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Original Research Article

Gene encoding bacterial wilt related protein as a target forstress resistant transgenic plants

ABSTRACT

Background &Aim: Bacterial Wilt is an important and crucial disease of tomato causing decrease in crop yield per year. In order to produce wilt resistant varieties exploration of defense mechanism against bacterial wilt is important. Aim of study is to confirm the expression of some of suspected defense genesreported to be produced in bacterial wilt resistant varieties in comparison to susceptible verities.

Place and Duration of Study: Study was done in Department of Biochemistry and Molecular Biology, University of Gujrat Pakistan for one year for fulfillment of masters of Philosophy degree of research student.

Study design: Salicylic acid was deliberated on tomato var. Roma (medium resistant) and Riogrande (resistant variety) for three consecutive days. Expression analysis studies of iron ATP-binding cassette (ABC) transporters, ATP synthase and chaperonin using RT-PCR along with measurement of antioxidants activities.

Results: Iron ABC transporters was expressed in control and within 1st day of SA treatment in both varieties, while chaperonin was expressed after two days in Roma and after three days in Riogrande of SA treatment. ATP synthase was expressed after three days in Riogrande while started from 1st day in Romaafter SA treatment. Peroxidase and catalase activity increased in both varieties after one day;with little decrease after two days ofSA treatment.

Conclusion: Expression of iron ABC transporters, ATP synthase and chaperonin in response to SA application signifies importance of these genes in response to bacterial wilt in tomato.

Keywords: Salicylic acid, Tomato, ATP synthase, Iron ABC transporter, Chaperonin.

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1. INTRODUCTION

Tomato (*Solanum lycopersicum)* is second world most significant vegetable crop, and for last 25 years total world production and consumption of this important crop has raisedmoderately. Small genome, short life cyclesimilar to many commercially vital plants (potato, eggplant, peppers and tobacco), made it important asstudies conducted on tomatoes are equally valid to these plants[1; 2].Itis challenged by various biotic stresses and abiotic stresses; became chief reason for tomato loss worldwide [3]. Tomato respond to biotic/abiotic stresses by multiple defense mechanisms including cellular reactions generated by the pathogens leads to signaling pathways [4; 5].Plant immune systems dependent on resistant/susceptible varietyand their defense mechanisms [6].

Main objective of studying plant pathogen interactions isto reveal the signaling moleculesthat leadsto defense related pathways[7].Signaling molecules/hormones includingauxins, cytokinins, salicylic acid (SA), abscisic acid (ABA), gibberellic acid (GA), ethylene (ET), jasmonic acid (JA), secreted by plants in response to environmental stresses have important functions in production of protein related to defense, growth and development [8; 9]. SA has an important role in plant defense against biotic and abiotic stresses, along with regulation of various physiological and biochemical processes (Plant growth, photosynthesis, respiration, thermos tolerance, stomatal responses, nodulation, nitrate metabolism, flowering), and ET biosynthesis [10; 11; 12].Increased SA in plant cells deliberately/naturally is also coupled with augmentation of pathogenesis related proteins (PR) proteins along with defense and metabolism related proteins resulted in systemic acquired resistance (SAR) [13; 14; 15]. It had been reported forits critical role in defense aided with organization of SAR afterbiotrophic and hemi-biotrophic pathogens infections specifically in model plants[16]. SAR is a potent form of innate immunity which is efficient and a long-term resistance effect against a wide variety of pathogens [17].SA had been also reported to produce oxidative stress in plants at high concentration butat low concentrations enhanced antioxidative capabilityhelp to cope with stresses [18]. Antioxidant enzymes such as polyphenol oxidase, peroxidase (POD), catalase (CAT)were reported to increase4 days afterSA treatment (War et al. 2011). Antioxidant activity after SA treatment can be a measure ofresistance or susceptibility of the varieties.

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Taking into account the mechanisms which plants employ to defend themselves against pathogens may enable the researchers to design novel strategies to improve disease resistance in crop plants. ATP synthase, iron ATP-binding cassette (ABC) transporters and chaperoninrelated to protein destination, storage synthesis and photosynthesis expression were some of the important proteins expressed in plants resistant to biotic stress in response to SA treatment [19; 20]. Previously SA induces proteins were explored in susceptible and resistant varieties of tomato (Roma and Pant Bahr)[15]. In current study ATP synthase, iron ABC transportersand chaperonin were confirmed using RTPCR analysis in response to SA in Riogrande (resistant) and Roma (medium resistant) to bacterial wilt.Along with these protein encoding genes, antioxidant enzymatic assays(POD, CAT) were carried out afterone, two and three days of SA treatment.

2. MATERIALS AND METHODS

2.1. Plant Material

Tomato (*Solanum lycopersicum*) Riogrande (resistant) and Roma (mediumresistant) cultivars were used. The seeds of Riogrande and Roma were obtained from National Agriculture Research Centre (NARC), Islamabad. Seeds were surface sterilized in 0.8 % (v/v) “Clorox” bleach (Sodium hypochlorite) as reported [15]. Seedlings were planted in pots containing sand and soil, grown in growth chamber under white fluorescent light (600 mol m^{-2} s^{-1}; 16 h light/8 h dark) at 25°C and 70% relative humidity.

2.2. SA Treatment, RNA Isolation and cDNA Synthesis

SA (Wako) was used at 1 mM concentration prepared in water. Three-week-old seedlings of the Roma and Riogrande were treated with SA for 1, 2 and 3 days andRNA extraction was done for 3 consecutive days.Total RNA was isolated from leaf cells of controlled and treated tomato plants using the TRI Reagent (Cat. No. TRI. 118; Lot No. 4221).RNA was precipitated by mixing it with isopropanol, centrifuged (12000 g) for 8 min at 4-25° C. RNA pellet was washed with 75% ethanol, centrifuged, dissolved in nano-pure water andstored.cDNA was prepared from total RNA samples using cDNA synthesis kit (Invitrogen, USA).

2.3. RT-PCR Analysis

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Accession number of five proteins(given in Table 1) were analyzed using bioinformatics tools, and relatively suitable length of aligned region of PCR primers were designed using Primer3 online software. Forward and reverse primers were designed for Iron ABC transporter, ATP synthase and 60-KDa chaperonin. cDNAwas amplified with gene-specific primers using PCR. Confirmation of the desirable sequences in extracted cDNA from all species will be done by running PCR reaction with five set of primers under specific conditions (Table 1). 20 µl PCR reaction mixtures contains 1.5 of the template cDNA, with 2 µl Taq buffer, 1 µl $MgCl_2$, 0.5 µl dNTP mixture, 0.5 µl of each forward and reverse primer, 1 µl Taq polymerase and 13 µl of nanopurewater. Initial denaturation step for PCR is restricted to (95°C) for 4 minutes, followed by 40 cycles of denaturation step at (95°C) for 30 seconds, annealing temperature (55°C for ATP synthase, 53°C 60-kDa chaperonin and iron ABC transporter) for 1.5 minutes and extension (72°C) for 30 sec, and a final extension cycle of 7 min (72°C).The amplified PCR products were analyzed by 1 % (w/v) agarose gel electrophoresis.

2.4. Enzymatic Assays

2.4.1. PODactivity

PODactivity was determined following the dehydrogenation of guaiacol as a substrate [21]. Plant material (2g)is grinded in pre-chilled mortar with 0.1 M phosphate buffer (pH 7). Homogenate is centrifuged at 18000 g for 15 min at 5℃. Supernatant is used as enzyme source within 2-4 h.3 ml of the buffer solution is pipetted along with 0.05 ml guaiacol solution (20 mM), 0.1 ml enzyme extract and 0.03 ml hydrogen peroxide solution (12.3 mM) in cuvette. Absorbance was taken on spectrophotometer at 750 nm.

2.4.2. CAT Assay

CAT was assayed by monitoring the decrease of absorbance as described by Aebi[22]. The reaction mixture consisted of 50 µL of enzyme, 2.5 mL potassium phosphate buffer(50 mM; pH 7.6), 200 ulhydrogen peroxide (12.5 mM) and homogenate sample (50 µl), along with 250 µl of distilled water were taken in a cuvette. Absorbance was measured at 240 nm three times for each sample with a time interval of 30 seconds. One unit activity of CAT is described as an absorbance change of 0.01 as unit per minute.

3. Results and Discussion

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Bacterial wilt is one of the important bacterial stresses faced by tomato, restraining its production [23]. Different genes had been identified which are expressed in tomato varieties resistant and susceptible to bacterial wilt [24; 25].SA mediated defense pathway play a key role in response to biotic stresses[26]. RTPCR is widely used for confirmation of gene or protein expressionwas used for confirmation of Iron ABC transporter, ATP synthase and chaperonin 60 in Riogrande and Roma [27]. RNA extraction was done with 3-W-Old seedlings leaves, followed by cDNA synthesis and RTPCR ofIron ABC transporter, ATP synthase and chaperonin 60 in resistant and mediumresistant varieties of tomato (Fig S1A; S1B).

3.1. Morphological variation

Leaves became light green after 1, 2 and 3 days of SA treatment in comparison to controlin Roma (Fig 1A, 1B, 1C, 1D). Riogrande leaves almost remain same as control (Fig 1E, 1F, 1G, 1H).Reduction in chlorophyll in semi-resistant variety (Roma) show its more sensitiveness to water stress, as the leaves were treated with aqueous solutions of SA in comparison to control. It is in line with observation for Roma [15].

3.2. Antioxidant enzymes assay

PODand CAT activity was increased one day after treatment of SA, decrease after 2 days and again increased after 3 days of SA treatment in Riogrande and Roma(Fig 2A; 2B).CAT and POD help to scavenge the stress damage were confirmed in these two varieties primarily. Antioxidant activity was more prominent in resistant variety suspects its role to reduce the ROS damage. Higher induction of antioxidant enzymes (POD, PPO) along with generation of H_2O_2 were reported in response to SA (1, 1.5, 2 mM) in *Cicer arietinum L.* [28]. Antioxidative enzymes POD, thioredoxin, superoxide dismutase, glutathione S-transferase were reported in tomato after SA treatment to protect plant from damage[29; 30]. H_2O_2 in plants is generated during photorespiration, mitochondrial electron transport and β-oxidation of fatty acids, and its intracellular level are stringently controlled for maintaining homeostasis in the cell [31]. Immune response is suppressed by inhibition of host cell's POD activity, thereby inhibiting ROS production [32].

3.3. Iron ABC transporter

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Iron ABC transporter was found to be expressed within one day of SA treatment in both Roma and Riogrande (Fig 3A). SA enhanced Iron ABC transporter expression in Roma and in Riogrande after 1, 2 and 3 days of SA treatment (Fig 3A). Iron ABC transporter, was also upregulated in the proteomics analysis of tomato cvs and soybean in response to application of either JA or SA [15; 20].Iron ABC transporter had a role in stress resistance in plants, and induced in response to bacteria, fungus, heavy metal stress, ET, SA, JA, ABA, resistance to HR, iron homeostasis, cell viability and iron acquisition in plants[30; 33; 34].Iron ABC transporter, photosynthesis related gens, defense genes, antioxidant enzymes were induced in response to SA treatment can be used as genetic tools to investigate disease resistance [35]. ABC transporter genes reduction indirectly resulted in reduced photosynthesis, and production of stress in apple [36]. Iron ABC transporter is also induced in JA dependent pathway in response to infection by bacterial and fungal infectionsin tobacco [37].It expression in response to JA, SA, bacterial and fungal pathogens; made it favorite gene responsible for susceptibility or resistance of tomato cultivars Roma and Riogrande.

3.4. ATP Synthase

ATP synthase was found to be expressed within one day of SA treatment in Roma along with control, while in Riogrande it is expressed in control and 3 days after SA treatment (Fig 3B).It is line with observation that insusceptible cultivars ATP synthase is up-regulated and in resistant cultivar it is expressed after 2 days [19]. Proteomic approach show reduced expression of ATP synthase with 1, 2 and 3 days of SA treatmentin same cultivar Roma [15].Wu et al. [10]via gel free techniques (LC MS/MS) reported phosphoproteins such as ATP synthase and iron ABC transporters are upregulated by SA in maize. ATP synthase is expressed in response to biotic/abiotic stresses for production of resistance in plants by suppressing HR response, along with increased metabolism of glucose [38].Janda et al. [39] reported regulatory effects of SA on photosynthetic electron transport processes. Chloroplastic-ATP synthase was found to be important to trigger ET, SA/JA pathway in response to insect herbivory [40]. Transgenic tobacco with ATPase exhibited resistance in response to *P. syringae*, suggesting its role in defense via JA or SA [41].Upregulation of ATP synthase after 1st day of treatment in susceptible (Pant Bahr) and medium resistant varieties of tomato and after 2 days in resistant variety of tomato implied its importance as defense gene in response to biotic stresses,

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insect herbivory, and suppression of HR made it a good candidate transgenic tomato with enhanced resistance to bacterial wilt.

3.5. Chaperonin

Chaperonin was expressed in control and 2 days after SA treatment in Roma (Fig 3C)and after 3 days of SA treatment in Riogrande (Fig 3D). SA enhanced Chaperoninexpression in Roma and in Riogrande after 2 and 3 days of SA treatment. Proteomic approach show enhanced expression of Chaperonin in susceptible variety (Pant Bahr) and unchanged expression in medium resistant variety (Roma) after SA treatment. Wu et al. [27] reported that after inoculation with *P. solanacearum*;ATP synthase was expressed in susceptible and chaperonin was expressed in resistant maize cultivar in SA pathway. Moshe et al. [42] reported up regulation of chaperonin in resistant tomato cultivar in response to tomato leaf curl virus infection in comparison to susceptible cultivar. Proteomics analysis of *Arabidopsis* associate chaperonin-60 with photosynthesis for increasing duration of stress [43].Cueto-Ginzo et al. [44] also reported SA balance photosynthetic reduction during infection of potato virus in tomato by increasing mesophyll conductance.Molecular Chaperone is another candidate found to be important for resistance in resistant cultivars in response to stresses in comparison to accumulation of ROS species in susceptible cultivars. Chaperone was expressed in resistant, medium resistant and in susceptible cultivars; more prominent in resistant variety (Riogrande)(Fig 3D).

4. **CONCLUSION**

Proteomics technique was used for identification of iron ABC transporter, ATP synthase and chaperonin in resistant and susceptible varieties of tomato [15]. Proteomics analysis data show chaperonin, ATP synthase, iron ABC transporter which were upregulated/remain unchanged in one variety in comparison to other in response to application of SA are candidates proteins that can be responsible for susceptibility or resistance of tomato cultivars. RTPCR show iron ABC transporter expression remain same in resistant and medium resistant cultivars. While chaperonin expression was important; as expressed strongly after 3 days of SA application in resistant cultivars also

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192 supported by previous reports. Similarly ATP synthase expression was after 2 days in Riogrande.
193 CAT and POD enzymatic activity was increased in both cultivars after one and more after 3 days of
194 SA treatment little more in Riogrande. Genes encoding proteins related to protein folding and
195 photosynthetic genes are targets for biotic stress related pathway in tomato.

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Abbreviations: Salicylic acid (SA), Systemic acquired resistance (SAR), Abscisic acid (ABA), Gibberellic acid (GA), Ethylene (ET), Jasmonic acid (JA), Pathogenesis related (PR), Catalase (CAT), Peroxidase (POD), ATP-binding cassette transporters (ABC transporters).

302 **Table 1. Primers for genes encoding protein sequences, melting temperature ™ and their functional category.**
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Protein Name	Protein database/ Tm	Forward Primer	Functional Category
ATP synthase epsilon chain (F)	gi|114610/ 55°C	5-3' TGACCTTAAATCTTAGTGTACTGACC	Glycolysis pathway
ATP synthase epsilon chain (R)		5-3' TTATGAAATCGGATTGATAGCC	
60 kDa chaperonin (F)	gi|603912/ 53°C	5-3' GAGATTGTGTTCGACCAGGAG	Protein destination and storage
60 kDa chaperonin (R)		5-3' ACACGCCATCGTTGATAACC	
Iron ABC transporter (F)	gi|57504871/ 53°C	5-3' ATGAAAAAAATTTTAATCATTATGAGTTTA	Electron transport and photosynthesis
Iron ABC transporter (R)		5-3' TTAAAAATTTTGATTTTCTAATCTTGTTTT	

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Figures

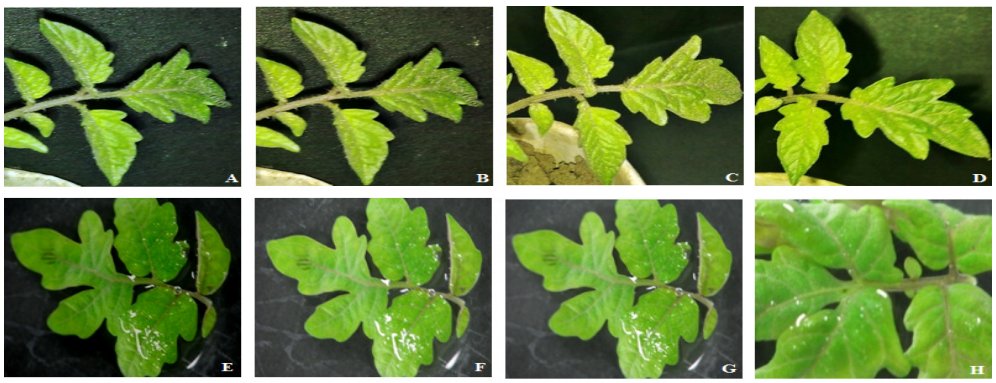

Fig 1

Fig 1. Morphological variation of *Solanum lycopersicum* cvs Roma (a, b, c and d) and Riogrande (e, f, g & h) in control and 1, 2 and 3 days after SA treatment.

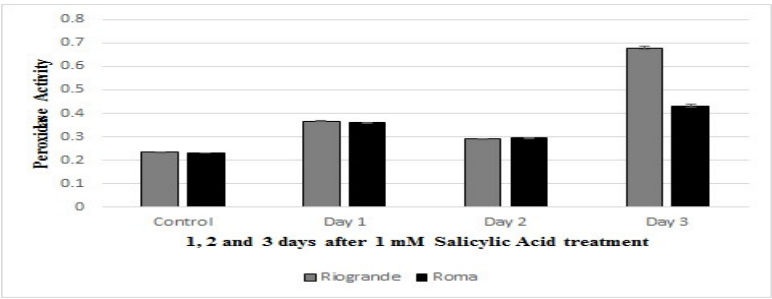

Fig 2A

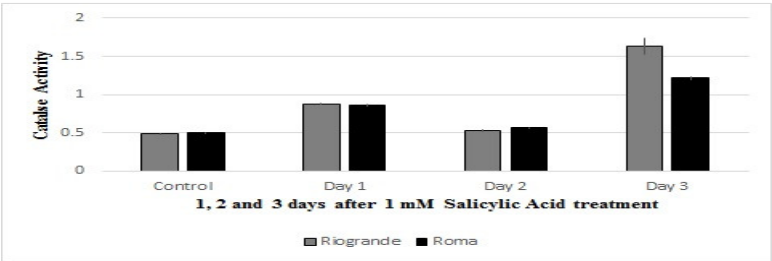

Fig 2B

Fig 2A. Peroxidase activity in *S. lycopersicum* cvs Roma and Riogrande 1, 2 and 3 days after 1 mM SA treatment. 2B. Catalase activity in tomato cvs Roma and Riogrande 1, 2 and 3 days after 1 mM SA treatment.

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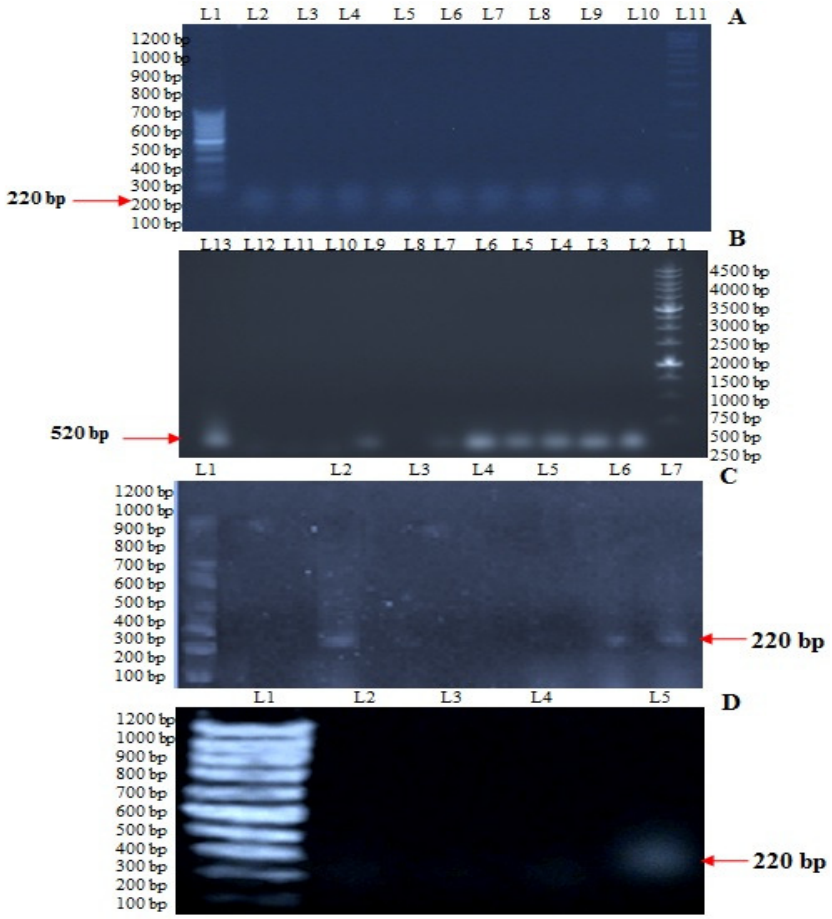

Fig 3

Fig 3. RTPCR of 3-W-Old *S. lycopersicum* leaves after 1, 2 and 3 day treatment of SA using **3A.** ABC transporter primer (220 bp). L 2-5 containing PCR products of Roma control, 1, 2 & 3 days after treatment of SA. L 6-9 containing PCR products of Riogrande control, 1, 2 & 3 days after treatment of SA. L1 100 bp marker.

3B. With ATP Synthase primer (520 bp). L 1: 1 Kb ladder, L2-L5: PCR product of control, 1, 2 & 3 days after treatment of SA of Roma respectively, L6-L9 contain PCR product of control, 1, 2 & 3 days after treatment of SA of Riogrande respectively, L11- 13 contain PCR product of Riogrande 1, 2 & 3 days after SA treatment respectively as repeat. **3C.**With Chaperonin primer (220 bp). L 1: 100 bp ladder, L2: PCR product of control, L5, L6, L7 1, 2 & 3 days after treatment of SA of Roma respectively. **3D**. With Chaperonin primer (220 bp). L 1: 100 bp ladder, L2: PCR product of control, L3, L4, L5 1, 2 & 3 days after treatment of SA of Riogrande respectively.

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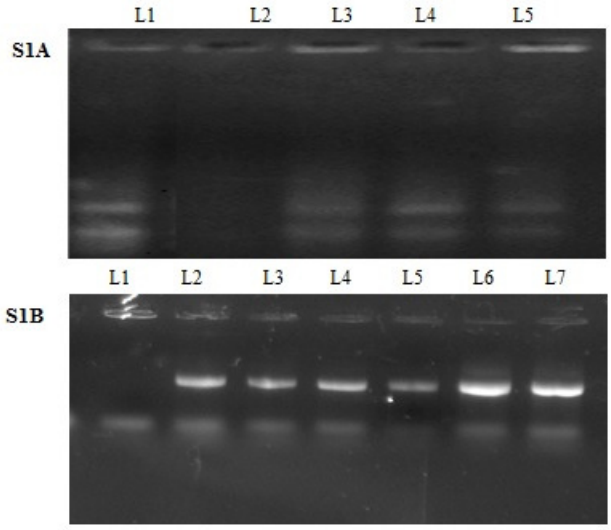

Fig S1

Fig S1. (S1A) RNA of 3-W-old *S. lycopersicum* seedlings leaves of Roma.RNA extracted from 3-W-Old tomato leaves. L 1 is showing the bands of RNA of control while L 2, 3, 4 & 5 are showing of treated (S1B) cDNA samples from RNAs extracted after 1, 2 & 3 day of SA treatment of Roma and Riogrande.