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Proteomic analysis of salicylic acid enhanced disease resistance in bacterial wilt affected chilli (*Capsicum annuum*) crop

B. Chandrasekhar [a, **], S. Umesha [a, *], H.N. Naveen Kumar [b]

[a] Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India
[b] Department of Studies in Biochemistry, Kuvempu University, Shankaraghatta, Shimoga, Karnataka, India

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ABSTRACT

Salicylic acid (SA) is an important endogenous chemical signal that plays a key role in enhancing plant defense responses. Exogenous application of 0.5 mM SA for 3 days enhanced resistance to bacterial wilt (BW) disease (by reduction in disease incidence level upto 64% under greenhouse conditions) without any detrimental effect on plant growth. To understand the dynamics of protein in SA-primed chilli during BW infection, proteomic approach using 2DE-SDS PAGE was performed. Proteomic analysis revealed 25 differentially expressed proteins (which were more prominent in SA primed-challenge inoculated chilli samples), of which 20 were successfully identified by Nano-LC MS/MS analysis. The differential expression pattern revealed that proteins associated with stress and defense, energy and metabolism, protein synthesis, protein destination and storage and transcription related were upregulated indicating the involvement of SA induced disease resistance in chilli seedlings. This suggests the complexity of the proteome and inter-connected pathways responsible for SA induced resistance in chilli. Correlation in the differential expression of catalase and EF-1A from proteomic as well as semiquantitative RT-PCR suggests this probable use as biomarkers in screening susceptibility of chilli cultivars for wilt disease. Findings from this study will serve as basis for designing disease-management strategies based on resistance conferred by SA, which could applicable to other biotic stress affected staple crops.

1. Introduction

Chilli (*Capsicum annuum* L.) is a spice belonging to family solanaceae, and it is the most popular and widely cultivated vegetable all over the world. In India, chilli production has reached 1.29 million tones and it is grown in an area of 0.079 million hectares with the productivity of 16.329 mt ha-1 [1]. In India, *Capsicum annuum* and *Capsicum frutescens* are the only two species grown and majority of the cultivated varieties belong to *Capsicum annuum*. Chilli, besides imparting pungency to the dishes, also is a rich source of vitamins A, C, E and P and assists in digestion. Solanaceous crops are prone to a number of bacterial diseases, which are the major limiting factors for production in many countries. These diseases include bacterial leaf spot, bacterial speck, bacterial canker and bacterial wilt. Among them, bacterial wilt caused by *Ralstonia solanacearum* Smith (*Pseudomonas solanacearum*=*Burkholderia solanacearum*) is a very destructive and harmful disease, resulting in complete loss of the crop [2]. *R. solanacearum* significantly reduces the *C. annuum* yield and thus bacterial wilt is the fourth major disease of *C. annuum* globally [3]. The bacterial wilt symptoms are characterized by initial wilting of upper leaves and within a few days complete wilting of plant takes place followed by signs of stunting and adventitious root development in main stem. *R. solanacearum* is aerobic, Gram negative, rod shaped, motile, non flourescent with a polar flagellar tuft. Virulent colonies are white fluidal with pink centers whereas red colonies are avirulent. Bacterial wilt disease is most common in northern regions, it is more severe in tropical, subtropical and temperate regions with hot humid summers. *R. solanacearum* is seed-borne and persists for many years in soil and crop residues [4]. *R. solanacearum* strains were classified into four phylotypes based on geographical isolation [5]. These phylotypes are restricted to geographical regions: Strains from Asia were constituted as phylotype I, Strains from America were constituted as phylotype II, Strains from Africa and surrounding islands in the Indian Ocean were constituted as phylotype III and Strains from Indonesia were constituted as phylotype IV.

* Corresponding author.
** Corresponding author.
E-mail address: su@appbot.uni-mysore.ac.in (S. Umesha).

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Each phylotype is further divided into sequevars (isolates with less than 1% nucleotide variation) in the endoglucanase (*egl*) locus. *R. solanacearum* can rapidly adapt to environmental changes and new hosts to counteract the host immune responses, one such recent emerging strains are the phylotype IIB-4NPB (non pathogenic on banana) strains from Martinique [6]. T3SS (Type III secretion system) is the main pathogenic determinant of *R. solanacearum*, syringe like membrane transport protein that injects virulence/effectors into host [7].

Disease control has mainly dependent on breeding disease resistant cultivars. However, frequent appearance of new species/races of *R. solanacearum* [8] has increased the susceptibility of resistant cultivars in the field. In our previous study, we reported a new evolutionary isolate of *R. solanacearum* which showed complete divergence [9] and it was not possible to control it with regular measures. Development of new disease control strategies based on plant innate defense mechanisms may offer promise for sustainable production and environmental pollution abatement. In contrast to mRNA expression profiling, proteomic profile monitors the actual protein composition of the cell/tissue. In the present scenario, protein studies of *C. annuum* gains prominence after its complete genome has been sequenced [10].

Salicylic acid (SA) is a central signalling molecule during plant innate immune response. It is usually induced by infection with biotrophic and hemibiotrophic pathogens, and serves as an important component of Pathogen associated molecular pattern-triggered immunity (PTI), effector triggered immunity (ETI) and systemic acquired resistance (SAR). SA acts as an endogenous resistance signal in plants, the rapid increase in ROS (H_2O_2) following pathogen infection activates SA synthesis which in turn activates the defense gene expression [11]. Some plants inherently elevate the salicylic acid production under stress which in turn increases disease resistance. The role of SA in plant pathogen interactions has been studied extensively. Exogenous SA application significantly enhances plant tolerance to abiotic stress (cold, heat, salt, drought and U.V) [12] and biotic stress by virus [13] and bacteria [14]. SA promotes higher expression of catalase in tobacco plants in response to Tobacco mosaic virus (TMV) [15]. ATPsynthase cf_1 β subunit expression was elevated in chilli pepper (*Capsicum annuum*) leaves in response to *Fusarium oxysporum* infection [16]. Exogenous application of SA induces SAR, is a type of whole-plant resistance developed after an initial infection which leads to downstream expression of pathogenesis-related proteins (PR-proteins) [17] which regulates the defense status of plants with a wide range of antimicrobial properties. PR proteins were classified into different families ranging from PR1 to PR17 [18]. SA upregulated expression of different classes of PR proteins viz., PR1 (antifungal) in arabidopsis [19], PR5 (Thaumatin-like) in tobacco [20], PR8 (chitinase class III) in chinese crab apple [21]. Several families of PR proteins and proteins responsible for programmed cell death (PCD) and a type of hypersensitive response mechanism were characterized in chilli plant induced by TMV and pepper mild mottle virus (PMMoV) [22]. The expression of PR proteins is a significant mechanism that plants have developed to protect themselves from pathogen. PR proteins were upregulated during both compatible and noncompatible interactions of PMMoV in *Capsicum chinense* plant [23]. So far no work has been reported on protein profile of SA treated chilli seedlings in response to BW disease.

The objective of the present study was to identify/characterize the SA induced defense related protein expression from *C. annuum* plants upon different treatments *viz*., SA treatment, *R. solanacearum* treatment, SA + *R. solanacearum* treatment along with untreated controls using 2DE-LCMS/MS and also to evaluate the various levels of SA on chilli disease incidence. Some, selected proteins were evaluated for their transcript accumulation by semiquantitave RT-PCR at fixed time points after treatments of chilli cultivar to support the mass spectrometric data.

2. Materials and methods

2.1. Plant and pathogen

Bacterial wilt susceptible seeds (cv. *Pusajwala*) were purchased from the Vegetable and fruit promotion council keralum, Kerala. Seeds were grown in pots containing sterilized potting mixture (mixture of sand, clay and farm yard manure in 1:1:1 ratio). *R. solanacearum* inoculum (Virulent isolate KF220588) was prepared by growing cells of the bacterium on Kelman's TZC agar medium and incubated at 28 ± 2 °C for 24–48 h. Bacterial cells were harvested in sterile distilled water by centrifugation (Eltek, Bangalore, India) at 12,000 rpm for 10 min. The pellet was resuspended in 5 ml distilled water and bacterial suspension was adjusted to OD_{600} = 0.01 using UV–visible spectrophotometer (Beckman Coulter DU 100, Maharashtra, India) to obtain the concentration of 1×10^8 cfu ml^{-1}. Pathogenicity test was performed on 3 week old seedlings.

2.2. SA treatment on growth characteristics

Chilli seedlings at the fully developed fourth-leaf stage (3 week old seedlings) were pretreated with 0.1, 0.25, 0.5, 0.75, 1 and 1.25 mM of SA aqueous solution, by spraying until run-off, and subsequently challenged with *R. solanacearum* on 2nd day. Control plants were sprayed with equal volume of sterilized distilled water and inoculated similarly as the *R. solanacearum* treated plants. Plants were kept in humidity chamber at 25 ± 1 °C, disease symptoms were recorded 7 days after inoculation and as per the scoring of Tans-kersten et al. [24]. Disease indices were calculated by the formula: Disease Index = ([no. of plants of each grade × disease grade]/[total no. of plants × the highest disease grade]) × 100. The experiment had 3 treatments and 5 replicates.

2.3. SA treatment on pathogen tolerance

0.5 mM SA (Sigma, Bangalore) was administered exogenously 2 days before bacterial inoculation. 3 week old chilli plants of similar size were selected and divided into four groups of five replications (each replication consists of 1 pot with 5 healthy plants); Group 1: healthy control, plants sprayed with water; Group 2: infected control, plants inoculated with *R. solanacearum*; Group 3: plants treated with 0.5 mM SA; Group 4: Plants treated with 0.5 mM SA + challenge inoculation with *R. solanacearum*. Plants whose uppermost leaves were fully expanded in each treatment, 2 days after initiation of pathogen stress were collected, immediately frozen in liquid nitrogen, and stored at −80 °C to measure physiological parameters.

2.4. Proteome isolation and 2-DE

At four-leaf stage of chilli seedlings, 0.5 mM SA solution was sprayed onto the chilli leaves using an air sprayer until run-off. The leaf samples of all groups were harvested at 24 h after SA treatment. The leaf samples were frozen in liquid nitrogen and stored at −80 °C. The control plants were sprayed with sterilized water.

Proteins were extracted from *C. annuum* leaves comprising of all groups by phenol method [25] with slight modifications. Approximately 1 g of leaf tissue was homogenized in mortar and pestle (complete protein extraction step was carried out at 4 °C to limit the protease activity). Homogenized leaf tissue sample was transferred into microfuge tube containing 800 μl of extraction buffer