Chitosan nanoparticles - based approach to study induced defense responses, bioavailability and localization of harpin$_{Pss}$ in tomato

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by

Sravana Sandhya Rani Nadendla

(Regd. No. 13LPPH16)

Department of Plant Sciences

School of Life Sciences

University of Hyderabad

Hyderabad 500 046

India

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University of Hyderabad

(A Central University established in 1974 by an act of parliament)

HYDERABAD-500 046, INDIA

DECLARATION

I, Sravana Sandhya Rani Nadendla, hereby declare that this thesis entitled **"Chitosan nanoparticles - based approach to study induced defense responses, bioavailability and localization of harpin$_{Pss}$ in tomato"** submitted by me under the guidance and supervision of **Prof. Appa Rao Podile** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Sravana Sandhya Rani Nadendla

(13LPPH16)

Prof. Appa Rao Podile

(Research Supervisor)

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad – 500046, Telangana, India

CERTIFICATE

This is to certify that this thesis entitled **"Chitosan nanoparticles - based approach to study induced defense responses, bioavailability and localization of harpin$_{Pss}$ in tomato"** is a record of Bonafide work done by **Mrs. Sravana Sandhya Rani Nadendla**, a research scholar for Ph. D. programme under the Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. This thesis is free from plagiarism and has not been submitted in part or in full to this or any other University or institution for the award of any degree or diploma.

Parts of the thesis have been:

A. Published in the following publications:
   1. **Appa Rao Podile and Sandhya Rani Nadendla** (2017) Biopesticide compositions comprising stable harpin$_{Pss}$ loaded chitosan nanoparticles and methods thereof. Indian patent 201741034005.

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Sandhya

Dedicated to

My mother & husband

CONTENTS

ABBREVIATIONS

°C	: degree centigrade/degree Celsius
•OH	: hydroxyl radicals
4CL	: 4-coumarate-CoA ligase
cDNA	: complementary DNA
CSNPs	: chitosan nanoparticles
C-terminal	: carboxy terminal
Cyt C	: cytochrome C
DAMPs	: danger-associated molecular patterns
DNA	: deoxy ribonucleic acid
dNTPs	: deoxy nucleotide triphosphates
EDTA	: ethylene diamine tetra acetic acid
ETI	: effector-triggered immunity
FE-SEM	: field emission scanning electron microscopy
FNR	: ferredoxin NADP-reductase
FTIR	: fourier transform infrared spectroscopy
g	: gram
GFP	: green fluorescent protein
GST	: glutathione S-transferase
h	: hour(s)
H_2O_2	: hydrogen peroxide
H-CSNPs	: harpin$_{Pss}$-loaded chitosan nanoparticles
hpi	: hours post inoculation
HR	: hypersensitive response
Hrp	: hypersensitive response pathogenicity
hrpZ	: gene encoding harpin
HSP	: heat shock protein
HXT	: hexose transporter

IPTG	: isopropyl β-D-thiogalactoside
ISR	: induced systemic resistance
JA	: jasmonic acid
Kb	: kilobase pair
kDa	: kilodalton
L	: litre
LB	: Luria-Bertani
LOX	: lipoxygenase
M	: molar
MALDI-TOF	: matrix-assisted laser desorption/ionization-time of light
MAPK	: mitogen-activated protein kinase
mg	: milligram
min	: minute
mL	: milliliter
mM	: millimolar
NBS-LRR	: nucleotide-binding site leucine-rich repeat
Ni-NTA	: nickel-nitroacetic acid agarose
nm	: nanometers
NO	: nitric oxide
NPs	: nanoparticles
N-terminal	: amino terminal
$O_2^{\cdot -}$	: superoxide radicals
OD	: optical density
PAGE	: polyacrylamide gel electrophoresis
PAL	: phenylalanine ammonia lyase
PAMPs/MAMPs	: pathogen/microbe-associated molecular patterns
PBS	: phosphate buffered saline
PCD	: programmed cell death
PCR	: polymerase chain reaction

PFLP : plant ferredoxin-like protein

PMSF : phenylmethylsulfonylfluoride

POD : peroxidase

PR proteins : pathogenesis-related proteins

PRRs : pattern recognition receptors

Pss : *Pseudomonas syringae* pv. *syringae*

Pst : *Pseudomonas syringae* pv. *tomato*

PTI : PAMP triggered immunity

RNA : ribonucleic acid

RNase : ribonuclease

ROS : reactive oxygen species

rpm : revolutions per minute

RT-PCR : reverse transcriptase-polymerase chain reaction

SA : salicylic acid

SAR : systemic acquired resistance

SDS : sodium dodecyl sulphate

TPP : sodium tripolyphosphate

TTSS : type three secretion system

V : volts

µg : microgram

µM : micromolar

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Introduction

1.1. Immune system in plants

Plants lack adaptive immunity unlike animals. But, at cellular and molecular level, plants do have innate immune system with systemic signaling capability from infection sites. We now know the recognition capacity of the plant immune system. At the same time, research on pathogen biology begun to unravel how these organisms manipulate host immunity to cause disease. In a co-evolution process, where both plants and pathogens are evolving, plants are reported to respond to infection using a two branched innate immune system that respond to pathogen-derived molecules. Among all classes of pathogen-derived molecules, pathogen associated molecular patterns (PAMPs) such as fungal chitin or bacterial flagellin have been considered as very important components. PAMPs present on host cell surface are conserved microbial elicitors that are identified by receptor proteins called pattern recognition receptors (PRRs). The stimulation of PRRs results in PAMP-triggered immunity (PTI) a relatively broad spectrum immunity. Response of plants to endogenous molecules released by invasion of pathogen, such as cell wall or cuticular fragments called danger-associated molecular patterns (DAMPs). The second class of perception involves recognition by intracellular receptors of pathogen virulence molecules called effectors which is more specific and is often strong. The immunity triggered by such recognition induces effector-triggered immunity (ETI).

1.1.1. Pathogen-associated molecular patterns (PAMP) and PAMP-triggered Immunity:

Plants have a significant capacity to recognize microbes through strategies involving both conserved and variable microbial molecules often referred as elicitors. Pathogens secrete virulence factors to manipulate the plant defense responses. Plants use their pre-existing physical barriers like trichomes, needles and thorns which check the invasion of potential attackers. In addition, plants inhibit the pathogen growth by producing secondary metabolites.

But, many pathogens are able to cross this first line of defense and succeed in spreading their infection in plants. However, as a second line of defense, a broad-spectrum of inducible plant defenses become active to prevent further damage caused by the pathogen, either by damaging physiology of pathogens or by inhibiting its colonization. For this second line of defense, plants have developed sophisticated strategies to recognize their attacker and to develop further effective defense response (Jones and Dangl, 2006). This primary immune response recognizes common microbial features, such as extracellular structures like flagellin and cell wall components like chitin, lipopolysaccharides and glycoproteins (Bittel and Robatzek, 2007). Such determinants of microbial pathogens are referred to as PAMPs or elicitors (Chisholm et al., 2006; Jones and Dangl, 2006; Bittel and Robatzek, 2007).

PAMPs are recognised by PRRs, particular type of receptors on host cell-surface, which in turn initiate various downstream signaling pathways that eventually result in the activation of a basal resistance that is called PTI (Chisholm et al., 2006; Jones and Dangl, 2006; Boutrot and Zipfel, 2017). Both plants and animals share some common features of downstream immune responses in PAMP perception, including the production of antimicrobial compounds and the activation of conserved mitogen-activated protein kinase (MAPK) signalling cascades (Nürnberger et al., 2004; Ausubel, 2005). In plants, different MAMPs likely activate convergent defense responses including stomatal closure to prevent bacterial entry, deposition of callose to reinforce the cell wall, changes in cytoplasmic Ca^{2+} levels, the production of reactive oxygen species (ROS), phytoalexin and nitric oxide (NO), induction of defense-related genes and activation of MAPK cascades (He et al., 2007). In general, PAMPs are evolutionarily conserved molecules present in many microbes and are often crucial for their survival, fitness or virulence. In order to avoid recognition by host, pathogens modify PAMPs by acquiring active mechanisms. The modification of individual PAMPs by microbes will not significantly dilute the overall PTI, because most microbes are recognized by plant

innate immune systems through the perception of multiple and distinct PAMPs. However, just as plants have evolved the ability to recognize PAMPs, so fungi have evolved ways to outwit plants. They have developed small molecules called effector proteins that bind to PAMPs, in effect hiding them from the plant receptors. The tomato fungus *Cladosporium fulvum*, for example, secretes an effector protein called Ecp6, which contains lysin motifs just like those in the plant receptors. By binding chitin fragments, Ecp6 helps the fungus to avoid detection by its host plant (Sánchez-Vallet et al., 2013).

1.1.2. Type III secretion system (TTSS) and ETI:

Gram negative bacterial pathogens such as *Pseudomonas syringae*, use a molecular needle, the type III secretion system, to inject a set of bacterial virulence proteins (type III effectors; T3Es) directly into the host cytoplasm to suppress PTI and promote pathogen proliferation (Lindeberg et al., 2012). Plant pathogenic bacteria deliver 15–30 effectors per strain into host cells using TTSS. Bacterial effectors contribute to pathogen virulence, often by mimicking or inhibiting eukaryotic cellular functions. Hence, the T3Es from any successful bacterial pathogen dampen PTI sufficiently to allow successful colonization. In response, plants evolved a second class of immune receptors, the intracellular nucleotide-binding site leucine-rich repeat (NBS-LRR) receptors (NLRs). NLRs directly or indirectly detect the presence of T3Es and induce second layer of robust resistance response called ETI (Dodds and Rathjen, 2010). According to Flor hypothesis (Flor, 1971), resistant plants have an *R* gene in their genome and recognize pathogen encoded *avr* gene. It is sufficient to trigger a signalling cascade that leads to plant immunity in resistant plants.

1.1.3. Subcellular localization of effectors:

T3Es use TTSS to secrete proteins into the host cells and then manipulate host defense responses against bacteria (Büttner, 2016). Previous studies on human pathogenic bacteria

such as *Salmonella* and *Yersinia* made us to understand the detailed molecular and biochemical properties of the TTSS machinery (Galán et al., 2014). The first TTSS-associated filamentous structure was discovered in *P. syringae*, a plant pathogen with a broad host range including several important crop species (Xin and He, 2013; Galán et al., 2014; Büttner, 2016). T3Es are important for the pathogenicity of the bacteria and they play significant role in suppressing the first line of plant defense responses (Xin and He, 2013; Büttner, 2016). Several studies have confirmed that the effector proteins from pathogenic bacteria are targeted to specific subcellular organelles in plants and suppress innate immunity by altering the physiological properties of the cell (Alfano and Collmer, 2004; Kay and Bonas, 2009; Choi et al., 2013; Aung et al., 2017). Thus, the localization of the effectors is important for their function inside the host cell.

A number of *P. syringae* T3Es localize to discrete subcellular plant compartments. Plasma membrane is the common localization site for several effectors within plant cells (Block and Alfano, 2011). For example, the well-studied T3Es AvrPto and AvrRpm1 are both localized to the plasma membrane (Nimchuk et al., 2000). Two *P. syringae* T3Es, HopI1 and HopN1, localize to chloroplasts using uncharacterized non-cleavable transit peptides (Jelenska et al., 2007; Li et al., 2014). When expressed in plants, HopG1 and HopM1 localize to mitochondria and trans-Golgi network, respectively (Block et al., 2010; Nomura et al., 2011). HopK1 and AvrRps4, T3Es from *P. syringae* pv. *tomato* suppress PTI-induced immune responses depending on their chloroplast transit peptides, indicating the significant role of chloroplast in plant immunity (Li et al., 2014). Chloroplasts play a central role in integrating multiple environmental stimuli and accommodate many biosynthetic pathways, including those for plant hormones. A common strategy deployed by pathogens to hijack host immune signaling is to alter the phytohormone balance. Chloroplasts also produce ROS that are potentially damaging but which also act as signaling molecules and may have a direct

antimicrobial role. Considering the importance of ROS and hormone balance to plant–pathogen interactions, the chloroplast represents a prime target for manipulation by pathogens (Zabala et al., 2015).

1.1.4. Hypersensitive response (HR) in plants:

Depending on the nature of the interaction of plant and pathogen, the cell death can be related with disease resistance or susceptibility. The HR is the well-known cell death response in plants during disease resistance. In some cases, it can occur without or with very little cell death (Bendahmane et al., 1999). The interaction of avr proteins from pathogen and R proteins from plants commonly results in HR. The HR can be the outcome of several signaling pathways. HR serves as growth inhibitor of the infecting pathogen by killing infected and uninfected cells thereby creating a physical barrier of dead cells. At the site of HR, it turns dark brown due to the accumulation of phenolic compounds and exhibits auto fluorescence. However, these are the ultimate steps in the death process. HR involves two phases - Phase I involves ion fluxes with efflux of hydroxide and potassium and influx of calcium and hydrogen ions into the cell. Phase II includes oxidative burst by producing ROS, superoxide anions, NO, hydroxyl radicals and hydrogen peroxide.

Producing a ‘ladder’ of DNA by disruption of membrane and cleavage of nuclear DNA into fragments was one of the crucial events of cell death. The HR induced by bacteria, viruses and fungi finally result in the plant DNA cleavage (Levine et al., 1996; Ryerson and Heath, 1996; Mittler et al., 1997), but only the fungus-induced response results in a DNA laddering (Ryerson and Heath, 1996). Caspases are the key executioners of cell death in animals during apoptosis (Green and Reed, 1998). Even though caspases have not been found in plants, caspase-like activities have been identified from biochemical and inhibitor studies (del Pozo and Lam, 2003). For induction of HR, minimum levels of both H_2O_2 and NO are required in

the host (Delledonne et al., 2001). As the infection proceeds, generation of NO results in the spreading of cell death by giving cell to cell signalling. In support of this role of NO, Zhang et al. (2004) showed that, attenuation of HR takes place by inhibiting the NO synthesis.

1.2. Harpin$_{Pss}$:

Harpins are one group of proteinaceous elicitors of HR in non-host plants produced by plant pathogenic bacteria like *Pseudomonas, Erwinia*, *Xanthomonas* and *Ralstonia*. Harpin protein was identified and isolated in an attempt to recognize bacterial factors involved in HR in *E. amylovora,* a causative agent for fire blight disease and the corresponding gene was designated as *hrpN* (Wei et al., 1992). Harpins are secreted only upon interaction with plant and subsequently delivered into plant apoplast *via* bacterial TTSS. But, as a characteristic feature of proteins secreted through the TTSS, harpins lack N-terminal signal peptide (Wei et al., 1992). In some of the plant pathogenic Gram negative bacteria, the ability of causing disease in host plants or inducing HR in non-host plants is controlled by a cluster of genes referred as hypersensitive response and pathogenesity (*hrp*). Some of the conserved *hrp* genes are involved in the secretion of one or more proteins that elicit the HR in non-host plants (Lindgren et al., 1986). Indication of local lesions upon exposure to harpins is not 'necrosis' due to the toxicity to plant cells, rather a programmed cell death as a result of active metabolic changes in response to harpin recognition (He et al., 1993).

Plants activate defense response accompanied by expression of pathogenesis-related (PR) proteins and systemic acquired resistance (SAR) when infiltrated with an aqueous solution of harpin (Strobel et al., 1996; Dong et al., 1999). Harpin$_{Pss}$, a 34.7 kDa extracellular protein that is encoded by *hrpZ* gene of *P. syringae* pv. *syringae* which rapidly elicits HR upon infiltration in tobacco and few other plants. Ca^{2+}-dependent association of harpin$_{Pss}$ with tobacco cell walls was revealed in immunolocalisation studies. Further, depolarization of

plasma membrane on treatment with harpin suggests that the cell wall could be the harpin binding site (Pike et al., 1998). However, disease resistance in plants is confirmed both by apoplastic and cytoplasmic localisation of harpin (Sang et al., 2012). Harpin$_{Pss}$ is polydispersic in nature (Tarafdar et al., 2014) and the multimeric forms include dimer, trimer, tetramer and octamer, but the possibility of other forms is not restricted (Chen et al., 1998). Several partial deletion mutants also elicited a strong HR that is undistinguishable from that of HR elicited by intact harpin$_{Pss}$ (Alfano and Collmer, 1996) and the mechanism behind this HR elicitation by intact harpin$_{Pss}$ and deletion mutants is not clear. Recently, Anil et al. (2014) investigated some deletion mutants of HrpZ$_{Pss}$, and suggested that some of the leucine zipper-like motifs of HrpZ$_{Pss}$ are not essential to induce HR in tobacco. The HR induced by the mutants was faster than that of HR induced by intact HrpZ$_{Pss}$, possibly due to better exposure of the HR inducing region of the protein, for interaction with the plant receptors. Alkalinization was induced immediately after addition of different concentrations of harpin$_{Pss}$. The pH change caused by the same concentrations of deletion mutants is similar in magnitude with that caused by the harpin$_{Pss}$.

1.2.1. Harpin induces broad-spectrum disease resistance in non-host plants:

Harpin triggers growth and induces defense responses in plants against broad range of pathogens. When harpin protein is applied as foliar spray, it may bind to the receptor proposed to be present on cell wall exterior and activates a set of complex defense signals. The signals mainly include SAR-mediated by salicylic acid (SA) and few other defense mechanisms mediated by methyl jasmonate (MJ) and ethylene (ET). The actual mechanism behind disease resistance could be the elevated levels of metabolic alteration which comprises increased photosynthesis, nutrient uptake, root development that leads to increased biomass of vegetative parts.

An alternative approach to the use of bio-hazardous pesticides is the foliar application of elicitor molecules like harpin. Along with enhanced disease resistance in crop plants, harpin application also resulted in an increase of crop yield (Chen et al., 2008). In *Arabidopsis*, harpin from *E. amylovora* induced SAR genes like PR-1 and PR-2 and elicited resistance to *P. syringae* pv. *tomato* and *Peronospora parasitica*. On the other hand, harpin failed to elicit resistance in *Arabidopsis* plants over expressing the *nahG* gene which prevents accumulation of SA, defective in regulation of SAR and responding to SA. Thus, the harpin-induced resistance in *Arabidopsis* is SAR and is mediated by NIM1 that requires the involvement of SA (Dong et al., 1999). A specific fragment of $HpaG_{Xooc}$ from *X. oryzae* pv. *oryzicola* $HpaG_{10\text{-}42}$ was more efficient in inducing disease resistance as well as promoting growth of the rice crop which resulted increased grain yield. This fragment was effective, than full-length peptide, against bacterial blight, rice blast and sheath blight in field with 60 to 90 % decrease of diseases and 27% increase in grain yield (Chen et al., 2008). Harpin, in combination of pathogen specific bacteriophage, decreased the incidence of tomato bacterial spot disease in field, more effectively than that of bactericide copper-mancozeb treatment, illustrates that the cost effectiveness as well as environment-friendly use of harpin protein (Obradovic et al., 2004).

Harpin is known as a plant elicitor from the past several years. But the mechanism of harpin interaction with host plants and mode of action have remained largely unclear. When infiltrated into nonhost plants, harpin trigger disease resistance associated responses, such as HR, expression of PR genes and SAR (Dong et al., 1999). In higher plants the HR is associated with disease resistance and is characterized by the swift localized death of cells with subsequent interaction with a microbial pathogen. It was reported that harpin initiates an intracellular signaling cascade after binding with receptors on the plant cell membrane (Lee et al., 2001), that results in the activation of non-specific plant defense responses. When

applied in plants according to the dosage, harpin activates the PR genes expression, peroxidase (POD), phenylalanine ammonia lyase (PAL) and inducing programmed cell death (PCD). The HR elicited by harpins results in three kinds of effects. The first is improving plant disease-resistance by inducing PCD, activating signaling pathways associated with PR genes expression, JA and PAL, etc. The products of PR genes expression include chitinase, POD, lysozyme, and pathogen aggression directly. PAL associates with the accumulation of lignin, phytoalexins and phenols to enhance plant anti-pathogen ability directly and indirectly (Andi et al., 2001). Second, harpin can stimulate the expression of genes related to growth, such as growth factors and elements of ET signaling pathway, etc. (Akbudak et al., 2006). Harpin also confers resistance to stress of plant by inducing expression of cold-regulated genes and heat shock genes (Bleecker and Kende, 2000).

Bacterial harpin, elicits ROS which eventually lead to HR (He et al., 1993; Chen et al., 2008) in plants. During pathogens infection, harpin-mediated HR is found to be an efficient mechanism related with plant disease resistance. In sweet pepper, one of the photosynthetic ferredoxin types has been identified as plant ferredoxin-like protein (PFLP). It shares 48–75% identity with photosynthetic-type ferredoxin found in other plants, such as tomato, pea, spinach, rice, maize and *Arabidopsis* (Dayakar et al., 2003). This PFLP intensified harpin mediated ROS. It was also observed that PFLP-transgenic tobacco enhanced harpin-mediated HR (Dayakar et al., 2003; Huang et al., 2004). Previous studies have also revealed that the mutant PFLP, defective in the CK2P domain, fails to enhance the harpin-mediated HR in *Arabidopsis* (Lin et al., 2011). The transgenic expression of PFLP in *Arabidopsis* and tobacco lead to the harpin mediated HR and other resistance mechanisms, that are dependent on the protease mediated pathway (Dayakar et al., 2003; Huang et al., 2004; Ger et al., 2014).

Foliar treatment with elicitor molecules could be an alternative to the use of conventional ecologically hazardous chemicals for the control of plant diseases. As a biotic

pesticide, plants need higher dosage of harpin compared to chemical pesticides and continuous application of harpin is required when disease occurs. When sprayed on leaves, only a few harpin molecules would be able to interact with the putative receptors due to the unique architecture of leaves. Thus, bioavailability of harpin gets significantly reduced. To overcome such difficulties, scientific advancement under nanotechnology provide novel tools for management of biotic and abiotic stresses, rapid disease detection and increasing the ability of plants to absorb pesticides or nutrients that can change agriculture and related sectors (Khot et al., 2012; Kah et al., 2014). Nano-encapsulation of pesticides and herbicides could facilitate the successful intrusion through cuticles and tissues, allowing slow and regular discharge of the active substances (Pérez-de-Luque and Rubiales, 2009). Nano-formulations of pesticides and herbicides have been developed with added advantage of increased efficacy, specificity and reduced toxicity during field application (Grillo et al., 2014; Pereira et al., 2014). Similar way we hypothesize that nanoparticles usage can minimize the dose of harpin. Sustained target delivery of harpin in plants can also be achieved.

1.3. Nanotechnology in agriculture:

Nanotechnology, has a very significant impact on world's economy, industry, and people's life. It deals with the physical, chemical, and biological properties of matter considered at nanoscale (1–100 nm), which results in the development of innovative and novel properties like increase in the surface area of the particles that can be utilized to address numerous technical and societal issues.

Agricultural production has been challenged by many diseases, weeds and insect pests resulting in loss of crop yield. For increasing the crop productivity, farmers are using high dose of chemicals which may leads to soil corrosion, deprivation of agro-ecosystems,

deposition of excess chemicals, environmental pollution and insects and pathogens gaining resistance to chemical pesticides (Pimentel, 1995). So, there is a need to seek alternative ways for use of agrochemicals. Changes can include (i) decrease of use of pesticides and fertilizers, (ii) fast and accurate detection of pests, pathogens and nutrient levels in soil, and (iii) cheering soil health by reducing the agrochemicals usage. Nano-based smart delivery systems currently gaining importance in the agricultural sector aid in combating crop pathogens, reducing nutrient losses in fertilization, increasing crop productivity through optimized water, nutrient management and enhancing the efficiency of pesticides at lower dosage rates (Parisi et al., 2015; Fraceto et al., 2016).

A wide scope of budding applications of nanotechnology has been envisaged in agriculture, leading to extensive research in both academic and industrial institutions (Chen and Yada, 2011; Parisi et al., 2015; Thomas et al., 2017). Certainly, the unique properties of substances at nanoscale make them suitable candidates to create and develop novel tools for sustainable agriculture (Fig. 1.1). Nanotechnology derived devices are also being explored in the field of plant breeding and genetic transformation (Torney et al., 2007). One of the most capable and practicable options for handling current challenges for sustainable agriculture and food safety is encapsulating active ingredients, such as herbicides, fertilizers, insecticides, fungicides, and micronutrients in controlled release matrices. Nanoparticles (NPs) encapsulated with active ingredients enhance the efficacy of chemical ingredients, reduces their volatilization, and decreases toxicity and environmental contamination.

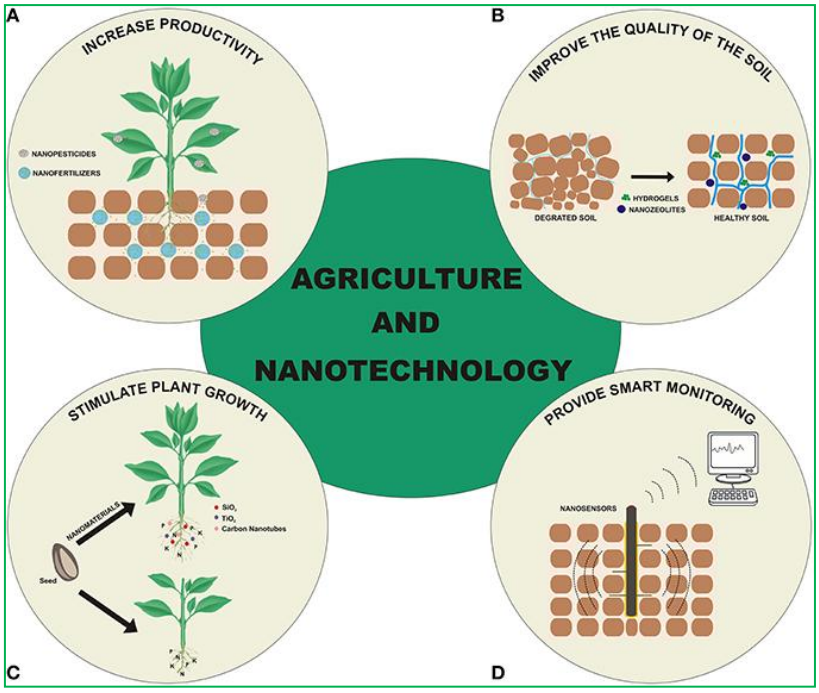
1.3.1. Nanoscale carriers:

Effective release of pesticides, fertilizers, herbicides and plant growth regulators is possible by nanoscale carriers (Ram et al., 2014; Pereira et al., 2017). Polymer based encapsulation by weak ionic bond attachments are in use for efficient delivery, controlled release and improve

the stability of active ingredients against degradation in the environment (Sawant et al., 2006). Low use efficiency is the prominent issue with the use of traditional chemical fertilizers, which increases the application cost and causes environmental pollution (Wilson et al., 2008). Nanomaterials with large surface area could solve this problem due to their nanosize, ensuring their surface protection, controlled release, and ultimately boosting up their use efficiency. Compared to ordinary fertilizers, nano-fertilizers proved to be more effective in reducing nitrogen loss due to emissions and leaching. Moreover, controlled release of fertilizers minimizes the over usage of chemical fertilizers and thereby reduces the related toxic effects (Pereira et al., 2015). Biodegradable chitosan NPs have been used for controlled release of the fertilizers like urea, potassium chloride and calcium phosphate (Corradini et al., 2010).

1.3.2. Nanoparticles (NPs) and plant protection:

The application of nanotechnology as well as the introduction of nanomaterials in agriculture, can greatly contribute to address the issue of sustainability. In fact, the efficient use of fertilizers and pesticides can be enhanced by the use of nanoscale carriers and compounds, reducing the amount to be applied without impairing productivity. By using nano materials like nanosilica, the targeted genes can be easily transferred into the cells (Torney et al., 2007). This technique could also be used in the formulation of pesticides, insecticides, and insect repellents (Barik et al., 2008). Moreover, pesticides formulation against various insect pests can be done by nanoemulsions like oil in water (Gan and Wang, 2007). Pesticide loaded



Fraceto et al. (2016) Frontiers in Environmental Science

Fig 1.1: Potential applications of nanotechnology in agriculture.

(A) Increase the productivity using nanopesticides and nanofertilizers; **(B)** Improve the quality of the soil using nanozeolites and hydrogels; **(C)** Stimulate plant growth using nanomaterials (SiO_2, TiO_2, and carbon nanotubes); **(D)** Provide smart monitoring using nanosensors by wireless communication devices.

porous hollow silica nanoparticles have been employed successfully as an efficient and controlled release water soluble formulation. Nano-silica has been utilized as a sole nano-insecticide due to its ability to absorb into the insect cuticular layer which acts as a protection barrier (Barik et al., 2008). NPs can be effectively used to manage a variety of agricultural insect pests and ectoparasites of animals as they are small in size, possess modified surface charge and hydrophobicity (Schurmann et al., 2005).

Introduction of nano-encapsulation of chemicals like, insecticides, herbicides and fertilizers is another remarkable feature of nanotechnology. Nano-encapsulation ensures not only the targeted delivery of the chemical but also helps in controlled release and the processes like diffusion, dissolution, biodegradation, and osmotic pressure with specific pH are the basics for the controlled release of the nano-chemicals (Torney et al., 2007). Nano-encapsulation could facilitate the successful intrusion of herbicides through cuticles and tissues, allowing slow and sustained delivery of the active substances (Pérez-de-Luque and Rubiales, 2009). Thus, nano materials have been proved to be effecient carrier materials in crop plants for prolonged delivery of pesticides. In comparison with commercially available insecticides, NPs provides cost effective and an eco-friendly control of pathogenic microbes. These studies may increase the frontiers for nanoparticle based technologies in pest management (Yoon et al., 2007). With the growing public concern on the nanotoxicity and its direct or indirect impact on environment, extensive research is required for application of biosynthesized nanoparticles in agriculture (Mishra et al., 2017).

1.4. Chitosan for crop protection:

Chitosan, poly [β-(1–4)-linked-2-amino-2-deoxy-d-glucose], is an N-1deacetylated product of chitin, the chief component of arthropod and crustacean shells, such as shrimps, lobsters, cuttlefishes and crabs. Chitosan has many significant biological and chemical properties, such

as biodegradable, biocompatible, bioactive, and polycationic in nature. The use of chitosan as an antimicrobial agent and also an effective plant elicitor of SAR against pathogens (Iriti and Varoni, 2015) was reported. Chitosan alone induce defense responses in plants like strawberry (Ghaouth, 1992) and tomato (Benhamou et al., 1997). Chitosan can activate innate immunity by stimulating production of hydrogen peroxide (H_2O_2) in rice (Lin et al., 2005) and NO in tobacco (Zhang et al., 2011). Chitosan can also trigger defense-related gene expression like change in the expression of genes related to jasmonic acid–ethylene (JA/ET) signalling and mitogen-activated protein kinases (MAPKs) which cause changes in protein phosphorylation (Chen and Yada, 2011). Chitosan when applied on plants enhances the efficacy of biological control agents, for pathogen control (Abro et al., 2014; Vallance et al., 2011). Chitosan was shown to be fungistatic against both necrotrophic and biotrophic fungal pathogens. Thus, it is evident that chitosan is more effective and can be used in several ways to reduce the severity of disease in plants and to improve the crop productivity in an eco-friendly and sustainable manner.

1.4.1. Chitosan as a potential delivery system:

Chitosan is one of the most extensively used polymers and has many significant biological and chemical properties, such as biodegradable, biocompatible, bioactive, polycationic and solubility in aqueous acidic media (Sinha et al., 2004). As it is a weak base, the chitosan is not soluble at physiological pH. Chitosan synthesized by deacetylating chitin through amide hydrolysis using NaOH or through enzymatic hydrolysis in the presence of chitin deacetylase (Suh and Matthew, 2000). Chitosan's amine groups readily complex with a variety of oppositely charged polymers such as sodium salt of poly (acrylic acid), xanthan, carboxymethyl cellulose, carrageenan, pectin, alginate etc. (Sonia and Sharma, 2011). Chitosan provides flexibility for development of variety of formulations as it is obtained with different degrees of acetylation and molecular weights (500–1400 kDa). Chitosan in the form

of NPs gets easily absorbed by plant surfaces (e.g. leaf and stems) and helps in prolonging the contact time between agrochemicals and the target absorptive surface. Chitosan NPs also improve the molecular bioavailability of the active ingredients penetrating through the cell membrane and enhancing absorption effect (Nagpal et al., 2010). Taken together, these advantages indicate that chitosan has an intense future as a nano-carrier in the field of sustainable agriculture.

1.4.2. Approaches for preparation of chitosan nanoparticles (CSNPs):

Various techniques are used to synthesize CSNPs (Fig. 1.2) *viz*., ionotropic gelation, emulsion cross-linking, emulsion droplet coalescence, precipitation, reverse micellar method, sieving and spray drying (Kashyap et al., 2015). The selection of method for synthesis of CSNPs is based on requirements such as the particle size and shape, release time of the active ingredients, thermal stability and residual toxicity of the final product. Among these methods ionotropic gelation was most efficient due to simple and mild usage of chemicals for cross-linking. This procedure will reduce the side effects of toxic chemicals or reagents and has an excellent capacity to associate with the proteins.

1.5. Outline of the thesis:

To explore the ways in which NPs enhance bioavailability of harpin$_{Pss}$ to induce defense responses in plant, we studied and compared the responses of tomato to the harpin$_{Pss}$ with harpin$_{Pss}$-loaded CSNPs (H-CSNPs). We described the histological, biochemical and molecular mechanisms in relation to the activation of defense response during the interaction of tomato with harpin$_{Pss}$ and H-CSNPs. Harpin$_{Pss}$ has a crucial role in both host immunity and growth of plants, but the cellular localization of protein and molecular details of the signalling mechanism are poorly understood. In this work, we studied the cellular localization of harpin$_{Pss}$ by transient expression of harpin$_{Pss}$ in tomato leaves. The expression and

subcellular localization of harpin$_{Pss}$ was analyzed by confocal microscopy. For this study we framed three major objectives.

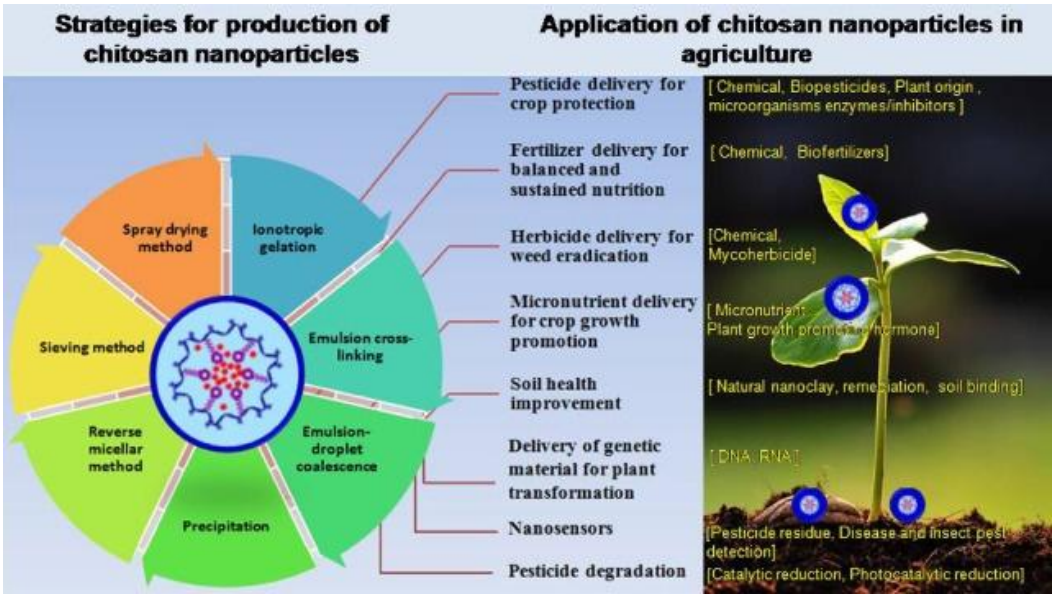


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Fig 1.2: Strategies for the production of chitosan naoparticles and their applications as a delivery system in agriculture.

1.6. Objectives:

The work is sub-divided into three parts as given below:

- to prepare, test and characterize H-CSNPs for induction of disease resistance in tomato,
- to track route of entry of CSNPs and localization of harpin$_{Pss}$ in tomato leaves, and
- to identify interacting protein partner of harpin$_{Pss}$ in tomato.

Materials & Methods

2.1. Materials and plants:

Water soluble chitosan (MW: < 200 kDa with 90% degree of deacetylation) was a gifted from Mahtani Chitosan (India). Sodium tripolyphosphate (TPP) was obtained from Loba chemie Pvt. Ltd and BSA from Himeida (India). Molecular biology kits and enzymes were procured from Simga-Aldrich (USA), Qiagen (Germany), MBI Fermentas (Germany) and Takara bio (Japan). All other chemicals used in the work were of analytical grade and obtained from Sigma-Aldrich (USA), GE health care (USA), Promega Life Science (USA), Fermentas (Germany), Himedia (India), and Qualigens fine chemicals (India). Tomato (*Lycopersicon esculentum* cv. Arka vikas) plants were grown in soil in a growth chamber with a 16 h photoperiod at 24^0C and constant (70%) humidity.

2.2. Harpin$_{Pss}$ expression and purification:

The *hrp*Z gene (1.02 kb) encoding full length harpin$_{Pss}$ was cloned under *Nde* I and *Xho* I sites of pET 28a vector as described earlier (Tarafdar et al., 2009). *Escherichia coli* BL 21 (DE3) cells transformed with pET28a-*hrp*Z were grown in Luria Bertani (LB) broth containing kanamycin (50 µg mL^{-1}) to OD_{600}~0.5 and induced with 0.5 mM isopropyl thiogalactoside. Harpin$_{Pss}$ was purified using Ni-NTA column. The concentration of harpin$_{Pss}$ was determined using BCA protein assay.

2.3. Preparation of CSNPs and harpin$_{Pss}$-loaded CSNPs:

CSNPs were prepared as described by Zhang et al. (2004) with slight modification. To 5 mL of 0.1% (w/v) CS, pH 5.5, 1.0 mL of 0.1% TPP (w/v) was added, drop-by-drop, with continuous stirring on magnetic stirrer at 1200 rpm to avoid agglomeration of NPs. This resulted in an opalescent solution indicating the formation of CSNPs. The CSNPs were separated at 20,000 g for 30 min, washed twice and re-suspended in milliQ water. Harpin$_{Pss}$-

loaded CSNPs (H-CSNPs) were prepared by mixing Harpin$_{Pss}$: CS weight ratio as 0.1:1, *i.e.*100 µg mL^{-1}.

2.4. Physicochemical characterization and stability of NPs:

2.4.1. Physicochemical characterization of CSNPs:

The particle size and surface morphology of the NPs were analyzed using field emission scanning electron microscopy (FE-SEM). To determine the functional groups involved in reduction and stabilization between the polymer and the protein molecules, samples for fourier transform infrared spectroscopy (FTIR) analysis were prepared by grinding 1 to 2 mg of lyophilized NPs suspension along with 100 mg KBr and pelletized using the hydraulic press at 20,000 prf.

2.4.2. Stability of CSNPs:

The physicochemical stability of the NPs was evaluated using measurements of size, zetapotential, encapsulation efficiency and *in vitro* release of harpin$_{Pss}$ with the NPs stored for a period of 90 days. The samples were analyzed in triplicate at 26 ± 2^{0}C.

The entrapment efficiency of harpin$_{Pss}$ within the CSNPs was determined by pelletizing the sample at 20,000 g for 20 min. The amount of free harpin$_{Pss}$ in the supernatant was analyzed at A_{562} using the micro BCA protein assay (as above) and resolved on 12% SDS-PAGE. The supernatant of non-loaded CSNP suspension was used as a blank. Entrapment efficiency was calculated based on the ratio of the amount of protein present in the NPs to the amount of harpin$_{Pss}$ used for loading.

In vitro release profile of harpin$_{Pss}$ from CSNPs was measured by direct dispersion method (Anitha et al., 2011). Initially, free harpin$_{Pss}$ in the supernatant was removed by centrifugation at 30,000 g for 40 min and the pellet was transferred to another tube containing phosphate

buffer saline (pH 7.4) and measured the release of harpin$_{Pss}$ at an interval of 24 h up to 120 h at A_{562}.

2.5. Enzyme assays:

Four week-old tomato plants were sprayed with harpin$_{Pss}$ and H-CSNPs to observe the efficiency of CSNPs in delivering harpin$_{Pss}$ to elicit the defense responses in tomato. The final concentration of protein used for all treatments in this experiment was 100 µg/mL. Leaf samples were collected at regular intervals of 24 h, up to 96 h, immediately frozen in liquid N_2 and stored at -80^{0}C. The induced defense response was assessed by assay of defense-related enzymes. All the experiments were performed in triplicates.

2.5.1. Preparation of leaf extract:

All steps were carried out at 4^{0}C as per the procedure described earlier (Rani et al., 2015). Tomato leaf tissue (0.5 g) was crushed in liquid N_2 and extracted with 2 mL of 100 mM homogenization buffer (100 mM potassium phosphate pH7, 0.5 mM EDTA, 0.1 mM PMSF and 2% PVP). The suspension was homogenized for 1 min and centrifuged at 10000g for 30 min. The supernatant was used as plant extract for assay of defense related enzymes like peroxidase (POD) and phenyl alanine ammonia lyase (PAL).

2.5.2. Peroxidase (POD):

POD was assayed in a reaction mixture consisting of 5 mM guaiacol, 50 mM sodium acetate buffer (pH 7.0), 0.03% H_2O_2 and the enzyme extract (0.1 mL) with a final volume of 1 mL. Increase in absorbance due to oxidation of guaiacol to tetraguaiacol was measured at A_{470} as described by Rani et al., (2015). The POD activity was calculated using molar extinction co-efficient of 26.61 $mM^{-1}cm^{-1}$ for tetra-guaiacol at 470 nm.

2.5.3. Phenylalanine ammonia lyase (PAL):

PAL was assayed in a reaction mixture consisting of 200 mM Tris-HCl (pH 7.0), 20 mM L-phenylalanine and the enzyme extract (0.1 mL) with a final volume of 1 mL. The reaction was carried out at 37^0C for 60 min. Increase in absorbance due to formation *t*-cinnamic acid was measured at A_{290} as described earlier (Podile and Laxmi, 1998). The PAL activity was calculated using molar extinction co-efficient 17.4 $mM^{-1}cm^{-1}$ for *t*-cinnamic acid at 290 nm.

2.6. Experimental design and GeneChip analysis for transciptome study:

2.6.1. Sample collection:

All samples were collected in two independently repeated experiments at 24, 48 and 72 h post spray application (Fig. 2.1). For each sample, leaf material was harvested from three plants treated with harpin$_{Pss}$, H-CSNPs and CSNPs and pooled separately and flash frozen in liquid nitrogen. The final concentration of protein taken for all treatments in this experiment was 100 µg/ml. Leaves collected from buffer-inoculated plants were used as the reference sample to which all other samples were compared.

Total RNA was isolated from 100 mg of the leaf tissue using Qiagen's RNeasy Plant Minikit. RNA quality and quantity were analyzed with a NanoDrop spectrophotometer and an Agilent 2100 bioanalyzer prior to GeneChip hybridization. RNA with RNA integrity number (RIN) ≥7.0 was considered to be of good quality. RNA labeling, hybridization and analysis were done as described by Uma and Podile, (2014).

2.6.2. RNA labelling:

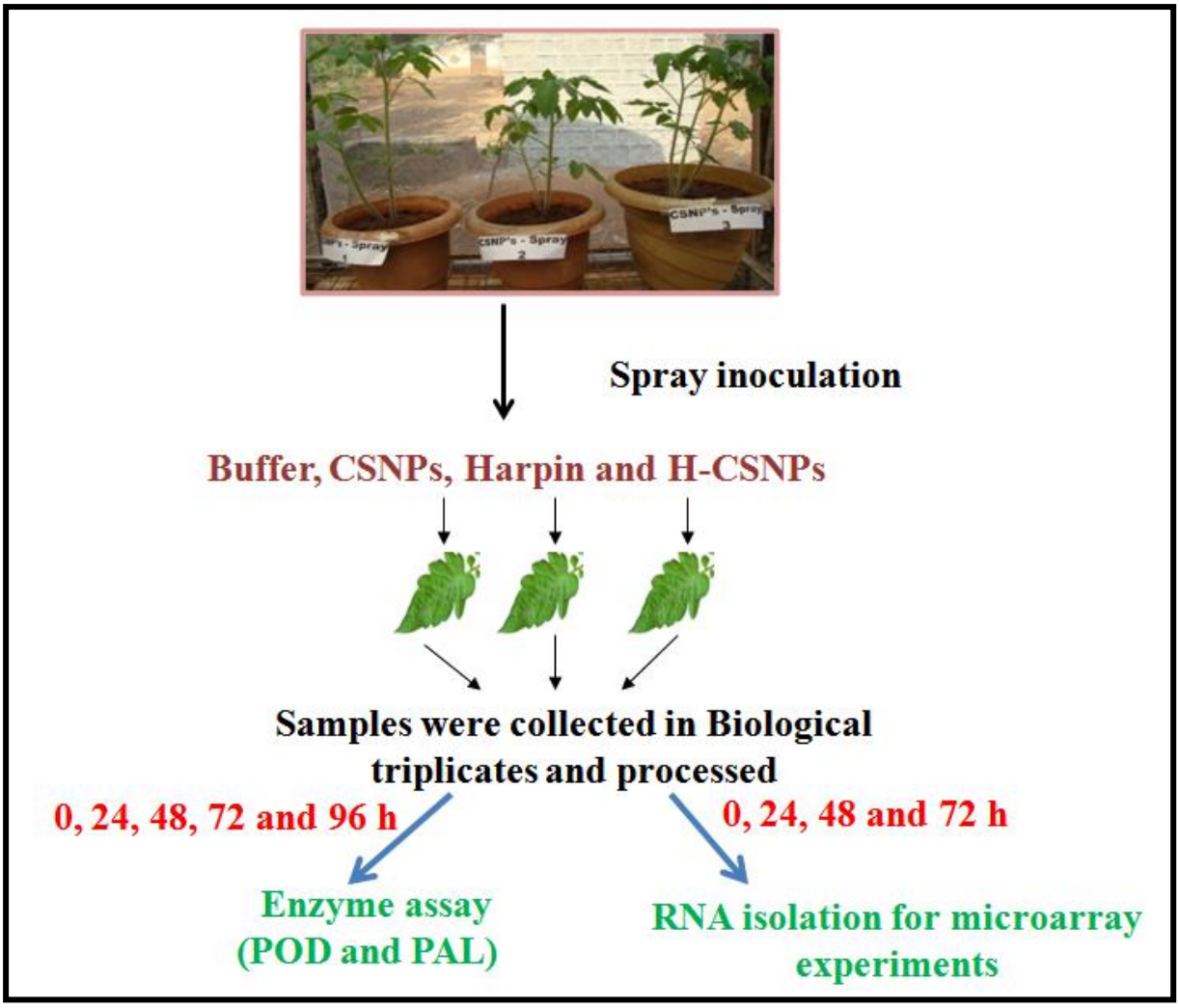


Fig 2.1: Experimental design for the transcriptome and biochemical analysis of tomato.

Three different sets of tomato plants consisting three plants per set were individually treated with harpin$_{Pss}$, CSNPs and H-CSNPs. Leaf samples were collected from all three plants of each set and total RNA was isolated and pooled to minimize the background noise. Buffer treated leaves served as control.

Double-stranded cDNA was synthesized from poly(A)+ mRNA present in the total RNA using MMLV-reverse transcriptase (Agilent Quick Amp Kit, USA) and a primer encoding a T7 RNA polymerase promoter sequence fused to (dT) 24. The double-stranded cDNA was purified and used as a template in the subsequent *in vitro* transcription reaction. Fluorescent complimentary RNA (cRNA) was generated from cDNA for one-color processing using Agilent's Quick Amp Labeling Kit (USA). The amplification of cRNA was carried out in the presence of T7 RNA polymerase, cyanine 3-labeled CTP and NTPs mix.

2.6.3. Hybridization and data collection:

The labeled target cRNA was purified, fragmented, and hybridized to a whole genome tomato 4X44K AMADID: 22270 gene chip arrays according to protocols provided by the manufacturer (Agilent, USA). Fragmentation of labeled cRNA and hybridization was done using the Gene expression hybridization kit of Agilent (Part Number 5188-5242). Hybridization was carried out in Agilent's surehyb chambers at 65 °C for 16 h. The hybridized slides were washed using Agilent's gene expression wash buffers (Part No. 5188-5327) and scanned using the Agilent microarray scanner G Model G2565BA at 5 µ resolution.

2.6.4. Data analysis:

The scanned images were manually verified and found to be devoid of uneven hybridization, streaks, blobs and other artifacts. Hybridization across the slide was good based on number of feature that were "g is PosAndSignif" which indicates feature is positive and significantly above background. Feature Extraction (FE) 9.5.3 supported extraction of one-color .tif images of Agilent microarrays scanned on Agilent Scanner. Normalization was done using Gene Spring GX version 12.0 software. Intra-array normalization, which deals with variability within a single array, was done among the controls using Percentile Shift

Normalization method. In intra-array normalization, gProcessed signal (dye normalized background subtracted signal intensity) was log transformed, and for each of the array, the 75th percentile value was calculated separately. In each sample, the log transformed intensity value for each probe was subtracted by the calculated 75th percentile value of the respective array and expression values were obtained. Feature extracted data was analyzed using Gene Spring GX version 12.0 software from Agilent (USA). Signal quantification and data analysis were achieved using Gene Spring GX version 12.0 software. Following local background subtraction, the signal for each spot was normalized based on the median value of the median intensity of all the spots for each array.

Genes for which the hybridization signal was greater than the average value plus two standard deviations of the controls only were analyzed. Each ratio was converted to its $\log_2$ value, and the average $\log_2$ value for each gene of the two independent arrays corresponding to each experiment was calculated. Statistical significance of the gene expression differential over the course of the replicate experiments was calculated by using a Student's t test analysis. Only genes with high levels of significance ($P < 0.05$) and a minimum absolute value of $\log_2 > 2$ were systematically considered in this study, to minimize the false positive as up- or down-regulated. Expression profiles from each time point were clustered based on their similarity in expression pattern using a hierarchical average linkage clustering algorithm and Pearson correlation distance.

2.6.5. Annotation of probe set:

Differentially expressed transcripts were annotated using the BLAST hit from the non-redundant database of NCBI (http://www.ncbi.nlm.nih.gov) against *Solanum tuberosum* total genome. For gene ontology, we used potato gene model for each probe set.

2.7. Labeling of harpin$_{Pss}$ and CSNPs to track NPs:

2.7.1. Fusion of *hrpZ*$_{Pss}$ and *GFP*:

Overlap extension/fusion PCR was performed to generate *hrp*Z and *GFP* fusion chimeras. Plasmid templates p*hrp*Z-pET28a (+) and p*GFP*-pET28a (+) with appropriate combinations of primers were used to generate harpin$_{Pss}$ fusion chimeras. Based on the fusion of auxiliary domains to either 'N' or 'C' termini of the *hrp*Z gene, the chimeras were designated as *GFP-hrpZ* and *hrpZ-GFP*. The amplicons (*GFP+hrpZ* and *hrpZ+GFP* of 1.75 kb) were double-digested and ligated to *Eco*R I & *Xho* I sites of pET-28a (+). All the ligation reactions were performed at 16°C for 16 h, using T4 DNA ligase. Highly efficient competent cells of *E. coli Rosetta-gami* II (DE3) were used for transformation. Positive clones were selected on appropriate antibiotic plates and confirmed by both double digestion and sequencing.

2.7.2. Heterologous expression and purification:

E. coli Rosetta-gami cells containing appropriate *HrpZ* and *GFP* fusion chimeras were used for over expression of proteins. Single colony was inoculated on to 5 mL of LB broth with antibiotics and incubated at 37°C over night with shaking. Overnight culture was diluted 50 times with fresh LB broth with antibiotics and incubated at 37°C. After reaching OD_{600} to 0.5, cells were induced with 500 µM IPTG to express the proteins. Cells were harvested after 4 h of induction and washed with 100 mM Tris-Cl pH 8.0 with 500 mM NaCl and resuspended in same buffer. Cells were disrupted by sonication and insoluble debris was removed by centrifugation. Protein preparations were compared with un-induced controls to check the protein expression by SDS-PAGE.

Five ml of Ni-NTAgarose (Sigma-Aldrich) was packed in a polypropylene column and equilibrated with 25 mL of Tris-Cl buffer with 10 mM imidazole. Sonicated supernatant was applied to the column and flow through was stored at -20°C. The column was washed with 20 mL of washing buffer (20 mM imidazole). Finally, the protein was eluted with 15 mL of

elution buffer (200 mM imidazole) and fractions were collected. Purity of the protein was analyzed by SDS-PAGE. The purified protein in 50 mM sodium phosphate, pH 7.4, was used for localization studies.

2.7.3. Labeling of chitosan with rhodamine-123:

Five mL of 0.1% CS solution was incubated for 1 h with 25 µL of sterile rhodamine-123 (1 mg mL^{-1}), the pH was adjusted to 5.5 using 10N NaOH (Snima et al., 2012). Later NPs were prepared by drop-wise addition of 0.1% (w/v) TPP into CS solution till the solution became opalescent. GFP-HrpZ-loaded RCSNPs (GH-RCSNPs) were prepared by taking GFP-HrpZ: CS weight ratio as 0.1:1, *i.e.*100 µg ml^{-1}. The preparation of NPs was similar (described above) with the addition of GFP-HrpZ and rhodamine labeled CS, in place of $harpin_{Pss}$ and CS, respectively.

2.7.4. Sub-cellular localization of nanoparticles and proteins:

Four week-old tomato leaves were infiltrated with 1 mL of GFP (20 µg mL^{-1}), GFP-HrpZ (20 µg mL^{-1}) and GFP-HrpZ RCSNPs (GH-RCSNPs)**.** After 24 h, the abaxial side of the leaf was directly visualized under a laser scanning confocal microscope (ZEISS LSM 880). The localization of GFP fused $harpin_{Pss}$ was visualized with sequential imaging of GFP at 488 nm excitation and 505–525 nm emission and chloroplast auto fluorescence at 633 nm excitation and 660 nm emission.

2.8. Deletion of chloroplast transit peptide (ΔN_{1-30}) and cloning of *hrpZ-GFP* or ΔN_{1-30}*hrpZ-GFP* fusion gene in binary vector pCB302 and *in planta* transient expression:

Binary vector pCB302 was used for the construction of translational *hrpZ-GFP* or ΔN_{1-30}*hrpZ-GFP* fusion. Chimera was made as 'N' or 'C' terminus of *hrpZ* or Δ*hrpZ* fused with *GFP* and primers were designed with appropriate restriction sites (*NcoI* and *Xba I*)

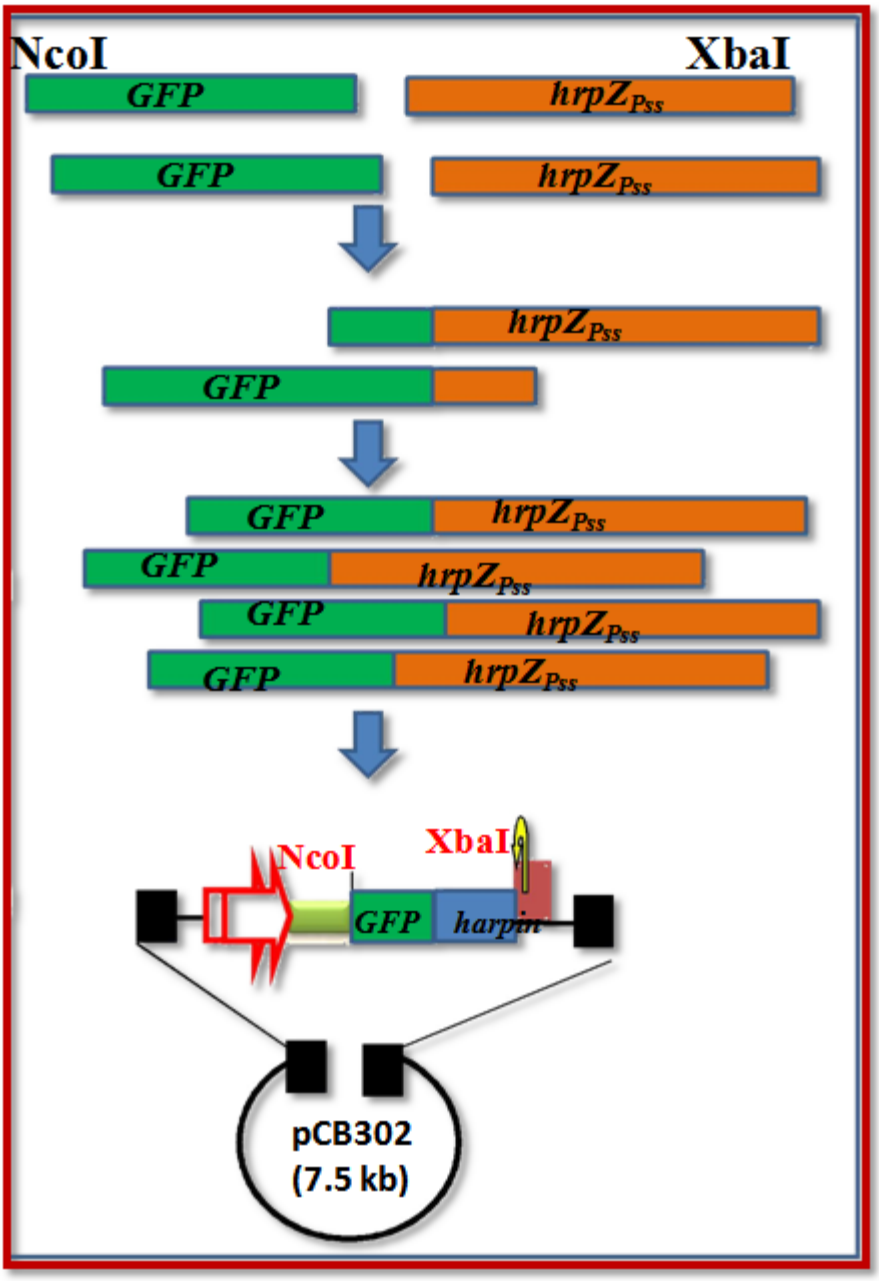


Fig 2.2: Overview of cloning of *GFP-hrpZ* fusion gene in binary vector pCB302.

Individual genes (*hrpZ* and *GFP*) were amplified using gene specific primers. Overlap-extension PCR was performed using individual *hrpZ* and *GFP* as templates with overlapping region. Resulting fusion product was cloned into a binary vector pCB302 in *NcoI* and *XbaI* restriction sites.

compatible to binary vector (Fig. 2.2). Directional cloning was performed and *E. coli* DH5α was transformed with recombinant plasmid. Clones were confirmed by colony PCR, restriction digestion and sequencing. *Agrobacterium tumifaciens* EHA105 was mobilized with plasmid of *hrpZ-GFP* and ΔN_{1-30}*hrpZ-GFP* and selected on Rif+Kan plate and pCB302 with *GFP* alone served as EVP (empty vector preparation). Single colony of *hrpZ-GFP*/ΔN_{1-30}*hrpZ-GFP* was inoculated in LB broth with antibiotics, incubated at 28°C with shaking. Overnight grown culture was diluted with fresh LB at 1:100 dilution and incubated at 28°C with agitation for 18 h. Cells were harvested by centrifugation, re-suspended in infiltration buffer (10 mM MES pH 5.4, 10 mM $MgCl_2$ with 150 µM acetosyringone) to a final OD_{600} of 0.8 and incubated at 28°C for 6 to 8 h. Culture suspension was infiltrated into the leaf of five fully expanded leaf stage tomato plants. Leaf samples were collected after 48 h and the localization of GFP fusions was visualized by laser scanning confocal microscopy, using a ZEISS LSM 880 microscope, with sequential imaging of GFP at 488 nm excitation and 505–525 nm emission and chloroplast auto fluorescence at 633 nm excitation and 660 nm emission.

2.9. Defense responses:

2.9.1. Harpin/H-CSNPs induced cell death in tomato leaves:

Purified recombinant $harpin_{Pss}$ was used to evaluate the HR and cell death. Five to six-leaf stage green house-grown tomato (*S.lycopersicum* 'Arka vikas') leaves were infiltrated with $harpin_{Pss}$, H-CSNPs, CSNPs, $\Delta N_{1-30}harpin_{Pss}$ and ΔN_{1-30}H-CSNPs using hypodermic syringe. The final concentration of protein taken for all treatments in this experiment is 20 µg/ml. Buffer infiltration alone served as negative control. The plants were maintained at 28°C. After 48 h the HR developed in control was compared with the above treatments.

Simultaneous detection of H_2O_2 accumulation and cell death in a single tomato leaf was done by a combination of DAB (3, 3'-diaminobenzidine tetrahydrochloride dihydrate) and evans blue staining methods as described by Pogany et al. (2009). Intact leaves were infiltrated with 2.5 mM DAB solution (dissolved in 2.5 mM TRIS-phosphate buffer, pH 7.8). DAB-infiltrated leaves were incubated in light for 2 h and further vacuum-infiltrated with 1.3 mM Evans blue (Fluka) solution (0.125 g of Evans blue dissolved in 100 mL of distilled water). After 15 min of incubation in the dark, leaves were decolorized by soaking in a clear solution (80% ethanol and 20% chloroform) containing 0.15% trichloroacetic acid for 24 h.

2.9.2. Measurement of *Rhizoctonia*-infection in tomato:

The tomato plants, treated with harpin$_{Pss}$, H-CSNPs, CSNPs, ΔN_{1-30}harpin$_{Pss}$ and ΔN_{1-30}H-CSNPs and buffer treated plants served as control. The final concentration of protein taken for all treatments in this experiment was 100 µg/ml. Three leaves from each plant were excised and sterilized with 70 % alcohol for 10-20 sec, washed twice with sterile water and placed on moist sterile whatman no 3 paper in petri plates under sterile conditions in dark. *Rhizoctonia solani* agar plugs of 5 mm diameter were excised from fully grown fungal mycelium were placed on adaxial surface of the leaves and incubated in dark at 28°C. Symptoms were evaluated seven days post inoculation (dpi) and representative images were acquired (Dey et al., 2014).

2.9.3. Estimation of chlorophyll content:

Five to six-leaf stage green house-grown tomato leaves were treated with harpin$_{Pss}$, H-CSNPs, CSNPs, ΔN_{1-30}harpin$_{Pss}$ and ΔN_{1-30}H-CSNPs. The final concentration of protein taken for all treatments in this experiment was 100 µg/ml. Buffer treatment alone served as negative control and the plants were maintained at 28°C. After 48 h leaves were detached for

chlorophyll extraction. Chlorophyll was extracted from the leaves and estimated according to the method of Arnon. (1949).

Extraction:

About 400 mg of fresh leaf material was ground with 10 ml of 80% acetone at 4°C in a pestle and mortar and centrifuged at 2500 g for 10 min at 4°C. The residue was re-extracted with 80% acetone until the green color disappeared in the residue and the extracts were pooled and transferred to a graduated tube and made up to 20 ml with 80% acetone and assayed immediately.

Estimation:

About 3 ml aliquots of the extract were transferred to a cuvette, and the absorbance was read at 645, 663 and 480 nm in a spectrophotometer against 80% acetone as a blank.

Chlorophyll content was calculated using the formula of Arnon (1949)

Total chlorophyll (mg/ml) = $(0.0202 \times A_{645}) + (0.00802 \times A_{663})$

Chlorophyll "a" (mg/ml) = $(0.0127 \times A_{663}) - (0.00269 \times A_{645})$

Chlorophyll "b" (mg/ml) = $(0.0229 \times A_{645}) - (0.00468 \times A_{663})$

2.9.4. Defense-related gene expression analysis:

All samples were collected in three independently repeated experiments at 6, 12 and 24 h post infiltration (hpi). For each sample, leaf material was harvested from three plants infiltrated with harpin$_{Pss}$, H-CSNPs, CSNPs, ΔN_{1-30}harpin$_{Pss}$ and ΔN_{1-30}H-CSNPs, and flash frozen in liquid nitrogen. The final concentration of protein taken for all treatments in this experiment is 20 µg/ml. Leaves collected from buffer-infiltrated plants were used as the reference sample to which all other samples were compared. Total RNA was isolated from 100 mg of the

frozen leaves using Qiagen's RNA plant kit. The quality and concentration of RNA samples were examined by ethidium bromide-stained agarose gel electrophoresis and NanoDrop. A total of 7 defense related genes were selected to study their expression pattern in treated tomato leaves.

Reverse transcription (RT) PCR was performed using cDNA synthesis kit from Takara Bio (Japan) as per the suggested protocol. Briefly, 4 µg of total RNA, 1 µl of 50 µM Oligo-dT primer and 1 µL of 10 mM dNTP were mixed and made to a toal of 10 µL using nuclease-free water. The mixture was incubated at 65°C for 5 min and cooled immediately by keeping in ice bath. To the mixture 4 µL of 5X RT buffer, 1 µL of Blue Print RTase and 0.5 µL of recombinant RNase inhibitor (40 U μL^{-1}) were added and made up to 20 µL using nuclease-free water. The reaction mixture was incubated at 37 °C for 10 min followed by 60 min at 42 °C. The enzyme was inactivated by heat denaturation at 95 °C for 5 min. One micro litre of RT reaction mixture was used as a template for PCR using gene specific primers (Table 2.1). PCR was carried out in 25 µL containing 200 µM dNTP mix, 2 mM MgCl2, 0.8 pmol each of forward and reverse primers, 1X PCR buffer and 1 unit of *Taq* polymerase. PCR was performed with varying annealing temperatures with cycling parameters of 94°C for 3 min for initial denaturation followed by 40 cycles of 94°C for 1 min (denaturation), 1 min for annealing with gene specific temperature and 72°C for 1 min (extension), a final extension at 72°C for 10 min in an Eppendorf Mastercycler Gradient Thermal cycler, Germany. PCR products were resolved in 1.2% TAE agarose gel and compared with loading control actin.

2.10. Identification of protein interacting partner of harpin$_{Pss}$ in tomato:

2.10.1. Isolation of total leaf proteins of tomato and *in vitro*-pull down assay:

S. No	Gene product	Forward primer (5' →3')	Reverse primer (5' →3')
1	PAL	CGACTTGAGGCATTTGGAGGA	CAGGGGTCATCAGCATAGGT
2	POD	GCTGACATTCTTGCTCTTGCT	CGCTTGAAACTCGTCCATCT
3	Actin	TGATGGTGGGTATGGGTCAA	AGGGGCTTCAGTTAGGAGGAC
4	CHIT	TCACACAACTACAACTATGGGC	GCTTTGGGGATTGAGGAGTCA
5	PPO	ATTGGCGGGAAAAGAAGGGA	GTGGGCATTGGCGGGTAA
6	MYB	AGATGGAGGAGTTAGCAATGATG	ATGTGAATGGAGGAGGACTTG
7	WRKY	CAAGAATCCAGCCAAGCAAATG	CCCGTTCGTCAATGTCCCTTC
8	FNR	GGTTGGAATCCATAAATATC	AAAAAAGAACAAGATATT

Table 2.1: List of genes and respective primers used for the RT-PCR analysis of defense-related transcripts altered during harpin$_{Pss}$-induced resistance.

Total protein was extracted in homogenization buffer containing 50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 20 mM 2-mercaptoethanol and the EDTA-free complete miniprotease inhibitor cocktail (Roche). Insoluble debris was removed by centrifugation at 14,000 *g* for 10 min at 4°C and in supernatant protein concentration was quantified (Rani et al., 2015).

Pull-down assay using his-tagged harpin$_{Pss}$ was performed in binding buffer (20 mM HEPES, pH 7.6, 150 mM KCl, 5 mM MgCl2, 10 mM imidazole, 0.005% [v/v] Tween 20, 10% [v/v] glycerol, and 1 mM DTT). In parallel, 10 µg of harpin$_{Pss}$ was immobilized in 20 µL of Ni-NTA agarose for 1 h with rotation, at 4°C in binding buffer. To pull down the interacting protein, approximately 200 µg of leaf protein was incubated with the immobilized harpin$_{Pss}$, overnight, with rotation at 4°C, in a final volume of 1 mL of binding buffer. Then, the Ni-NTA agarose was carefully loaded on a column and washed with 3 mL of binding buffer. The bound proteins were eluted with 100 µL binding buffer supplemented with 300 mM imidazole after short 5 min incubation. A similar assay was conducted in the absence of the His-tagged protein in order to assess for any nonspecific interaction between total protein and the Ni-NTA agarose. Eluted proteins were analyzed by coomassie brilliant blue staining and differential bands were subjected to MALDI-TOF analysis.

2.10.2. Confirmation of interaction of harpin$_{Pss}$ with FNR using yeast two-hybrid system:

Full-length PCR-amplified products of *HrpZ* and *FNR* were inserted in the Gal4 activating domain of yeast vector (pGAD-C1) and binding domain of yeast vector (pGBDU-C1), respectively, and transformed into competent PJ269-C yeast cells. The transformed mixture was plated on double-dropout (-uracil, -leucine) medium as a control for measuring

transformation efficiency and then plated on triple dropout (-uracil, -leucine, -histidine) medium to confirm the protein-protein interaction, and assessed after 2–4 days at 30°C.

Results

3.1. Preparation and characterization of CSNPs:

CSNPs were prepared using ionic gelation method. The addition of TPP to chitosan (CS) solution at pH 5.5 facilitated electrostatic interaction between amino groups of CS and anionic TPP. The CS/TPP (5/1) ratio led to efficient cross-linking of amino groups producing the most compact particle structure. The size of the spherical CSNPs was in the range of 100±10 nm (Fig. 3.1A). Later the harpin$_{Pss}$ was entrapped within the NPs by cross-linking with the TPP. The size of the H-CSNPs was in the range of 120±10 nm (Fig. 3.1B). Zeta potential of CSNPs and H-CSNPs showed +32 mV and +49 mV, respectively suggesting a high degree of stability. Increase in zeta potential in the presence of harpin$_{Pss}$, indicated that the presence of harpin$_{Pss}$ probably led to better stabilization of the charges and the groups responsible for intermolecular interactions among the components of the NPs.

Several characteristic features were identified in the FT-IR spectrum of pure CS (Fig. 3.2A). At 3452 cm^{-1}, the characteristic peak of the hydroxyl group (OH) was recorded, overlapped with N–H stretch. At 1645 cm^{-1} the characteristic peaks of CS with high intensity that corresponds to the vibration of amide I. At 1384 cm^{-1}, the C–N stretch was recorded and finally at 1093 cm^{-1}, the peak of C–O stretch group appeared. In the spectrum of CSNPs prepared with TPP (without protein), the amino group absorption shifted at 1640 cm^{-1}, suggesting that these groups interacted with TPP creating ionic bonds (Fig. 3.2C). The absorption band at 1563 cm^{-1} was assigned to the protonated amino groups of CS at pH 5.5. The phosphate absorption band at 1212 cm^{-1} in TPP shifted to 1210 cm^{-1} in CSNPs, indicating conjugation with CS. At 1021 cm^{-1} and 904 cm^{-1} characteristic peaks of C-N stretch and O-H bend, respectively appeared in CSNPs. Many characteristic peaks of harpin$_{Pss}$ shifted to different wave numbers when it was entrapped in the NPs (Fig. 3.2E),

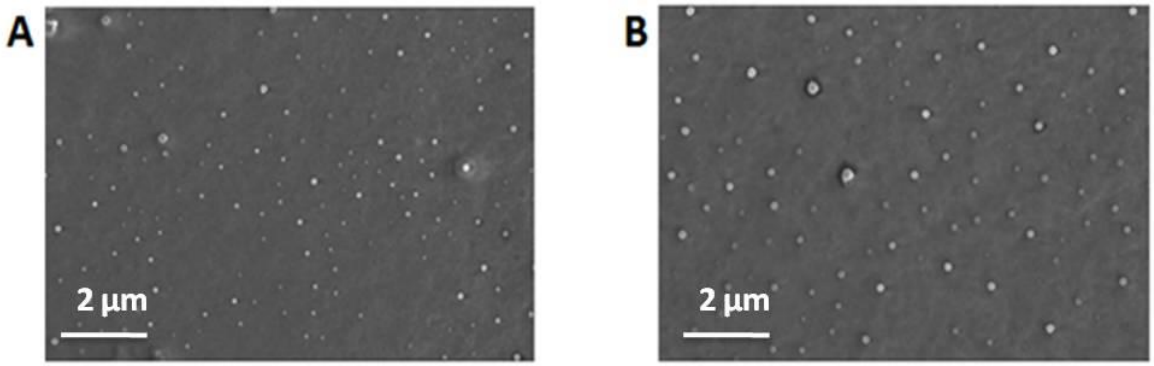


Fig 3.1: Field Emission Scanning Electron Microscopy of CSNPs and H-CSNPs.

(A) CSNPs were prepared with 5:1 ratio of CS and TPP at a pH of 5.5. (B) H-CSNPs prepared at 0.1:1 ratio of harpin$_{Pss}$: CS. The chitosan and TPP were taken as 1 mg mL^{-1}. Image mag: 25 KX.

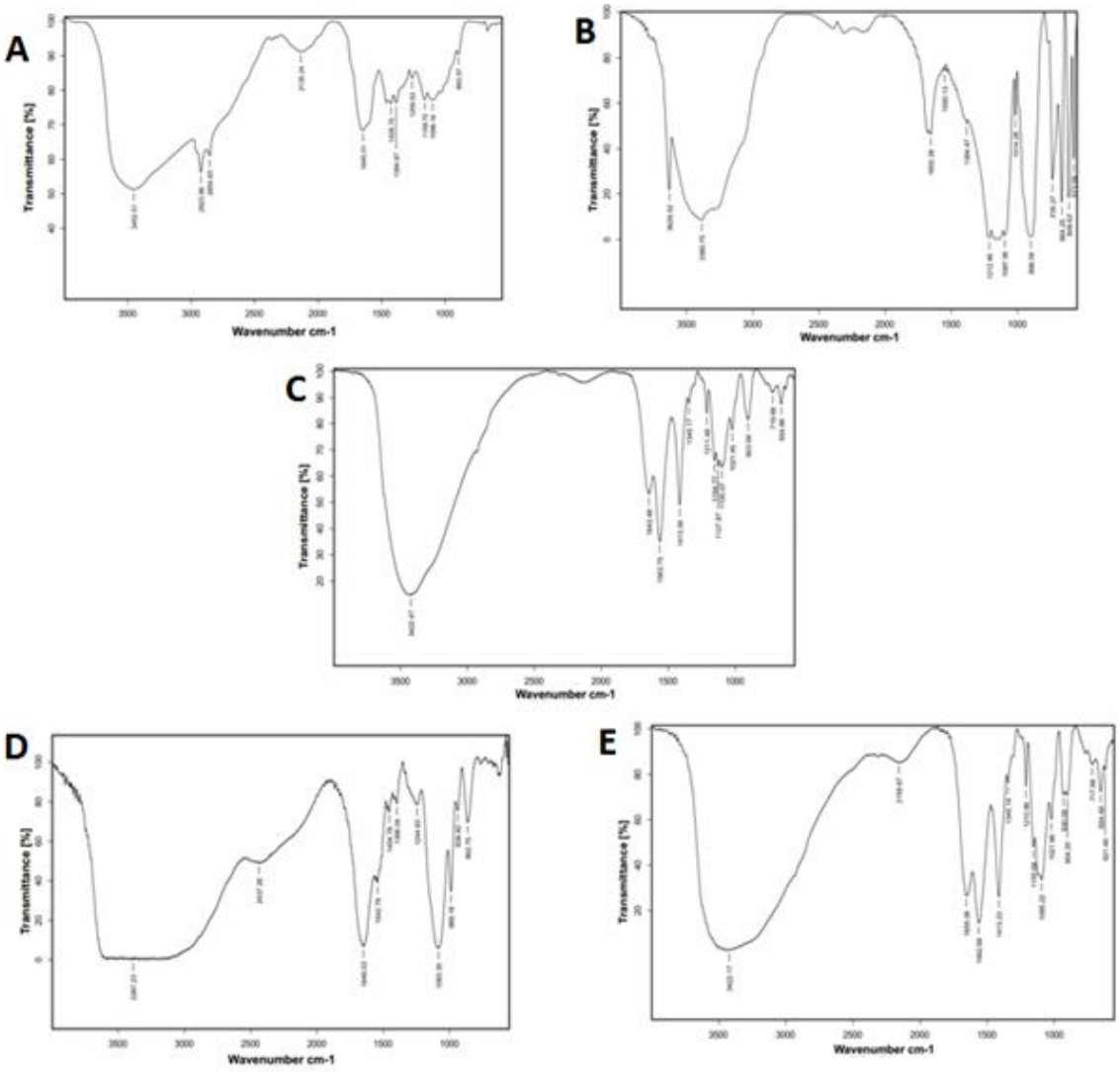


Fig 3.2: FTIR Spectra of: (A) CS, (B) TPP, (C) CSNPs, (D) harpin$_{Pss}$ and (E) H-CSNPs.

Interaction of CS and TPP resulted C-N stretch (1021 cm^{-1}) and O-H bend (904 cm^{-1}), absorption band (1563 cm^{-1}) of protonated amino groups observed in CSNPs spectrum and many characteristic peaks of harpin$_{Pss}$ shifted when it was entrapped in the CSNPs, suggesting strong interaction with CSNPs.

suggesting a strong interaction between harpin$_{Pss}$ and NPs matrix. A few other differences were also recorded at the wave numbers 1649 cm^{-1} that shifted to 1666 cm^{-1} and at 1454 cm^{-1}, which shifted to 1413 cm^{-1}. Absorption band of harpin$_{Pss}$ at 1398 cm^{-1} assigned to C-N stretch shifted to 1345 cm^{-1} and 1063 cm^{-1} of C-O stretch shifted to 1095 cm^{-1} in H-CSNPs.

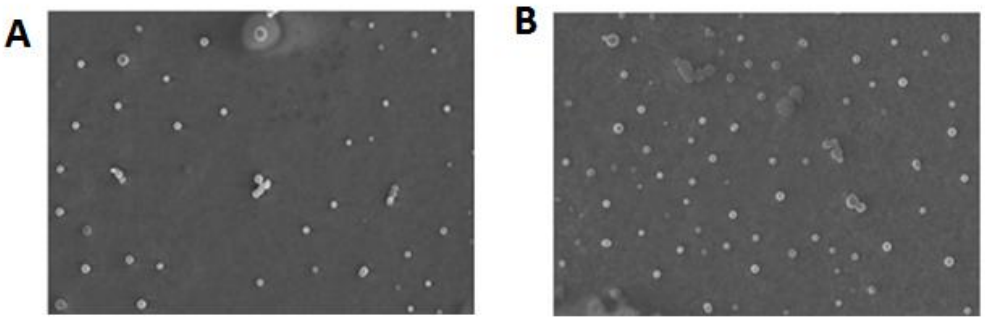
3.2. Stability of NPs:

3.2.1. Size and zeta potential:

Stability of the NPs was evaluated over a period of 90 days. There was an increase in size with slight agglomeration during prolonged incubation. The initial zeta potential values obtained for the CSNPs and H-CSNPs were +32 mV and +49 mV, respectively, with the values changing to +25 mV and +41 mV, respectively, after 90 days (Fig. 3.3). The agglomeration of NPs could be due to a decrease in charge, with time, which enabled enhanced interactions between the particles, after storage of CSNPs for 90 days.

3.2.2. Encapsulation efficiency and *in vitro* release of H-CSNPs:

Encapsulation efficiency of the NPs increased with increase in the concentration of the protein up to an optimum level (150 µg ml^{-1}) that later decreased with increase in protein concentration (Fig. 3.4A). Highest encapsulation efficiency of CSNPs was 90%. From the electrophoretic analysis of the protein, a small amount of protein was detected in the supernatant i.e. free protein fraction. However, maximum protein entrapped within the NPs could be detected in the pellet fraction of bound protein (Fig. 3.4C). To determine the shelf life of CSNPs, the encapsulation efficiency was calculated for H-CSNPs for every 30 days, till 3 months. The encapsulation efficiency of CSNPs decreased sharply after 30 days but remained moderately stable thereafter up to 90 days (Fig. 3.4B).



C

Size (nm) and Zeta potential (mV)		
	Size/zeta (Day 1)	Size/zeta (Day 90)
CSNPs	100±5/+32	150±5/+25
H-CSNPs	120±5/+49	180±5/+41

Fig 3.3: Stability of the NPs during a period of 90 days.

Morphological stability of (A) CSNPs and (B) H-CSNPs was observed under FE-SEM after storage for 90 days and (C) change in zeta potential. SEM micrograph of CSNPs and H-CSNPs showing slight aggregation after storage of 90 days and decrease in zeta potential was observed. SEM image mag: 25 KX.

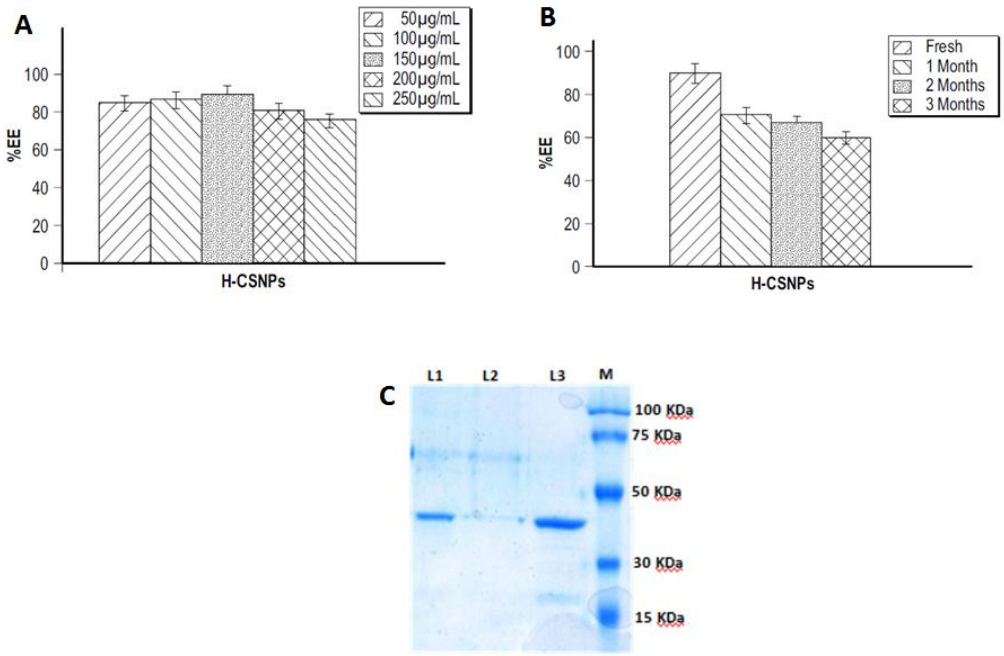


Fig 3.4: Encapsulation efficiency of (A) CSNPs loaded with different concentrations of harpin$_{Pss}$, (B) H-CSNPs stored for different time periods and (C) SDS-PAGE, lane 1-H-CSNPs pellet (150 µg mL^{-1}) , lane 2-H-CSNPs supernatant, lane 3-Harpin$_{Pss}$ (150 µg mL^{-1}) and lane 4-protein marker. Error bars represent the standard deviation of the mean from three independent experiments.

The *in vitro* release of harpin$_{Pss}$ from the CSNPs occurred in two phases. In the first phase, 30% of harpin$_{Pss}$ was burst released (Fig. 3.5), while in the second phase, it was slowly released up to 120 h, resulting in a cumulative harpin$_{Pss}$ release. A sequential time frame of SEM images of H-CSNPs during the release confirmed that the H-CSNPs maintained their shape, size and integral structure up to 6 h (Fig. 3.6). The H-CSNPs had an increased particle size due to swelling after 6 h, and by 12 h the NPs lost shape, a process that progressed till 96 h. At 96 h, there was a distinctive pattern of the structural disintegration of NPs into fractions of smaller particles, with the loss of compact structure.

3.3. POD and PAL in tomato leaves:

The activity of POD and PAL were measured to compare the effect of harpin$_{Pss}$, when loaded in the NPs, in inducing the defense response in tomato. POD activity increased sharply with a peak at 24 h in all the three treatments. However, harpin$_{Pss}$ treatment resulted in an increase of POD activity to the maximum at 24 h followed by CSNPs and H-CSNPs. POD activity sharply declined after 24 h in case of harpin and reached to the level of control plants by 96 h. However, steady increase of POD was observed for CSNPs and H-CSNPs up to 48 h and 72 h, respectively (Fig. 3.7A). Like POD, PAL also increased sharply at 24 h for harpin$_{Pss}$ and gradually decreased up to 96 h. But, in case of CSNPs and H-CSNPs the activity reached a maximum at 48 h and 72 h, respectively that decreased later (Fig. 3.7B). Changes in the activities of POD and PAL are thought to be key components in SAR.

3.4. Transcriptional changes in tomato:

We studied the transcriptome changes in tomato against treatment with harpin$_{Pss}$, CSNPs, and H-CSNPs. We used the GeneChip ® Tomato Genome Array (Agilent) to measure and compare the difference in transcript accumulation (44,000 probes) between control- and treated tomato leaves. To study the nature of the transcriptome changes, we have annotated

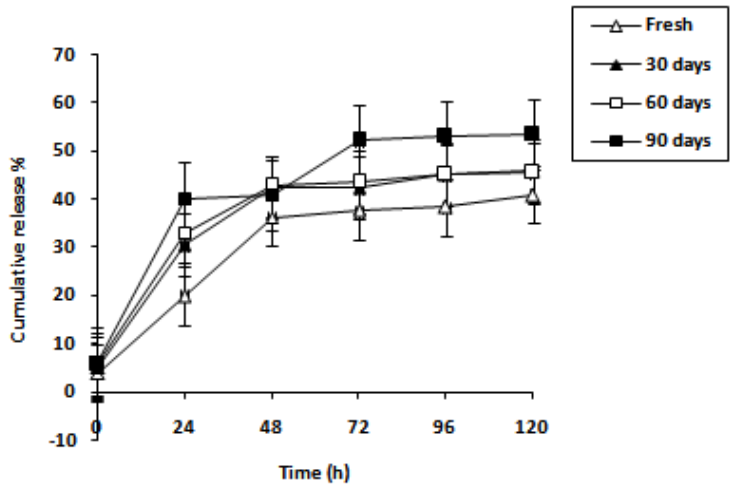


Fig 3.5: Effect of storage of H-CSNPs on subsequent harpin$_{Pss}$ release profile.

The cumulative release of harpin$_{Pss}$ from CSNPs stored for different time periods (30, 60 and 90 days) were compared with freshly prepared NPs. Particle preparation conditions: CS & TPP concentration = 1.0 mg/ml, harpin$_{Pss}$ concentration = 0.1 mg mL^{-1}. Release medium: phosphate buffer saline, T=37±1°C, pH 7.4. Error bars represent the standard deviation of the mean from three independent experiments.

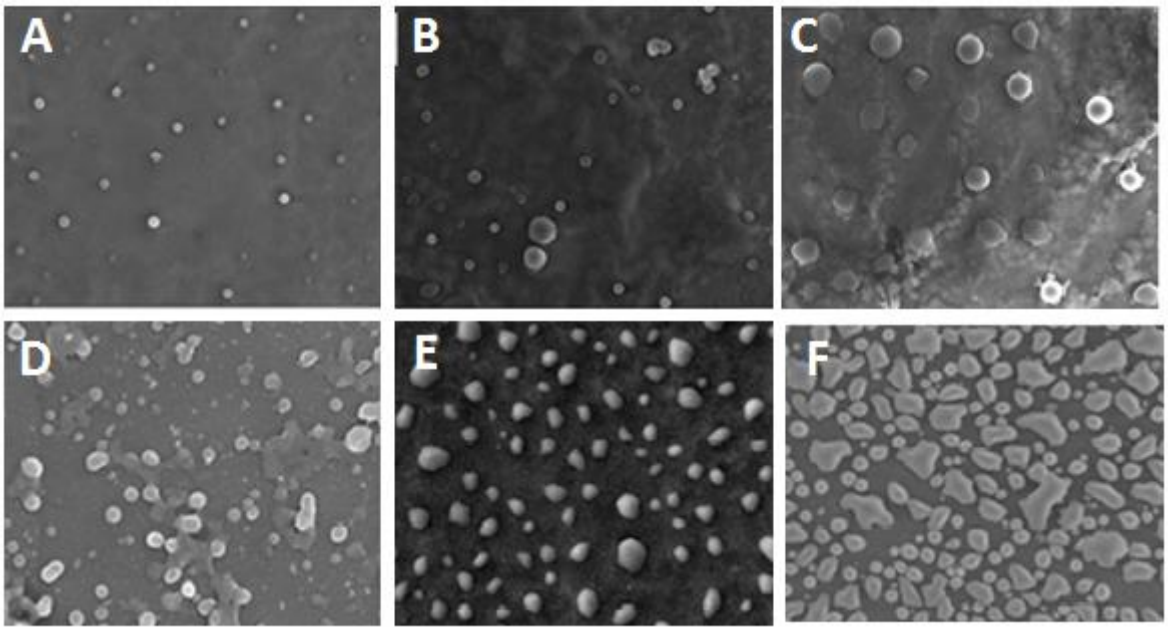


Fig 3.6: Time sequential FE-SEM images of H-CSNPs during harpin$_{Pss}$ release studies.

(A) 0 h (B) 6 h (C) 12 h (D) 24 h (E) 72 h and (F) 96 h. Particle preparation conditions: CS and TPP concentration = 1.0 mg mL^{-1}, harpin$_{Pss}$ concentration = 0.1 mg mL^{-1}, CS to TPP mass ratio = 5:1, release medium T=37±1°C, pH 7.4. Mag: 25 KX.

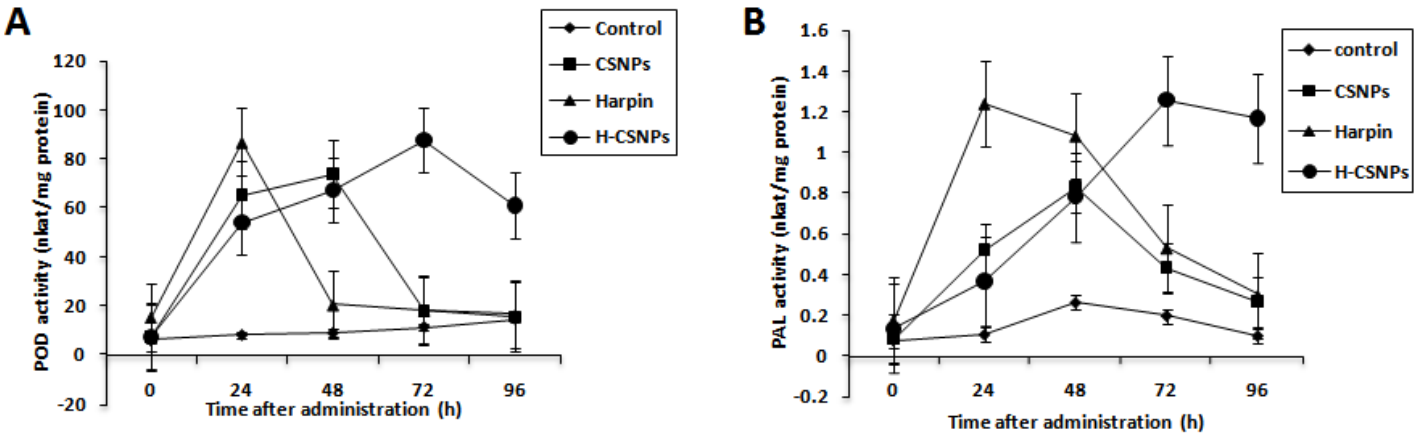


Fig 3.7: Biochemical responses of tomato during elicitor treatment.

(A) Peroxidase (POD), (B) Phenylalanine ammonia lyase (PAL) activity (nkat/mg protein) in a time course after treatment of tomato leaves with CSNPs, harpin$_{Pss}$ and H-CSNPs. Error bars represent the standard deviation of the mean from three independent experiments.

the ESTs provided in the array by making use of NCBI-BLAST programme, considering appropriate homology with minimal error.

Tomato genes that were differentially regulated (more than two-fold change with a $P \leq 0.05$) upon all three treatments were identified at 24, 48 and 72 h, using samples harvested at 0 h as reference samples, to which the samples from all other time points were compared. In the harpin$_{Pss}$ treatment, the number of up-regulated genes was 634, 388 and 263 at 24 h, 48 h and 72 h, respectively, while the number of down-regulated genes was 441, 248 and 542 at 24 h, 48 h and 72 h, respectively. In CSNPs treatment of tomato, the number of genes up-regulated was 476, 582 and 375 at 24 h, 48 h and 72 h, respectively, while the number of genes down-regulated was 362, 673 and 389 at 24 h, 48 h and 72 h, respectively. In H-CSNPs treated tomato, 862, 277 and 209 genes were up-regulated at 24 h, 48 h and 72 h, respectively and 764, 288 and 242 genes were down-regulated at 24 h, 48 h and 72 h, respectively.

The genes commonly expressed at different time intervals in each treatment were shown in venn diagram (Fig. 3.8). Only genes with high level of significance ($P < 0.05$) and a minimum absolute value of $\log_2 > 2$ were systematically considered, to minimize the false positive as up- or down-regulated. We performed an orthology prediction (majorly with *S. tuberosum*) for all gene probe sets that can be probed by the GeneChip and assigned gene ontology annotation to the differentially regulated transcripts. These were used to identify the major differentially regulated biological processes. To gain insight into the function of the differentially expressed genes, we have categorized their putative biological function of the genes essentially based on previous literature and subdivided into six different functional groups (Fig. 3.9) *i.e.*, implicated in defense response (I), signal transduction (II), transport (III), transcription (IV), photosynthesis and housekeeping (V) and aromatics biosynthesis (VI).

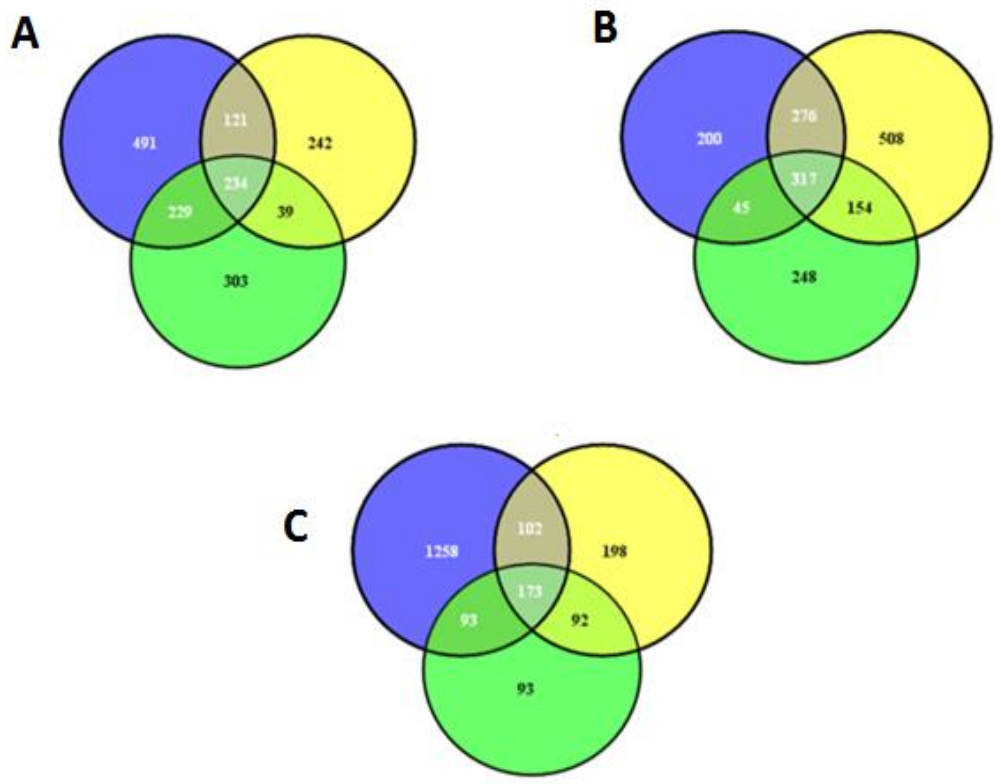


Fig 3.8: The number of differentially expressed transcripts in tomato treated with harpin$_{Pss}$ and NPs treatments.

Venn diagrams showing the number of transcripts that were differentially expressed in tomato leaves treated with harpin$_{Pss}$, CSNPs and H-CSNPs at a level of log_2 expression value of ≥2 and ≤-2 with P<0.05. (A) Number of differentially regulated transcripts during harpin treatment after 24 h (blue), 48 h (yellow) and 72 h (green). (B) Number of differentially regulated transcripts during CSNPs treatment after 24 h (blue), 48 h (yellow) and 72 h (green). (C) Number of differentially regulated transcripts during H-CSNPs treatment after 24 h (blue), 48 h (yellow) and 72 h (green).

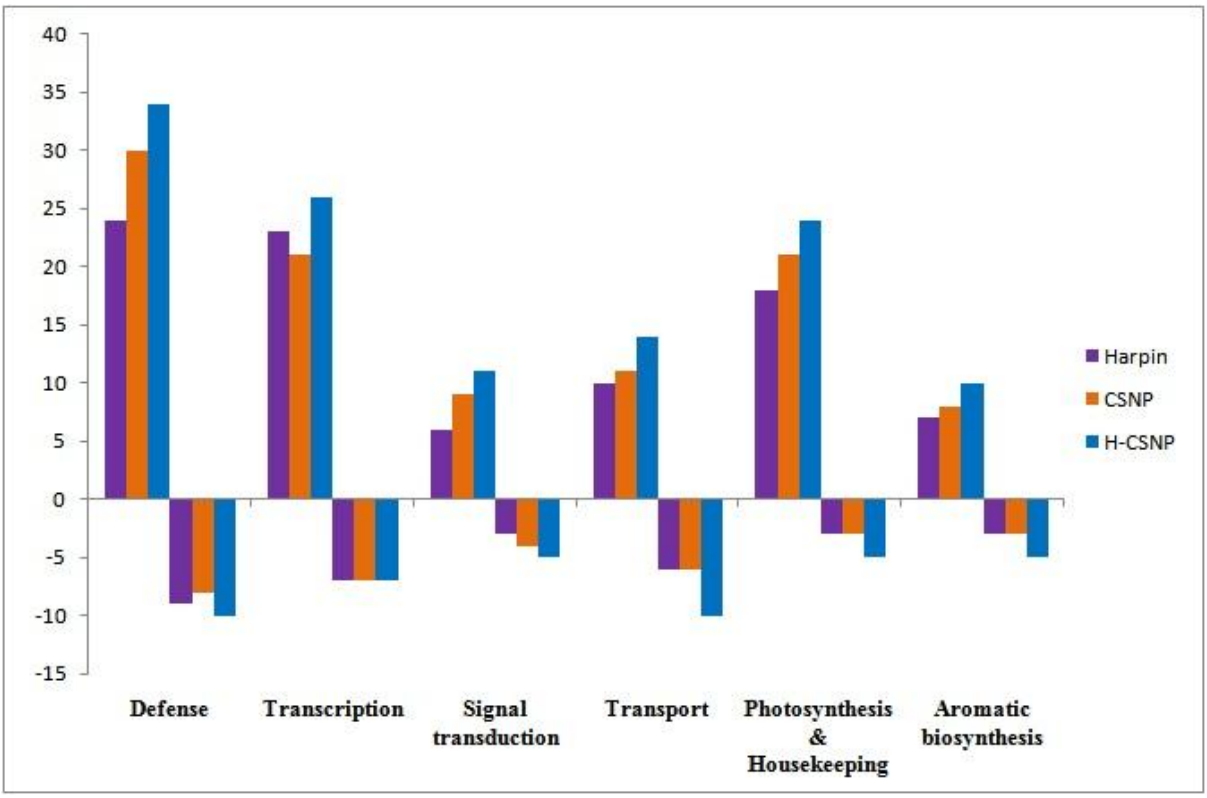


Fig 3.9: Functional categories of differentially expressed transcripts in tomato treated with harpin$_{Pss}$, CSNPs, and H-CSNPs.

Assigned functional categories of differentially expressed transcripts using cut-off statistical parameter P <0.05 with $\log_2$ expression value of ≥ 2 and ≤ -2. Differentially expressed transcripts in harpin$_{Pss}$, CSNPs and H-CSNPs treated tomato leaves compared to mock-inoculated tomato leaves.

Harpin$_{Pss}$-tomato combination resulted in the alteration of a huge number of transcripts that are mostly involved in the oxidative response, host defense, ion transport and jasmonic acid signaling. Treatment with H-CSNPs resulted in alteration of comparatively large number of transcripts among other treatments. Down-regulation was most prominent for genes related to photosynthesis and transport, which may be related to the senescence-promoting activity of JA.

3.4.1. Defense response:

Differentially regulated transcripts in the defense category included several genes annotated as being related to fungal cell-wall degradation, PR proteins, antioxidant enzymes and putative *R*-genes. Transcripts encoding β-1,3-glucanase were highly up regulated (5.6-fold) in plants treated with harpin$_{pss}$ and H-CSNPs when compared to CSNPs treated sample (3-fold). Transcript of antioxidant enzyme POD was highly up regulated (5.75-fold) by H-CSNPs treatment compared to CSNP (4.67-fold) and harpin$_{Pss}$ (4.77-fold). Other transcripts which express during stress conditions superoxide dismutase, polyphenol oxidase, catalase, proline dehydrogenase and heat shock 70 kDa protein were commonly up regulated in all the three treatments (Table 3.1). Whereas antioxidant defense-related transcript encoding glutaredoxin was highly up regulated (4.51-fold) only by H-CSNPs treatment but not expressed in rest of the two treatments.

Transcript encoding PAL 1, enzyme involved in phenyl proponoid pathway was commonly up regulated in all the three treatments. Protein family PR-5 (osmotin or thaumatin-like proteins) was up-regulated at 24 h by all three treatments but not expressed in remaining time points. Transcript of wound-induced protein like WIN1 was up-regulated in all the three treatments. Transcript encoding chitin binding lectin was down-regulated in all the treatments (Table 3.1).

3.4.2. Signal transduction:

Several genes involved in signal transduction events were differentially expressed in all the three treatments such as receptor kinases, protein kinases and calcium-mediated signal transduction proteins. The transcript encoding phosphatidylinositol 4-kinase, the enzyme involved in inositol phosphate metabolism was up-regulated in all treatments. Genes related to protein kinases like calcium-dependent protein kinase and serine threonine protein kinase are only induced by H-CSNPs treatment. Transcript encoding calmodulin-like protein was up regulated in H-CSNPs and CSNPs treatments and not in harpin$_{Pss}$ treatment. Whereas gene encoding MAPK was up-regulated by both harpin$_{Pss}$ and H-CSNPs and not by CSNPs. (Table 3.1).

3.4.3. Transcription factors:

The transcriptional factors belonging to different families like WRKY, MYB and NAM/NAC factors were differentially regulated. Transcript annotated as CUP-SHAPED COTYLEDON3 (CUC3) which encodes a putative NAC-domain transcription factor was highly up-regulated in both harpin$_{Pss}$ and H-CSNPs treatments but not in CSNPs treatment. WRKY transcription factors 2, 4, and 6 were up-regulated in all the treatments (Table 3.1). We also found that transcripts encoding ethylene response factor 3 (ERF 3), auxin response factor 1, Dof zinc finger protein, TATA binding protein associated factor, MYC transcription factor and transcription factor R2R3-MYB were commonly up-regulated in all the three treatments at 24 h but gradually decreased in later time points. Transcripts of double WRKY type transfactor and auxin/indole-3-acetic acid were up-regulated only by H-CSNPs treatment but not by other two treatments.

Transcript of MADS-box protein 5 was down regulated (-7 fold) in all treatments and also transcripts encoding multiprotein bridging factor 1, ethylene-responsive transcriptional

coactivator, DNA binding protein ACBF-like and mitochondrial SBP40 were commonly down regulated by all the three treatments (Table 3.1).

3.4.5. Transport:

Transcripts encoding genes involved in transportation in regular cellular processes were differentially regulated. Transcripts annotated as hexose transporters (HXT), inorganic phosphate transporter (PT4) and H(+)-transporting ATPase were up-regulated in all the treatments. Transcript encoding amino acid transporter was highly up-regulated by H-CSNPs treatment (5.13-fold) when compared to CSNP (3.5-fold) and harpin$_{Pss}$ (4.3-fold) treatment (Table 3.1). Transcripts encoding NADH dehydrogenase and membrane channel were commonly up-regulated by both CSNPs and H-CSNPs treatments but not by harpin$_{Pss}$. Transcripts related to Ras-related GTP binding protein and P-glycoprotein were up-regulated only by H-CSNPs treatment and not expressed in other two treatments. Whereas transcript encoding small GTP-binding protein Sar1BNt-like protein was up-regulated by both harpin$_{Pss}$ and H-CSNPs treatments. Transcripts of sucrose transporter 4, major intrinsic protein 2, cytochrome b, tyramine hydroxycinnamoyl transferase and putative inward rectifying potassium channel were commonly expressed in all the three treatments.

Transcripts encoding proton pump interactor 1, plastidic ATP/ADP-transporter and triose phosphate/phosphate translocator, chloroplastic (cTPT) were down-regulated in all three treatments (Table 3.1). Transcripts encoding sulfate transporter and ABCG subfamily transporter protein were up-regulated by both harpin$_{Pss}$ and H-CSNPs treatments and not expressed in CSNPs treatment. Whereas transcripts encoding alternative oxidase and non-specific lipid-transfer protein were up-regulated by both CSNPs and H-CSNPs treatments but not by harpin$_{Pss}$. Transcripts encoding NADH-quinone oxidoreductase and putative AAA

ATPase were up-regulated only by H-CSNPs treatment and not expressed in other two treatments.

3.4.6. Photosynthesis and house-keeping:

Transcripts encoding primary metabolism include carbohydrate, fatty acid and amino acid metabolism were differentially regulated in all the three treatments. Transcripts encoding 1,3-β-glucan glucanohydrolase, fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase, phytoalexin-deficient 4-2 protein, gamma-tocopherol methyltransferase and L-galactono-1,4-lactone dehydrogenase were up-regulated by both CSNPs and H-CSNPs treatments and not by harpin$_{Pss}$. Transcript encoding fructokinase which regulates starch synthesis, was up regulated by both harpin$_{Pss}$ and H-CSNPs treatment. Transcript encoding pyruvate kinase, is an enzyme involved in glycolysis was up regulated in H-CSNPs treated leaves (3.68-fold) but not in rest of the two treatments (Table 3.1). Transcripts encoding enzymes that regulate carbohydrate metabolism *i.e.*, sucrose-phosphatase, isoamylase, β-amylase PCT-BMYI, glyceraldehyde-3-phosphate dehydrogenase, putative glycosyl transferase, glycolate oxidase and 1,4-α-glucan branching enzyme were up-regulated in all the three treatments. Transcripts encoding fatty acid metabolism regulating enzymes which include sterol reductase and HMG-CoA reductase were up-regulated by all the three treatments. Also transcripts encoding amino acid metabolism regulating enzymes like arginine decarboxylase and dehydro ascorbate reductase were up-regulated by all the three treatments.

Transcript encoding phosphoenolpyruvate carboxylase and 4-alpha-glucanotransferase were down-regulated by all the three treatments. Transcript encoding alpha-amylase was down-regulated when treated with H-CSNPs and not in other two treatments. Whereas transcript encoding hexokinase-1 is down-regulated by both CSNPs and H-CSNPs treated samples and not by harpin (Table 3.1).

Down-regulation as a consequence of H-CSNPs treatment was observed for transcripts encoding proteins involved in photosynthesis (Photosystem II protein, Transketolase, NADH-quinone oxidoreductase), whereas ferredoxin-thioredoxin-reductase was up regulated (Table 3.1).

3.4.7. Aromatics biosynthesis:

The phenylpropanoid biosynthetic pathway, is involved in biosynthesis of secondary metabolites like isoflavinoid, flavonoid and anthocyanins. PAL is a key biosynthetic catalyst in phenyl propanoid pathway. PAL catalyses the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid. The transcript related to PAL1 was up-regulated in all the three treatments (Table 3.1).

Transcripts belonging to the flavonoid synthesis were differentially expressed in abundance. For example, the transcripts of flavonoid 3-glucosyl transferase and flavanone 3 β-hydroxylase increased in abundance in all the treatments. Whereas flavonol synthase was down-regulated in H-CSNPs treatment. Transcript encoding flavonoid 3',5'-hydroxylase was up regulated by both harpin$_{Pss}$ and H-CSNP treatments but not expressed in CSNPs treatment. Cinnamoyl CoA reductase, the enzyme responsible for diverting the phenylpropanoid pathway into the branch of lignin synthesis was also up regulated in all the three treatments. In addition, other genes involved in the lignin synthesis pathway increased clearly in abundance in all the three treatments such as 4-coumarate-CoA ligase 1 and HMG coenzyme A synthase. Transcript annotated as Gibberellin 3-β-hydroxylase 2, enzyme that participates in diterpenoid biosynthesis and caffeoyl-CoA-O-methyl transferase, enzyme participates in phenyl proponoid biosynthesis were specifically up-regulated by H-CSNPs treatment. Transcript annotated as vetispiradiene synthase, enzyme that participates in monoterpenoid pathway was up-regulated by CSNPs treatment but not by other two treatments (Table. 3.1).

3.5. Localization of harpin$_{Pss}$ and CSNPs:

To locate the specific target site of harpin$_{Pss}$ and CSNPs in tomato, harpin$_{Pss}$ was translationally fused to GFP (GH or HG) and encapsulated into RCSNPs (GH-RCSNPs or HG-RCSNPs). GFP alone served as a control. Confocal images revealed that GFP alone migrated to the cytoplasm, whereas a strong GFP signal was prominently found in chloroplasts in GFP-HrpZ treated tomato leaves (Fig. 3.10A), which was confirmed by detecting the chloroplast auto fluorescence. Overlay with chloroplasts indicated the strong and specific localization of harpin$_{Pss}$ in chloroplasts. In the GH-CSNPs treated tomato leaves GFP fluorescence localized to chloroplast along with the plasma membrane indicating localization of harpin$_{Pss}$ in chloroplasts (Fig. 3.10B). We tracked the H-CSNPs entry into plant cells and harpin$_{Pss}$ localized in chloroplasts. PSORT and TargetP also predicted that the transit peptide of harpin$_{pss}$ (amino acids 1–30 at N-terminus) target this protein to chloroplasts. To further confirm the localization of harpin$_{Pss}$ and ΔN_{1-30}harpin$_{Pss}$ in plant tissue, *hrpZ*/ΔN_{1-30}*hrpZ* was fused with *GFP* and transiently expressed HrpZ-GFP in tomato leaves through *Agrobacterium*-mediated transformation and GFP alone served as a control. From the confocal data it was found that a strong GFP signal was prominently found at chloroplast in HrpZ-GFP infiltrated leaves, which was confirmed by overlay of GFP with chloroplast auto fluorescence (Fig. 3.11). After deletion of chloroplast transit peptide (ΔN_{1-30}harpin$_{Pss}$) was unable to target chloroplast and GFP fluorescence was observed in cytoplasm in case of ΔN_{1-30}HrpZ-GFP (Fig. 3.11).

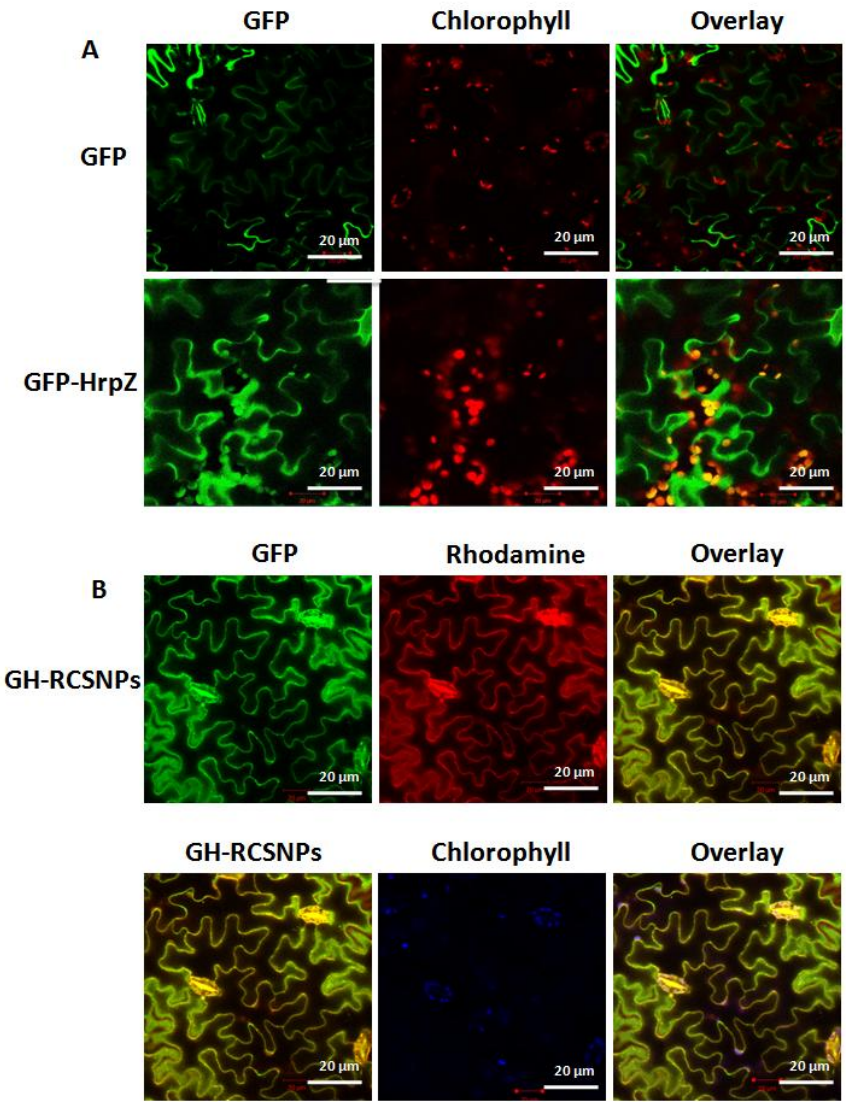


Fig 3.10: Subcellular localization of GFP-HrpZ fusion proteins and GH-RCSNPs.

(A) The subcellular localization of N-terminal GFP fusion construct (100 µg mL $^{-1}$of *E.coli* purified GFP-HrpZ) was examined in tomato leaves. Confocal microscopy images illustrating that free GFP fluorescence was observed in the cytoplasm whereas a strong GFP signal was prominently found in chloroplasts along with cytoplasm in GFP-HrpZ. Fluorescence in the green channel represents the GFP signal; fluorescence in the red channel represents the chloroplasts autofluorescence. The overlay of GFP and chloroplasts is shown (right). (B) Subcellular localization of GFP-HrpZ (100 µg mL $^{-1}$) encapsulated in rhodamine labeled CSNPs (GH-RCNPs). Confocal microscopy images illustrating that rhodamine fluorescence was observed in the cytoplasm whereas a strong GFP signal was prominently found in chloroplasts along with cytoplasm in GH-RCSNPs. Fluorescence in the green channel represents the GFP signal; fluorescence in the red channel represents the rhodamine signal and fluorescence in the blue channel represents chloroplasts autofluorescence. The overlay of GFP and chloroplasts is shown (right bottom). All scale bars are 20 µm.

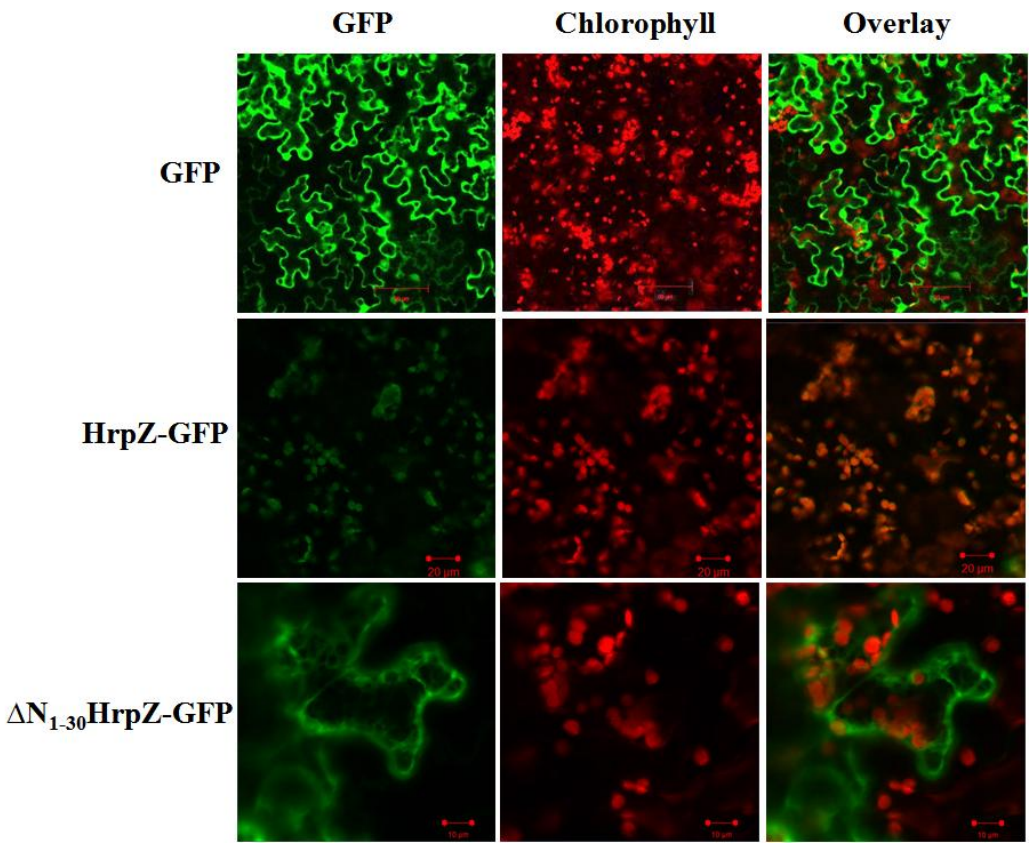


Fig 3.11: Transient expression of HrpZ-GFP and localization in tomato leaf cells.

Confocal microscopy images of the tomato leaf transiently expressed with GFP, HrpZ-GFP and ΔN_{1-30}HrpZ-GFP are shown. Confocal microscopy images illustrating HrpZ-GFP localized in chloroplasts and ΔN_{1-30}HrpZ-GFP localized at cytoplasm. The overlay of GFP and chloroplasts is shown (right). All scale bars are 20 µm. GFP pseudocolored - green, chloroplast autofluorescence pseudocolored - red.

3.6. Defense responses:

3.6.1. Micro- and macroscopic changes in tomato leaves following harpin$_{Pss}$ and H-CSNPs treatments:

H-CSNPs were infiltrated into tomato leaves to see whether there was difference in their ability to induce cell death due to the encapsulation of harpin$_{Pss}$ in CSNPs. In the leaves infiltrated with H-CSNPs, cell death symptoms developed faster. The size of the necrotic lesion was larger than in the harpin$_{Pss}$, followed by CSNPs treatment. There was no cell death in control and ΔN_{1-30}harpin$_{Pss}$ treated leaves (Fig. 3.12B). Simultaneous detection of H_2O_2 and cell death revealed that ROS accumulation and cell death were enhanced in the H-CSNPs infiltration, compared with harpin$_{Pss}$ treatment. It was evident that cellular H_2O_2 accumulation and occurrence of cell death were co-localized, and that H_2O_2 accumulation preceded cell death (Fig. 3.12A).

3.6.2. Disease severity in tomato treated with harpin$_{Pss}$ or H-CSNPs:

To study the role of harpin$_{Pss}$ and H-CSNPs in reducing the severity of disease caused by *Rhizoctonia solani* in tomato, we performed detached leaf bioassays. When harpin$_{Pss}$ and H-CSNPs pretreated tomato leaves were inoculated with *R. solani*, plants were highly tolerant to the fungus (Fig. 3.12C). In H-CSNPs treated leaves the severity of infection was less. In contrast, plants treated with ΔN_{1-30}harpin$_{Pss}$ and buffer were highly susceptible to the fungus. *Rhizoctonia* growth ceased in H-CSNPs treated tomato leaves. Upon extended incubation, the fungus failed to colonize and infect tomato leaves. In contrast, the fungus completely colonized and macerated leaf tissue during the same time in buffer treated leaves.

3.6.3. Determination of chlorophyll content:

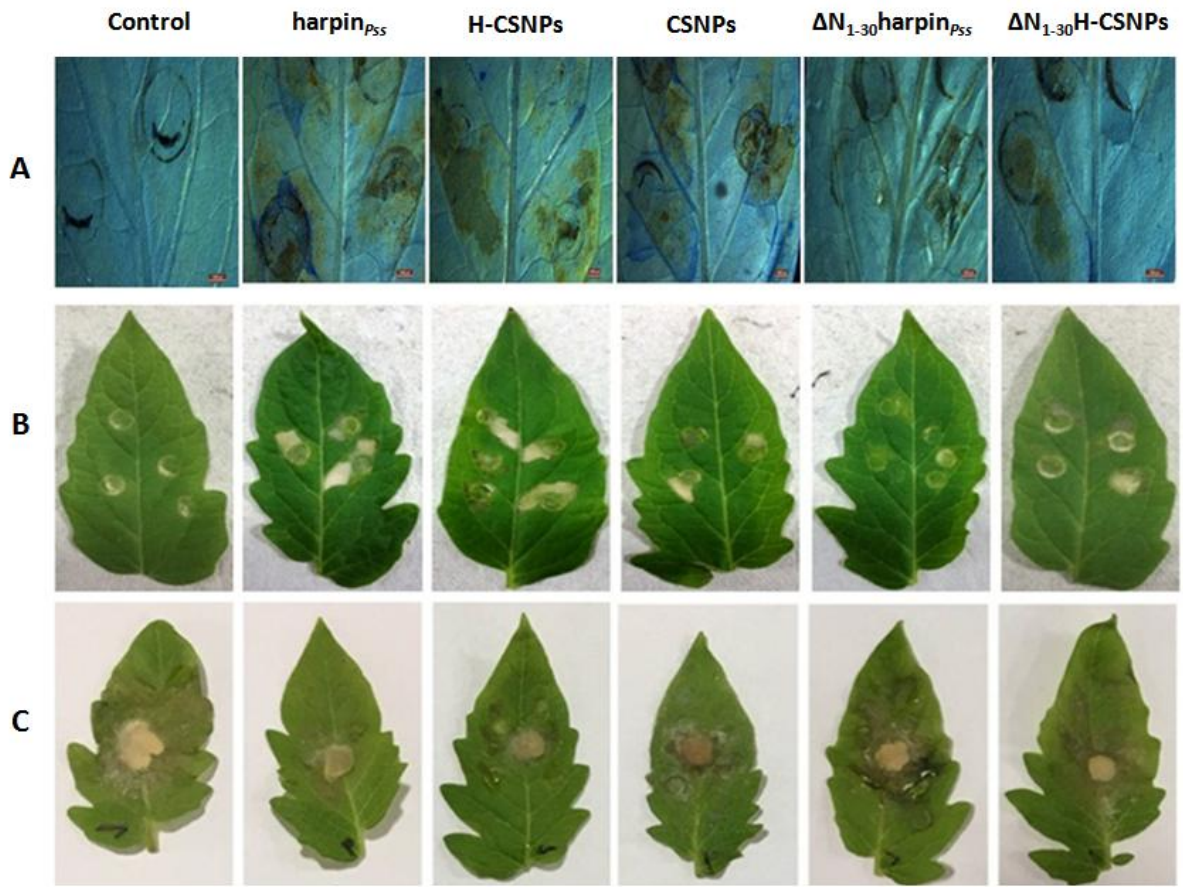


Fig 3.12: Micro- and macroscopic changes in tomato leaves treated with harpin$_{Pss}$ and H-CSNPs.

(A) Microscopic detection of H_2O_2 accumulation and cell death together (DAB + Evans blue). H_2O_2 accumulation detected with DAB (brown spots) and cell death with evans blue (blue spots). H_2O_2 bursts and cell death colocalize in cells of tomato leaves infiltrated with harpin$_{Pss}$ and H-CSNPs, and the accumulation of H_2O_2 precedes cell death. (B) Cell death in leaves. (C) Bioassay of harpin$_{Pss}$/H-CSNPs for resistance against *Rhizoctonia solani* in tomato. Detached tomato leaves were inoculated with *Rhizoctonia solani*. Leaves were wounded prior to inoculation. Photographs were taken seven days post-inoculation.

When compared with control, increased total chlorophyll content was observed in all treatments. Maximum chlorophyll content was observed in H-CSNPs treated leaves followed by harpin$_{Pss}$. Leaves infiltrated with harpin$_{Pss}$, H-CSNPs, CSNPs, ΔN_{1-30}harpin$_{Pss}$ and ΔN_{1-30}H-CSNPs showed 2.16, 2.30, 1.87, 1.97 and 1.82-fold of increase in chlorophyll content than control, respectively (Fig. 3.13). Similar pattern of increase in chlorophyll 'a' and chlorophyll 'b' contents were observed in all treatments.

3.6.4. Effect of deletion of chloroplast transit peptide of harpin$_{Pss}$ on induction of defense response genes in tomato:

Semi-quantitative RT-PCR analysis was carried out to assess the expression of defense-related genes in tomato on foliar application of harpin$_{Pss}$ and ΔN_{1-30}harpin$_{Pss}$. Seven defense related genes (POD, PAL, polyphenol oxidase, ferredoxin NADP reductase, WRKY, MYB and chitinase) were selected to evaluate transcript profile under different treatments. The cDNA, prepared using RNA of the tomato leaves with the above treatments, showed differential pattern of gene expression of the selected genes at 6 h, 12 h and 24 h when compared with the control (Fig. 3.14). Harpin$_{Pss}$ treatment resulted in the up-regulation of all the above genes, while there was no change in expression with ΔN_{1-30}harpin$_{Pss}$ treatment, when compared with control.

3.7. Interaction of harpin$_{Pss}$ with chloroplastic FNR protein:

To know the interacting protein of harpin$_{Pss}$ in tomato, *in-vitro* pull down assay was performed. Two proteins - PR-10 and ferredoxin NADP-reductase (FNR) were identified as possible interacting proteins of harpin in tomato through MALDI-TOF. To further confirm the interaction of harpin$_{Pss}$ with FNR, yeast two hybrid assay was performed by cloning harpin$_{Pss}$ and FNR in yeast vectors and co-expressed in yeast. Growth of yeast colonies

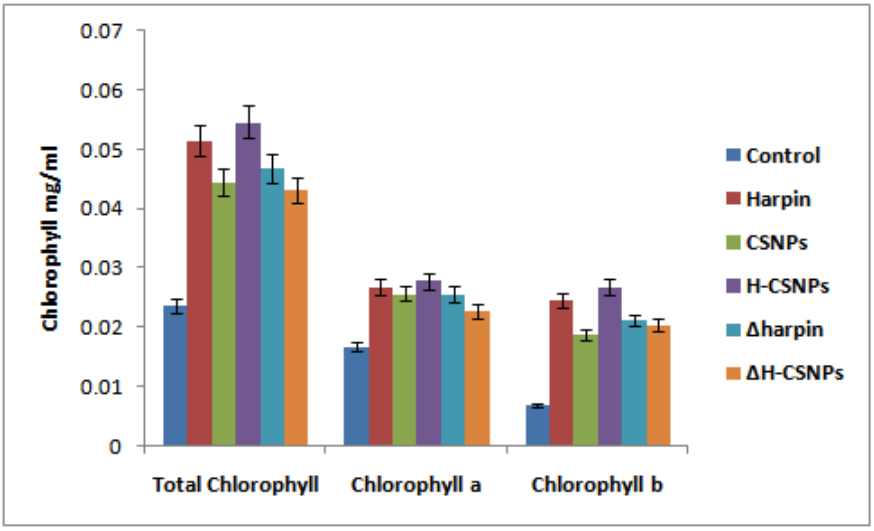


Fig 3.13: Effect of different forms of harpin on total chlorophyll, chlorophyll a and chlorophyll b.

Relative chlorophyll content in tomato leaves from control plants and after treatment with harpin$_{Pss}$, CSNPs, H-CSNPs, ΔN_{1-30}harpin$_{Pss}$ and ΔN_{1-30}H-CSNPs. Values represent mean ± SD of three separate experiments, each in triplicate.

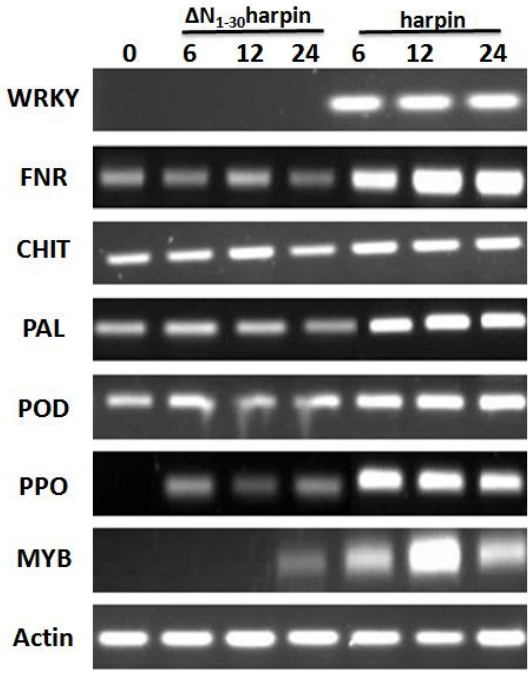


Fig 3.14: Analysis of harpin$_{Pss}$-induced defense gene expression in tomato.

Tomato leaves infiltrated with purified recombinant harpin$_{Pss}$ and ΔN_{1-30}harpin$_{Pss}$ (20µg/ml). Leaf samples were collected at 0, 6, 12 and 24 h post infiltration. Total RNA was isolated using trireagent and the cDNA was synthesized. RT-PCR was performed using gene specific primers and the products were resolved on 1.8% agarose gel.

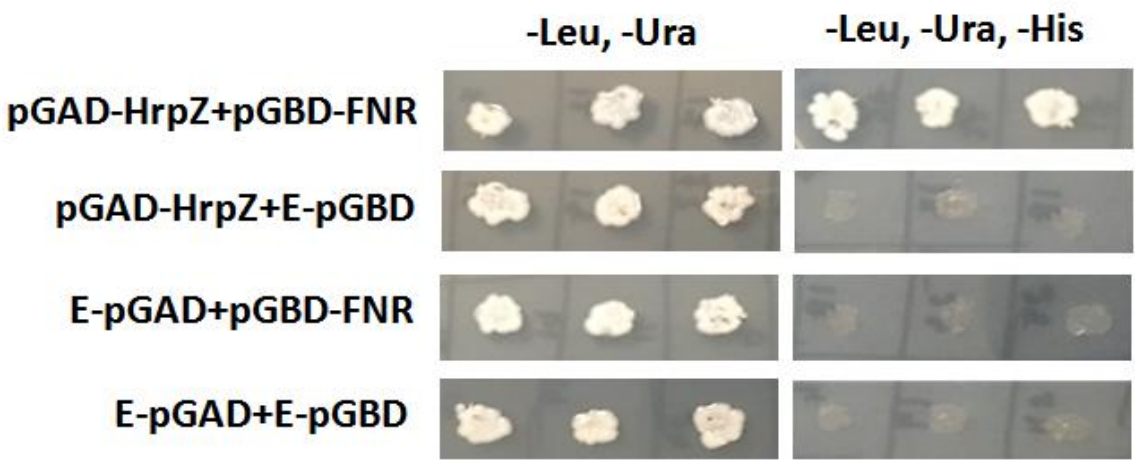


Fig 3.15: Yeast 2-hybrid analysis showing the interactions among harpin and FNR.

Yeast transformed with plasmids containing harpin$_{Pss}$ and FNR was plated onto agar plates lacking essential nutrients: uracil (Ura), leucine (Leu), and/or histidine (His). For each pair, the *hrpZ* was fused to the GAL4-activating domain in pGAD-C1, and *FNR* was fused to the GAL4-binding domain in pGBDU-C1. *Left*, control plate lacking two essential nutrients (uracil and leucine) to demonstrate transformation efficiency. *Right*, yeast survival exists only upon interaction between the GAL4 fusion proteins translated. Each experimental condition was repeated twice with identical results.

transformed with harpin$_{Pss}$ and FNR on histidine drop out medium proved their interaction in yeast (Fig. 3.15).

Discussion

A common feature of induced resistance to disease is the priming of plant tissues by elicitors that allows rapid deployment of active defense mechanisms against invading pathogens. Elicitors are environmentally safe biological molecules that can induce effective defense responses in plants. Harpin$_{Pss}$, an elicitor from *P. syringae* pv. *syringae*, induces SAR in non-host plants, thereby providing resistance to pathogen infection. Harpin constitute a major group of proteins exported by the TTSS of plant pathogens like *Pseudomonas*, *Ralstonia* and *Erwinia* species. The role of harpin during colonization of host plants and their site of action is not known completely. However, infiltration of harpin into non-host plants triggers disease resistance related responses, such as HR, accumulation of PR proteins and SAR (Dong et al., 1999).

Foliar treatment with elicitor molecules like harpin could be an alternative to the use of conventional chemicals for the control of plant diseases. The effect of harpin was transient in plant organs. When sprayed on leaves, only a few harpin molecules would be able to interact with the putative receptors due to the unique architecture of leaves. Thus, the bioavailability of harpin gets significantly reduced. Nano-encapsulation of pesticides and herbicides could facilitate the successful intrusion through cuticles and tissues, allowing slow and regular discharge of the active substances (Pérez-de-Luque and Rubiales, 2009). Nano-formulations of pesticides and herbicides have been developed with added advantage of increased efficacy, specificity and reduced toxicity during field application (Grillo et al., 2014; Pereira et al., 2014).

The nano-encapsulation technique has many advantages. The NPs not only can carry protein, drill through epidermis but also can release protein continuously *in situ*, improving the bioavailability. Here our data suggested that the nanoencapsulation could be used in the preparation of a new form of protein insecticide, which could prolong the duration of elicitor effect, enhance bioavailability and avoid repeated application. NPs could help protein

pervade through epidermis and cell wall mainly from stoma. Applied in agriculture, harpin proteins are always used of an excessive dose to obtain the best effect, while continuous application is still required when disease occurs. Furthermore, it is significant to develop nanoscale pesticide form, such as harpin$_{Pss}$ loaded CSNPs (H-CSNPs) to promote application of protein elicitor pesticides. Here, we demonstrated improved bioavailability of harpin$_{Pss}$ and induced defense responses in tomato by encapsulating harpin$_{Pss}$ in CSNPs.

4.1. Physicochemical characterization of harpin$_{Pss}$-loaded chitosan nanoparticles (H-CSNPs):

To develop a nanoscale biopesticide, such as H-CSNPs and promote the application of harpin$_{Pss}$ was challenging. The ionic interaction between CS and TPP was dependent on the solution pH, owing to the variation in ionization degree of CS and TPP. The pH of CS solution may affect protein interaction and encapsulation. The CS molecular chain is fully extended in solution at pH 5.5 because of electrostatic expulsion between amine groups present along the molecular chain. Stable, non-toxic and organic solvent free synthesis of CSNPs was developed using ionic gelation technique (Agnihotri et al., 2004). The ratio between CS and TPP was critical and control the size of the NPs. The biological performance of CSNPs mainly depends on size (Pan et al., 2002). It was evident that the increase in CS/TPP ratio resulted in the generation of NPs with smaller size. Furthermore, an optimum CS/TPP ratio of 5/1 (w/w), was observed to give a maximum yield of mono-disperse NPs. The weight ratio of CS and TPP (5/1) lead to the most efficient cross-linking of amino groups of CS with TPP and resulting the most compact particle structure (Zhang et al., 2004). Interestingly chitosan forms colloidal particles and encapsulate biological macromolecules like protein, DNA, and RNA etc. both inside and on its surface. In the present study, the particle size of the H-CSNPs was affected by loading of harpin$_{Pss}$ (Fig. 3.1)

The nature of interactions between the protein and CS or TPP was analyzed with FT-IR spectroscopy since the physicochemical interactions like the formation of hydrogen bonds between the drugs and CS or TPP, will automatically lead to frequency shifts in absorption peaks (Papadimitriou et al., 2008). Shifts of characteristic peaks of amino groups in the FT-IR spectra of CSNPs indicated that these groups interacted with TPP, creating ionic bonds (Figure 3.2). Many characteristic peaks of harpin$_{Pss}$ shifted to different wave numbers when it was encapsulated in the NPs, suggesting a strong interaction between harpin$_{Pss}$ and NPs matrix.

The CSNPs synthesized by ionotropic gelation lose their integrity in aqueous media. Most protein release profiles from CSNPs exhibit an initial burst release, apparently from the particle surface, followed by a sustained release driven by diffusion of protein through the polymer wall and polymer erosion. The burst is more likely a consequence of rapid surface desorption of large amounts of protein molecules from a huge specific surface area provided by large numbers of particles at nanoscale (Gan and Wang, 2007). We observed a similar pattern of harpin$_{Pss}$ release from the NPs in a biphasic pattern, characterized by an initial burst release period followed by a period of slower release. The initial fast release might be the result of the rapid desorption of harpin$_{Pss}$ located on the surface of the NPs. After the burst release, the rate of release changed to sustained diffusion through the matrix (Fig. 3.5). This is in agreement with a study of CSNPs containing lysozyme (Deng et al., 2006) that was attributed to the increased porosity of the NPs generated during protein dissolution.

4.2. HCSNPs and harpin$_{Pss}$ induced defense responses and disease inhibition in tomato:

Microbial elicitors or attempted infection with an avirulent pathogen causes the rapid production of reactive oxygen intermediates. Thus, H_2O_2 from the oxidative burst plays a key role in the orchestration of a localized HR during the expression of plant disease resistance.

Our results in simultaneous detection of H_2O_2 and cell death revealed that ROS accumulation and cell death were enhanced in the H-CSNPs infiltration, compared with harpin$_{Pss}$ treatment (Fig. 3.12A).

Harpin proteins from different sources protect crop plants by inducing defense responses. The infiltration or spray of harpin protein triggers disease resistance–associated responses such as HR, transcript accumulation of PR protein genes, and SAR (Dong et al., 1999; Strobel et al., 1996) in non-host plants. Cucumber plants were protected from bacterial, fungal and viral infection with exogenous application of harpin (Strobel et al., 1996). Harpin-induced resistance protected the cucumber plants from anthracnose, bacterial angular leaf spot and tobacco necrosis virus infections. Transgenic tobacco plants expressing harpin gene remarkably reduced the severity of TMV infection (Peng et al., 2003). We have treated the tomato plants with harpin$_{Pss}$/H-CSNPs and evaluated the response for resistance against a *R. solani*. Pre-treatment with harpin$_{Pss}$ to *Rhizoctonia* inoculation resulted harpin$_{Pss}$-induced resistance against the fungus. Such resistance was enhanced by encapsulation of harpin$_{Pss}$ in CSNPs (Fig. 3.12C). The plants treated with H-CSNPs, resisted *R. solani* infection to a longer period (4-5 weeks) when compared with those treated with harpin$_{Pss}$.

Increased POD activity is typical of the process of disease resistance, and is one of the oxidative enzymes known as “wound or oxidative-burst enzymes” (Wojtaszek, 1997). POD is implicated in a variety of functions, such as defense mechanisms (Bradley et al., 1992). POD also involved in phenolics production in tomato plants, as an effective resistance mechanism in tomato-*R. solani* pathosystem (Nikraftar et al., 2013). PAL is a key defence-related enzyme of the phenylpropanoid pathway. Increased PAL activity is a key response to pathogen challenge in many plant species and is closely correlated with resistance (Pallas et al., 1996). PAL regulates secondary metabolism in plants, leading to the biosynthesis of

phenylpropanoids as well as the signalling molecule, SA. Enhancement of PAL activity was reported in response to *R. solani* inoculation in cowpea pretreated with SA (Chandra et al., 2007). PAL activity increased in inoculated leaves of the resistant melon cultivar, resulting in extensive and locally intense deposition of phenolic compounds and lignin surrounding epidermal cells (Ge et al., 2013). Improved POD and PAL activity in our study indicated that the tomato plants responded by harpin$_{Pss}$ and H-CSNPs treatments and role of these enzymes in disease resistance has been reported in many earlier cases.

4.3. Role of some important transcripts differentially expressed in tomato on harpin$_{Pss}$ or H-CSNPs treatment:

A spotted microarray was used to analyze harpin$_{Pss}$-induced changes in the transcriptome of tomato plants. Harpin$_{Pss}$ or H-CSNPs treatment of tomato plants resulted in massive changes in gene expression, causing transcript accumulation or depletion. Harpin$_{Pss}$-tomato combination resulted in the alteration of a huge number of transcripts that are mostly involved in the oxidative response, host defense, ion transport and jasmonic acid signaling. Chuang et al., (2014) also demonstrated that harpin$_{Pss}$ modulates plant gene expression, most likely through its effects on ROS and JA signaling pathways. Treatment with H-CSNPs resulted in alteration of comparatively large number of transcripts among other treatments. Down-regulation was most prominent for genes related to photosynthesis and transport, which may be related to the senescence-promoting activity of JA.

4.3.1. Regulation of defense-related genes:

Differentially regulated transcripts in the defense category included several genes related to antifungal proteins, PR proteins, antioxidant enzymes and putative *R*-genes. Transcripts encoding β-1,3-glucanase and POD were up-regulated by H-CSNPs in high fold when compared to CSNP and harpin$_{Pss}$ treatments. Other transcripts which express during stress

conditions superoxide dismutase, polyphenol oxidase, catalase, proline dehydrogenase and Hsp70 protein were commonly up regulated in all the three treatments (Table 3.1). Whereas, antioxidant defense related transcript encoding glutaredoxin is specifically up regulated by H-CSNPs treatment. Several other studies have also demonstrated the effectiveness of harpin for the modulation of the expression of genes related to defense responses. Transcript encoding PAL 1, enzyme involved in phenyl proponoid pathway is commonly up regulated in all the three treatments. Protein family PR-5 (osmotin or thaumatin-like proteins) was up-regulated at 24 h by all three treatments but not expressed in remaining time points. Pre-treatment with harpin has been reported to activate enzymes involved in defense responses in muskmelon fruit, including the activation of POD, PAL, β-1,3-glucanase, and chitinase (Wang et al., 2011). The overexpression of harpin proteins from *X. oryzae* pv. *oryzae* in rice resulted in the activation of genes involved in the phytoalexin biosynthesis pathway and ROS scavenging (Li et al., 2012).

4.3.2. Defense signaling pathways:

In our study, in addition to activation of genes involved in biotic stress responses, harpin also induced the expression of genes involved in the biosynthesis of JA. JA plays important role in defense against herbivores (Woldemariam et al., 2012). Based on these results, we conclude that, in addition to activating common elements involved in the regulation of disease resistance, treatment with harpin also affects biosynthetic pathways related to herbivore-induced defense responses. The genes encoding enzymes involved in JA-biosynthesis (lipoxygenase, methyl jasmonate esterase, and allene oxide synthase) were up-regulated after harpin$_{Pss}$ and H-CSNPs treatments. The JA is a positive regulator of resistance to necrotrophic fungi (Glazebrook, 2001) and may be part of the basal defense response. JA, often interacting with ET, plays a key role orchestrating the defense response against arthropods, and is strongly associated with downregulation of photosynthesis genes. JA

originates from α-linolenic acid released from chloroplast membranes and enzymes early in the octedecanoid pathway producing JA, LOX and AOS, are localized in the chloroplast (Wasternack, 2007).

An active involvement and importance of protein kinases in elicitation of defense responses was known (Zhang and Klessig, 1998; Romeis, 2001). Transcript encoding calmodulin-like protein was up regulated by H-CSNPs and CSNPs treatments and not by harpin$_{Pss}$ treatment. Whereas, MAPK gene was up-regulated by both harpin$_{Pss}$ and H-CSNPs. Components of MAP kinase cascades were differentially regulated in line with the evidence that MAP kinase modules play important roles in plant immunity (Pedley and Martin, 2004). Earlier study has suggested that the interactions between harpin and plant cells induces the defense response through activation of the MAPK signaling pathway (Lee et al., 2001). Hence, the regulation of processes involved in both plant defense and growth by harpin are most likely attributed to the activation of the MAPK pathway, a signaling pathway implicated in diverse cellular events, including the ET and disease resistance signaling pathways (Yang et al., 2001; Yoo and Sheen, 2008).

4.3.3. Regulation of transcriptional factors:

Transcript annotated as CUC3 which encodes a putative NAC-domain transcription factor was highly up-regulated in both harpin$_{Pss}$ and H-CSNPs treatments but not in CSNPs treatment. The NAC are a family of genes specific to plants and play a role in defense and abiotic stress responses as well as in a diverse set of developmental processes. The barley NAC gene HvNAC6 was implicated in basal defense against the barley powdery mildew pathogen, *Blumeria graminis* f.sp. *hordei* (Jensen et al., 2007). Transcripts of double WRKY type transfactor were up regulated only by H-CSNPs treatment but not by other two treatments. WRKY transcription factors regulate expression of surveillance genes at the top

of the defense-signaling cascade, including the positive regulation of an *R* gene by one or more WRKY proteins (Mohr et al., 2010). Transcripts encoding ethylene response factor 3, auxin response factor 1, Dof zinc finger protein, TATA binding protein associated factor, MYC transcription factor and transcription factor R2R3-MYB were found to be up-regulated in all the three treatments at 24 h and then decreased in later time points. Members of ERF gene family contain highly conserved DNA-binding domain which recognize the *cis* acting element for ethylene-inducible defense genes (Ohme-Takagi, 1995). The ET-responsive signaling pathway plays a crucial role in the harpin-induced defense response (Dong, 2004). The R2R3-MYB transcription factor plays central roles in the control of plant-specific processes, including primary and secondary metabolism, cell fate and identity, development, response to abiotic and biotic stresses (Dubos et al., 2010).

4.3.4. Transport and photosynthesis related gene expression:

Transcripts annotated as hexose transporters, inorganic phosphate transporter and H(+)-transporting ATPase were up-regulated in all the treatments. These hexoses are transported into the cell by hexose transporters where they are believed to fulfill the energy and carbon requirements for the resistance response (Truernit et al., 1996). Down-regulation as a consequence of H-CSNPs treatment was observed for transcripts encoding proteins involved in photosynthesis (Photosystem II protein, Transketolase, NADH-quinone oxidoreductase) and ferredoxin-thioredoxin-reductase was up regulated. The repression of photosynthesis-related genes has also been observed in many plant-pathogen interactions (Matsumura et al., 2003). The transcriptional up regulation of ferredoxin may reflect its direct participation in pathogen defence.

4.3.5. Differential activation of aromatics biosynthesis -related genes:

The activation of the phenylpropanoid pathway produces many secondary metabolites, such as lignins, flavonoids, and isoflavonoids (Whitbred and Schuler, 2000). The transcript related PAL1 was up-regulated in all the three treatments (Table. 3.1). Transcript annotated as gibberellin 3-β-hydroxylase 2 (involved in diterpenoid biosynthesis) and caffeoyl-CoA-O-methyltransferase (involved in phenyl proponoid biosynthesis) were specifically up regulated by H-CSNPs treatment.

4.4. Localization of harpin$_{Pss}$ and CSNPs in tomato:

The reality that NPs passed through the epidermal cell wall opens up the possible ways of nanotechnology-based applications for agriculture. Known special characteristics of the epidermic outer cell wall, specifically its considerable thickness, and the presence of protective waxes, a possible particle penetration point could be through the stomata. In fact, this aperture is a route used by pathogens of different species. Interestingly, water-suspended 43 nm hydrophilic particles have been described as infrequently penetrating *Vicia faba* leaves through stomatal pores (Eichert et al., 2008). We tracked the H-CSNPs entry into plant cells and harpin$_{Pss}$ localized in chloroplasts. PSORT and TargetP also predicted that the transit peptide of harpin$_{pss}$ (amino acids 1–30) targets this protein to chloroplasts. To further confirm the localization of harpin$_{Pss}$, we transiently expressed harpin$_{Pss}$ in tomato leaves through *Agrobacterium*-mediated transformation and confocal analysis proved that harpin$_{Pss}$ specifically targeted to chloroplasts (Fig. 3.11). Deletion of chloroplast transit peptide resulted in failure of harpin$_{Pss}$ to target chloroplasts and remained in cytoplasm and also reduced the harpin$_{Pss}$ induced defense responses in tomato (Fig. 3.12).

Several *P. syringae* type-III effectors have been shown to localize to distinct subcellular plant compartments. Plasma membrane is common localization site for most of the effectors within plant cell (Block and Alfano, 2011). For example, the well-studied type-III effectors

AvrRpm1 and AvrPto are both localized to the plasma membrane (Nimchuk et al., 2000). HopI1 is a ubiquitous *P. syringae* virulence effector that acts inside plant cells. When expressed in plants, HopI, HoPG1, and HopM1 localize to chloroplasts, mitochondria and trans-Golgi network, respectively (Jelenska et al., 2007; Block et al., 2010; Nomura et al., 2011). Harpin constitute another group of effector proteins exported by the TTSS, but the cellular localization of the protein and molecular details of the signalling mechanism are not understood completely. We, therefore, fused harpin$_{Pss}$ to GFP at the N terminal region and detected that the haprin$_{Pss}$ directed GFP to the chloroplasts.

4.5. Harpin$_{Pss}$ interacting with chloroplastic ferredoxin NADP reductase:

In the present work, *in vitro* pull down experiment and yeast two hybrid assay revealed that chloroplastic ferredoxin NADP reductase could be the protein interacting with the harpin$_{Pss}$ in tomato. We also observed transcriptional up-regulation of ferredoxin-thioredoxin-reductase on H-CSNPs treatment. In sweet pepper, one of the photosynthetic ferredoxin (Fd) types has been identified as plant ferredoxin-like protein (PFLP) because it assists the harpin-mediated HR. This PFLP shares 48%–75% identity with photosynthetic-type Fd found in other plants, such as tomato, pea, spinach, rice, maize and *Arabidopsis*. Dayakar et al., (2003) observed a synergy between a PFLP and harpin, from *P. syringae*. It was possibly by altering cellular redox state, the PFLP enhanced the ability of harpin to induce production of active oxygen species to mount a HR. It was also observed that over-expression of ferredoxin in tobacco conferred resistance to *P. syringae* and *E. carotovora* (Lin et al., 2011). The up regulation of Fd and FNR gene expression following biotic assault may be related to the role of these proteins in defence rather than a response of photosynthesis. In plants, biotic stress results in the up-regulation of genes coding for defense and the down regulation of genes coding for photosynthesis proteins (Hermsmeier et al., 2001). Thus the chloroplastic ferredoxin NADP

reductase (FNR) was the interacting partner of harpin$_{Pss}$ in the chloroplast revealed through this study.

Summary & Conclusions

Plants have high susceptibility to pathogens in the seedling stage. However, with the development of secondary cell wall, it turns more difficult to be infected by pathogens. Applied as a form of biopesticide, harpin-like proteinaceous elicitors can induce SAR pathway, which induce PAL activity to enhance lignin accumulation, and accelerate secondary cell wall development in plants. Unfortunately, this effect lasts rather short. Repeated application is needed when diseases occur. On the other hand, the cell wall has poor osmosis to macromolecule, and the osmosis will decrease with the accumulation of lignin, thus the harpin effect plays down after repeated application.

Furthermore, it will be useful to develop nanoscale pesticide form, such as CSNPs using ionic gelation technique to promote application of protein elicitor as nano-pesticide. Stable, non-toxic and organic solvent free synthesis of CSNPs was achieved using ionic gelation technique.

Harpin$_{Pss}$ (encoded by *hrp*Z of *P. syringae* pv. *syringae*) with a molecular weight of 34.7 kDa was expressed heterologously in *Escherichia coli* and purified using Ni-NTA column. CSNPs were prepared using ionic-gelation method and analyzed using FE-SEM. The size of the spherical CSNPs was in the range of 100±10 nm. Later the harpin$_{Pss}$ was encapsulated within the NPs by cross-linking with the TPP. The prepared H-CSNPs of 120±50 nm with spherical morphology exhibited a protein dependent increase of their size compared with the blank (non-loaded) NPs. The nature of interactions between the protein and chitosan or TPP was established with FT-IR spectroscopy since any kind of physicochemical interaction that may take place, like the formation of hydrogen bonds between the protein and CS or TPP, will automatically lead to frequency shifts in absorption peaks. Many characteristic peaks of harpin$_{Pss}$ shifted to different wave numbers, when it was entrapped in the NPs, suggesting strong interaction between harpin$_{Pss}$ and NPs matrix. The physicochemical stability of the NPs was evaluated using measurements of size, zetapotential, encapsulation efficiency and *in*

vitro release of harpin$_{Pss}$ with the NPs stored for a period of 90 days. The stability and efficiency of H-CSNPs was found to be comparable to freshly prepared NPs up to 90 days. Zeta potential of CSNPs and H-CSNPs showed +32 mV and +49 mV, respectively suggesting a high degree of stability. Increase in zeta potential in the presence of harpin$_{Pss}$, indicating that the presence of harpin$_{Pss}$ probably led to better stabilization of the charges and the groups responsible for intermolecular interactions among the components of the NPs.

The *in vitro* release profile of harpin$_{Pss}$ from CSNPs exhibited an initial burst release, apparently from the particle surface, followed by a sustained release driven by diffusion through the polymer wall and polymer erosion. Here, harpin$_{Pss}$ release from the nanoparticles follows a biphasic pattern, characterized by an initial burst release period followed by a period of slower release. The initial fast release might be the result of the rapid desorption of the protein located on the surface of the nanoparticles. After the burst release, the rate of release changed to sustained diffusion through the matrix.

Our study on POD and PAL activity indicated that the tomato plants responded by harpin$_{Pss}$ and H-CSNPs treatments and role of these enzymes in disease resistance has been reported in many earlier cases. Transcriptome data strongly suggested that a majority of the altered transcripts belonged to the defense, signaling and metabolism confirmed that the harpin$_{Pss}$-induced effects are through the metabolic alteration of the host plant. Transcript encoding calmodulin-like protein was up regulated in H-CSNP and CSNP treatments and not in harpin$_{Pss}$ treatment. Whereas, MAPK gene was up-regulated by both harpin$_{Pss}$ and H-CSNPs and not by CSNPs treatment. Components of MAP kinase cascades were differentially regulated in line with the evidence that MAP kinase modules play important roles in plant immunity. In tomato, harpin$_{Pss}$ and H-CSNPs treatments resulted in the activation of JA-biosynthesis genes (lipoxygenase, methyl jasmonate esterase, and allene oxide synthase) and JA hormone was known to induce basal defense response, in particular

give resistance against necrotrophic fungi. Transcript annotated as CUC3 which encodes a putative NAC-domain transcription factor was highly up-regulated in both harpin$_{Pss}$ and H-CSNPs treatments but not in CSNPs treatment. Transcript of double WRKY type transcription factor was up regulated only by H-CSNPs treatment but not by other two treatments. WRKY transcription factors regulate the expression of *R* genes, which are playing significant role in defense-signaling cascade.

We tracked the H-CSNPs entry into plant cells and harpin$_{Pss}$ localized in chloroplasts. PSORT and TargetP also predicted that the transit peptide of harpin$_{pss}$ (amino acids 1–30 at N-terminus) target this protein to chloroplasts. Further *in-planta* transient expression of harpin$_{Pss}$ in tomato confirmed localization of harpin$_{Pss}$ in chloroplasts. It was also observed that harpin$_{Pss}$ localised in the cytoplasm after deletion of chloroplast transit peptide (ΔN_{1-30}harpin$_{Pss}$). When harpin$_{Pss}$ and H-CSNPs pretreated tomato leaves were inoculated with *R. solani*, plants were highly tolerant to the fungus. In H-CSNPs treated leaves the severity of infection was less. In contrast, plants treated with ΔN_{1-30}harpin$_{Pss}$ were highly susceptible to the fungus. Simultaneous detection of H_2O_2 and cell death revealed that ROS accumulation and cell death were enhanced in the harpin$_{Pss}$ and H-CSNPs infiltration, whereas there was no cell death observed in control and ΔN_{1-30}harpin$_{Pss}$ treated leaves. Also ΔN_{1-30}harpin$_{Pss}$ treated tomato plants showed decreased chlorophyll content than plants treated with full length harpin$_{Pss.}$ Semi-quantitative RT-PCR analysis was carried out to assess the expression of defense-related genes in tomato on foliar application of harpin$_{Pss}$ and ΔN_{1-30}harpin$_{Pss}$. Harpin$_{Pss}$ treatment resulted in the up-regulation of all the seven defense-related genes (POD, PAL, polyphenol oxidase, ferredoxin NADP reductase, WRKY, MAPK and chitinase), while there is no change in expression with ΔN_{1-30}harpin$_{Pss}$ treatment when compared with control. Hence chloroplastic localization of harpin$_{Pss}$ resulted in induction of various defense responses in tomato. We hypothesized that harpin$_{Pss}$ may interact with the protein inside the

plant cell and inducing further defense pathways. To find out the interacting protein partner of harpin$_{Pss}$ in tomato, we performed *in vitro* pull down experiment and yeast two hybrid assay. These two experiments revealed that chloroplastic ferredoxin NADP reductase is the protein interacting with the harpin$_{Pss}$ in tomato. We further observed transcriptional up-regulation of ferredoxin-thioredoxin-reductase on H-CSNPs treatment. The ferredoxin-like protein may enhance the ability of harpin$_{Pss}$ to induce production of active oxygen species to mount a HR. The up regulation of Fd and FNR gene expression following biotic assault may be related to the role of these proteins in defense rather than a response of photosynthesis.

CONCLUSIONS:

- Harpin$_{Pss}$ loaded CSNPs (H-CSNPs) were prepared by ionic-gelation method. The H-CSNPs were round with good glomeration and high encapsulation efficiency. The prepared H-CSNPs of 120±10 nm with spherical morphology exhibited controlled *in vitro* release of harpin$_{Pss}$.

- The interaction between the harpin$_{Pss}$ and the encapsulating polymer was deduced using FTIR spectroscopy. The stability and efficiency of H-CSNPs was comparable to freshly prepared NPs up to 90 days.

- Foliar application of H-CSNPs on tomato exhibited no visible HR, but increased the expression of defense related enzymes like POD and PAL which demonstrated the slow and steady release of harpin$_{Pss}$ from the H-CSNPs.

- H-CSNPs showed enhanced HR and reduced severity of *Rhizoctonia* infection in tomato.

- The transcripts for several proteins involved in defense response were differentially expressed when treated with harpin$_{Pss}$, CSNPs and the H-CSNPs in

tomato. Genes implicated in biosynthesis of JA and aromatic amino acids were up-regulated, while the genes related to photosynthesis were down-regulated on H-CSNPs treatment.

- The CSNPs entered into cells and released harpin$_{Pss}$ in the chloroplasts.
- Transient expression of harpin$_{Pss}$ in tomato leaves further confirmed the localization of harpin$_{pss}$ specifically in chloroplasts.
- PSORT and TargetP predicted that the amino terminus of harpin$_{Pss}$ contained chloroplast transit peptide (N_{1-30}).
- Deletion of chloroplast transit peptide (ΔN_{1-30}harpin$_{Pss}$) resulted in failure of harpin$_{Pss}$ to target chloroplasts and remained in cytoplasm and also reduced the harpin$_{Pss}$ induced defense responses.
- *In vitro* pull-down assays revealed that chloroplastic ferredoxin NADP reductase (FNR) was the possible interacting partner of harpin$_{Pss}$ in tomato.
- Co-expression in yeast further confirmed the interaction of harpin$_{Pss}$ with FNR.

Bibliography

Abro, M.A., Lecompte, F., Bardin, M., Nicot, P.C., 2014. Nitrogen fertilization impacts biocontrol of tomato gray mold. Agron. Sustain. Dev. 34, 641-648.

Agnihotri, S.A., Mallikarjuna, N.N., Aminabhavi, T.M., 2004. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J. Control. Release. 100, 5-28.

Akbudak, N., Tezcan, H., Akbudak, B., Seniz, V., 2006. The effect of harpin protein on plant growth parameters, leaf chlorophyll, leaf colour and percentage rotten fruit of pepper plants inoculated with *Botrytis cinerea*. Sci. Hortic. (Amsterdam). 109, 107-112.

Alfano, J.R., Collmer, A., 1996. Bacterial pathogens in plants: Life up against the wall. Plant Cell. 8, 1683-1698.

Alfano, J.R., and Collmer, A.,2004. Type III secretion system effector proteins: double agentsin bacterial disease and plant defense. Annu. Rev. Phytopathol. 42, 385-414.

Andi, S., Taguchi, F., Toyoda, K., Shiraishi, T., Ichinose, Y., 2001. Effect of methyl jasmonate on harpin-induced hypersensitive cell death, generation of hydrogen peroxide and expression of PAL mRNA in tobacco suspension cultured BY-2 cells. Plant. Cell. Physiol. 42, 446-9.

Anil, K., Raju, B.V., Podile, A.R., 2014. Leucine zipper-like motifs of $HrpZ_{Pss}$ are not essential to induce hypersensitive response in tobacco. J. Plant Pathol. 96, 1-6.

Anitha, A., Deepagan, V.G., Divya Rani, V.V., Menon, D., Nair, S.V., Jayakumar, R., 2011. Preparation, characterization, in vitro drug release and biological studies of curcumin loaded dextran sulphate-chitosan nanoparticles. Carbohydr. Polym. 84, 1158-1164.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24, 1-15.

Ausubel, F.M., 2005. Are innate immune signaling pathways in plants and animals conserved? Nat. Immunol. 6, 973-9.

Aung, K., Xin, X., Mecey, C., and He, S.Y.,2017. Subcellular localization of *Pseudomonas syringae* pv. *tomato* effector proteins in plants. Methods Mol. Biol.1531,141-153.

Barik, T.K., Sahu, B., Swain, V., 2008. Nanosilica—from medicine to pest control. Parasitol. Res. 103, 253-258.

Bendahmane, A., Kanyuka, K., Baulcombe, D.C., 1999. The Rx gene from potato controls separate virus resistance and cell death responses. Plant Cell. 11, 781-92.

Benhamou, N., Rey, P., Chérif, M., Hockenhull, J., Tirilly, Y., 1997. Treatment with the mycoparasite pythium oligandrum triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology. 87, 108-122.

Bittel, P., Robatzek, S., 2007. Microbe-associated molecular patterns (MAMPs) probe plant immunity. Curr. Opin. Plant Biol. 10, 335-41.

Bleecker, A.B., Kende, H., 2000. Ethylene: a gaseous signal molecule in plants. Annu. Rev. Cell Dev. Biol. 16, 1-18.

Block, A., Alfano, J.R., 2011. Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? Curr. Opin. Microbiol. 14, 39-46.

Block, A., Guo, M., Li, G., Elowsky, C., Clemente, T.E., Alfano, J.R., 2010. The *Pseudomonas syringae* type III effector HopG1 targets mitochondria, alters plant development and suppresses plant innate immunity. Cell. Microbiol. 12, 318-30.

Boutrot, F., Zipfel, C., 2017. Function, discovery, and exploitation of plant pattern

recognition receptors for broad-spectrum disease resistance. Annu. Rev. Phytopathol. 55, 257-286.

Bradley, D.J., Kjellbom, P., Lamb, C.J., 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell. 70, 21-30.

Büttner, D.,2016. Behind the lines-actions of bacterial type III effector proteins in plant cells.FEMS Microbiol. Rev.40,894-937.

Chandra, A., Saxena, R., Dubey, A., Saxena, P., 2007. Change in phenylalanine ammonia lyase activity and isozyme patterns of polyphenol oxidase and peroxidase by salicylic acid leading to enhance resistance in cowpea against *Rhizoctonia solani*. Acta Physiol. Plant. 29, 361-367.

Chen, H., Yada, R., 2011. Nanotechnologies in agriculture: New tools for sustainable development. Trends Food Sci. Technol. 22, 585-594.

Chen, L., Qian, J., Qu, S., Long, J., Yin, Q., Zhang, C., Wu, X., Sun, F., Wu, T., Hayes, M., Beer, S. V, Dong, H., 2008. Identification of specific fragments of HpaG Xooc, a harpin from *Xanthomonas oryzae* pv. *oryzicola*, that induce disease resistance and enhance growth in plants. Phytopathology. 98, 781-91.

Chisholm, S.T., Coaker, G., Day, B., Staskawicz, B.J., 2006. Host-microbe interactions: shaping the evolution of the plant immune response. Cell. 124, 803-14.

Choi, H.W., Brooking-Dixon, R., Neupane, S., Lee, C.J., Miao, E.A., Staats, H.F., and

Abraham, S.N.,2013. *Salmonella typhimurium* impedes innate immunity with a mastcell-suppressing protein tyrosine phosphatase, SptP. Immunity.39,1108-1120.

Chuang, H., Chang, P.Y., Syu, Y., 2014. Harpin protein, an elicitor of disease resistance, acts as a growth promoter in *Phalaenopsis* orchids. J. Plant Growth Regul. 33, 788-797.

Corradini, E., de Moura, M.R., Mattoso, L.H.C., 2010. A preliminary study of the incorparation of NPK fertilizer into chitosan nanoparticles. Express Polym. Lett. 4, 509-515.

Dayakar, B.V., Lin, H.J., Chen, C.H., Ger, M.J., Lee, B.H., Pai, C.H., Chow, D., Huang, H.E., Hwang, S.Y., Chung, M.C., Feng, T.Y., 2003. Ferredoxin from sweet pepper (*Capsicum annuum L.*) intensifying harpin(pss)-mediated hypersensitive response shows an enhanced production of active oxygen species (AOS). Plant Mol. Biol. 51, 913-24.

Del Pozo, O., Lam, E., 2003. Expression of the baculovirus p35 protein in tobacco affects cell death progression and compromises N gene-mediated disease resistance response to Tobacco mosaic virus. Mol. Plant. Microbe. Interact. 16, 485-94.

Delledonne, M., Zeier, J., Marocco, A., Lamb, C., 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc. Natl. Acad. Sci. 98, 13454-13459.

Deng, Q., Zhou, C., Luo, B., 2006. Preparation and characterization of chitosan nanoparticles containing lysozyme. Pharm. Biol. 44, 336-342.

Dey, D., Uma, B., Padmaja, G., Podile, A.R., 2014. Pathogen-induced expression of harpinpss increases resistance in tobacco against *Fusarium oxysporum* f. sp. *nicotianae*. J. Plant Pathol. 96, 335-342.

Dodds, P.N., Rathjen, J.P., 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. Nat. Rev. Genet. 11, 539-48.

Dong, H.P., 2004. Downstream divergence of the ethylene signaling pathway for harpin-stimulated *Arabidopsis* growth and insect defense. Plant Physiol. 136, 3628-3638.

Dong, H., Delaney, T.P., Bauer, D.W., Beer, S. V., 1999. Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. Plant J. 20, 207-215.

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., Lepiniec, L., 2010. MYB transcription factors in *Arabidopsis*. Trends Plant Sci. 15, 573-81.

Eichert, T., Kurtz, A., Steiner, U., Goldbach, H.E., 2008. Size exclusion limits and lateral heterogeneity of the stomatal foliar uptake pathway for aqueous solutes and water-suspended nanoparticles. Physiol. Plant. 134, 151-60.

Flor, H.H., 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9, 275-296.

Fraceto, L.F., Grillo, R., de Medeiros, G.A., Scognamiglio, V., Rea, G., Bartolucci, C., 2016. Nanotechnology in agriculture: which innovation potential does it have? Front. Environ. Sci. 4.

Galán, J.E., Lara-Tejero, M., Marlovits, T.C., and Wagner, S.,2014. Bacterial Type IIISecretion Systems: Specialized Nanomachines for Protein Delivery into Target Cells. Annu. Rev. Microbiol. 68**,** 415-438.

Gan, Q., Wang, T., 2007. Chitosan nanoparticle as protein delivery carrier-systematic examination of fabrication conditions for efficient loading and release. Colloids Surf. B. Biointerfaces. 59, 24-34.

Ge, Y., Bi, Y., Guest, D.I., 2013. Defence responses in leaves of resistant and susceptible

melon (*Cucumis melo* L.) cultivars infected with *Colletotrichum lagenarium*. Physiol. Mol. Plant Pathol. 81, 13-21.

Ger, M.J., Louh, G.Y., Lin, Y.H., Feng, T.Y., Huang, H.E., 2014. Ectopically expressed sweet pepper ferredoxin PFLP enhances disease resistance to *Pectobacterium carotovorum* subsp. carotovorum affected by harpin and protease-mediated hypersensitive response in Arabidopsis. Mol. Plant Pathol. 15, 892-906

Ghaouth, A. El, 1992. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. Phytopathology. 82, 398.

Glazebrook, J., 2001. Genes controlling expression of defense responses in *Arabidopsis*--2001 status. Curr. Opin. Plant Biol. 4, 301-8.

Green, D.R., Reed, J.C., 1998. Mitochondria and apoptosis. Science. 281, 1309-12.

Grillo, R., Rosa, A.H., Fraceto, L.F., 2014. Poly(ε-caprolactone) nanocapsules carrying the herbicide atrazine: effect of chitosan-coating agent on physico-chemical stability and herbicide release profile. Int. J. Environ. Sci. Technol. 11, 1691-1700.

He, P., Shan, L., Sheen, J., 2007. Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. Cell. Microbiol. 9, 1385-96.

He, S.Y., Huang, H.C., Collmer, A., 1993. *Pseudomonas syringae* pv. *syringae* harpinPss: a protein that is secreted *via* the Hrp pathway and elicits the hypersensitive response in plants. Cell. 73, 1255-66.

Hermsmeier, D., Schittko, U., Baldwin, I.T., 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-

related plant mRNAs. Plant Physiol. 125, 683-700.

Huang, H.E., Ger, M.J., Yip, M.K., Chen, C.Y., Pandey, A.K., Feng, T.Y., 2004. A hypersensitive response was induced by virulent bacteria in transgenic tobacco plants overexpressing a plant ferredoxin-like protein (PFLP). Physiol. Mol. Plant Pathol. 64, 103-110.

Iriti, M., Varoni, E.M., 2015. Chitosan-induced antiviral activity and innate immunity in plants. Environ. Sci. Pollut. Res. 22, 2935-2944.

Jelenska, J., Yao, N., Vinatzer, B.A., Wright, C.M., Brodsky, J.L., Greenberg, J.T., 2007. A J domain virulence effector of *Pseudomonas syringae* remodels host chloroplasts and suppresses defenses. Curr. Biol. 17, 499-508.

Jensen, M.K., Rung, J.H., Gregersen, P.L., Gjetting, T., Fuglsang, A.T., Hansen, M., Joehnk, N., Lyngkjaer, M.F., Collinge, D.B., 2007. The HvNAC6 transcription factor: a positive regulator of penetration resistance in barley and *Arabidopsis*. Plant Mol. Biol. 65, 137-50.

Jones, J.D.G., Dangl, J.L., 2006. The plant immune system. Nature. 444, 323-9.

Kah, M., Machinski, P., Koerner, P., Tiede, K., Grillo, R., Fraceto, L.F., Hofmann, T., 2014. Analysing the fate of nanopesticides in soil and the applicability of regulatory protocols using a polymer-based nanoformulation of atrazine. Environ. Sci. Pollut. Res. Int. 21, 11699-707.

Kashyap, P.L., Xiang, X., Heiden, P., 2015. Chitosan nanoparticle based delivery systems for sustainable agriculture. Int. J. Biol. Macromol. 77, 36-51.

Kay, S., and Bonas, U.,2009. How *Xanthomonas* type III effectors manipulate the host

plant.Curr. Opin. Microbiol.12, 37-43.

Khot, L.R., Sankaran, S., Maja, J.M., Ehsani, R., Schuster, E.W., 2012. Applications of nanomaterials in agricultural production and crop protection: A review. Crop Prot. 35, 64-70.

Lee, J., Klessig, D.F., Nürnberger, T., 2001. A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. Plant Cell. 13, 1079-93.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R., Lamb, C., 1996. Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. 6, 427-37.

Li, G., Froehlich, J.E., Elowsky, C., Msanne, J., Ostosh, A.C., Zhang, C., Awada, T., Alfano, J.R., 2014. Distinct *Pseudomonas* type-III effectors use a cleavable transit peptide to target chloroplasts. Plant J. 77, 310-321.

Li, W., Shao, M., Zhong, W., Yang, J., Okada, K., Yamane, H., Zhang, L., Wang, G., Wang, D., Xiao, S., Chang, S., Qian, G., Liu, F., 2012. Ectopic expression of Hrf1 enhances bacterial resistance *via* regulation of diterpene phytoalexins, silicon and reactive oxygen species burst in rice. PLoS One. 7, e43914.

Lin, W., Hu, X., Zhang, W., Rogers, W.J., Cai, W., 2005. Hydrogen peroxide mediates defence responses induced by chitosans of different molecular weights in rice. J. Plant Physiol. 162, 937-44.

Lin, Y.H., Huang, H.E., Chen, Y.R., Liao, P.L., Chen, C.L., Feng, T.Y., 2011. C-terminal region of plant ferredoxin-like protein is required to enhance resistance to bacterial disease in *Arabidopsis thaliana*. Phytopathology. 101, 741-9.

Lindeberg, M., Cunnac, S., Collmer, A., 2012. *Pseudomonas syringae* type III effector repertoires: last words in endless arguments. Trends Microbiol. 20, 199-208.

Lindgren, P.B., Peet, R.C., Panopoulos, N.J., 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168, 512-522.

Matsumura, H., Reich, S., Ito, A., Saitoh, H., Kamoun, S., Winter, P., Kahl, G., Reuter, M., Kruger, D.H., Terauchi, R., 2003. Gene expression analysis of plant host-pathogen interactions by SuperSAGE. Proc. Natl. Acad. Sci. U. S. A. 100, 15718-23.

Mishra, S., Keswani, C., Abhilash, P.C., Fraceto, L.F., Singh, H.B., 2017. Integrated approach of agri-nanotechnology: challenges and future trends. Front. Plant Sci. 8.

Mittler, R., Simon, L., Lam, E., 1997. Pathogen-induced programmed cell death in tobacco. J. Cell Sci. 110, 1333-44.

Mohr, T.J., Mammarella, N.D., Hoff, T., Woffenden, B.J., Jelesko, J.G., McDowell, J.M., 2010. The Arabidopsis downy mildew resistance gene RPP8 is induced by pathogens and salicylic acid and is regulated by W box cis elements. Mol. Plant. Microbe. Interact. 23, 1303-15.

Nagpal, K., Singh, S.K., Mishra, D.N., 2010. Chitosan nanoparticles: a promising system in novel drug delivery. Chem. Pharm. Bull. (Tokyo). 58, 1423-1430.

Nikraftar, F., Taheri, P., Falahati Rastegar, M., Tarighi, S., 2013. Tomato partial resistance to *Rhizoctonia solani* involves antioxidative defense mechanisms. Physiol. Mol. Plant Pathol. 81, 74-83.

Nimchuk, Z., Marois, E., Kjemtrup, S., Leister, R.T., Katagiri, F., Dangl, J.L., 2000.

Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. Cell. 101, 353-63.

Nomura, K., Mecey, C., Lee, Y.N., Imboden, L.A., Chang, J.H., He, S.Y., 2011. Effector-triggered immunity blocks pathogen degradation of an immunity-associated vesicle traffic regulator in *Arabidopsis*. Proc. Natl. Acad. Sci. 108, 10774-10779.

Nürnberger, T., Brunner, F., Kemmerling, B., Piater, L., 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. 198, 249-66.

Obradovic, A., Jones, J.B., Momol, M.T., Balogh, B., Olson, S.M., 2004. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. Plant Dis. 88, 736-740.

Ohme-Takagi, M., 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell online. 7, 173-182.

Pallas, J.A., Paiva, N.L., Lamb, C., Dixon, R.A., 1996. Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. Plant J. 10, 281-293.

Pan, Y., Li, Y., Zhao, H., Zheng, J., Xu, H., Wei, G., Hao, J., Cui, F., 2002. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin *in vivo*. Int. J. Pharm. 249, 139-147.

Papadimitriou, S., Bikiaris, D., Avgoustakis, K., Karavas, E., Georgarakis, M., 2008. Chitosan nanoparticles loaded with dorzolamide and pramipexole. Carbohydr. Polym. 73, 44-54.

Parisi, C., Vigani, M., Rodríguez-Cerezo, E., 2015. Agricultural nanotechnologies: What are the current possibilities? Nano Today. 10, 124-127.

Pedley, K.F., Martin, G.B., 2004. Identification of MAPKs and their possible MAPK kinase activators involved in the Pto-mediated defense response of tomato. J. Biol. Chem. 279, 49229-35.

Peng, J.L., Dong, H.S., Dong, H.P., Delaney, T.P., Bonasera, J.M., Beer, S.V., 2003. Harpin-elicited hypersensitive cell death and pathogen resistance require the NDR1 and EDS1 genes. Physiol. Mol. Plant Pathol. 62, 317-326.

Pereira, A.E.S., Grillo, R., Mello, N.F.S., Rosa, A.H., Fraceto, L.F., 2014. Application of poly(epsilon-caprolactone) nanoparticles containing atrazine herbicide as an alternative technique to control weeds and reduce damage to the environment. J. Hazard. Mater. 268, 207-15.

Pereira, A.E.S., Sandoval-Herrera, I.E., Zavala-Betancourt, S.A., Oliveira, H.C., Ledezma-Pérez, A.S., Romero, J., Fraceto, L.F., 2017. γ-Polyglutamic acid/chitosan nanoparticles for the plant growth regulator gibberellic acid: Characterization and evaluation of biological activity. Carbohydr. Polym. 157, 1862–1873.

Pereira, E.I., da Cruz, C.C.T., Solomon, A., Le, A., Cavigelli, M.A., Ribeiro, C., 2015. Novel slow-release nanocomposite nitrogen fertilizers: the impact of polymers on nanocomposite properties and function. Ind. Eng. Chem. Res. 54, 3717-3725.

Pérez-de-Luque, A., Rubiales, D., 2009. Nanotechnology for parasitic plant control. Pest Manag. Sci. 65, 540-5.

Pike, S.., Ádám, A.., Pu, X.A., Hoyos, M.., Laby, R., Beer, S.., Novacky, A., 1998. Effects of *Erwinia amylovora*harpin on tobacco leaf cell membranes are related to leaf necrosis

and electrolyte leakage and distinct from perturbations caused by inoculated*E. amylovora*. Physiol. Mol. Plant Pathol. 53, 39-60.

Pimentel, D., 1995. Amounts of pesticides reaching target pests: Environmental impacts and ethics. J. Agric. Environ. Ethics 8, 17-29.

Podile, A.R., Laxmi, V.D. V., 1998. Seed bacterization with *Bacillus subtilis* AF 1 increases phenylalanine ammonia-lyase and reduces the incidence of fusarial wilt in pigeonpea. J. Phytopathol. 146, 255-259.

Pogany, M., von Rad, U., Grun, S., Dongo, A., Pintye, A., Simoneau, P., Bahnweg, G., Kiss, L., Barna, B., Durner, J., 2009. Dual roles of reactive oxygen species and NADPH Oxidase RBOHD in an *Arabidopsis-Alternaria* pathosystem. Plant Physiol. 151, 1459-1475.

Ram, P., Vivek, K., Kumar, S.P., 2014. Nanotechnology in sustainable agriculture: Present concerns and future aspects. African J. Biotechnol. 13, 705-713.

Rani, T.S., Takahashi, D., Uemura, M., Podile, A.R., 2015. Proteins associated with oxidative burst and cell wall strengthening accumulate during *Citrus-Xanthomonas* non-host interaction. Plant Mol. Biol. Report. 33, 1349-1360.

Romeis, T., 2001. Protein kinases in the plant defence response. Curr. Opin. Plant Biol. 4, 407-14.

Ryerson, D.E., Heath, M.C., 1996. Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell. 8, 393-402.

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.J.,

Thomma, B.P.H.J., Mesters, J.R., 2013. Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. Elife. 2, e00790.

Sang, S., Li, X., Gao, R., You, Z., Lü, B., Liu, P., Ma, Q., Dong, H., 2012. Apoplastic and cytoplasmic location of harpin protein $Hpa1_{Xoo}$ plays different roles in H_2O_2 generation and pathogen resistance in *Arabidopsis*. Plant Mol. Biol. 79, 375-91.

Sawant, R.M., Hurley, J.P., Salmaso, S., Kale, A., Tolcheva, E., Levchenko, T.S., Torchilin, V.P., 2006. "SMART" drug delivery systems: double-targeted pH-responsive pharmaceutical nanocarriers. Bioconjug. Chem. 17, 943-949.

Schürmann, U., Hartung, W., Takele, H., Zaporojtchenko, V., Faupel, F., 2005. Controlled syntheses of Ag-polytetrafluoroethylene nanocomposite thin films by co-sputtering from two magnetron sources. Nanotechnology. 16, 1078-1082.

Sinha, V.R., Singla, A.K., Wadhawan, S., Kaushik, R., Kumria, R., Bansal, K., Dhawan, S., 2004. Chitosan microspheres as a potential carrier for drugs. Int. J. Pharm. 274, 1-33.

Snima, K.S., Jayakumar, R., Unnikrishnan, A.G., Nair, S. V., Lakshmanan, V.K., 2012. O-Carboxymethyl chitosan nanoparticles for metformin delivery to pancreatic cancer cells. Carbohydr. Polym. 89, 1003-1007.

Sonia, T.A., Sharma, C.P., 2011. Chitosan and its derivatives for drug delivery perspective. Vol. 243, Springer-Verlag, Berlin. pp. 23-53.

Strobel, N.E., Ji, C., Gopalan, S., Kuc, J.A., He, S.Y., 1996. Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZPss protein. Plant J. 9, 431-439.

Suh, J.K., Matthew, H.W., 2000. Application of chitosan-based polysaccharide biomaterials

in cartilage tissue engineering: a review. Biomaterials. 21, 2589-98.

Tarafdar, P.K., Vedantam, L.V., Kondreddy, A., Podile, A.R., Swamy, M.J., 2009. Biophysical investigations on the aggregation and thermal unfolding of harpin(Pss) and identification of leucine-zipper-like motifs in harpins. Biochim. Biophys. Acta. 1794, 1684-92.

Tarafdar, P.K., Vedantam, L.V., Sankhala, R.S., Purushotham, P., Podile, A.R., Swamy, M.J., 2014. Oligomerization, conformational stability and thermal unfolding of harpin, $HrpZ_{Pss}$ and its hypersensitive response-inducing C-terminal fragment, C-214-$HrpZ_{Pss}$. PLoS One. 9,e109871.

Thomas, E., Rathore, I., Tarafdar, J.C., 2017. Bioinspired production of molybdenum nanoparticles and its effect on chickpea (*Cicer arietinum L*). J. Bionanoscience. 11, 153–159.

Torney, F., Trewyn, B.G., Lin, V.S.Y., Wang, K., 2007. Mesoporous silica nanoparticles deliver DNA and chemicals into plants. Nat. Nanotechnol. 2, 295-300.

Truernit, E., Schmid, J., Epple, P., Illig, J., Sauer, N., 1996. The sink-specific and stress-regulated Arabidopsis STP4 gene: enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. Plant Cell. 8, 2169-82.

Uma, B., Podile, A.R., 2014. Overlapping sets of transcripts from host and non-host interactions of tomato are expressed early during non-host resistance. Plant Omics. 7, 19-27.

Vallance, J., Déniel, F., Floch, G., Guérin-Dubrana, L., Blancard, D., Rey, P., 2011. Pathogenic and beneficial microorganisms in soilless cultures. Agron. Sustain. Dev. 31,

191-203.

Wang, J., Bi, Y., Zhang, Z., Zhang, H., Ge, Y., 2011. Reduction of latent infection and enhancement of disease resistance in muskmelon by preharvest application of harpin. J. Agric. Food Chem. 59, 12527-33.

Wasternack, C., 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. 100, 681-97.

Wei, Z.M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A., Beer, S. V, 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science. 257, 85-8.

Whitbred, J.M., Schuler, M.A., 2000. Molecular characterization of CYP73A9 and CYP82A1 P450 genes involved in plant defense in pea. Plant Physiol. 124, 47-58.

Wilson, C.A., Goldstein, D.N., McCormick, J.A., Weimer, A.W., George, S.M., 2008. Tungsten atomic layer deposition on cobalt nanoparticles. J. Vac. Sci. Technol. A Vacuum, Surfaces, Film. 26, 430-437.

Wojtaszek, P., 1997. Oxidative burst: an early plant response to pathogen infection. Biochem. J. 322, 681-92.

Woldemariam, M.G., Onkokesung, N., Baldwin, I.T., Galis, I., 2012. Jasmonoyl-L-isoleucine hydrolase 1 (JIH1) regulates jasmonoyl-L-isoleucine levels and attenuates plant defenses against herbivores. Plant J. 72, 758-67.

Xin, X.F., and He, S.Y.,2013. *Pseudomonas syringae* pv. *tomato* DC3000: A model pathogen for probing disease susceptibility and hormone signaling in plants. Ann. Rev. Phytopathol. 51,473-498.

Yang, K.Y., Liu, Y., Zhang, S., 2001. Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. Proc. Natl. Acad. Sci. 98, 741-746.

Yoo, S.D., Sheen, J., 2008. MAPK signaling in plant hormone ethylene signal transduction. Plant Signal. Behav. 3, 848-9.

Yoon, K.Y., Hoon Byeon, J., Park, J.H., Hwang, J., 2007. Susceptibility constants of *Escherichia coli* and *Bacillus subtilis* to silver and copper nanoparticles. Sci. Total Environ. 373, 572-575.

Zabala, M., Littlejohn, G., Jayaraman, S., Studholme, D., Bailey, T., Lawson, T., Tillich, M., Licht, D., Bölter, B., Delfino, L., Truman, W., Mansfield, J., Smirnoff, N., Grant, M., 2015. Chloroplasts play a central role in plant defence and are targeted by pathogen effectors. Nat. Plants. 1, 15074.

Zhang, H., Oh, M., Allen, C., Kumacheva, E., 2004. Monodisperse chitosan nanoparticles for mucosal drug delivery. Biomacromolecules. 5, 2461-2468.

Zhang, H., Zhao, X., Yang, J., Yin, H., Wang, W., Lu, H., Du, Y., 2011. Nitric oxide production and its functional link with OIPK in tobacco defense response elicited by chitooligosaccharide. Plant Cell Rep. 30, 1153-1162.

Zhang, S., Klessig, D.F., 1998. The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. Proc. Natl. Acad. Sci. U. S. A. 95, 7225-30.

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(71)**Name of Applicant :**
1)UNIVERSITY OF HYDERABAD
Address of Applicant :Head of the Institution, University of Hyderabad, Hyderabad 500046, Telangana, India. Telangana India
(72)**Name of Inventor :**
1)PROF. APPA RAO PODILE
2)SRAVANA SANDHYA RANI NADENDLA

(57) Abstract :
BIOPESTICIDE COMPOSITIONS COMPRISING STABLE HARPINPss-LOADED CHITOSAN NANOPARTICLES AND METHODS THEREOF Exemplary embodiments of the present disclosure are directed towards biopesticide compositions for plants comprising stable harpin-loaded chitosan nanoparticles. The composition is a sustained release composition, and the chitosan nanoparticles improve the bioavailability of harpin by releasing harpin steadily over an extended period of time. The composition exhibits improved induction of disease resistance in plants as compared with native harpin. Furthermore, the invention disclosure is directed towards methods for making the biopesticide composition and application to the plants. In the present invention disclosure, harpin showed enhanced hyper sensitive reaction and disease inhibition in tomato (Lycopersicon esculentum) when conjugated with CSNPs. Genes related to the biosynthesis of jasmonic acid and aromatic amino acids, were found to be up-regulated. On the other hand genes related to photosynthesis were down-regulated.

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Table 3.1: Harpin or H-CSNPs-induced genes with known or putative roles in plant immune responses.

Genes identified as being up (down)-regulated upon harpin$_{Pss}$, CSNPs and H-CSNPs treatments were tentatively grouped according to the (putative) function of the gene products. Fold changes in up- (with no prefix) and down-regulated (with negative mark prefix) between mock- and treated tomato leaves are given.

Agilent probe ID	Gene Description	Fold change (*P*<0.05)								
		Harpin			CSNP			H-CSNP		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
	I. Defense-related genes									
A_96_P199044	β-1,3-glucanase	5.63	1.13	1.66	3.00	2.61	2.57	5.42	-2.11	0.31
A_96_P191249	Chitinase	3.25	1.29	1.65	-	-	-	3.33	0.73	1.34
A_96_P145621	Peroxidase	4.77	2.96	4.38	4.67	3.34	4.05	5.75	3.85	2.74
A_96_P202744	Resistance gene-like	2.38	2.11	1.93	-	-	-	2.25	1.77	2.00
A_96_P072789	Heat shock 70 kDa protein	2.74	2.21	2.34	2.38	2.28	3.06	2.44	1.48	2.48
A_96_P176614	Chaperone protein DnaJ 4	3.04	2.57	2.52	2.18	3.03	3.05	3.15	2.56	2.46
A_96_P095444	Anti-bacterial protein	-	-	-	1.45	1.26	0.74	2.22	0.66	1.13
A_96_P066321	CC-NB-LRR protein	-	-	-	-	-	-	2.16	0.08	0.50
A_96_P194734	Superoxide dismutase	2.74	2.91	2.61	3.04	2.64	3.02	1.54	2.81	3.23
A_96_P217879	Osmotin-like protein	4.67	-	-	2.78	-	-	3.60	-	-
A_96_P025531	PEN2-like protein	-	-	-	2.03	2.10	1.34	2.76	2.06	1.55
A_96_P027151	Polyphenol oxidase	3.57	1.61	3.28	3.15	2.44	3.43	4.13	1.31	2.81
A_96_P067211	PR- 2	2.36	2.85	2.19	2.84	2.30	2.56	2.40	2.73	2.73
A_96_P077804	PR-1a1 protein	4.25	0.46	-0.35	-	-	-	2.58	-0.65	-0.58
A_96_P168889	Catalase	2.64	2.01	2.41	1.87	2.66	2.81	3.07	1.65	2.22
A_96_P151671	PAL 1	2.25	1.90	2.12	2.44	2.45	2.32	2.09	1.70	2.16
A_96_P227544	Lipoxygenase chloroplastic	2.17	1.39	2.09	-	-	-	2.38	0.70	2.09
A_96_P120042	Methyl jasmonate esterase	1.85	1.81	2.25	-	-	-	2.10	1.52	2.50
A_96_P232704	Allene oxide synthase	4.81	2.08	1.81	-	-	-	4.43	-0.54	0.52
A_96_P109067	Glutaredoxin	-	-	-	-	-	-	4.51	-	-
A_96_P245300	Proline dehydrogenase	4.51	3.20	4.01	4.36	3.78	3.47	3.92	3.09	3.70
A_96_P051681	GST (PRP1)	-2.44	-3.79	-3.36	-3.63	-3.79	-3.03	-2.25	-4.01	-3.25
A_96_P090644	Heat shock protein 90	-2.27	-1.33	-1.42	-	-	-	-2.86	-1.43	-1.42
A_96_P074134	Proteinase inhibitor I	-2.87	-2.04	-2.68	-	-	-	-2.92	0.98	-0.95
A_96_P078899	Ascorbate peroxidase	-2.48	-2.28	-2.01	-2.93	-2.60	-1.77	-2.38	-2.08	-2.30
	II. Signal transduction									
A_96_P121512	Phosphatidylinositol kinase	3.38	3.28	3.00	3.02	3.69	3.06	3.78	3.00	2.33
A_96_P057841	Stpk1 protein kinase	-	-	-	-	-	-	2.24	0.94	1.21
A_96_P113332	LRR receptor-like kinase	2.17	1.43	1.99	2.17	1.90	2.20	2.38	1.65	1.78
A_96_P065726	MAP kinase	2.16	0.79	1.55	-	-	-	2.77	1.02	1.25
A_96_P049831	Calcium-dependent protein kinase 4 (CDPK 4)	-	-	-	-	-	-	2.02	1.11	0.87
A_96_P248437	NO synthase protein I	-	-	-	-1.96	-2.38	-2.10	-2.08	-1.97	-1.59

Table. 3.1 (Contd.)

Agilent probe	Gene Description	Fold change (*P*<0.05)								
		Harpin			CSNP			H-CSNP		
		24h	48h	72h	24h	48h	72h	24h	48h	72h
	III Transport-related									
A_96_P133767	Sucrose transporter 4	3.31	2.68	3.24	2.70	3.27	3.08	3.29	2.11	2.76
A_96_P152576	Cytochrome b	2.70	2.62	3.14	3.26	3.46	3.33	2.65	3.12	2.79
A_96_P119312	Hexose transporter	2.45	1.68	1.51	2.15	2.26	1.56	2.32	1.01	1.34
A_96_P067651	H(+)-transporting ATPase	4.72	2.82	4.24	3.98	4.57	3.62	5.09	2.95	3.72
A_96_P254692	Ras- GTP binding protein	-	-	-	-	-	-	2.49	0.11	0.06
A_96_P241629	Membrane channel protein	-	-	-	1.50	1.84	1.74	2.26	0.92	1.24
A_96_P047761	NADH dehydrogenase	-	-	-	2.07	2.22	2.53	2.74	1.03	2.01
A_96_P138562	P-glycoprotein	-	-	-	-	-	-	2.39	-	-
A_96_P011921	sulfate transporter	-3.21	-1.98	-3.05	-	-	-	-4.40	-2.03	-2.09
A_96_P073814	ABCG subfamily	-2.33	-1.73	-2.83	-	-	-	-2.44	-1.27	-1.11
A_96_P029906	Plastidic ATP/ADP-	-3.21	-0.78	-1.90	-3	-1.41	-1.69	-3.70	-1.39	-1.76
A_96_P013986	Alternative oxidase	-	-	-	-3.52	-3	-2.01	-3.34	-2.85	-3.24
A_96_P072844	Lipid-transfer protein	-	-	-	-5.16	-3.17	-3.48	-3.38	-6.16	-3.86
A_96_P055041	NADH-quinone	-	-	-	-	-	-	-2.19	-0.32	0.41
A_96_P251867	Putative AAA ATPase	-	-	-	-	-		2.34	-	-
A_96_P068246	Proton pump interactor 1	-2.75	-2.87	-2.86	-2.72	-3.74	-2.72	-2.82	-2.66	-3.44
	IV Transcription factors									
A_96_P098284	WRKY transcription factor 4	3.35	3.18	2.78	2.73	3.50	3.38	3.71	2.21	2.91
A_96_P199669	WRKY transcription factor 2	2.09	1.41	2.12	1.72	2.38	2.12	2.15	0.95	1.12
A_96_P216739	WRKY protein	2.27	0.89	1.43	1.28	1.22	1.65	2.27	1.18	0.96
A_96_P220009	MYC transcription factor	2.57	2.68	2.26	2.75	2.74	2.04	1.46	2.01	2.50
A_96_P242274	WRKY transcription factor 6	2.19	1.04	1.87	2.41	1.94	1.83	2.49	0.68	1.22
A_96_P061051	TATA binding protein	2.24	1.67	1.92	1.82	2.03	1.75	2.73	1.00	1.53
A_96_P217644	Dof zinc finger protein	2.56	1.60	2.00	1.73	2.16	2.35	2.86	1.15	1.81
A_96_P090584	Double WRKY protein	-	-	-	-	-	-	2.28	1.38	1.15
A_96_P086494	Ethylene response factor 3	3.24	2.53	2.76	3.79	3.41	2.14	3.43	2.40	2.14
A_96_P021866	Auxin response factor 1	2.20	1.41	1.83	2.13	1.81	1.65	2.20	1.15	1.60
A_96_P063681	R2R3-MYB	2.82	1.42	2.36	1.40	2.22	2.42	3.52	1.09	1.38
A_96_P000181	MADS-box protein 5	-6.90	-7.04	-6.54	-5.76	-6.48	-6.79	-7.29	-7.33	-5.76
A_96_P263622	Multiprotein bridging	-2.38	-2.07	-1.88	-2.69	-2.15	-1.85	-2.84	-1.92	-1.92
A_96_P043281	DNA binding protein ACBF-	-2.64	-1.92	-2.02	-2.51	-1.92	-1.56	-2.58	-1.64	-2.06

Table. 3.1 (Contd.)

Agilent probe	Gene Description	Fold change (*P*<0.05)								
		Harpin			CSNP			H-CSNP		
		24h	48h	72h	24h	48h	72h	24h	48h	72h
	V Photosynthesis and Housekeeping									
A_96_P067073	Sterol reductase	3.14	2.74	3.09	3.22	3.73	2.91	3.38	1.83	2.61
A_96_P027076	Sucrose-phosphatase	2.55	3.00	2.55	2.83	2.50	2.65	2.16	2.12	2.25
A_96_P011131	Isoamylase isoform 2	2.22	1.66	1.98	1.25	2.75	2.40	2.24	1.05	1.44
A_96_P089580	Arginine decarboxylase	3.17	2.73	2.44	2.87	2.91	2.63	2.86	2.15	2.85
A_96_P069754	Dehydroascorbate reductase	2.31	1.13	2.13	1.77	2.02	1.99	2.59	0.75	1.44
A_96_P265587	β-amylase	3.36	1.97	2.93	2.78	2.78	2.68	3.57	1.64	2.18
A_96_P190619	HMG-CoA reductase	3.36	2.49	2.85	3.14	2.59	1.30	2.97	1.79	2.21
A_96_P067051	Ga20 oxidase	3.04	2.11	3.04	2.92	3.14	2.33	2.40	0.37	2.53
A_96_P040361	Isovaleryl-CoA dehydrogenase 2,	2.29	1.49	1.89	1.61	1.65	1.63	2.11	1.17	1.40
A_96_P264462	Fructokinase	2.89	0.24	1.49	-	-	-	3.54	-0.47	1.17
A_96_P172729	1,4-α-glucan branching enzyme	2.18	1.20	1.50	1.43	2.11	2.02	2.74	1.17	1.17
A_96_P027866	Phytoalexin-deficient 4-1 protein	2.28	1.87	1.77	2.07	2.12	1.51	2.32	1.46	1.49
A_96_P135717	Phosphoenolpyruvate carboxylase	-	-	-1.89	-	-2.82	-	-1.94	-1.54	-2.37
A_96_P013146	Hexokinase-1	-	-	-	-	-1.77	-	-2.17	-1.71	-1.66
A_96_P137002	Transketolase, chloroplastic	2.33	1.59	1.22	-	-	-	2.31	0.25	1.46
A_96_P227454	Ferredoxin-thioredoxin-reductase	-	-	-	-	-	-	2.31	0.78	0.81
A_96_P055041	NADH-quinone oxidoreductase	-	-	-	-	-	-	-2.19	-0.32	0.41
A_96_P049851	NADH-quinone oxidoreductase	-	-	-	-	-	-	2.26	2.01	1.93
A_96_P027381	Oxygen-evolving enhancer protein 2	-	-	-	-	-	-	-2.06	-1.25	-1.22
A_96_P100959	PSII polypeptide	-	-	-	-	-	-	-2.27	-0.71	-0.94
A_96_P262427	Photosystem II protein I	-	-	-	-	-	-	-2.37	-1.17	-1.21
	VI Aromatics biosynthesis									
A_96_P058091	Flavonoid 3-glucosyl transferase	2.47	1.35	2.08	2.19	1.72	1.87	2.87	0.61	1.53
A_96_P111342	Flavonoid 3',5'-hydroxylase	3.12	-	2.49	-	-	-	3.84	-	2.57
A_96_P085949	4-coumarate—CoA ligase	3.29	3.25	3.51	3.47	4.44	3.09	2.21	1.86	3.03
A_96_P212304	Flavanone 3 β-hydroxylase	2.70	1.98	2.45	2.45	2.92	2.64	2.74	1.97	1.91
A_96_P254977	4-coumarate:coenzyme A ligase	2.49	1.54	2.34	1.95	2.08	2.14	2.78	1.23	2.01
A_96_P029671	Gibberellin 3-β-hydroxylase 2	-	-	-	-	-	-	2.42	-	-
A_96_P151671	Phenylalanine ammonia-lyase 1	2.25	1.90	2.12	2.44	2.45	2.32	2.09	1.70	2.16
A_96_P084479	Cinnamoyl CoA reductase	3.12	1.70	1.59	3.54	1.92	2.00	3.11	0.69	1.50
A_96_P063931	chalcone synthase	-	-	-3.28	-	-4.54	-	-4.53	-3.70	-3.02
A_96_P056571	Flavonol synthase	-	-	-	-	-	-	-2.62	-2.09	-2.23