive promoters over total promoters in the genome were detected as putative *cis*-elements. Further analyses of these candidates revealed that some elements correspond to another type of putative *cis*-regulatory elements, REG, which is suggested to function in a position-sensitive fashion. The other predicted elements are thus suggested to be of a position-insensitive type (s). Total 16 synthetic promoters have been constructed in this experiment. Cross-detection of the same elements by SA, H_2O_2, or CF suggests their possible crosstalk. Elzbieta et al. (2000) reported that, pathogenic infection enhances ROS-dependent signaling system to induce H_2O_2 and SA that activate transcription factors for the expression of defense genes in SAR pathways. On the other hand, PGPF induces ROS-dependent signaling system to activate SA and H_2O_2 that stimulate transcription factors for the defense gene expression in ISR pathway (David et al., 2008). This suggests that PGPF induced systemic resistance (ISR) might share SAR signaling pathway

involving SA and H_2O_2 responsive *cis*-regulatory elements in the transcriptional network of disease resistance.

Transcriptional profiling is particularly informative to understand transcriptional responses. In my laboratory, microarray analysis to see transcriptional response of Arabidopsis treated with GP17-2 in roots has been performed. Taking advantage of the in house data, I analyzed the microarray data in detail, by comparing selected public microarray data of pathogen, phytohormones, hydrogen peroxide (H_2O_2), and wound responses. Results showed that there is a peak of SA/H2O2 response at 6 hours post GP17-2 treatment, and another peak of abscisic acid (ABA) response at 24 hours post GP17-2 treatment. These results indicate that the GP17-2 treatment causes a sequence of responses from SA/H_2O_2 to ABA.

Utilizing the microarray data, I achieved *in silico* promoter analysis in order to reveal participating *cis*-regulatory elements involved in the GP17-2-mediated ISR. An octamer-based frequency comparison method that has been developed in our laboratory was used for the prediction. Special care was taken for cross-detection by prediction of the SA/H_2O_2 response.

This study was composed of two parts: prediction of putative transcriptional regulatory elements by analyzing *Arabidopsis* microarray data with the help of bioinformatics study, and preparation of synthetic plant promoters by using the predicted putative *cis*-regulatory elements to diagnose the regulatory responses of the elements in transcriptional network. Promoters responsive to SA, H_2O_2 and culture filtrate (CF) of PGPF, identified by the corresponding microarray data, were subjected to the prediction.

CHAPTER 5

Construction of luciferase based vectors using synthetic promoters and their functional analysis *in planta*

Construction of luciferase based vectors using synthetic promoters and their functional analysis *in planta*

5.1 INTRODUCTION

Environmental stresses such as water deficit, high salinity, and low temperature adversely affect the productivity and quality of agriculturally important crops (Bartels and Sunkar , 2005). Genetic transformation has been become a powerful tool for the improvement of stress tolerance of plants, and many stress-tolerant plants have been produced (Jaglo-Ottosen et al. 1998; Apse et al. 1999; Yusuke et al. 2000; Hsieh et al. 2002; Kasuga et al. 2004; Zhang et al. 2004; He et al. 2005; Zhang et al. 2011).

Some promoters are known to be activated by osmotic stress, high salt, drought, or ABA treatment (Yamaguchi-Shinozaki and Shinozaki 1994; Wang et al. 1995). Moreover, different cis-acting elements in these promoters are involved in stress-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki 2005). ABRE (ABA-responsive element) and DRE/CRT (dehydration-responsive element/C repeat) are major cis-acting elements in abiotic stress-inducible gene expression. DRE/CRT elements with the core sequence C/DRE (GCCGAC) play an important role in regulating gene expression in ABA-independent regulatory systems and can be found in promoter regions of many dehydration-, high-salt-, and cold-stress inducible genes in Arabidopsis, such as rd29A, kin1, and cor15a (Baker et al. 1994; Wang et al. 1995; Kim et al. 2002). Various types of ABRE-like sequences have been reported, including the G-box sequence (CACGTG), which is present in a large number of environmentally regulated genes (Menkens et al. 1995). Other cis-regulatory elements, such as MYB (C/TAACNA/G), MYC (CANNTG), LTRE (CCGAC) play key roles in activating gene expression in response to osmotic stress

and/or ABA (Baker et al. 1994; de Bruxelles et al. 1996; Abe et al. 2003; Nakashima et al. 1997).

Applications in plant genetic engineering with transcription factors driven by stress-induced promoters provide an opportunity to improve the stress tolerance of crops (Viswanathan and Zhu 2002). However, the activities of native promoters identified so far have certain limitations, such as low expression activity and low specificity. A series of synthetic promoters for higher-level expression of foreign genes has been reported in the literature (Mitsuhara et al. 1996; Rushton et al. 2002; Shin et al. 2003; Kobayashi et al. 2004; Bhullar et al. 2011). With the information currently available on the regulatory mechanisms of abiotic stress tolerance in plants, it is now feasible to construct strong inducible promoters artificially. Thus, in the current study, I have selected *cis*-regualtory elements derived from stress-induced promoters (e.g. PGPF, phytohormone) in Arabidopsis, to construct artificial promoters. The pattern of inducibility driven by these artificial synthetic promoters was characterized in stable transgenic Arabidopsis by monitoring expression of the luciferase (LUC) reporter gene, upon exposure of these plants to various stress conditions. In addition, promoter activity was assessed through luminescence estimation of LUC expression in transgenic plants under various stress conditions (biotic and phytohormone) as compared to the wild type Col-0 and /or vector control.

5.2 MATERIALS AND METHODS

5.2.1 Construction of LUC vector

LUC+/tNOS from yy327(Yamamoto et al. 2003) and pNOS::BAR/tOCS from SL J75515 (http://www.tsl.ac.uk/research/jonathan-jones/plasmids.htm) were inserted into the BamHI/HindIII and EcoRI sites of a binary vector, pPZP200, respectively (Hajdukiewicz et al., 1994) to make yy326. A modified CIP7 intron with a 12-bp deletion was prepared as a cassette for copy number estimation by competitive PCR (Yamamoto et al., 2003), and inserted into the SacI site of yy326. Subsequently, -46 to +8 region of the 35S promoter of Cauliflower Mosaic Virus (CaMV 35S) (Benfey and Chua, 1990), containing a TATA box, was inserted into the BamHI site. Map of the final construct, yy447, is shown in Fig. 5.1.

5.2.2 Construction of synthetic promoters

A synthetic promoter construct (CF+P-1) was produced by annealing sense and antisense oligonucleotides of a circadian controlled regulatory element (data not shown), and inserted into the SacI/BamHI site of yy447 to make yy462. Prepared vectors, including yy462 were used for transformation of Arabidopsis *via Agrobacterium tumefaciens* GV3101 pMP90 (Koncz and Schell, 1986)

5.2.3 Plant transformation

Arabidopsis thaliana Col were transformed by the floral dipping method (Clough and Bent, 1998). Dipped plants were places in a plastic tray and covered with a tall clear-Plastic dome to maintained humidity. Plants were transferred in a low light or dark location overnight and returned to the growth chamber on the next day; care was taken to keep domed plants out of

direct sunlight. Plastic domes were removed approximately 12-24 h after transformation and plants were grown for a further 3-5 weeks until siliques turn to brown color and dry.

5.2.4 T1 selection and copy number estimation of T-DNA

T_1 transgenic seeds were sown on GM –agar plates (Valvekens et al., 1988) supplemented with cefotaxime at 100 mg/L and BASTA (gluphosinate ammonium, Crescent Chemical, Co., Hauppauge, NY) at 10mg/L. BASTA tolerant green plants with a long primary root were transferred to soil after 7-10 days (Fig. 5.2).

BASTA-resistant T1 plants were subjected to estimation of copy number of T-DNA by PCR. Preparation of PCR template from plant tissue was essentially followed by the alkali-boiling method (Klimyuk et al., 1993). A leaf from a T1 plant was put into a well of 96-hole PCR plates. After addition of alkaline solution (40µl of 0.25M NaOH), samples were incubated at 95 ^{0}C for 5 min. Then 40µl of 0.25M of HCl were added to the leaf sample together with 20 µl of 0.5M Tris-HCl (0.25% NP-40) and incubated for more 2 min at 95 ^{0}C. 1 µl of that T-DNA sample was used for PCR to estimate the copy number.

Primers used for the PCR were CIP7SF (5'-TCT GTT CAC TCT CTT AGA TGC CAA A-3') and CIP7SR (5'-CAC AGA GTC CAC AAC AAT TGA AA-3') (Fig. 5.3). Cycle conditions of the competitive PCR was (94 ^{0}C for 1 min, 80 ^{0}C for 4 min) x 1 cycle, (94 ^{0}C for 15 sec, 50 ^{0}C for 15 sec, 72 ^{0}C for 30 sec) x 40 cycles, and (72 ^{0}C for 5 min) x 1 cycle. The PCR products were diluted with equal volume of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), mixed with ¼ volumes of the loading dye solution containing 1 µg /ml VistraGreen (GE Healthcare Japan, Hachioji), and separated by electrophoresis in 15% polyacrylamide gel with the electrophoresis buffer (modified TBE: 50 mM Tris-Cl, 50 mM Borate, 1 mM EDTA). After electrophoresis, gels

were scanned with a confocal laser scanner (Typhoon 9400, GE healthcare Japan, Hachioji). Signal strength of each band was expressed numerically with ImageQuant 5.2 (GE healthcare Japan, Hachioji) and calculated relative intensity of each pair of bands. The *in vitro* mix experiments were performed with a DNA template of total Arabidopsis DNA and Plasmid DNA (yy447) in different proportions, both of which had been digested with EcoRI to equalize their template activity. Arabidopsis genomic DNA was isolated from Col-0 plants following CTAB method (Murray and Thompson, 1980).

5.2.5 Selection of transgenic lines:

T_1 transformants with single copy T-DNA were let to self and the resultant T_2 seeds were subjected to segregation analysis of the herbicide resistance. Single copy insertion at the T1 generation was confirmed by the comparative PCR analysis at the T2 generation.

5.2.5 *in vivo* luciferase assay

Seeds of transgenic lines containing synthetic promoter and vector control were sown on GM plates supplemented with 0.8% Bactoagar (Japan BD, Tokyo) and 1% sucrose. Plates were kept in the dark for 2-4 days at 4 ^{o}C for vernalization and then grown in a growth chamber at 22 ^{o}C under 18L/ 6D cycle at the light intensity of 6-8 W m^{-2} for 14 days. Plants were sprayed with 1 mM luciferin solution 1 day prior to the assay (Kimura et al., 2001).

To analyze the effects of circadian rhythm on the expression of the synthetic promoter, automated monitoring of bioluminescence was performed by using an automated 2-channel photomultiplier system (Ishiura et al., 1998) inside the growth cabinet with the same growing condition. During analysis, photons were counted under 18 h day/ 6 h night cycle for the first 2

days followed by continuous low light for the next 3 days. The data from assay were logged into a text file and subsequently analyzed using Excel software (Microsoft Japan, Tokyo, Japan).

5.2.6 *in vivo* luciferase assay of synthetic promoter in response to pathogen infection

Synthetic promoter (CF+P-1) inserted in to yy447 was brought to *in vivo* analysis to analyze its response against the bacterial leaf speck pathogen *P. syringae* pv. tomato (*Pst*) DC3000 infection. About 60 seeds of 3 transgenic lines (T2) containing synthetic promoter were sown on GM plates supplemented with 0.8% Bactoagar (Japan BD, Tokyo) and 1% sucrose. Plates were kept in the dark for 2-4 days at 4 ^{o}C for vernalization and then grown in a growth chamber at 22 ^{o}C under continuous low light (6-8 W m^{-2}) for 10 days. Plants were sprayed with 1 mM luciferin solution 1 day prior to the assay. *Pst* DC3000 inoculum was prepared following the method as mentioned on chapter 2. About 1.5 ml of inoculum was sprayed to each plate. Control was maintained by spraying with same amount of $MgSO_4$ (10mM) solution. Then the plants were placed in the photomultiplier to measure luciferase luminescence. Col-0 and yy447 plants were used as control line.

5.2.7 *in vivo* luciferase assay of synthetic promoter in response to Abscisic acid

Synthetic promoter (CF+P-1) inserted in to yy447 was brought to *in vivo* analysis to analyze its response to Abscisic acid. Seeds of T2 lines containing synthetic promoter were sown on GM plates supplemented with 0.8% Bactoagar (Japan BD, Tokyo) and 1% sucrose. Plates were kept in the dark for 2-4 days at 4 ^{o}C for vernalization and then grown in a growth chamber at 22 ^{o}C under continuous low light (6-8 W m^{-2}) for 2 weeks. Plants were then transferred to a 24 wel plate (1 seedling/wel) filled with 500 µl of GM broth in each and grown for more 1 week. 1 mM of luciferin solution was added to each wel 1 day prior to the assay. 100 µM ABA was added in

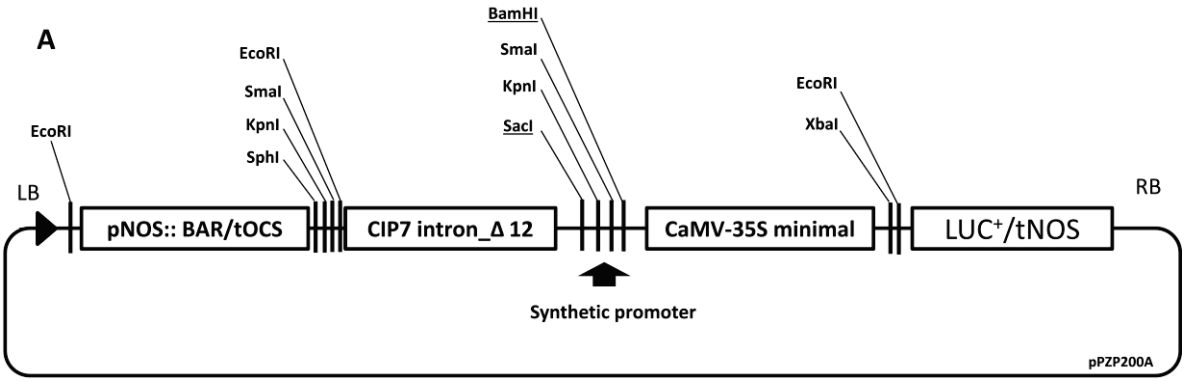
each wel. Control was maintained by adding with 0.1% methanol (Me-OH) solution. Then the plants were placed in the photomultiplier to measure luciferase luminescence.

5.2.8 *in vivo* luciferase assay of synthetic promoter in response to SA acid

Followed by the ABA treatment analysis, synthetic promoter (CF+P-1) was then brought to analyze its response to SA acid treatment. The similar method was followed to grow plants as in case of ABA treatment. Then 500 μM SA was added in each wel. Control was maintained by adding with 0.1% Et-OH solution. Then the plants were placed in the photomultiplier to measure luciferase luminescence.

5.2.9 *in vivo* luciferase assay of synthetic promoter in response to H_2O_2

Followed by the SA and ABA treatment analysis, synthetic promoter (CF+P-1) was then brought to analyze its response to H_2O_2 treatment. The similar method was followed to grow plants as in case of other hormone treatment. Then 3 % $H_2O_2$was added in each wel. Control was maintained by adding with same volume of SDW. Then the plants were placed in the photomultiplier to measure luciferase luminescence.



B

CCCCAAATGAAGTGCAGGTCAAACCTTGACAGTGACGACAAATCGTTGGGCGGGTCCAG

GGCGAATTTTGCGACAACATGTCGAGGCTCAGCAGGAATTCGAGCTGGAGTTTTTTTCT

CIP7 intron_Δ12

GTTCACTCTCTTAGATGCCAAAACTTAATGTTTCAATTGTTGTGGACTCTGTGATGTGCC

CaMV_35S_minimal

AACTTGGGAGCTCGGTACCCGGGGATCCCAAGACCCTTCCTCTATATAAGGAAGTTCATTTC

LUC+

ATTTGGAGAGGACTAAACCGATCCAAACAATGGCTATGGCTGAAGACGCCAAAAACATAAA

GAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAA
G

Fig 5.1. Construction of synthetic promoters

(A) Illastration of LUC vector yy447. Elements were inserted between the SacI and BamHI sites upstream of the minimal region of 35S promoter of *Cauliflower mosaic virus* (CaMV_35S). LB: Left border, RB: Right border, BAR: BASTA (Phosphinothricin) resistance gene, selection marker for plants, CIP7 intron_ Δ 12: COP1-interacting protien 7(CIP7) intron with a 12-bp deletion, CaMV-35S minimal: -46 to +8 region of Cauliflower mosaic virus (CaMV_35S), LUC^+ : firefly luminescent reporter, pNOS: nopaline synthase gene promoter, tOCS: octopine synthase gene terminator, tNOS: nopaline synthase terminator.

(B) Nucleotide sequence of the vector (yy447)

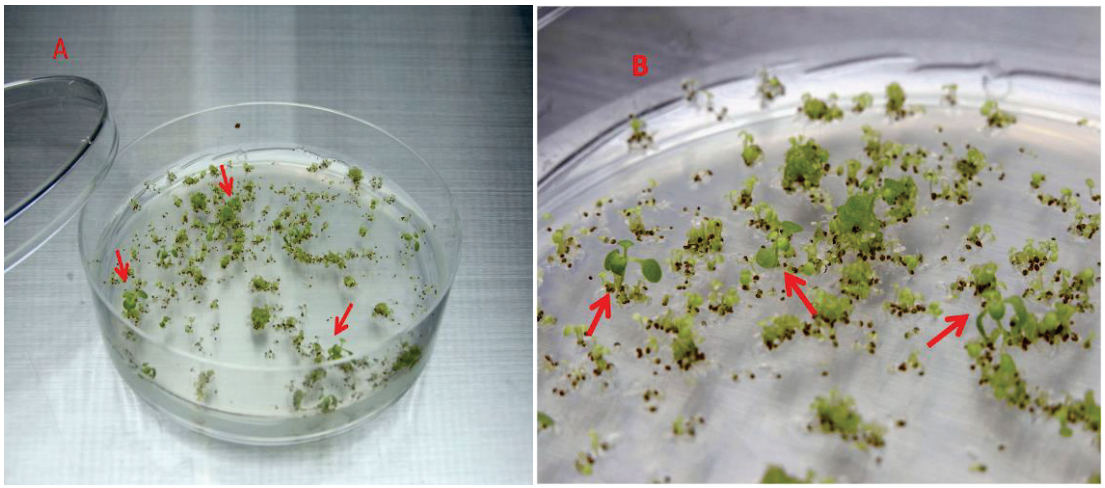


Fig. 5.2. Selection of BASTA resistant plants on selective media. A. The whole plate showing BASTA positive and negative plants. B. Part of plate with clear view of the BASTA resistant *Arabidopsis* plants. White arrows indicate BASTA positive more healthy and green plants.

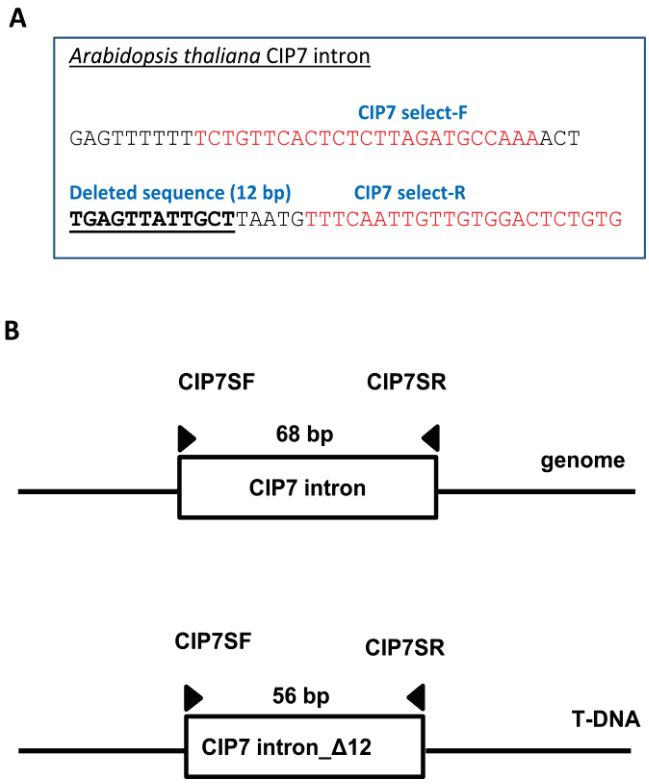


Fig. 5. 3 : Estimation of T-DNA copy number by competitive PCR

A. *Arabidopsis thaliana* CIP7 intron

B. CIP7 Select-F and CIP7 Select-R are PCR primers used for the competitive PCR anneal with CIP7 intron.

5.3 RESULTS

5.3.1 Construction of synthetic promoter containing vector yy447:

Luciferase reporter based vector yy447 was constructed using yy326 as the core vector in pPZP200. Figure 5.1A showing the map of the vector. A minimal promoter (-46 to +8 region of CaMV-35S) containing TATA box was inserted as transcriptional enhancer. For the selection of Arabidopsis transformants, a herbicide BASTA resistance gene *BAR* was introduced also. A cassette for copy number estimation (12 bp deleted CIP7 intron) was inserted to screen the single copy inserted transgenic plant. The firefly luciferase reporter genes *LUC* was introduced to evaluate the gene expression. Finally, a circadian controlled *cis*-regulatory containing synthetic promoter (data not shown) was inserted between BamHI and SacI restriction site. Prepared vector was sequenced before transformation to *Agrobacterium tumefaciens* and nucleotide sequences of all the inserts were carefully checked (Fig. 5.1B).

5.3.2 Estimation of the copy number of T-DNA

To estimate the T-DNA copy number inserted, *in vitro* DNA mix experiments were performed with a DNA template of total Arabidopsis genomic DNA and Plasmid DNA (yy447) mixed in different proportions: 1:0, 0:1, 1:1, and 2:1. For this analysis, competitive PCR was done using a CIP7 intron with 12 base-deletion marker . T-DNA contains CIP7 intron which is 12 bp shorter than *Arabidopsis thaliana* genomic CIP7and thus PCR product for T-DNA is 12-bp shorter than that one for genomic DNA (Fig. 5.3 A&B). Therefore, the lower band was indicating the T-DNA amplification where as the upper band indicating the genomic DNA. Figure 5.3 (C&D) showing the results of *in vitro* DNA mix experiments. When input (T-DNA/genome) was 1/2, that means single copy of T-DNA was mixed with double copy of genomic DNA, the upper band showed more intensity than the lower one (Fig. 5.3 C). Similarly, when input (T-DNA/genome)

was 1/1, both the bands showed similar intensity. On the other hand, if the band intensity of T-DNA is thicker than genomic DNA, it means, the plant inserted with multi copy (2 or 3 copies) of T-DNA. The band intensity was calculated by using Image Quant 5.2 and numerical data of the entire input ratio is shown in Fig. 5.3 D. Results showing that the relative intensity was equally proportionate to the T-DNA/genome mixture ratio.

5.3.3 Selection of transgenic lines

T0 transformants were let to be selfed and T1 transformants were screened with BASTA (herbicide) and greens seedlings with long root systems were separated to soil medium and leaves were sampled for T-DNA preparation. To identify the single copy inserted plants, competitive PCR was done using CIP7 intron with 12 bp deletion markers. Transgenic plants with single copy insertion were identified on the basis of the results of the *in vitro* mix experiments (Fig. 5.3C). Band intensity was compared between T-DNA and genomic DNA and the bands similar to input mix T-DNA/genome: 1/2 were counted as single copy inserted plant (Fig. 5.3). T1 transformants with single copy T-DNA insertion were let to be self-pollinated and T2 seedlings were brought to perform PCR again with same primers of CIP7 intron to get homozygous transgenic lines. Figure 5.4 is showing the gel picture of the segregation of single copy T-DNA in T2 generation giving a 1:2:1 ratio. Positive homozygous lines were identified by observing the bands with similar intensity while negative homozygous (not inserted with T-DNA) was the line showing only one upper band (Fig 5.4). Heterozygous lines were counted as the upper band is thicker than the lower. T3 seeds from the homozygous lines were used for further assay. Total 48 seeds were sown for this screening, where the segregation pattern more or less followed 1:2:1 ratio.

5.3.4 Analysis of the activity of synthetic promoter:

Synthetic promoter inserted in the yy447 was brought to *in vivo* analysis to analyze its activity by biological experiments. Since a circadian regulated synthetic promoter was introduced in to the vector, we conducted a representative experiment controlling the seedling growth under LD 18:6 photoperiods followed by a continuous low light (LL) (6-8 W m^{-2}) to observe the effect of circadian clock on reporter gene expression. Fig. 5.5 showing the *LUC* gene expression controlled by circadian clock comparing with the wild type (Col-0) Arabidopsis plant and the vector control yy447 seedlings. Reporter gene expression was analyzed by observing the luciferase luminescence that was expressed as countable photons after treatment with luciferin. A diurnal rhythm was found in the transgenic plants under LD 18:6 photoperiods, while a circadian oscillation of the reporter gene was observed under continuous low light (Fig. 5.5) with the peak levels occurring around 6-7 hrs after subjective dawn.

5.3.5 *in vivo* analysis of synthetic promoters in response to pathogen infection

Synthetic promoter (CF+P-1) inserted in yy447 was brought to in vivo analysis to analyze its response to pathogen infection. Bacterial leaf speck pathogen *P. syringae* pv. tomato (*Pst*) DC3000 was used in this experiment as pathogen inoculum. Single copy synthetic promoter containing T2 transgenic lines (3 lines) were subjected for this analysis. Data showed that all the 3 lines showed a repression of gene expression after few hours of pathogen treatment (Fig. 5.6). Pathogen treatment was also found to disturb the circadian rhythm that showed by the control plants.

5.3.6 *in vivo* analysis of synthetic promoters in response to phytohormone treatment

Synthetic vector containing the element CF+P-1 was subjected to *in vivo* analysis to observe reporter gene expression in response to phytohormone ABA and SA treatment. Fig. 5.7 and 5.8 showing that reported gene expression was repressed after treating with ABA and SA in CF+P-1 element containing transgenic plants. Circadian rhythm of the transgenic plants was also disturbed after phytohormone treatment. In both cases, a slight up-regulation was found immediately after treatment compared to mock followed by sharp repression. In case of SA treatment, reporter gene expression was slightly recovered after about 30 hrs of treatment. But in case of ABA treatment, no such pattern was found.

5.3.7 *in vivo* analysis of synthetic promoters in response to H_2O_2

Transgenic plants containing synthetic promoter of the element CF+P-1 were treated with H_2O_2 and reporter gene expression was observed. Fig 5.9 shows that H_2O_2 also repressed gene expression like phytohormone and pathogen did in CF+P-1 containing synthetic promoter.

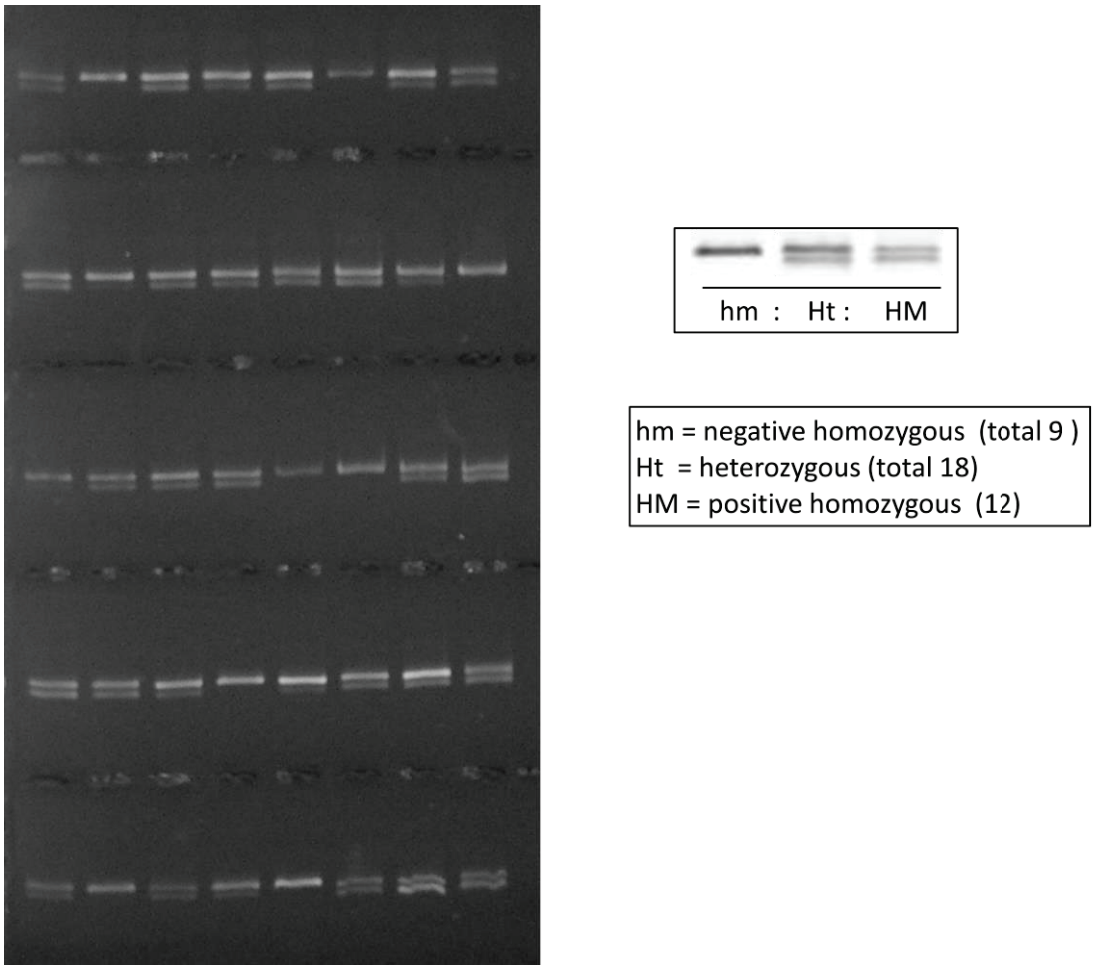


Fig 5.4. Segregation of single copy of synthetic vector in T2 transformants

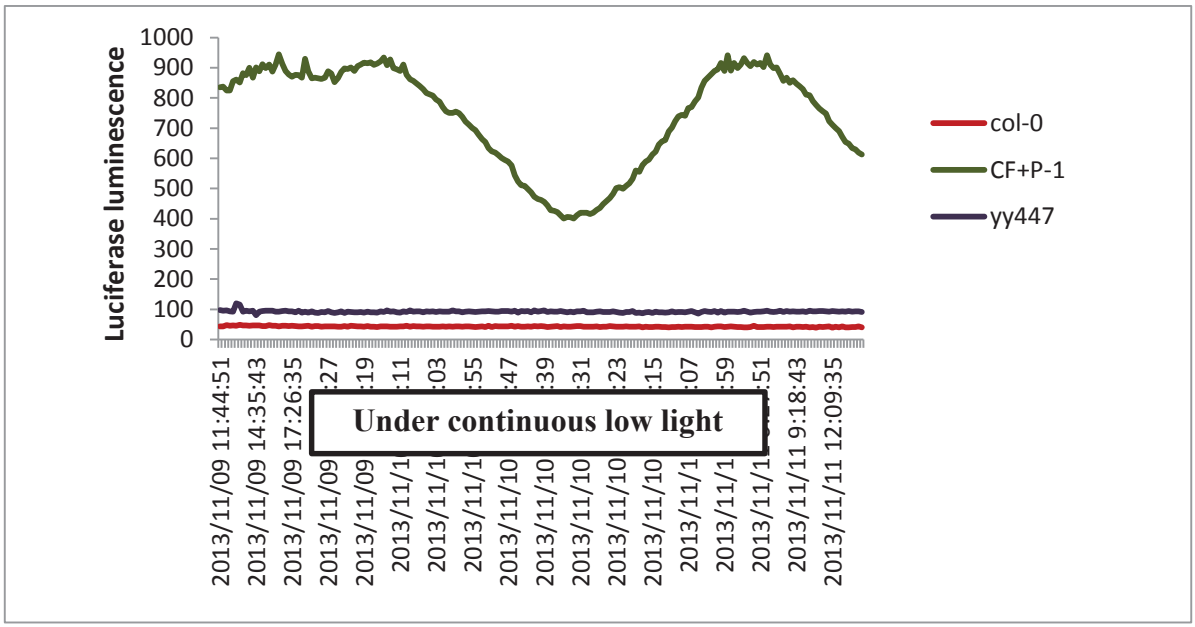


Fig. 5.5 Synthetic promoter showing circadian rhythm of reporter gene expression. Synthetic vector (CF+P-1) was subjected to *in vivo* analysis at 19 days after sowing. Average luciferase luminescence of 9 seedlings was recorded under continuous low light for 2 days. Seedlings of wild type (Col-0) plants were used as negative control where yy447 was used as vector control.

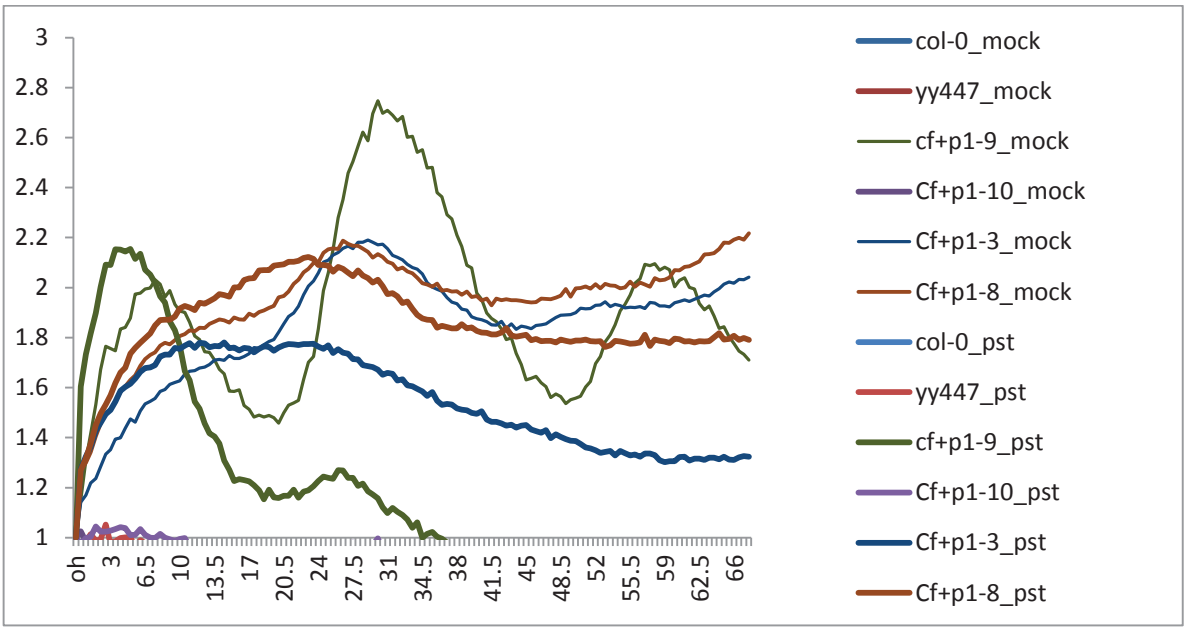


Fig. 5.6 Synthetic promoter showing reporter gene repression in response to the pathogen *Pst* DC3000 infection. Synthetic vector (CF+P-1) was subjected to *in vivo* analysis at 10 days after sowing. Data shows the average luciferase luminescence of 60 seedlings recorded under continuous low light. Seedlings of wild type (Col-0) plants were used as negative control where yy447 was used as vector control.

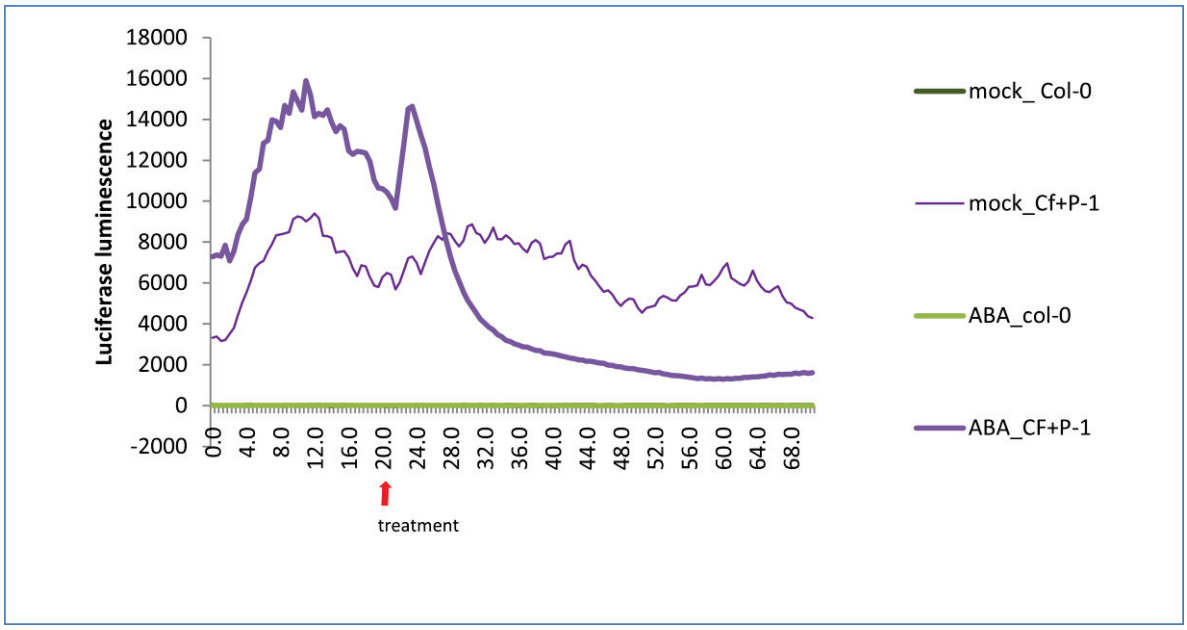


Fig.5.7 Synthetic promoter showing reporter gene repression in response to ABA. Synthetic vector (CF+P-1) was subjected to *in vivo* analysis at 3 weeks after sowing. Plants were treated with 1 00 µM ABA solution, where 0.1% Me-OH was used as mock treatment. Average luciferase luminescence of 20 seedlings was recorded under continuous low light. Seedlings of wild type (Col-0) plants were used as negative control.

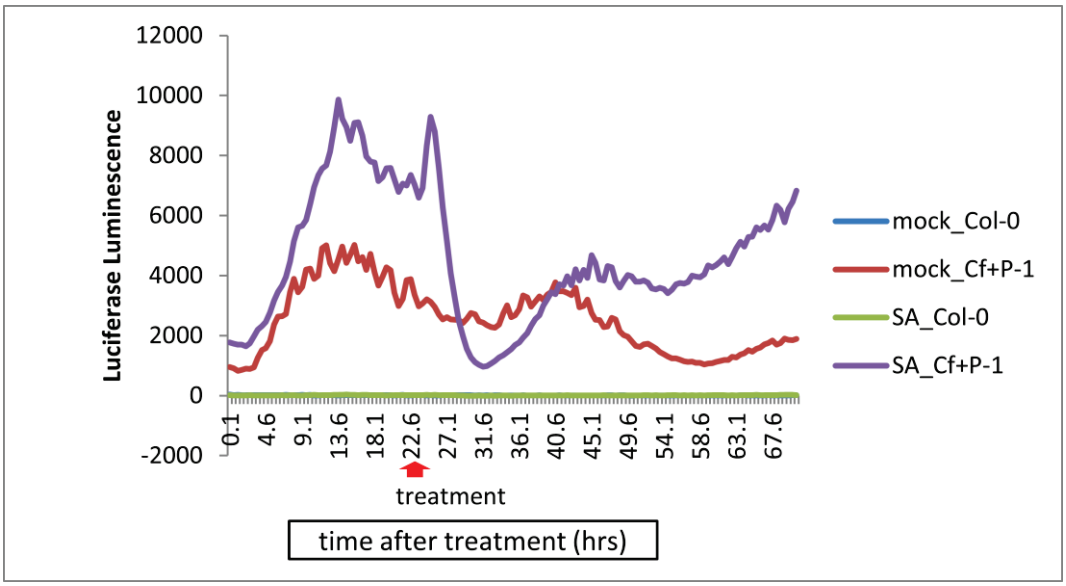


Fig. 5.8 Synthetic promoter showing reporter gene repression in response to SA treatment. Synthetic vector (CF+P-1) was subjected to *in vivo* analysis at 3 weeks after sowing. Plants were treated with 500 µM SA solution, where equal amount of 0.1 % Et-OH was used as mock treatment. Average luciferase luminescence of 20 seedlings was recorded under continuous low light. Seedlings of wild type (Col-0) plants were used as negative control.

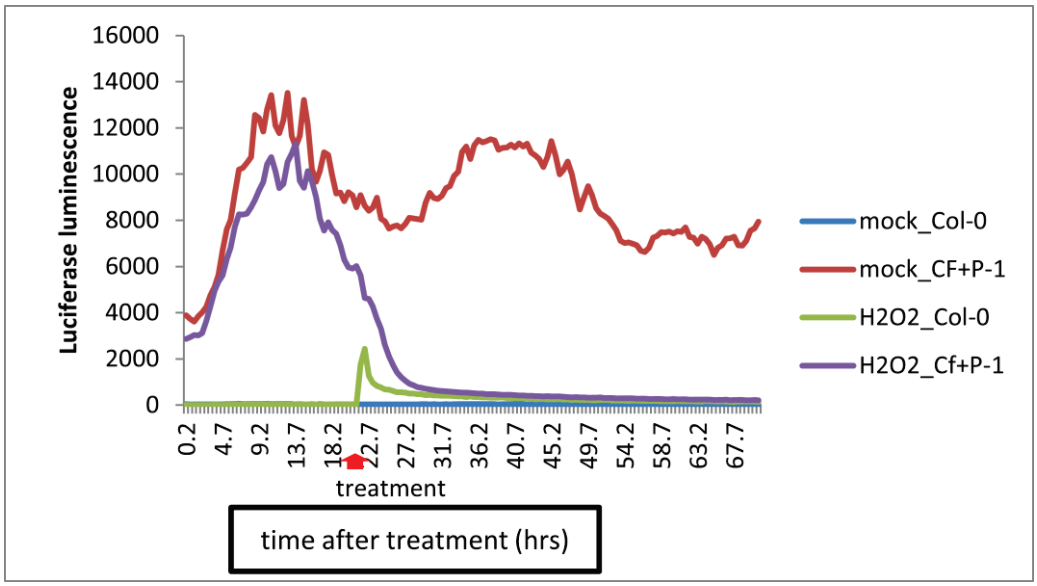


Fig. 5.9 Synthetic promoter showing reporter gene repression in response to H_2O_2 treatment. Synthetic vector (CF+P-1) was subjected to *in vivo* analysis at 3 weeks after sowing. Plants were treated with 3% H_2O_2 solution, where equal amount of SDW was used as mock treatment. Average luciferase luminescence of 20 seedlings was recorded under continuous low light. Seedlings of wild type (Col-0) plants were used as negative control.

5.4 DISCUSSION

From our previous study in chapter 4, examination of local and systemic gene expression revealed that culture filtrate of *Penicillium simplicissimum* GP17-2 modulate the expression of genes involved in both the SA, ABA signaling pathways. Phytohormones are acting on this signal transduction alone or interact each other in a cooperative, competitive, or interdependent way. This relationship between phytohormones is a part of the transcriptional network for complex phytohormones responses. These transcriptional networks are biologically important for plants to respond against any kind of environmental stress. Promoter regions of stress-inducible genes contain *cis*-acting elements involved in stress-responsive gene expression. Precise analysis of *cis*-acting elements and their transcription factors can give us an accurate understanding of regulatory systems in stress-responsive gene expression. Microarrays have already been used to characterize genes involved in the regulation of circadian rhythms, plant defense mechanisms, oxidative stress responses, and phytohormone signaling (Aharoni and Vorst, 2002). Microarray data can serve a long list of up-regulated as well as genes with no response to stresses, and thus has a potential to identify corresponding *cis*-regulatory elements. In *Arabidopsis* plant, thousands of genes have been found as up-regulated and down-regulated from microarray analysis of the stress-inducible genes (Kubota et al. unpublished). In chapter 4, I have analyzed the in house microarray data of GP17-2 treated Arabidopsis and compared with the public microarray data of different phytohormones. It was found that the PGPF involved both SA, ABA and H_2O_2 in the transcriptional regulatory network to induce systemic resistance in plants against pathogen infection.

Utilizing the microarray data, I achieved *in silico* promoter analysis in order to reveal participating *cis*-regulatory elements involved in the GP17-2-mediated ISR. Later, an octamer-

based frequency comparison method was used for the prediction of *cis*-regulatory elements. In this study, the predicted promoter elements were subjected to functional analysis *in planta*, using an approach of preparation and utilization of synthetic promoters. A luciferase-based new vector (yy447) has been developed for the purpose.

Among prepared synthetic promoters, one was found to show physiological responses in assays *in vivo*. This promoter, containing only one kind of *cis*-element, is controlled by circadian rhythm and showed repression by pathogen (*Pst* DC3000), ABA, SA, and H_2O_2 treatments.

Although the underlying molecular mechanisms behind the expression pattern of synthetic promoter are not yet clear, our findings showed that synthetic promoters can be responsive to various stress conditions, and induce/or repress gene expression. Since the roots of transgenic plants were treated by the phytohormone and H_2O_2, the gene was expressed systemically in all organs and tissues of plants,

SUMMERY AND CONCLUSION

Plants respond to adverse environmental conditions and pathogen attack by expressing specific genes and synthesizing a large number of anti-stress proteins that have roles in stress adaptation and plant defense. Biotic stress responses are known to include systemic responses to protect the whole plant body against invasion by pathogens. Systemic responses include cell-to-cell signaling, and at least three molecular species, salicylic acid (SA), H2O2, and jasmonic acid (JA) have been revealed to be involved in the intercellular signaling. Communication between these plant hormones might modulate the expression of biotic and also abiotic stress–responsive genes in plants. While molecular mechanisms for action of each plant hormone have been intensively studied for decades and thus have been gradually understood, interactions between these hormone-mediated signaling pathways and molecular mechanisms governing their cross-regulation generally remain unresolved.

The signal transduction pathway through SA accumulation is found in the systemic acquired resistance (SAR) induced by pathogen attack, while it is thought that JA and ET are the signal-transducing molecules for induced systemic resistance (ISR) by biocontrol agents (BCAs). From previous studies, non-necrotizing rhizosphere microorganisms can effectively trigger induced resistance. Colonization of roots with plant growth-promoting fungi (PGPF) can also lead to systemic resistance. In relation to the fact above discussed, here, I analyzed the effects of PGPF on the growth and disease suppression of plants to disclose the molecular mechanisms of the favorable behavior of PGPF.

In order to analyze growth promotion by PGPF, volatile organic compounds (VOC) were recovered from about 100 fungal strains and their effects on tobacco plant were examined. I

found that VOC preps from *Phoma* sp. GS8-3, significantly enhance the growth. VOC preps that showed growth promotion were then subjected to GC-MS analysis for molecular identification.

In the next step, VOC isolations were examined for ability of stimulation of plant defense responses to protect plants from infection by diseases. In this step, VOC isolations from *Phoma* sp. (GS8-3), *Cladosporium* sp. (D-c-4), *Ampelomyces* sp. (F-a-3) were tested for protection of *Arabidopsis thaliana* from infection of bacterial leaf speak pathogen *Pseudomonas syringae* pv. *tomato* (*pst*) DC3000. Among the three fungal isolates, significant effects of protection have been observed by VOC from D-c-4 and F-a-3. As major active volatile compounds for ISR, two novel volatile compounds; *m*-cresol and methyl benzoate (MeBA) have been identified from *Ampelomyces sp.* and *Cladosporium sp*., respectively.

Subsequently, signaling mechanisms for disease suppression by the VOC were investigated. Arabidopsis plants impaired in SA or JA/ET signaling pathways were treated with the VOC molecules, *m*-cresol and MeBA followed by challenge inoculation with *Pst*. Results showed that activation by MeBA or *m*-cresol treatments was impaired in the JA- or ET-disrupted mutants, indicating involvement of these plant hormones in the ISR primed by the volatiles. Analysis of defense-related genes by real-time qRT-PCR showed that both the SA- and JA-signaling pathways are involved in the *m*-cresol-primed ISR, whereas MeBA-activated ISR is mainly mediated by the JA-signaling pathway with partial recruitment of the SA-signaling.

Another PGPF, *Penicillium simplicissimum* GP17-2, acts on plants via unidentified soluble compounds, not VOC, to give ISR. In order to understand molecular mechanisms on ISR by GP17-2, I decided to analyze transcriptional responses, taking advantage of genomics tools and information that have been recently developed.

Transcriptional profiling is particularly informative to understand transcriptional responses. In my laboratory, microarray analysis to see transcriptional response of Arabidopsis treated with GP17-2 in roots has been performed. Taking advantage of the in house data, I analyzed the microarray data in detail, by comparing selected public microarray data of pathogen, phytohormones, hydrogen peroxide (H_2O_2), and wound responses. Results showed that there is a peak of SA/H2O2 response at 6 hours post GP17-2 treatment, and another peak of abscisic acid (ABA) response at 24 hours post GP17-2 treatment. These results indicate that the GP17-2 treatment causes a sequence of responses from SA/H_2O_2 to ABA.

Utilizing the microarray data, I achieved *in silico* promoter analysis in order to reveal participating *cis*-regulatory elements involved in the GP17-2-mediated ISR. An octamer-based frequency comparison method that has been developed in our laboratory was used for the prediction. Special care was taken for cross-detection by prediction of the SA/H_2O_2 response. The predicted promoter elements were subjected to functional analysis *in planta*, using an approach of preparation and utilization of synthetic promoters. A luciferase-based new vector (yy447) has been developed for the purpose. Among prepared synthetic promoters, one was found to show physiological responses in assays *in vivo*. This promoter, containing only one kind of *cis*-element, is controlled by circadian rhythm and showed repression by pathogen (*Pst* DC3000), ABA, SA, and H_2O_2 treatments. These results are expected to provide new knowledge to understand the transcriptional network of GP17-2-mediated ISR and plant hormone signaling.

ACKNOWLEDGMENT

To the almighty ALLAH, the wind beneath my wings, for everything.

This study would not have been completed if not for the tremendous help of the following individuals and institutions:

My esteemed supervisor Dr. Mitsuro Hyakumachi, Professor, Laboratory of Plant Pathology, Faculty of Applied Biological Sciences, Gifu University, for his invaluable guidance and continuous inspirations during the conduct of this study.

My honorable co-supervisor, eminent scientist Dr. Yoshiharu Y. Yamamoto, Professor, Laboratory of Plant Molecular Physiology, Faculty of Applied Biological Sciences, Gifu University, Japan, for his scholastic guidance, technical assistance and continuous encouragement for all the time.

Member of my advisory committee: Dr. Akio Morita, Professor, Faculty of Agriculture, Shizuoka University, for his constructive comments which greatly helped to improve the manuscript.

Dr. Masafumi Shimizu, Associate professor, Laboratory of Plant Pathology, Faculty of Applied Biological Sciences, Gifu University, for his assistance afforded in numerous ways during the duration of this study.

Mr. Yohei Yoshioka, Miss. Ayaka Hieno and my lovely friend Mary Grace, Doctoral course student, United Graduate School of Agricultural Science, Gifu University, Japan, for their cordial help and continuous support in every step of my research.

My contemporary lab-mates at the Laboratory of Plant pathology, Gifu University, for the assistance rendered in various ways.

The Ministry of Education, Culture, Sports, Science, and Technology (Monbukagakusho), Government of Japan, for the financial support.

The staff of the United Graduate School of Agricultural Science and the International Student Center, Gifu University, for their valuable assistance to foreign students.

My parents, my husband Dr. Md. Nojebuzzaman Nukta, my son Nene and daughter Nayeera and all other family members and friends for their prayers, unconditional love, and support.

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