Role of cytokinins in plant immunity

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Vorgelegt von
Muhammad Naseem (M.Phil)
Aus
Allai, Pakistan

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Mitglieder der Promotionskommission

Vorsitzender: Prof. Dr. M. J. Müller

1. Gutachter: Prof. Dr. T. Roitsch

2. Gutachter: Prof. Dr. W. Kaiser

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……TO MY UNCLE HABEEB-UR-REHMAN……

List of Abbreviations

°C	**Degree Celsius**
A	**Adenine**
Agro	***Agrobacterium tumefaciens***
A. thaliana	***Arabidopsis thaliana***
ABA	**Abscisic Acid**
Amp	**Ampicillin**
ATP	**Adenosin-Triphosphate**
b	**Base**
bp	**Basepairs**
BR	**Brassinosteroids**
BSA	**Bovines Serum Albumin**
C	**Cytosine**
CKs	**Cytokinins**
CFU	**Colony Forming Units**
Col-0	**Columbia**
cv.	**Cultivar**
D	**Day**
DEPC	**Diethylpyrocarbonate**
DMSO	**Dimethylsulfoxide**
ET	**Ethylene**
E. coli	***Escherichia coli***
egfp	**Enhanced green fluorescent protein**
et al.	**along with other co-workers**
EtOH	**Ethanol**
f	**Femto (10^{-15})**
G	**Guanin**
GA	**Gibberellic Acid**
g	**gram**
h	**Hour**
IPT	**Isopentenyltransferase from *Agrobacterium tumefaciens***
JA	**Jasmonic acid**
4x-JERE	**4 times Jasmonate and Elicitor Response Element**
k	**Kilo**
Kan	**Kanamycin**
KB	**King's Medium B**
LB	**Luria-Bertani-Medium**
M	**molar (mol/l)**
m	**Milli (10^{-3}), Meter**
µ	**Micro (10^{-6})**
min	**Minute(n)**
mRNA	**Messenger-RNA**

n	**Nano (10^{-9})**
N. tabacum	***Nicotiana tabacum***
OD	**Optical density**
p	**Pico (10^{-12})**
PCR	**Polymerase chain reaction**
P. syringae	***Pseudomonas syringae***
P.s tabaci	***Pseudomonas syringae* pv. *tabaci***
pv.	**Pathovar**
rRNA	**Ribosomal RNA**
rpm	**Revolutions per minute**
RT	**Room Temperatur, Reverse Transkription**
SA	**Salicylic acid**
sec	**Second**
SAG12	**Senescence associated protein**
SR1	**Streptomycin Resistant 1**
T	**Thymin**
Tab.	**Table**
TDZ	**Thidiazuron**
Tet	**Tetracycline**
Ti-Plasmid	**Tumor Inducing Plasmid**
TMV	**Tobacco Mosaic Virus**
O/N	**Overnight**
UV	**Ultraviolet**
V	**Volt**
W38	**Wisconsin**
WT	**Wild type**

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1. Summary

Phytohormones are known for their pivotal roles in promoting normal growth and development of the plants and contributing to the mechanism of defense. Although an over simplification, however, they may be categorized as stress specific and growth promoting. SA and JA/Ethylene are implicated in stress responses while auxins, cytokinins and gibberellins are involved in developmental processes. Phytohormones from the above perspective got much attention in the last few decades; however their reciprocal role is currently in focus. It is because of the reason that plant pathogens cause overall hormonal imbalance at host pathogen interface and alter host physiology for the sake of pathogenecity. Despite their importance in growth and development, cytokinins are among the most neglected phytohormones that are usually noticed as consequence rather than a cause of pathogen infection.

Results presented in this thesis are based on the hypothesis that elevated levels of CKs embody plants with resistance against hemibiotrophic pathogens. To explore a connection between the spread of *P. syringae* and its tobacco host, CKs over producing transgenic plants were investigated whereby bacterial *IPT* gene was expressed under the control of pathogen inducible, tetracycline inducible and developmentally inducible promoters. To further validate the out-come of transgenic plants, various types of cytokinins were exogenously fed to detached tobacco leaves. Mentioned transgenics and exogenous CKs feeding approaches unanimously resulted in, **"more cytokinins less disease symptoms"** and vice versa. This state of cytokinins mediated resistance was further substantiated with various cellular, signaling, biochemical and microbial approaches wherein levels of SA and JA remained unaffected. Conversely, *PR1* gene expression was strongly up-regulated in enhanced cytokinins accumulating samples. Moreover, less accumulation of ROS was observed in *IPT* expressing sites of the plants as compared to their corresponding controls. Additionally, we neither noticed any direct effect of cytokinins on the growth of *P. syringae* pv. *tabaci* nor found presence of anti-microbial peptides in cytokinins enriched extracts. Interestingly, enhanced accumulation of phtyoalexins in elevated CKs status of the plant proved to be a possible gesture in jeopardizing the spread of pathogen. Contrarily, no reduction was observed in the spread of fungal necrotrophic pathogen *Sclerotinia sclerotiorum* when leaves of elevated CKs were inoculated.

Besides host-pathogen interaction in perspective of elevated cytokinins, impact of modulated sugar status of the plant on the spread of pathogen was also investigated. For this purpose,

previously generated modulated invertase enzyme tobacco transgenic plants were analyzed. We showed that repression and de-repression of *CIN1* gene under the control of tetracycline inducible-promoter did not affect the growth of *P. syrinage* pv. *tabaci* in Tet::CIN1 transgenic plants. Moreover, invertase inhibitor tobacco lines expressing *NtCIF* gene under the control of the same promoter failed to exhibit differential pathogenic responses in induced and non induced status of the plant. Similar was the case of tomato transgenic plants expressing *Nt*CIF gene under the control of invertase gene Lin6 promoter in Lin6:: *Nt*CIF plants for *P.syringae* pv. *tomato DC 3000.* Interestingly, when challenged Lin6:: *Nt*CIF tomato plants with *Botrytis cinerea*, severe disease symptoms were observed on transgenic leaves as compared to control plants. To dissect a potential link between cytokinins and sugar metabolism with its effect on the growth of pathogen, invertase transgenic plants with elevated CKs were probed. When expressed exogenous *IPT* gene under the control of pathogen inducible promoter (4xJERE::IPT) in transgenic background of Tet::CIN1, we observed localized differences in symptom development for *P.syringae* pv. *tabaci*. Similarly, when exogenously fed with kinetin, detached leaves of Tet::CIN1 exhibited retarded growth of *P.syringae* pv. *tabaci* as compared to the tetracycline induced leaves. These results led to the conclusion that extracellular invertase may not play an essential role in cytokinins mediated disease resistance against hemibiotrophic pathogens.

2. Summary (German)

Phytohormone spielen eine zentrale Rolle in der Regelung normalen Wachstums, der Entwicklung und der Mitwirkung an Abwehrmechanismen in Pflanzen. Allgemein betrachtet können Phytohormone in zwei Klassen unterteilt werden – in solche, die in Beziehung zu Stressreaktionen stehen und in jene, die das Wachstum begünstigen. Salizylsäure, und Jasmonsäure sind in erster Linie an der Stressresonanz, Ethylen, Auxine, Cytokinine (CKs) und Gibberilline an Entwicklungsprozessen beteiligt. In den letzten Jahrzehnten wurde den Phytohormonen aus diesem Betrachtungswinkel starke Aufmerksamkeit gewidmet und heute stehen ihre wechselseitigen Beeinflussungen im Fokus. Die Tatsache, dass Pflanzenpathogene ein hormonelles Ungleichgewicht an der Wirtspflanzen-Pathogen Schnittstelle bedingen und es begleitend zu physiologischen Veränderungen kommt, wird dabei als Werkzeug für Erforschungen in Pflanzengeweben genutzt. Abgesehen von der bekannten Bedeutung, die Cytokinine für Wachstum und Entwicklung haben, sind sie bisher am meisten vernachlässigt worden und eher als Konsequenz denn als Grund von Pathogeninfektionen angesehen worden.

Die Ergebnisse dieser Arbeit basieren auf der Hyphothese, dass erhöhte Gehalte an CKs die Pflanzen mit einer Resistenz gegen hemibiotrophe Pathogene ausstatten. In diesem Zusammenhang wurden transgenetische Pflanzen untersucht, in welchen das bakterielle Gen *IPT* überexpremiert wurde. Kontrolliert wurde die Expression durch einen pathogen-induzierbaren, einen tetracyclin-induzierbaren oder durch einen wachstumsabhängigen Promotor. Für die weitere Validierung der an den transgenetischen Pflanzen gewonnenen Ergebnisse wurden unterschiedliche Cytokinin von abgeschnittenen Tabakblätter aufgenommen. Alle transgenetischen Ansätze und exogen applizierten Cytokiningaben zeigten ähnliche verringerte Krankheitsanzeichen. Diese Art der Resistenz wurde im Weiteren mit verschiedenen zellulären, biochemischen, mikrobiellen Techniken sowie durch Signalwirkungstests fundiert. Die Gehalte von SA und JA blieben unverändert, während die Expression des Gens *PR1* in Proben mit erhöhtem Cytokiningehalt stark hoch reguliert wurde. Darüber hinaus konnte eine verringerte Akkumulation von ROS in *IPT* exprimierenden Blättern gegenüber der entsprechende Kontrolle beobachtet wurden. Zusätzlich konnte weder ein direkter Effekt im Wachstum von *P. syringae* pv. *tabaci* noch die Präsenz von antimikrobiellen Peptiden in Cytokinin-angereicherten Extrakten festgestellt werden. Interessanterweise ist die verstärkte Akkumulation von

Phytoalexinen bei erhöhtem CKs-Status der Pflanze als ein mögliches Anzeichen für die Gefährdung durch die Ausbreitung von Pathogenen belegt. Im Gegensatz dazu konnten wir keine Wachstumsverlangsamung für *Sclerotinia sclerotiorum* in Blättern mit erhöhten CKs-Gehalten feststellen.

Neben der Wirt-Pathogen Interaktion im Hinblick auf erhöhte CK-Gehalte wurden die Auswirkungen eines modulierten Kohlenstoffhaushalts auf das Wachstum von Pathogenen untersucht. Dafür wurden zuvor generierte transgenetische Tabakpflanzen, basierend auf ein regulierbarem Invertase Enzym verwendet. Es konnte gezeigt werden, dass induzierte und nicht-induzierte Expression von *CIN1* unter der Kontrolle des Tet-Promotors das Wachstum von *P. syringae* pv. *tabaci* nicht beeinflusst. Darüber hinaus zeigten Linien, welche den Invertaseinhibitor *NtCIF* unter Kontrolle desselben Tet-Promotors exprimieren, keine differenzielle Veränderung des Wachstums von *P. syringae* pv. *tabaci* bei induziertem und nicht-induziertem Status der Pflanze. Ähnlich waren die Resultate in der transgenetischen Tomaten-Linie Lin6::*Nt*CIF für *P.syringae* pv. *tomato DC 3000*. Interessanterweise zeigten die Blätter von Lin6::*Nt*CIF Tomatenpflanzen starke Symptome nach Behandlung mit *Botrytis cinerea* verglichen zum Wildtyp. Eine mögliche Verbindung zwischen Cytokininen und Zuckermetabolismus im Bezug auf die Wirt-Pathogen Beziehung wurde ebenfalls untersucht. Die Expression des IPT-Gens unter der Kontrolle des pathogeninduzierbaren Promotors (4xJERE::IPT) im transgenetischen Hintergrund von Tet::CIN1 ergab lokale Unterschiede in der Entwicklung der Symptom von *P. syringae* pv. *tabaci*. Bei exogen appliziertem Kinetin an abgeschnittenen Tabakblättern von Tet::CIN1 verzögerte sich ebenfalls das Wachstum von *P. syringae* pv. *Tabaci* im Vergleich zu Tet-induzierten Blättern. Diese Ergebnisse führen zu der Schlussfolgerung, dass die extrazelluläre Invertase keine essentielle Rolle in der Cytokinin-vermittelten Resistenz gegen hemibiotrophe Pathogene spielt.

3. Introduction

3.1 Role of phytohormones in host-pathogen interaction

Phytohormones play important roles in regulating developmental processes and signaling networks involved in plant responses to a wide array of biotic and abiotic stresses. Significant progress has been made in identifying the key components and understanding the role of salicylic acid (SA), jasmonates (JA) and ethylene (ET) in plant responses to biotic stresses. However, recent studies indicate that other phytohormones such as abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinins (CKs), brassinosteroids (BR) and the most nascent peptide hormones are also implicated in plant defence signaling pathways but their role in plant defence is less well explored.

Among stress specific hormones SA, JA and ET play distinct roles; SA has been associated with biotrophic resistance while JA and ethylene are predominantly implicated in necrotrophic resistance. A sort of antagonism also prevails between these two hormones where one suppresses the effect of other and thus pathogenic bacteria are getting the opportunity to cause infection in the invaded tissues (Feys and Parker 2000 and Kunkel and Brooks 2002). Reciprocally, synergistic interaction may also take place between SA and JA. Truman et al., (2007) while showing a contrasting role of JA in the development of systemic acquired resistance (SAR) substantiated that rapid induction of JA biosynthetic and responsive genes in systemic tissue is necessary for SAR against *Pseudomonas syringae* pv. *tomato DC3000* (PstDC3000) to occur. Adding to the complexity of apparent simplification regarding these stress hormones Mur et al. demonstrated that expression of salicylate hydroxylase downregulated SA without affecting JA during the hypersensitive response HR (Mur et al., 2006). However pathogen being interacting counterpart also co-evolved strategies of conferring susceptibility. In this regard, Cui et al. (2005) demonstrated that systemic induced susceptibility towards *PstDC3000* is dependent on coronatine, a JA mimic produced by the bacteria.

Not only stress specific but imbalance in growth promoting hormones upon pathogen inoculation is part of the defense or susceptibility against the invading pathogen. After challenging with two virulent pathogens: *Xanthomonas campestris* pv. *campestris* and (PstDC3000) Donnell et al (2003) found rapidly elevated levels of auxin apart from increase in usual stress hormones such as SA, JA and ET. Independently, Schmelz et al. (2003) demonstrated the induction of auxins

biosynthetic genes upon inoculation of PstDC3000 with concurrent induction of abscisic acid (ABA). Induction of auxins or auxins related genes is more generalized phenomenon and has been elaborated with multiple tools for various plant-patho systems. Down these lines, Marois et al. (2002) showed that AvrBs3, a type III effector from *X. campestris* pv. *vesicatoria*, can induce auxin-responsive genes, resulting in cell hypertrophy. Furthermore, *in planta* expression of AvrRpt2, a type III effector from *P. syringae* alters auxin physiology in the absence of the corresponding R-gene (Kunkel et al., 2004). Interestingly, upregulation of auxin signalling renders the plant more susceptible to PstDC3000 and attenuation of auxin signalling via overexpression line of miR393, microRNA that targets auxin receptors, increased resistance against the same bacteria (Navarro et al., 2006). ABA which is predominantly implicated in abiotic stresses also has a profound role in disease development. Virulence of PstDC 3000 in a T3SS-dependent manner was proved to be dependent upon a functional ABA biosynthetic pathway (Truman et al., 2006 and Zabala et al., 2007). They substantiated that conditional expression of the bacterial effector AvrPtoB was sufficient to induce accumulation of ABA and promote bacterial growth. Furthermore, Thilmony et al., (2006) confirmed that in T3SS and coronatine dependent manners challenge of PstDC3000 elevate the level of auxin and ABA in plants.

Gibberellic acid (GA) seems to have an opposite effect on plant defence. DELLA proteins which are negatively regulating plant growth, GA promote plant growth by inducing the degradation of the (Harberd 2003). It has recently been demonstrated that DELLA delays *Botrytis* induced H_2O_2 accumulation and plant cell death (Achard et al., 2008). Loss-of-function mutations in DELLA proteins render the plant more resistant to PstDC3000 through potentiation of the SA-dependent defence pathway (Navarro et al., 2008). Reciprocally, same mutants are hyper-susceptible to the necrotrophic pathogen A. *brassicicola*. This further emphasizes that DELLAs promote resistance to necrotrophs and susceptibility to biotrophs, partly by modulating the balance between SA mediated and JA/ET-mediated defence signalling pathways (Navarro et al., 2008). It is not an easy task to unite all relevant reports regarding both growth and stress specific hormones into a single model, while each and every plant-patho system has its won specifications however; the working model of Bari and Jones (2008) is a commendable effort in putting together all parts and parcels of hormonal interactions in biotic stress (Fig.1).

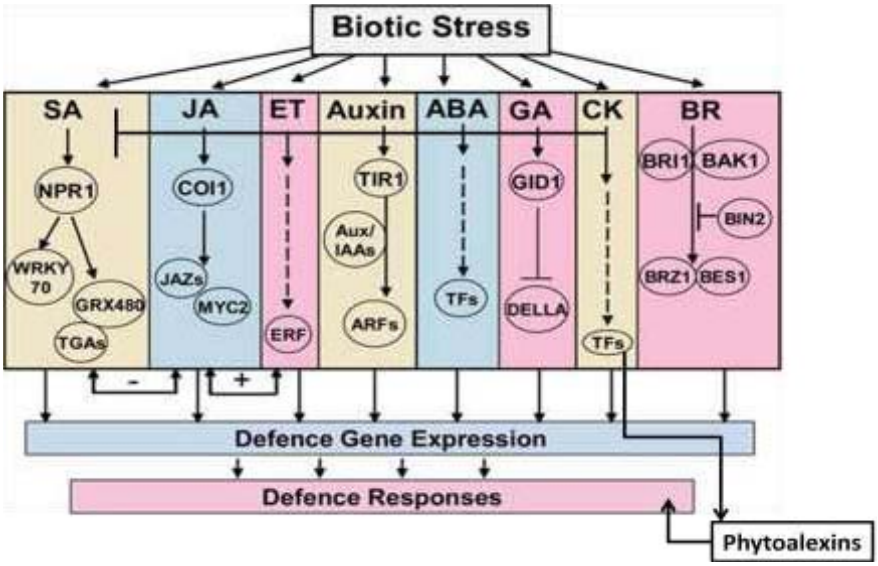


Fig.1 Overview of hormonal interplay during pathogen infection, with modifications in model of Bari and Jones (2008).

3.2 Cytokinins and its role in plant diseases

Cytokinins were discovered in the search for agents that enhanced division of plant cells in culture. Cytokinins are N^6-substituted adenine derivatives that contain an isoprenoid derivative side chain (Fig.2). These hormones influence numerous aspects of plant development and physiology, including seed germination, de-etiolation chloroplast differentiation, apical dominance, flower and fruit development, and leaf senescence and plant pathogen interactions. These processes are also monitored by various other stimuli e.g. light and other phytohormones. For example, the classical reports of Skoog and Miller (1957) revealed that undifferentiated callus cultures would form into roots or shoots depending on the ratio rather than the absolute amount of auxins and cytokinins.

The breakdown of tRNA was originally thought to be a mechanism for cytokinin synthesis. The released cis-zeatin could subsequently be converted to active trans-zeatin by the enzyme cis-transisomerase (Mok and Mok, 2001). Enzymatic activity that converts AMP and dimethylallyl-diphosphate (DMAPP) to the active cytokinin isopentenyladenosine-5-monophosphate (iPMP) was firstly discovered in *Dictyostelium discoideum* (Taya et al., 1978). Subsequently, the *tmr* gene (later designated as *ipt*) from *Agrobacterium tumefaciens*, was shown to encode an enzyme

with similar activity (Akiyoshi et al., 1984). *IPT* genes have also been identified in several other bacteria, and IPT activity was detected in crude extracts from a variety of plant tissues however, neither plant enzyme was purified nor did IPT gene was cloned.

Complete genome sequencing of *Arabidopsis* enabled researcher to identify total of nine IPT genes (Takei et al. 2001 and Kakimoto 2001). A phylogenetic approach revealed that *AtIPT2* and *AtIPT9* encode a putative tRNA-ipt while the other seven *AtIPTs* formed a distinct group more closely related to the bacterial *ipt/tmr* gene. When expressed in *Escherichia coli*, these seven genes resulted in the accumulation of the cytokinins iP and zeatin, substantiating that they encode cytokinin biosynthetic genes (Takei et al., 2001a). Ironically, in the absence of cytokinins, calli expressed *At*IPT4 gene under the control of CaMV 35S promoter, initiated shoot growth. However, CaMV 35S::*AtIPT2* calli were still dependent on cytokinin (Kakimoto, 2001). Surprisingly, unlike the bacterial IPT enzymes, purified AtIPT4 utilized ATP and ADP preferentially over AMP as a substrate (Kakimoto, 2001). The product of the plant enzyme is likely to be (iPTP) and (iPDP), which can be subsequently interconverted to zeatin.

Åstot et al., (2000) demonstrated the existence of alternative cytokinin biosynthesis pathway in plants. They compared the biosynthetic rate of zeatin riboside-5-monophosphate (ZMP) and iPMP in wild type and transgenic plants designed to inducibly overexpress the bacterial *ipt* gene. iPMP was the direct product of the transfer of DMAPP to AMP, and it can be converted to ZMP by an endogenous hydroxylase activity. In vivo deuterium labeling revealed a 66-fold higher biosynthetic rate of ZMP than that of iPMP, the product of IPT. By a feeding experiment with two tracers, which allowed the simultaneous determination of iPMP-hydroxylase activity and the de novo synthesis of ZMP, it was shown that the major precursor for ZMP was not cytoplasmic iPMP. The authors suggested the presence of an iPMP-independent pathway, in which ZMP is directly synthesized by IPT from AMP. To enhance the level of cytokinins in our host-pathogen interaction experiments, we expressed bacterial *IPT* gene under the control of inducible rather than constitutive promoters to avoid cytokinin mediated developmental phenotypes. Among them 4xJERE::IPT expression system is more promising and better tool for modulating the status of cytokinins at the host pathogen interface.

Although distinct but very heterogeneous class of phytohormones are the cytokinins (CKs), which have gained increasing attention since the classic experiments of Skoog and Miller (1957). The biosynthesis of these compounds is still mostly enigmatic (Mok and Mok 2001). A part from

their role in growth and development, CKs has also been linked to both resistance and susceptibility against herbivores and pathogens (Smigocki et al. 1993 and Siemens et al. 2006). These studies were either based on exogenous supply, mostly in the form of kinetin, or by introducing the bacterial *IPT* gene from *Agrobacterium* into plants. Depending upon the nature of interacting partners cytokinins elicit variable responses from plants against the invading pest. For instance, feeding of tomato plants with kinetin rendered plants susceptible to nematode *Meloidogyne incognita* which otherwise proved to be resistant against the infection of said pathogen (Dropkin et al. 1990). On the contrary, *IPT* expression in *Nicotiana plumbaginifolia* reduced viability of the insects *Manduca sexta* and *Myzus persicae*, moreover feeding of zeatin via petioles even enhanced the state of resistance (Smigocki et al. 1993).

Regarding the role of cytokinins in cessation of viral replication Sano et al. (1994) described that high endogenous CK-levels increased plants resistance to several viruses, and white clover mosaic potexvirus replication was significantly reduced after direct feeding of cytokinin (Jameson 2000). Incase of fungal infections the scenario is even more complex. Although it is widely accepted that green island around the infected area of fugal biotrophs contribute to their infection process. Contrarily, Sole and Fernandes (1970) described that floating tobacco leaf discs on kinetin-containing solutions inhibited the biotrophic fungus *Erysiphe cichoracearum*. Furthormore, it was found that increasing endogenous CK levels in barley reduced infection severity of *Erysiphe graminis* (Liu and Bushnell 1986). However, when constantly supplied with kinetin, potato tuber discs manifested hyper susceptibility during the infection of *Phytophthora infestans* (Beckman and Ingram 1994). Haberlach et al. (1978) demonstrated the interaction between tobacco tissue culture cells and the hemibiotroph *Phytophthora parasitica* var. *nicotiana*, it was found that 1µM kinetin drastically reduced fungal proliferation. Recently, overexpression of CK oxidase/dehydrogenase in *Arabidopsis thaliana*, which degrade CK contents, has been shown to enhance resistance against the biotrophic pathogen *Plasmodiophora brassicae* (Siemens et al. 2006). It is worth mentioning that fungal necrotrohic pathogens got substantially less attention than biotrophs.

Regarding bacterial pathogen it has been shown that during incompatible interaction between tobacco and *Pseudomonas pisi*, the pathogen multiplied in leaves treated with CK far better than in control. On the contrary, symptom development upon infection with the compatible *P. tabaci* was delayed, though in the latter case no data on bacterial growth countings have been provided

(Novacky et al. 1972). In another study, kinetin side directed spraying on tobacco leaves led to the failure of chlorosis development after infiltration with *P. tabaci* culture filtrate (Lovrekovich and Farkas 1963). Quite recently, Barna et al. (2008) demonstrated that the growth of incompatible *Pseudomonas* was slightly inhibited in *IPT*-expressing tobacco though the growth of *P. syringae* pv. *tabaci* remained unaffected.

Contrasting effects of higher CKs levels in the above mentioned plant-patho systems and lack of trend settings across the various nature of pathogens clealy depicts that role of CKs in plant immunity has apparently been ignored. To better understand the implications of CKs in plant-pathogen interactions we have used three independent transgenic approaches based on the expression of the bacterial *IPT* gene in tobacco plants. CKs levels were enhanced either by application of tetracycline in Tet-IPT lines (Redig et al. 1996) or after onset of senescence in SAG12-IPT plants (Gan and Amasino 1995). We also included a novel construct (4xJERE-IPT) whereby the expression of IPT gene is upregulated by pathogen infection. These approaches mimic the spatial and temporal deployement of endogenous CKs in tobacco plants. To compare the effect of increased endogenous CKs contents with exogenous supply we included a number of feeding experiments with detached tobacco leaves. For infections, hemibiotrophic and compatible bacterial pathogen *P. syringae* pv. *tabaci* and necrotrophic fugal pathogen *Sclerotinia sclerotiorum* was used.

3.3 *Pseudomonas syringae* being a model pathogen.

Pseudomonas syringae is a gram-negative bacterial pathogen and having relatively broader host range. Upon infection, it results in chlorosis followed by necrotic symptoms in leaves, stems, and fruits. It is considered to be a hemibiotrophic pathogen by virtue of its ability to obtain nutrients from living host cells in order to multiply in the apoplast and infect neighboring tissues. Hirano and Upper (2000) described it to be present either epiphytically or endophytically without eliciting typical disease symptoms in plant foliage. It gets an access to plant apoplast either through natural body openings e.g. stomata or hydathodes. Moreover, its delivery may also be facilitated due to mechanical wounding in the plants. Relatively low temperature and high humidity promote the multiplication of *P. syringae.* It has been studied extensively as a model pathogen to dissect the molecular mechanisms and co-evolution of pathogenesis and plant disease resistance.

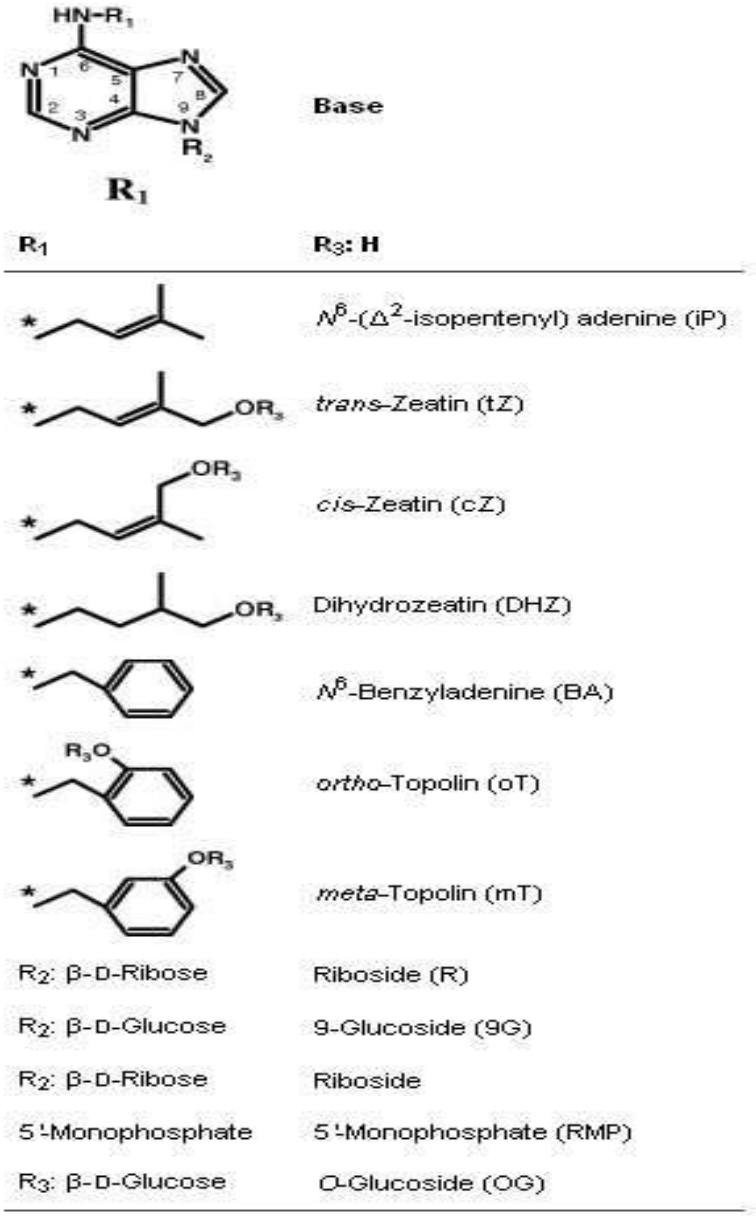


Fig.2 Types of cytokinins (Novák et al, 2008) with modification.

Rico and Gail (2008) reviewed that primary mechanism of plant defense against *P. syringae* is a basal defense response that is induced upon detection of microbe associated molecular patterns

(MAMPS) and is called as PTI (PAMPS-Triggered Immunity) (Mackey et al. 2003 and Navarro et al., 2006). *P.syringae* has the potential to suppress basal defenses in susceptible plants by secretion of effectors proteins into the cytoplasm of plant cells. Effector proteins combate plant surveillance mechanisms and signal transduction pathways, thereby allowing bacterial spread in the invaded tissue. Resistant plants frequently undergo a subsequent line of defence in which plant resistance proteins (R proteins) recognize either effectors or their effects on plant cells and trigger immune (Effectors Triggered Immunity) responses that block pathogen multiplication.

These immune responses include a localized cell death response known as the hypersensitive response (HR) (Alfano and Collmer 2004). R protein-dependent defenses have promoted further evolution of *P. syringae*, including the evolution of effectors that suppress effector-triggered immunity (Alfano and Collmer 2004; Chisholm et al. 2006; Jones and Dangl 2006; Mackey et al. 2003; Nomura et al. 2005 and Navarro et al., 2007). Although effector proteins are essential for suppressing host basal defenses in the first stages of invasion and for long-term survival in plant tissues, the initial success of *P. syringae* as a plant endophyte depends on its ability to survive and proliferate in the apoplast of healthy plants. According to Rico and Gail (2008), this success is likely to be determined at multiple fronts: its ability to tolerate preformed defense molecules, its ability to import and metabolize available nutrients, and ultimately, its ability to express pathogenecity and virulence factors that modulate host defenses and host metabolism. Boch and associates (2002) identified a wide range of plant-induced loci in *P. syringae* pv. *tomato* DC3000, which included not only virulence-associated genes such as *hrp* genes and coronatine biosynthesis genes but also genes involved in stress tolerance, polysaccharide synthesis, nutrient uptake, amino acid assimilation, and carbon metabolism.

During the course of pathogen attack not only monitoring of host responses is essential but physiological alterations inside the pathogen are also equally important. Most studies on the adaptation of *P. syringae* to the plant apoplast have focused on the expression and regulation of pathogenecity genes, such as the *hrp* genes that encode the type III protein secretion system (TTSS) that delivers effectors into plant cells. In vitro, *hrp* gene expression is induced by low PH; sugars such as sucrose, fructose, and mannitol; and a low N/C ratio. *hrp* expression is suppressed by amino acids and tricarboxylic acid cycle (TCA) intermediates, possibly due to catabolite repression (Huynh et al. 1989; Rahme et al. 1992 and Xiao et al. 1992 and Rico and Gail 2008). *hrp* genes are rapidly upregulated when bacteria are infiltrated into plant leaves

(Rahme et al. 1992; Xiao et al. 1992). Appoplastic fluid is instrumental in adequetly expressing *hrp* genes contributing to the virulence of the bacteria (Rico and Gail 2008). To get detailed insights into the spread of *P. syrinage*, we investigated the impact of elevated level of cytokinins in plants. Moreover, we also demonstrated the influence of altered carbohydrate status of the plant on the spread of pathogen by modulating invertase being a key metabolic enzyme in plants. To get a comparative understanding of the spread of pathogen in modulated invertase and cytokinins status of the plant besides *Pseudomonas*, we also worked with necrotrophic fugal pathogens i.e. *Botrytits cinerea* and *Sclerotinia sclerotiorum*.

3.4 Plant-pathogen interaction; cytokinins, primary and secondary metabolism

Plants are exposed to a variety of biotic stresses, however the mechanism of fight and flight is not very evident in them as does occur in animals. Animals are favored by the presence of mechanical articulation in their bodies and it's lacking in plants entitled them sessile in their locomotory behavior. Generally, plants respond to these stresses by the expression of defense genes and alterations in growth, development and metabolism.

One general stress response in plants is the induction of sink specific enzyme, the cell wall invertase (EC 3.2.1.26), and an enzyme which cleaves sucrose in the apoplast and plays an important role in regulating carbohydrate partitioning (Roitsch et al., 2003, Roitsch 2004). It has been shown that extracellular invertases are up regulated in response to different elicitors in suspension cultures and wounded green leaves (Godt and Roitsch 1977, Ehness et al., 1997). Extracellular Invertase Lin6 of tomato has been shown to be up-regulated in the photoautotraphic suspension cell culture of tomato in response to treatment with elicitors (Sinha et al., 2002). Recently, in a coordinated gene expression studies for defense, metabolism, and photosynthesis, it has been reported that Lin6 of *Arabidopsis* is up regulated upon pathogenic challenges (Berger et al., 2004).

Plants extracellular invertase genes represent small gene families, which are comprised of several members with specific expression patterns (Tymomska-lalanne and kreis, 1998, and Sherson et al., 2003). Under stressed conditions a fast and strong up regulation of extracellular invertase transcript level has been observed (Roitsch et al., 1995; Zhang et al., 1997; Strum and Chrispeels 1990; Sinha et al., 2002). The coordinated induction of momosccharide transporter and cell wall invertase during infection with fungal Biotroph (Photopolos et al., 2003) confirmed

an essential role of apoplastic sucrose cleavage in mediating defence responses. Both invertase and defence genes have been found to be co-induced by soluble sugars (Zhang et al., 1997; Roitsch et al., 1995; Sinha et al., 2002).

In recent years it become evident that sugars, and notably sucrose and its cleavage products are important metabolic signals that affect the expression of different classes of genes involved in primary metabolism and pathogen responses. These metabolic signals are generated by direct or indirect effects of invertases and were shown to regulate their own expression. Carbohydrate status of the plant influences the defense reactions and pathogen growth. This is documented in the Phenomenon of "high sugar resistance" and the inducibility of several PR-genes by sugars (Herbers et al., 1996). In addition transgenic tobacco plants over expressing a yeast invertase showed increase PR-gene expression and increased resistance against a virus (Herbers et al., 1996) supporting the proposed connection between carbohydrate status and pathogen response. To get an unequivocal picture of the underlying host pathogen interaction in modulated carbohydrate status of the plant, we challenged *P. syrinage* pv. *tabaci* against previously generated tobacco transgenic plants (Tet::CIN1) and (Tet::*Nt*CIF). Besides tobacco, tomato plants harboring tobacco cell wall invertase inhibitor (*Nt*CIF) under the control of Lin6 (Lara et al., 2004) cell wall bound invertase gene promoter have also been used in experiments.

Green islands formation around the infected regions due to attack of a biotrophic pathogen become visible during terminal stage of infection (Scott 1972). Whether generation of green islands is due to the re-greening or chlorophyll retention has been the subject of profound discussion. Importantly, although photosynthetic activity might be impaired in green island tissue, the region is still photosynthetically active (Scholes et al., 1986; Roberts and Walters 2007 and Walers et al., 2008). Long back, cytokinins are known to cause delay in senescence when applied to detached leaves or to leaves on intact plants or when endogenous potential of the synthesis was enhanced by expressing bacterial *IPT* gene in tobacco plants (Letham and Palmi 1993 and Gan and Amasino 1995). They are also known to play a role in the synthesis and maintenance of chlorophyll and are known to influence the structure, development and metabolism in chloroplast (Legocka and Szweykowska 1985). Indeed, cytokinins have also been shown to promote re-greening of senescent leaf tissue. Therefore, it is not surprising that cytokinins have been implicated in green island formation (Walters et al., 2008).

Cytokinins are known to induce invertase activity Roitsch et al., (2003) and have been found to co-induce hexose transporters and extracellular invertase in chenopodium rubrum (Ehness and Roitsch 1997). Recent work has shown that in tobacco, extracellular invertase is an essential component of the cytokinin mediated delay in senescence (Lara et al., 2004). Additionally, in tomato an increase in invertase activity upon infection of *P. syrinage* and *Botrytis cinerea* was also found (Berger et al., 2004). This increase was not only limited to these pathogen rather it was also observed for biotrophic fungal pathogens (Chou et al., 2000; Bonfig et al. 2006). Regarding interconnection between cytokinins and increase in invertase activity upon fungal biotrophic pathogen infection, Walters and Roberts (2007) proposed that in the early stages of the interaction, localized accumulation of cytokinin, leads to increased invertase activity. This in turn leads to: (i) nutrient mobilization towards infection sites (ii) reductions in photosynthetic metabolism; and (iii) the foundations of green island formation. However, *P. syringae* being a hemibiotrophic pathogen may not necessarily be following the same. Therefore, we exclusively analyzed the growth of *P.sryinage* pv. *tabaci* in transgenic plants with increased invertase activity and concomitantly with elevated levels of the cytokinins.

The role of phenolic compounds has long been implicated in biotic stress responses. One general stress response in elevated cytokinins status of the plant is to exhibit increased activities of antioxidant enzymes, peroxidases, several enzymes of intermediary metabolism and a presence of pathogenesis related (PR) proteins such as PR-1b protein and proteins with chitinase activity in extracellular fluid (Schnablová et al. 2006). The interaction between CKs and pathogenesis related proteins (PR protein) production was shown by (Sano et al. 1996). CKs interfered with the signal transduction mechanisms participating in PR proteins synthesis by controlling endogenous level of salicylic acid (SA) and jasmonic acid. SA belongs to a diverse group of secondary metabolites, generally called phenolic compounds, (e.g. flavonoids, tannins, hydrocinnamate esters, and lignin) that are synthesized normally during plant growth and development (Fig.3)

Phenolic compounds have been shown to serve as signaling molecules e.g. SA Dixion and Paiva (1995), to modulate the action of auxins Volpert and Osswald (1995), and to play an important role in the resistance of plants to biotic and abiotic stresses .There are still some unanswered questions regarding the exact role of phenolic substances in plant defense against the invading pathogen and herbivores. Antioxidative properties of polyphenols arise from their high reactivity

as hydrogen or electron donors and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron and from their ability to chelate transition metal ions, i.e. termination of Fenton reaction (Blokhena et al., 2003). Takahama and Oniki (1997) have proposed that the peroxidase-phenolics-ascorbic acid system can function as a hydrogen peroxide scavenging system in vacuoles and apoplast, because phenolics, ascorbic acid and peroxidase are normal components of those compartments.

Other phenolic biopolymers, lignins, are located in the primary and secondary walls of specific plant cells as well as in the middle lamella (Donaldson 2001) .They are synthesized for mechanical support and water transport of terrestrial vascular plants and in response to pathogen attack. The monomers of lignin derived from three hydroxycinnamyl alcohols or monolignols: pcoumaryl, coniferyl, and sinapyl are synthetized in the cytoplasm (Golgi or endoplasmic reticulum) and released into the cell wall from vesicles. Enzymes located within the cell wall during lignification, in either free or bound state, include various types of peroxidase (POD) and oxidase (including laccase). Oxidase activity may be associated with the earliest stages of lignification and POD with the later stages (Donaldson 2001). Recently, Schnablova et al, (2006) reported that transgenic plant expressing bacterial IPT gene under the control of *ssu* (Rubisco small sub unit) promoter, resulted in elevated cytokinins contents and concomitantly accumulation of most of the phenolic compounds raised except that of SA. They further demonstrated that besides no apparent incearse in the level of SA accumulation, production of PR1 was enhanced. They proposed that cytokinins may directly be involved in the accumulation of PR1. This further leads to the possibility that the action of cytokinins is independent of SA. Therefore, we also included PR1::GUS lines in our investigation and also analyzed the accumulation of SA and JA in elevated cytokinins status of the plant.

According to Dominik Grosskisnsky (2008), phytoalexins are plant secondary metabolites and are involved in defence against pathogenic microorganisms and also accumulating at the site of infection in hypersensitive responses (HR). First of all, in 1940 Müller and Börger discussed their role in resistance against *Phytophthora infestans* in potato. However twenty years later, first bonafide phytoalexin "Pisatin" was isolated by Cruickshank and Perrin (1960) from *Pisum sativum*. Untill now, many different types of phytoalexins have been identified from various plant families. Generally, they are low molecular weight lipophilic substance and are synthesized upon pathogen infection as a defence measure in plants. They are as important in the defence of

plant against an invading pathogen as antibodies in animals (Kuć and Rush 1985). Contrary to antibodies which are proteins and having the unique property of diversity, specificity and memory, phytoalexins are chemically very diverse, non-specific and do not accumulate before the onset of infection. However, they play a pivotal role in coordinated defence against the invading pathogen.

Regarding phytoalexins, Dominik Grosskinsky (2008) further reviewed that phytoalexins include various organic substances such as flavonoids, phenylpropanoids Isoflavonoide derivatives or sesquiterpenes. They are synthesized in pathways such as malonate actetate, acetate mevlanate and phenylpropanoid-biosythetic pathway (Kuć and Rush, 1985; Kuc, 1995 and Dixon et al., 2002). However these pathways link to each others directly or indirectly at the level of various intermediate products (Nugroho and Verpoorten, 2002). All plants posses the genetic infrastructure of producing phytoalexins however, specific families are producing specific compounds, for instance legumes mainly produces isoflavones, where as predominantly *Solanaceae* are liberating terpenoids (Kuć and Rush, 1985). Further studies showed that resistance in beans against *Colletotrichum lindemuthianum* is dependent upon isoflavon-phytoalexins (Durango et al., 2002). Spatial and temporal regulation of phytoalexins in pathogen dependent manners further underscores their implications in complex disease resistance mechanism. Therefore, we systematically investigate the potential role of phytoalexins as a mechanistic tool in cytokinins mediated resistance.

In tobacco (belongs to *Solanaceae*) two phytoalexins capsidiol and scopoletin have been identified (Fig. 4). Capsidiol are synthesized via sesquiterpen pathway while scopoletins are derivatives of coumarin and synthesized through phenylpropanoid pathway (Dominik Grosskinsky 2008). It has been demonstrated that the amount of capsidiol increases when plants are challenged with *lachrymans* (Guedes et al., 1982), TMV (Fuchs et al., 1983; Uegaki et al., 1988) and *P.* and also accumulated when treated with elicitors and methyl-JA (Chappell et al., 1987; Mandujano-Chavez et al., 2000). There is available evidence of the accumulation of capsidiol in pepper as well (Back et al., 1998 and Dominik Grosskinsky 2008).

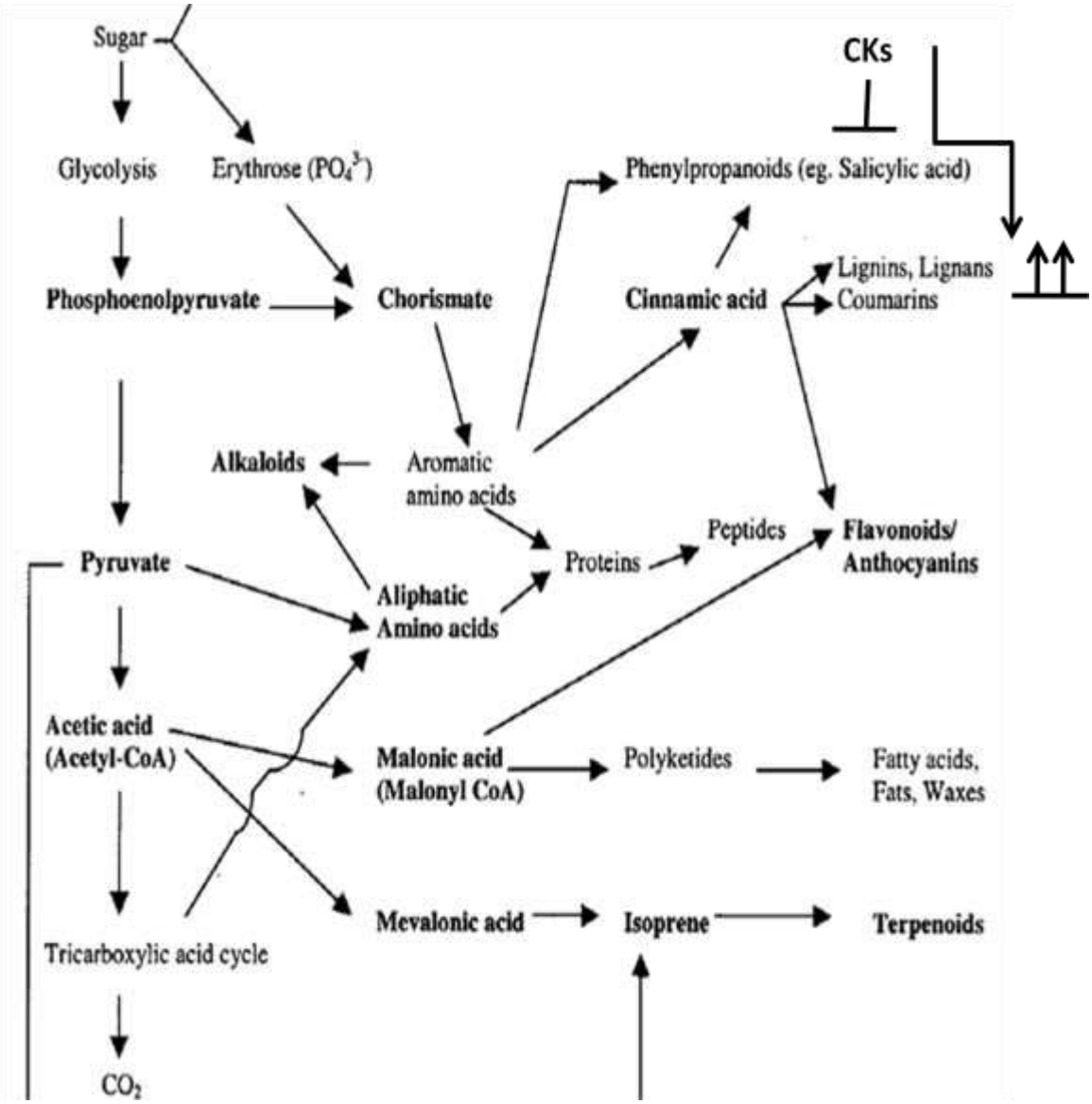
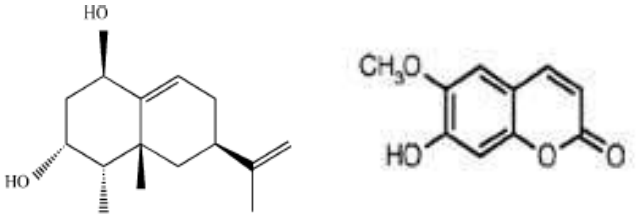


Fig. 3 Secondary metabolites, Nugroho und Verpoorte (2002), with modifications

Besides capsidiol, accumulation of scopoletin takes place in tobacco after infection with tobacco mosaic virus (Tanguy and Martin, 1972; Costet et al., 2002 and Dominik Grosskinsky 2008) and viral infections in potato (Nolte et al., 1993). Furthermore, in tobacco higher state of resistance was found against TMV with elevated scopoletin levels of the plant (Kim et al., 2000). Besides capsidiol and scopoletin, Dominik Grosskinsky (2008) described that the accumulation of other phytoalexins in tobacco such as debneyol, phytuberol and phytuberin (Burden et al., 1985; Moreau and Preisig, 1993), after infection with *P. syringae* pv. *tabaci* (Tanaka and Fujimori, 1985) and *Peronospora tabacina* (Stolle et al., 1988) or after treatment with bacterial elicitors (Moreau and Preisig, 1993) have been demonstrated. We, however, provide substantial evidence of a novel concept of the accumulation of phytoalexins in cytokinins dependent manner and proved that CKs mediated accumulation of scopoletin and capsidiol is sufficient in turning otherwise susceptible tobacco plants into resistant, against the infection of *P. syringae* pv. *tabaci*.



Capsidiol Scopoletin

Fig.4 Chemical structures of Capsidiol (Bohlmann et al., 2002) and Scopoletin (Chong et al., 2002).

3.5 Rationale for conducting this work

Classically, cytokinins (CKs) are plant hormones involved in various processes including differentiation, stem-cell control, chloroplast biogenesis, seed development, growth and branching of root, shoot and inflorescence, leaf senescence, nutrient balance and stress tolerance (Muller and Sheen 2007). Although, the exact role of CKs in plant defence is poorly understood, there are indications that CKs is involved in the regulation of plant defence responses against some pathogens. CKs play an important role in the development of club root disease caused by *Plasmodiophora brassicae* in Arabidopsis (Siemens et al. 2006). However, the molecular mechanism how CKs influences plant defences is not well known. Recently, infection with Rhodococcus fascians has been shown to modulate cytokinin metabolism in Arabidopsis (Depuydt et al. 2008). It has been shown that A. tumefaciens modifies CK biosynthesis by sending a key enzyme into plastids of the host plant to promote tumorigenesis (Sakakibara et al. 2005). Constitutive activation of a resistance (R) protein in Arabidopsis has been shown to display morphological defects through the accumulation of CK indicating the involvement of CK pathway in some R protein-mediated responses (Igari et al. 2008).

We, therefore, owned functional approaches to address the potential role of cytokinins in plant immunity against the model pathogen *P.syringae.* To elevate the level of cytokinins in plants, we express bacterial *IPT* gene under the control of inducible rather than constitutive promoters to avoid cytokinin mediated developmental phenotypes. In this context 4xJERE::IPT expression system is the most suitable tool for modulating the status of cytokinins at the host pathogen interface. To get detailed insights into the spread of *P. syrinage* , we investigate the impact of elevated level of cytokinins in plants. Moreover, we also demonstrate the influence of altered carbohydrate status of the plant on the spread of pathogen by modulating invertase being a key metabolic enzyme in plants. To get a comparative understanding of the spread of pathogen in modulated invertase and cytokinins status of the plant, apart from *Pseudomonas,* we also validate our hypothesis with necrotrophic fugal pathogens (*Botrytits cinerea* and *Sclerotinia sclerotiorum*). We demonstrate that higher levels of cytokinins in plants confer resistance against *P.syringae*. Moreover, we also explore the underlying mechanism of this resistance and substantiate the co-accumulation of higher levels of phytoalexins as a consequence of elevated levels of cytokinins in plants.

4. Results

4.1 Elevated level of cytokinins confer resistance in plants against *P. syringae*

4. 1.1 *P. syringae* impairs CKs-level at host-pathogen interface

*At*ARRs are A-type response regulator genes in *Arabidopsis thaliana*; among them *At*ARR5 is an early cytokinins response regulator and its promoter reporter (*At*ARR5::GUS) lines are frequently used to *in vivo* localize cytokinins in a tissue (Hwang and Sheen 2001 and Spichal et al., 2008). To get insights into the fate of cytokinins distribution upon pathogen infection, leaves of 5 weeks old *At*ARR5::GUS transgenic *Arabidopsis* plants were inoculated with *P. syringae* pv. *tomato DC3000* (10^6CFU/ml), while 10mM solution of $MgCl_2$ was infiltrated as a mock inoculation for comparison. GUS-staining of very low magnitude was observed on leaves of *At*ARR5::GUS with *P. syringae* pv. *tomato DC3000* compared to that of $MgCl_2$, which manifested relatively strong staining intensity for both time points of 24 and 48h (Fig.1). Moreover, staining intensity is even lesser at host pathogen interface than rest of the leaf in the underlying *P. syringae* pv. *tomato DC3000* and *At*ARR5::GUS interaction. Thus, *P. syringae* infection results in reduced level of cytokinins at host pathogen interface.

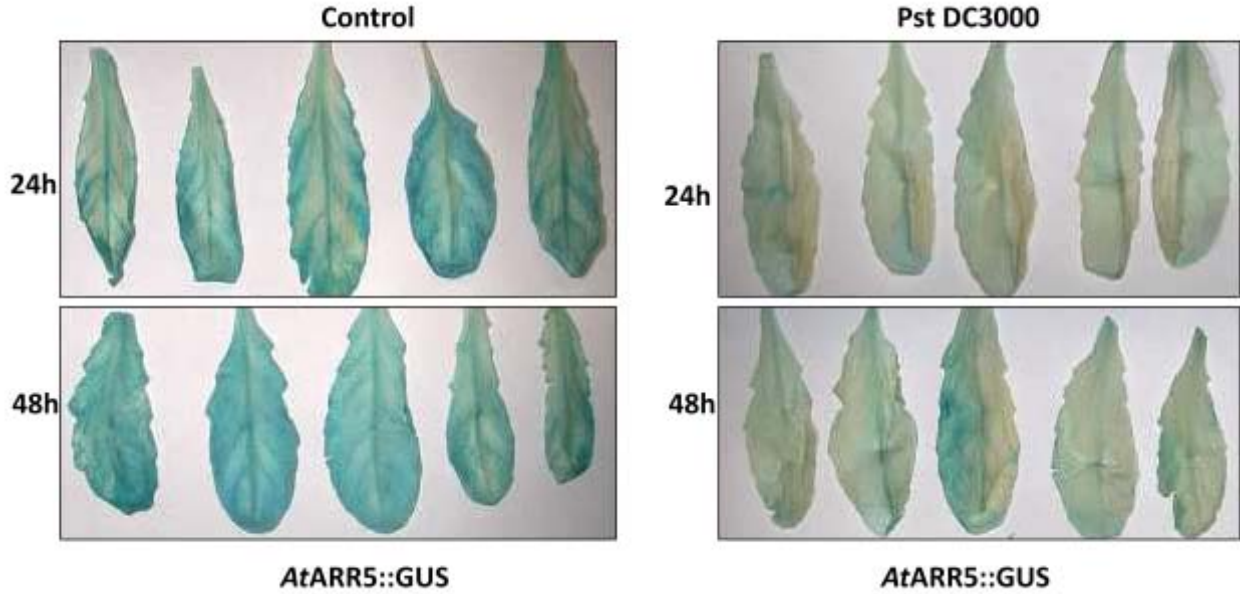
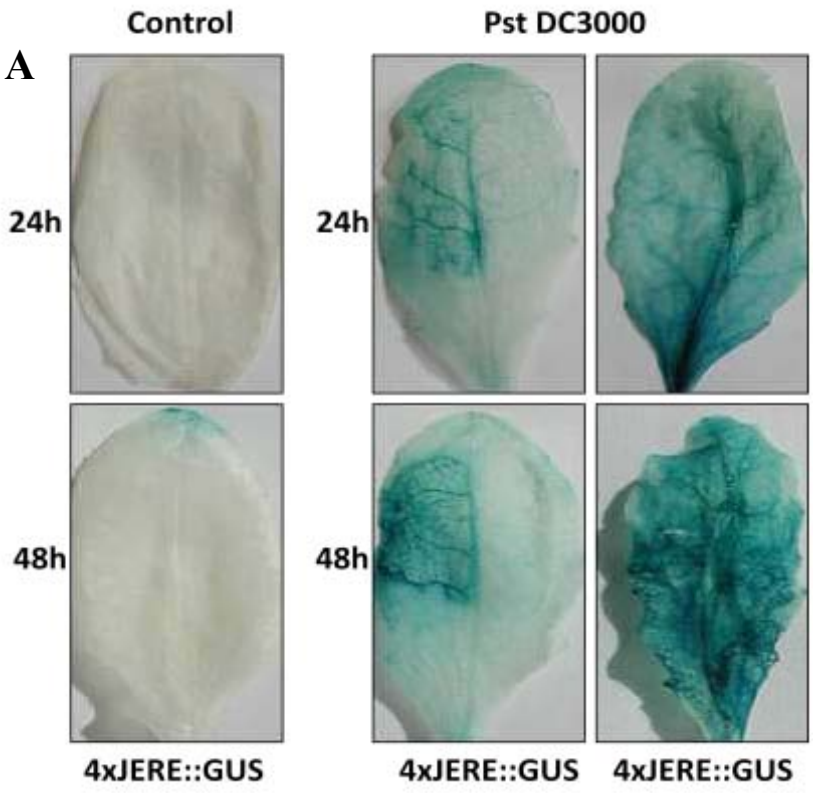


Fig. (1): *P. syringae* pv. *tomato* DC3000 affects CKs levels in leaves of *At*ARR5::GUS plants. Five-weeks old Arabidopsis *At*ARR5::GUS plants were syringe-infiltrated with *P. syringae* pv. *tomato* DC3000 (10^6CFU/ml) while for mock inoculation 10mM $MgCl_2$ was used. GUS-staining was performed at 24 and 48 hpi.

4.1.2 Development of a novel construct harboring *IPT* gene under the control of a pathogen inducible promoter

Functional approaches play an important role during the investigation of a biological phenomenon. Tight inducible expression of a transgene is more likely an unbiased and reliable tool in modulating physiological out-come of the plant. 4x-JERE (Jasmonate and Elicitor Response Element) is a synthetic promoter and very apt in a scenario of host pathogen interaction with its efficacy in manipulating and monitoring gene expression at host-pathogen interface (Rushton et al. 2002 and Bonfig et al. 2006). To get insights into the contextual expression of 4xJERE promoter, leaves of 5 weeks old 4xJERE::GUS *Arabidopsis* plants were inoculated with *P. syringae* pv. *tomato DC3000* (10^6CFU/ml), while 10mM solution of $MgCl_2$ was infiltrated as a mock inoculation for comparison. GUS-staining was observed only at the site of infection of *P. syringae* pv. *tomato DC3000* as compared to that of $MgCl_2$ which manifested no visible GUS staining in leaves of 4xJERE::GUS for both time points of 24 and 48hpi (Fig.2.A). Moreover, GUS staining was restricted to the host-pathogen interface in *Arabidopsis* leaves depending upon the nature of infiltration of the pathogen i.e. one sided or whole leaf based infiltration.

To generate transiently IPT expressing plants, bacterial *IPT* gene was first PCR amplified with restriction sites of XhoI and SacI from PUC19-IPT plasmid. *IPT* gene with inserted restriction sites was cloned into pGEMT-easy cloning vector as pGEMT-IPT. In pBT10-GUS vector which contains 4xJERE fragment. IPT was restricted from pGEMT-IPT, and cloned at the sites of XhoI and SacI by replacing GUS gene with it and thus pBT10-4xJERE-IPT plasmid was generated. Later on, both promoter and gene cassette was removed from pBT10 and cloned into pCambia 1380 at restriction sites of BgI II and HindIII resulting to a plasmid pCambia-4xJERE-IPT (Fig.2 B)



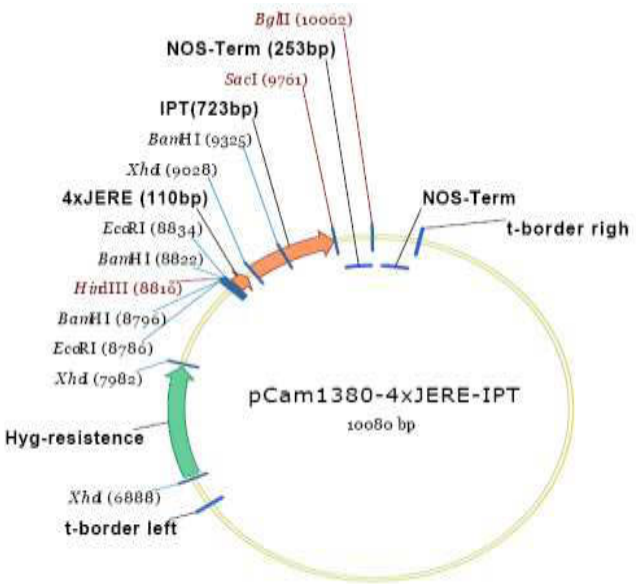


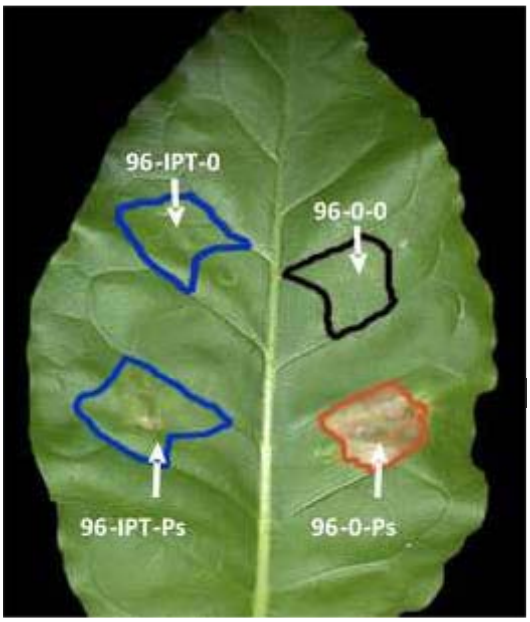
Fig.(2): Development of a novel construct for the expression of *IPT* gene under the control of a pathogen inducible promoter. (A) 4xJERE drives gene expression upon infection of *P. syringae* pv. *tomato DC3000* in leaves of promoter reporter (4xJERE::GUS) lines. 5-weeks old Arabidopsis 4xJERE::GUS plants were syringe infiltrated with *P. syringae* pv. *tomato DC3000* (10^6 CFU/ml) at the right half of the leaf as well as whole leaf, while for mock inoculation 10mM $MgCl_2$ was used. GUS-staining was performed at the time points 24 and 48 hpi. (B) Cloning of 4xJERE::IPT cassette into pCambia 1380 binary vector. *(pCambia1380-4xJERE-IPT was generated by Nicole Plickert, a former Diploma student in the working of Prof. Roitsch University of Wuerzburg*)

4.1.3 Transient expression of *IPT*-gene under the control of pathogen inducible promoter in tobacco plants abrogate the growth of *P. syringae* pv. *tabaci*.

To bring about very subtle modulations and enhance the level of CKs at host pathogen interface, the generated pCambia1380-4xJERE-IPT is a promising novel molecular biological tool to be used. Transient expression of bacterial *IPT* gene was achieved by transformation of restricted leaf areas of wild type tobacco by local infiltration with an *Agro.* strain ABL 4404 containing the plasmid pCambia 1380-4xJERE-IPT, 24 h before pathogen infection (Fig. 3A). When challenged with *P. syringae* pv. *tabaci*, an increase with time was found in the transcript levels of *ipt* such that earlier is the time point after pathogen infection, lesser is the transcript abundance of IPT and vice versa. (Fig.3B). Similar is the trend in the accumulation of CKs contents where Z-type of cytokinins are in abundance in IPT-expressing extract as compared to corresponding controls. However, OTR did not show any trend (Fig.3 F). Sites of the leaf expressing *IPT* gene upon the infection of *P. syrinage* pv. *tabaci* did not show visible disease symptoms. Contrarily, regions of the same leaf infiltrated with an *Agrobacterium* strain ABL4404 devoid of the plasmid pCambia 1380-4xJERE-IPT manifested symptoms of disease already 48 h after infiltration of *P. syringae* pv. *tabaci* (Fig. 3A).

To further substantiate the importance of this novel system, experiments were designed to observe the behaviour of *P. syringae* pv. *tabaci* at various growth stages i.e. early, late and very late phases of infection. Shown in Fig. 3C is the early stage of infection where *P. syringae* pv. *tabaci* exhibited chlorotic appearance at *non-IPT* expressing areas whereas such type of appearance at the *IPT*-expressing counterparts was not detected. Chlorotic areas turned into necrosis in the late phase of infection, however, still no visible disease symptoms appeared on *IPT* expressing areas (Fig. 3D). Eventually, the whole leaf got senescent except the *IPT*-expressing areas remained like a green-island still lacking disease (Fig. 3E). Delayed senescence in *IPT* expressing areas depict the presence of higher CKs contents as revealed in northern blotting analysis (Fig. 3B & E). These results show that induction of *IPT* gene at the site of infection is highly effective in preventing disease development.

A



B

C

D

E

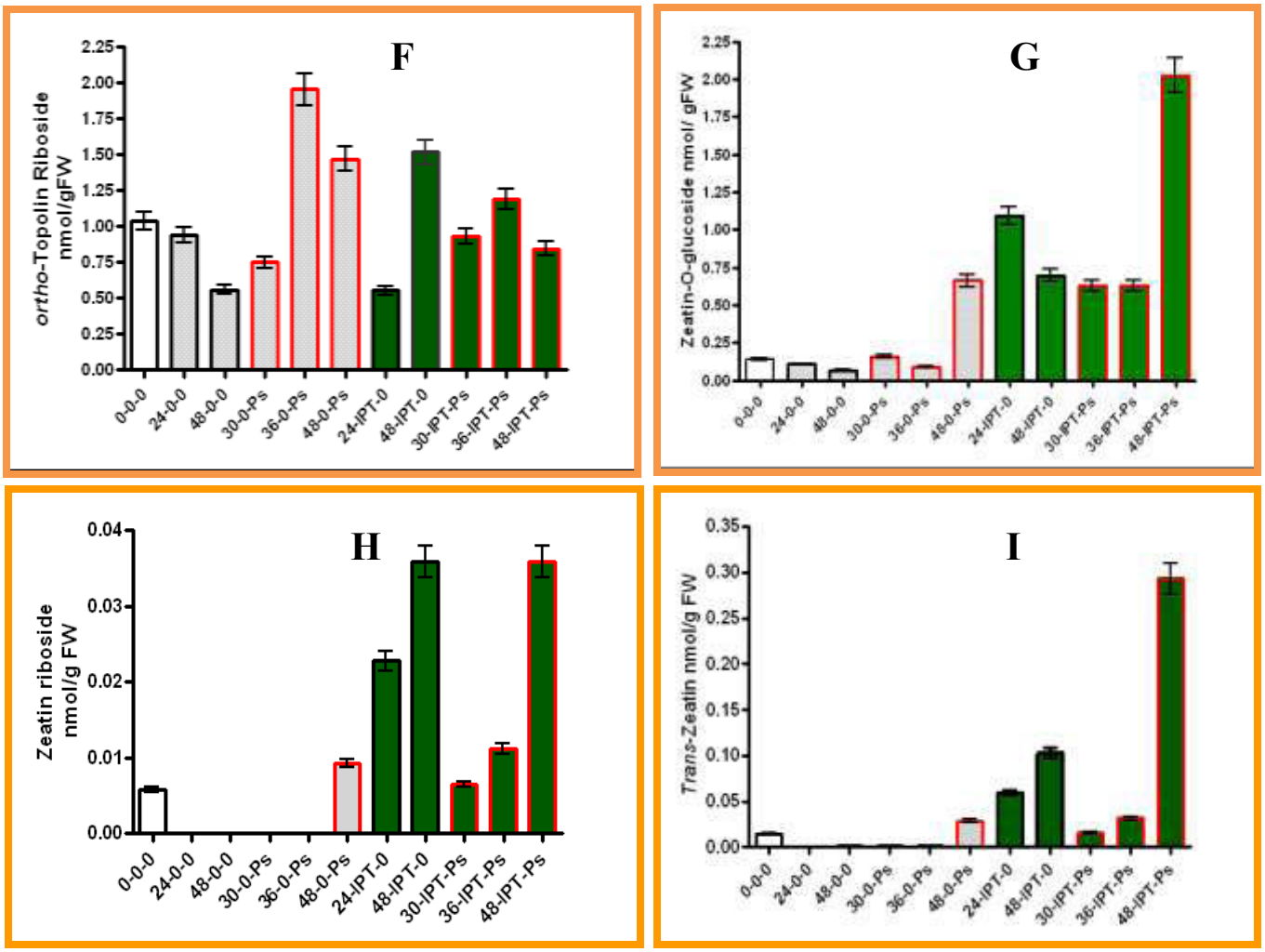


Fig.3 Pathogen inducible transient expression of bacterial *IPT* gene under the control of 4xJERE promoter results in elevated levels of CKs and resistance against *P. syringae* pv. *tabaci*. (A) Wild type tobacco (W38) leaf inoculated with *P. syringae* pv. *tabaci* (*P.s*.) or not (0). In the right half of the leaf infected sites has been infiltrated with *Agro*. strain ABL 4404 containing the plasmid pCambia 1380-4xJERE-IPT 24 h before pathogen infection. The equivalent sites in the left half has been given with the same, but empty, *Agro* strain. Picture was taken 10d after infection with *P. syringae* pv. *tabaci*. (B) Northern-Blotting showing the expression of *IPT* gene under the control of 4xJERE promoter in the presence and absence of *P. syringae* pv. *tabaci* (Ps) and (0) respectively, while control (0-0-0) did not contain *IPT* gene and hence no expression manifested. *(RNA isolation and Hybridization was performed by Dominik Grosskinsky former Diploma student in the working group of Prof. Roitsch University of Wuerzburg)* (C) Picture was taken 3 days after *P. syringae* pv. *tabaci* infiltration to show early phase of infection. (D) 15 days after *P. syringae* pv. *tabaci* infiltration. (E) 1-month after *P. syringae* pv. *tabaci* infiltration. (F) Determination of various types of cytokinins in 4xJERE::IPT tobacco samples *(Prepared samples were analysed by Dr. Markus Kirschke a co-worker in the working of Prof. Mueller University of Wuerzburg)* such as *Ortho*-Topolin riboside (G) Zeatin-O-glucoside (H) Zeatin riboside and (I) *Trans*-Zeatin.

4.1.4 Transient expression of *IPT*-gene under the control of 4xJERE-promoter in *Arabidopsis* plants jeopardize the growth of *P. syringae* pv. *tomato DC3000*

To further validate the resistance phenotype by virtue of 4xJERE::IPT mediated enhancement of CKs in tobacco leaves during its interaction with *P. syringae* pv.*tabaci* , *Arabidopsis* being a model plant system and *P. syringae* pv. *tomato DC3000* being a generalist pathogenic system was tested. Leaves of 5 weeks old wild type Col-0 *Arabidopsis* plants were infiltrated with *Agro.* strain ABL4404 containing the plasmid pCambia 1380-4xJERE-IPT. As a control similar, but non-transformed *Agro.* strain was used. 24h post *Agro.* infiltrations same areas of the leaves were inoculated with *P. syringae* pv. *tomato DC 3000* (10^6 CFU/ml). 4xJERE::IPT expressing leaves did not develop disease symptoms, however, *non-IPT* expressing control leaves exhibited normal disease symptoms 48h post *P. syringae* pv. *tomato DC3000* inoculation (Fig.4). These results further underscore the importance of 4xJERE::IPT system and also emphasize the cessation of the spread of *Pseudomona*s in a leaf with elevated CKs status.

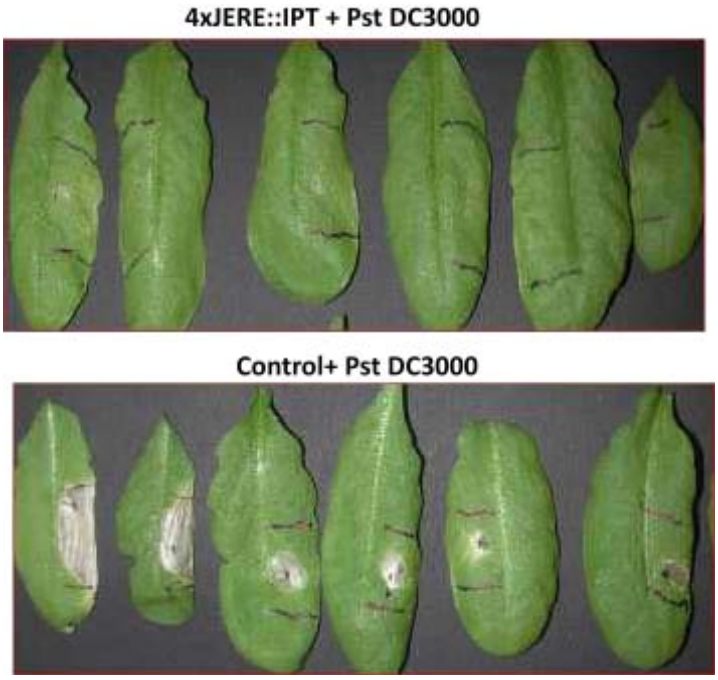


Fig. (4): Transient expression of bacterial IPT gene under the control of 4xJERE promoter in Arabidopsis leaves impart resistance against *P. syringae* pv. *tomato DC3000*. In upper inset 5-weeks old Col-0 wild type Arabidopsis leaves infiltrated with *Agro*. strain ABL 4404 containing the plasmid pCambia 1380-4xJERE-IPT and subsequent 24h treatment of *P. syringae* pv. tomato DC3000 (10^6 CFU/ml) show resistance to Pseudomonas, while the lower inset having the same accession with susceptible phenotype containing same agro strain devoid of pCambia 1380-4xJERE-IPT show necrosis after *Pseudomonas* infection. Picture was taken 6 days after *P.syringae* pv. *tomato DC3000* infiltration.

4.1.5 Chemically regulated expression of *IPT* gene in transgenic tobacco plants impedes the growth of *P. syringae* pv. *tabaci*

To investigate the spread of pathogen in modulated levels of cytokinins, Tet::IPT tobacco transgenic plants were investigated. Tet-promoter works on de-repression mechanism in terms of inducibility in Tet::IPT transgenic plants (Redig et al. 1996). 1mg/L of Cl_2-tetracycline was site infiltrated in leaves of Tet::IPT tobacco plants, while opposite site of the leaf was mock induced with 10mM $MgCl_2$. A delay of senescence was observed in tet-treated plants three weeks post induction, while no such delay noticed for mock induced site (Fig.5A). For investigating the spread of *P. syringae* pv. *tabaci* in Tet-IPT-tobacco plants, two-sites on right side of the leaf were induced with 1mg/L of Cl_2-tetracyclin and the opposite sites on left half of the same leaf were mock infiltrated with 10mM $MgCl_2$. 24 Hours later these allocated sites on the leaf were infiltrated with *P. syringae* pv. *tabaci* (10^6 CFU/ml). Visible symptoms appeared on mock-induced sites 48h post bacterial infiltration and turned into necrosis a week later when hardly very faint chlorosis appeared on Tet-induced sites of the leaf (Fig.5B).

To further strengthen this evidence, instead of site specific, more global and whole leaf-based induction of Tet::IPT was established by petiole feeding of Cl_2-teteracyclin (1mg/L) into detached leaves. Upon inoculation of *P. syringae* pv. *tabaci* 24h post Cl_2-Tet feeding, a very mild and delayed leaf chlorosis was observed on tet-induced leaves, whereas complete necrosis was observed on non-induced detached leaves one week PPI (Fig.5.B). Our experimental data revealed that *P. syringae* pv. *tabaci* has a high degree of sensitivity to Cl_2-tet even at a concentration of 1mg/L (the concentration used here for the induction of Tet::IPT transgenic plants). To fix this apprehension, wild type strain of *P. syringae* pv. *tabaci* was transformed with a plasmid harboring tet-resistance gene **(**Bloemberg et al, 2000), which rendered the said sensitive wild strain into resistant one even at a Cl_2-tet concentration of 50mg/L (Fig.5.D). Interaction of *P. syringae* pv. *tabaci* with Tet::IPT tobacco transgenic plants with a subsequent temporal delay in the appearance of the symptoms on induced sites and severity in disease on non-induced sites further supports the notion that higher levels of CKs cease the growth of invading pathogen.

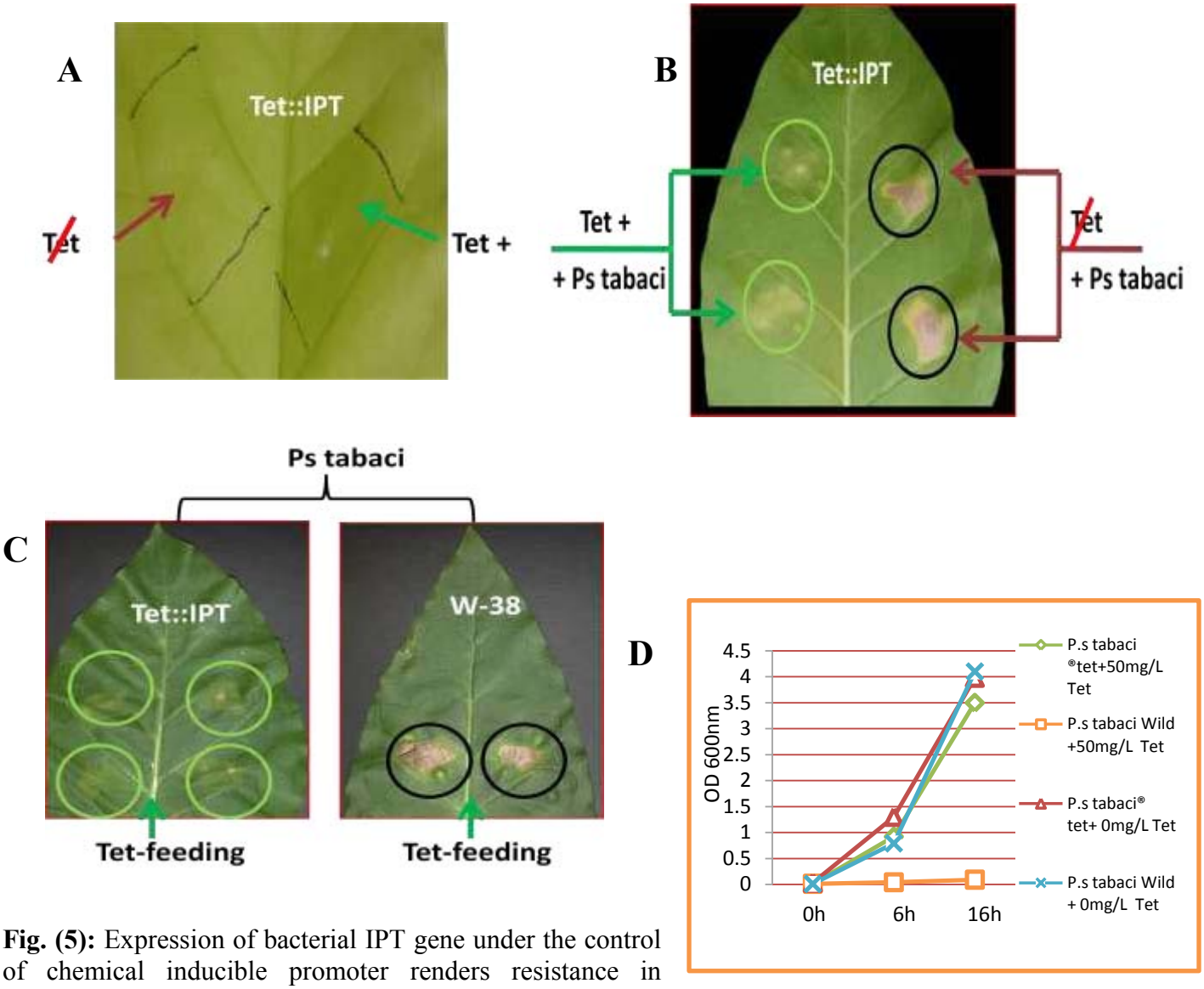


Fig. (5): Expression of bacterial IPT gene under the control of chemical inducible promoter renders resistance in tobacco plants against *P.syringae* pv. *tabaci*. (A)Tet::IPT tobacco leaves were induced with 1mg/L of Cl_2-Tetracycline at the right half and for mock induction 10mM $MgCl_2$ was used. Picture was taken 3 weeks after treatment; (B) Leaf of transgenic Tet::IPT tobacco plant was induced with Cl2-Tet on the left hand side while equivalent right sites were mock induced with 10mM MgCl2, 24h later both of the planes were infiltrated with *P.syringae* pv. *tabaci* (10^6CFU/ml). Picture was taken 1 week PPI. (C) Detached leaves of Tet::IPT transgenic plants were induced with Cl2-Tet via petiol feeding while for mock feeding 10mM MgCl2 was used. *P.syringae* pv. *tabaci* (10^6 CFU/ml) was infiltrated 24h post petiole feeding. Picture was taken 1 week PPI. (D) Transgenic strain of *P.syringae* pv. tabaci harboring Tet- resistance gene grows in liquid culture with 50mg/L Tet. X-axis is incubation time while y-axis depicts OD of growing cultures at 600nm.

4.1.6 Developmentally regulated expression of *IPT* gene in SAG12::IPT tobacco plants restricts the growth of *P.syringae* pv. *tabaci*

Level of cytokinins varies in a plant throughout its development. When it touches the minimum threshold, leaves are getting into senescence and SAG (Senescence Associated Genes) genes are getting an urge of regulation in plants (Gan and Amasino 1995). To investigate how fast is the spread of pathogen in various developmental stages of the leaf in perspective of cytokinins, we opted for a transgenic approach whereby bacterial *IPT* gene is developmentally regulated under the control of senescence associated gene promoter i.e. SAG12::IPT. To synchronize the onset of senescence in SAG12::IPT and corresponding wild accession of W-38, a detached senescence based assay approach was adopted (Lara et al, 2004). Independently but in a similar manner SAG12::GUS leaves were subjected to senescence and GUS-staining appeared on leaves 15 days post senescence imposition (Fig.6). On the onset of senescence in W-38 and comparable delayed senescence in SAG12::IPT, leaves of both categories were challenged with *P.syringae* pv. *tabaci* (10^6 CFU/ml). Rapid chlorosis followed by a necrosis appeared on control leaves than the manifestation of delayed and less severe disease symptoms on the leaves of SAG12::IPT plants (Fig.6).

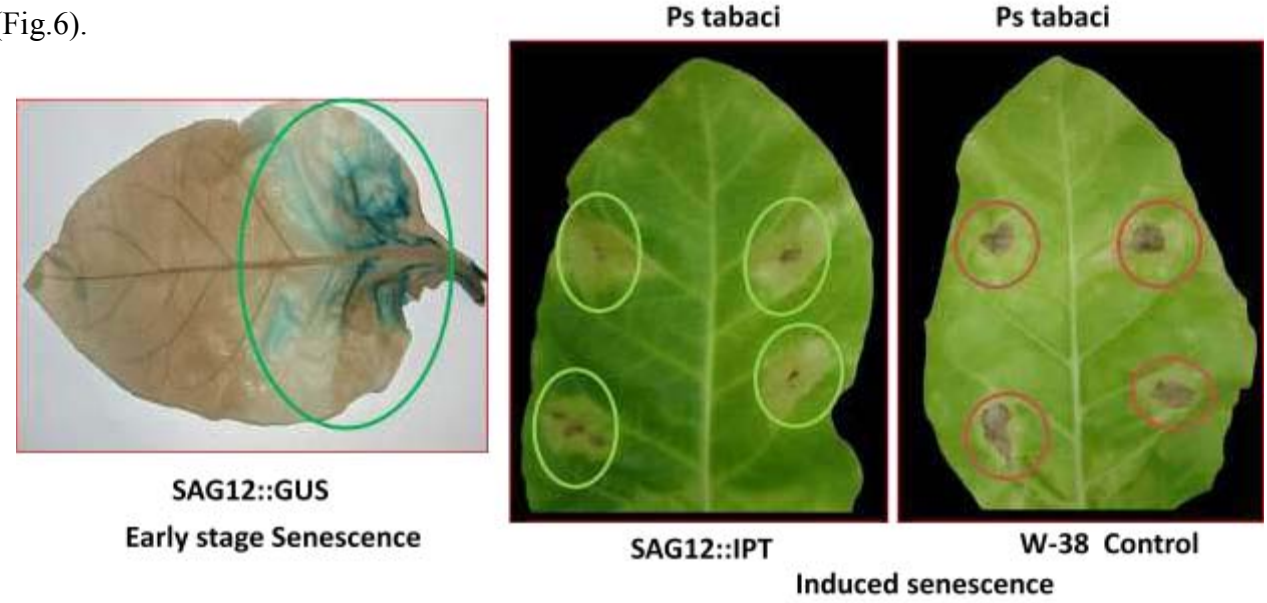


Fig.(6): Developmentally regulated expression of IPT gene impedes the growth of *P. syringae* pv. *tabaci*. (A) Detached leaf of transgenic SAG12::GUS (Promoter-Reporter) tobacco plant, GUS stained 15 days post senescence induction. Picture was taken after removal of chlorophyll in 70% ethanol. (B) 15 days post senescence induction leaves of both SAG12::IPT and that of wild type W38 infiltrated with *P. syringae* pv. *tabaci*; picture was taken 1 week PPI.

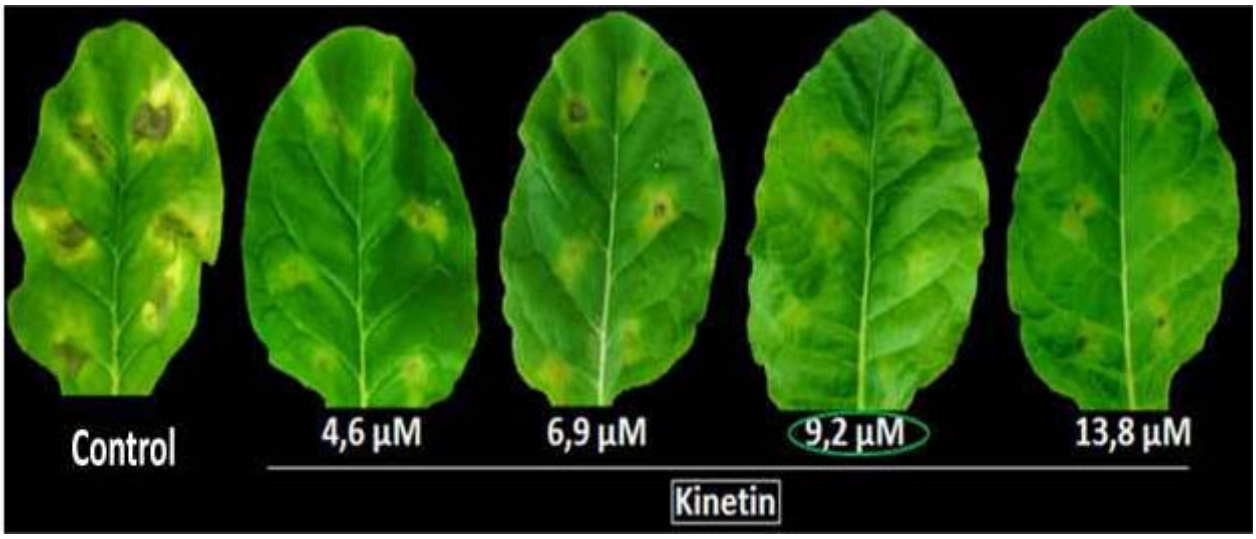
4.1.7 Exogenous feeding of cytokinins also cause increase in disease resistance

To augment the resistance response of elevated CKs level of the above described transgenic approaches, we additionally fed varying concentrations of cytokinins analogues into detached tobacco leaves. Kinetin, an adenine type derivative of cytokinins fed to detached leaves 24 h prior to infiltration with *P. syringae* pv. *tabaci*. 140 out of 196 analyzed individual leaves (71.4 %) treated with kinetin displayed substantially reduced disease symptom development (Fig.7 A). During the course of the experiment it was found that a concentration of 9.2 μM kinetin consistently gave the strongest resistance. It is known that high CK contents in leaves may trigger cell death (Carimi et al. 2003). Thus the more pronounced symptom development in leaves fed with kinetin concentration above 9.2 μM need not surprise.

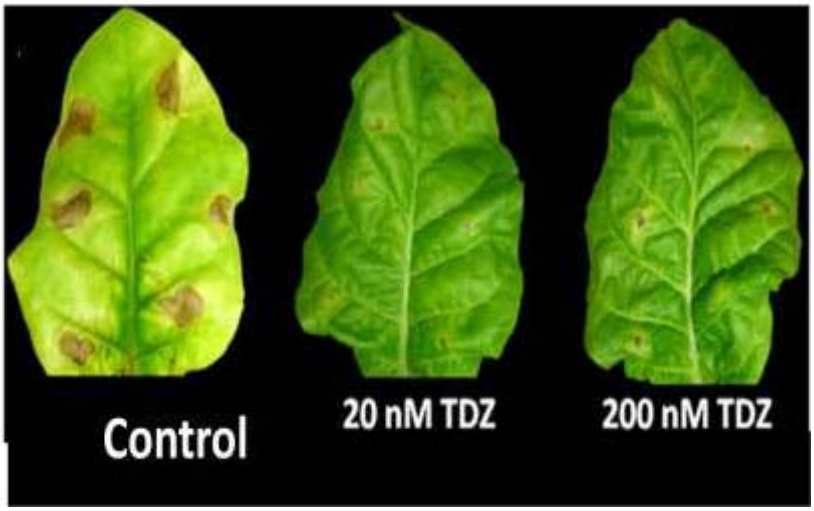
To test the ability of phenylurea derived CKs to cause resistance we also performed trials with 0.2 and 0.02 μM TDZ under the same conditions. TDZ is long known for its strong CKs like activity (Mok et al 1982). A significant reduction in *Pseudomonas* derived disease symptom development was observed in 46 out of 48 leaves (95.8 %) supplied with TDZ (Fig.7 B). Although unlikely, given the large number of replicates, variation in the physiological status between more and less resistant leaves might have caused the observed differences in symptom development. Therefore, we included a further test in which only one half of a leaf was dipped in kinetin for 90 s. Although differences in symptom formation were less pronounced than during feeding experiments, even such a short external kinetin supply was sufficient to help the leaf to fight against the pathogen. 21 out of 26 (80.8 %) dipped leaf halves had a less severe symptom development than the controls (Fig.7 C).

Additionally we analysed the impact of trans-zeatin on the growth of *P. syrin*gae pv. *tabaci* and found that 10μM of trans-zeatin petiole feeding also avoids disease symptoms in tobacco leaves in comparison to the control (Fig.7 D).

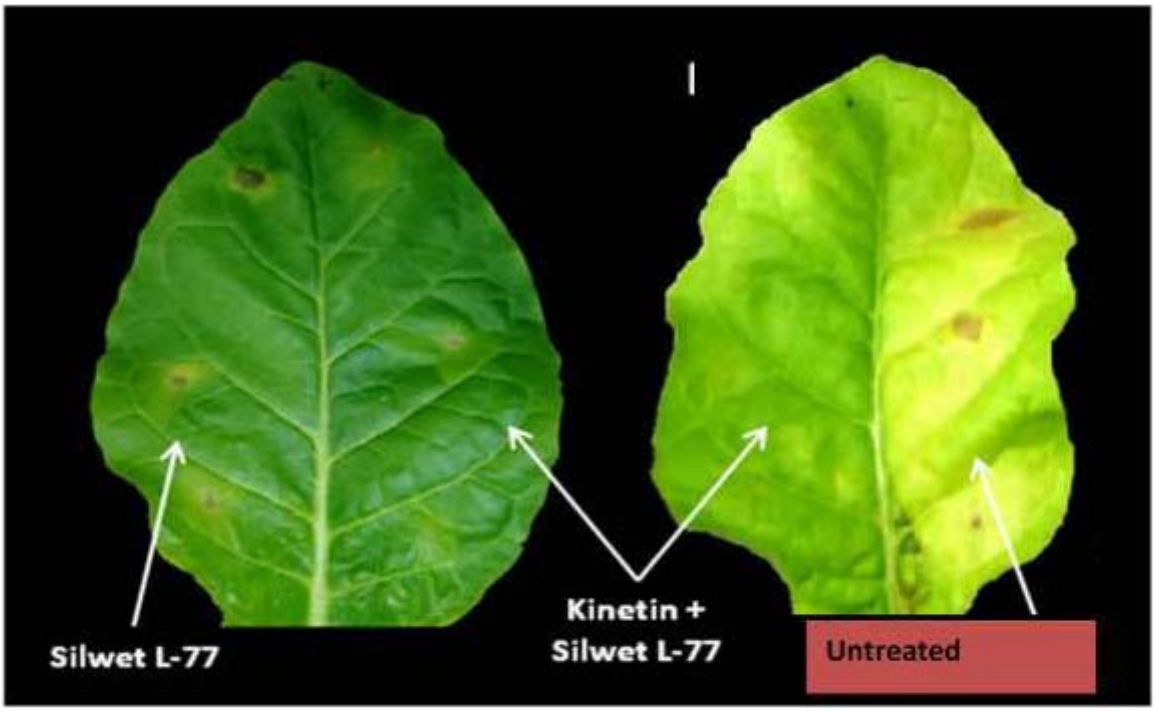
A


Control
4,6 µM
6,9 µM
9,2 µM
13,8 µM
Kinetin

B


Control
20 nM TDZ
200 nM TDZ

C



D

Fig.(7): Exogenous feeding of CKs also redistrict the growth of *P. syringae* pv. *tabaci.* (A) Representative figures of exogenous feeding of various concentrations of kinetin showing less symptoms than water control. 24h after kinetin feeding *P. syringae* pv. *tabaci* was infiltrated in 5-8 weeks tobacco SR1 plants. Picture was taken 7 days after *P. syringae* pv. *tabaci* inoculation. (B) Symptom development in tobacco SR1 leaves after exogenous feeeding of TDZ (thidiazuron) in two concentrations for 24 hours and infection of *P. syringae* pv. *tabaci*. Picture was taken 14 days after pathogen inoculation. (C) Kinetin dipping effect on the growth of *P. syringae* pv. *tabaci*. 90 sec. dipping in Silwet+Kinetin and only Silwet (left). 60 sec. dipping in Silwet+Kinetin and untreated non-dipped side (Right). *(A, B, C were performed and prepared by Dominik Grosskinsky former, Diploma student in the working group of Prof. Roitsch University of Wuerzburg)* (D) Symptoms development in tobacco SR1 leaves fed with 10µM zeatin followed by infiltration of *P. syringae* pv. *tabaci.* Water was used as mock feeding in control. Picture was taken 6 days post PPI.

4.1.8 Quantification of bacterial growth in elevated cytokinins status of the plant

Since all the tested transgenic and pharmaceutical approaches converge on the same basic conclusion that elevated CKs render plant resistance against *P. syringae* pv. *tabaci,* therefore, it is necessary to quantify the growth of the invading pathogen under altered CK concentration. Tobacco 4xJERE::IPT transient expression being a reproducible system was opted for monitoring *in planta* bacterial growth. Wild type leaves of W38 were previously infiltrated with *Agro*. strain ABL 4404 containing the plasmid pCambia 1380-4xJERE-IPT on one half of the leaf while other half got a treatment with same, but empty *Agro*. strain. Upon 24h of incubation, *P. syringae* pv. *tabaci* was introduced into the *IPT* expressing and the control regions (10^6cfu/ml). As illustrated in Fig.8.A samples were taken for quantification 0, 6, 9,12,15,24 and 48h PPI. *P. syringae* pv. *tabaci* multiplied almost at the same pace in early time points in both *IPT*-expressing and *non-IPT* expressing regions, however, after 15h PPI decline in bacterial growth started in *IPT*-expressing areas. Contrarily, *P. syringae* pv. *tabaci* grew exponentially higher and reached orders of magnitudes higher level in *non-IPT* than in *IPT* expressing sites 48h PPI (Fig.8 A).

To complement 4xJERE::IPT transient expression system *in planta,* bacterial growth was also monitored for exogenous kinetin feeding (being an analogous approach to elevate the level of CKs) in tobacco leaves. 9.2µM kinetin for 24h was fed to tobacco leaves of accession W-38, while for mock feeding water was used. Both treated and control leaves were inoculated with *P. syringae* pv. *tabaci* (10^6cfu/ml) and re-isolated for time points 0, 6, 12, 24 and 48h PPI. Bacterial plate counting results revealed a significant increase in the growth of *P. syringae* pv. *tabaci* 12h PPI for control leaves compared to that of kinetin treated ones. This difference is more significant for the later time points at 24h and 48h PPI (Fig.8 B).

Similar experimental out-comes from both these mutually exclusive approaches emphasize that CKs significantly hinder *in planta* bacterial growth and thereby augmenting the phenotypic observations based on disease symptom development in all mentioned transgenic and CKs analogues feeding experiments.

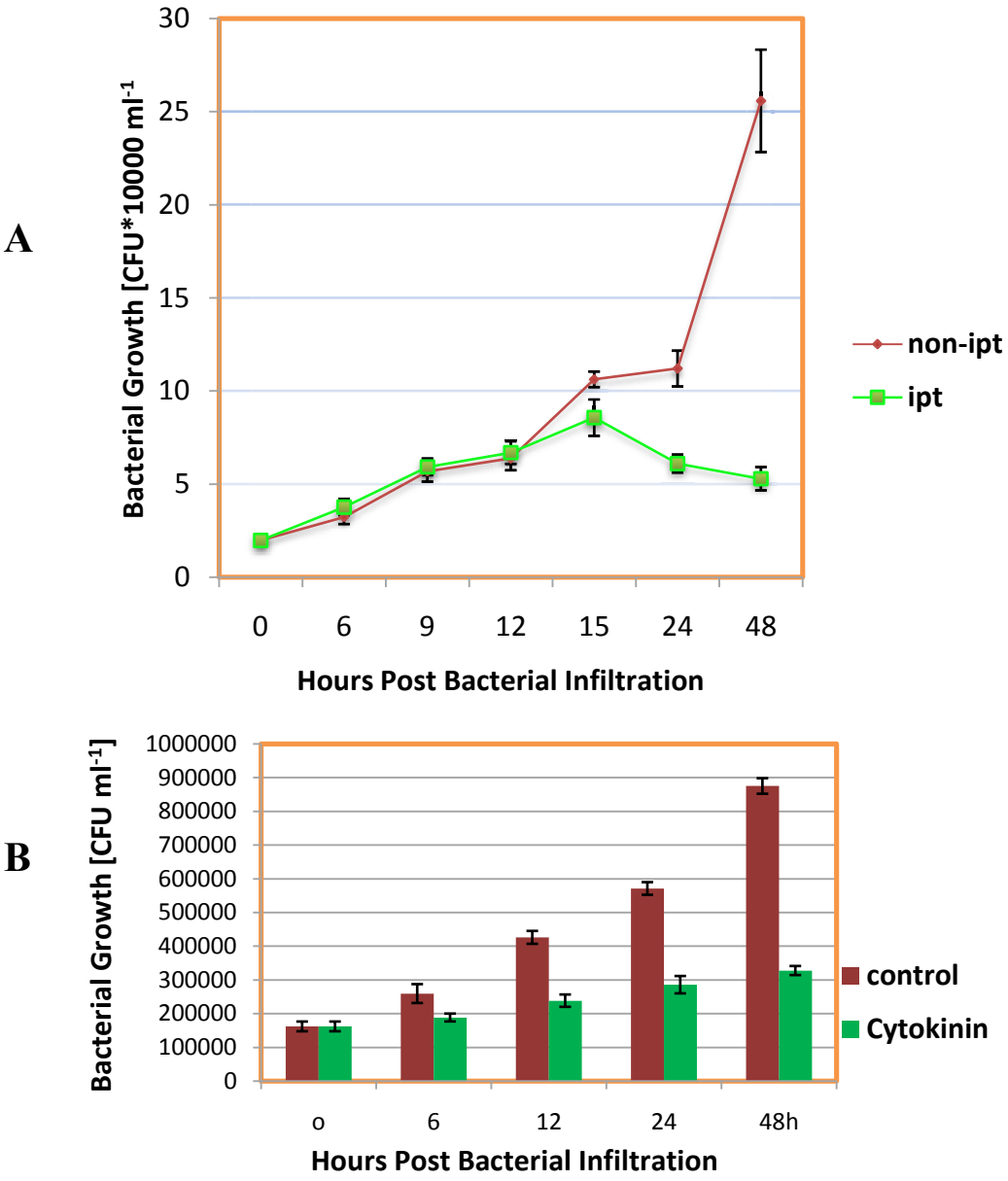


Fig. (8): Quantification of bacterial growth in elevated CKs status of the plant.
(A) Bacterial multiplication in transiently *IPT* expressing areas under the control of 4xJERE promoter and *non-IPT* expressing areas of tobacco W38 leaves inoculated with *P. syringae* pv. *tabaci* (10^6 CFU/ml). (Bacterial re-isolation and plate based counting was performed for time points on X-axis while colony number in CFU/ml is given at Y-axis.) Data points represent mean values of three independent experiments ± s.d.). (B) Bacterial growth in kinetin (9.2µM) and water fed tobacco SR1 detached leaves. Bacterial re-isolation and plate based counting was performed for time points on X-axis while colony number in CFU ml^{-1} is given at Y-axis. Data points represent mean values of individual experiments containing three replicates.

4.1.9 Elevated CK levels do not impede the spread of necrotrophic fungal pathogen

To investigate the range of effectiveness of CKs mediated resistance, inspite of biotrophic pathogen *Pseudomon*as, a necrotrophic fungal pathogen *Sclerotinia sclerotiorum* was inoculated on leaves containing higher levels of CKs . Tobacco leaves of wild accession W-38 were treated with *Agro*. strain ABL 4404 containing the plasmid pCambia 1380-4xJERE-IPT, which resulted in CKs producing spot on the leaf. Same but empty *Agro.* strain on other half of the leaf generated a corresponding control site on the leaf. When treated with *Sclerotinia* agar block of similar volume, no difference in symptoms development was observed (Fig.9 A). When exogenously fed via petiole with kinetin solution (9.2µM) and inoculated with agar block of *Sclerotinia*, in comparison to water control kinetin fed leaves proved to be slightly more sensitive to *Scleronitial* infection (Fig.9 B).

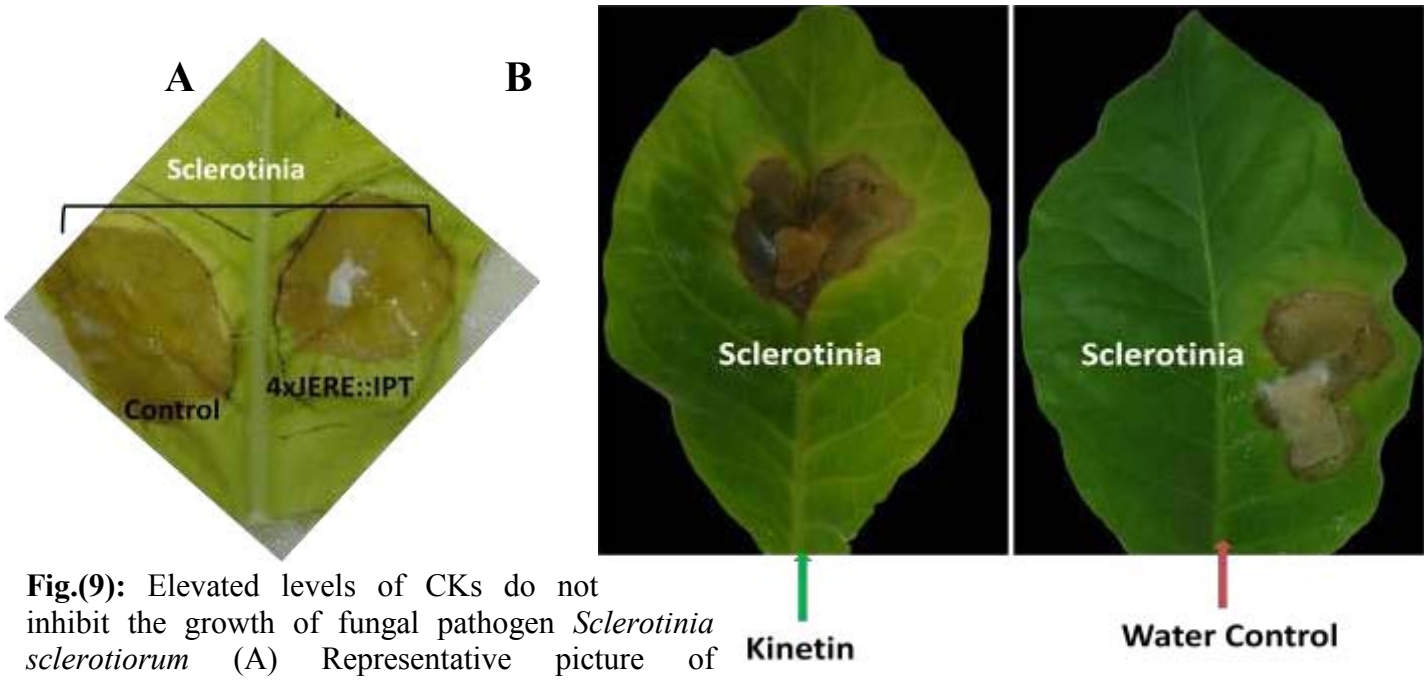


Fig.(9): Elevated levels of CKs do not inhibit the growth of fungal pathogen *Sclerotinia sclerotiorum* (A) Representative picture of experiment where right half contains transiently expressed IPT gene under the control of 4xJERE promoter while left half of the leaf does not contain *IPT* gene. 24 h post incubation, both the sites are given with agar block of *Sclerotinia sclerotiorum.* Picture was taken 1 week PPI. (B) 9.2µM kinetin solution for 24h is fed via petiole to wild type SR1 leaf, where as for control water was fed to same accession. 24 h post incubation, both the leaves are inoculated with identical volume of the agar block of *Sclerotinia sclerotiorum.* Picture was taken 1 week PPI. *(Performed by Dr Uwe Simon and Franziska Lang co-workers of the working group of Prof. Roitsch, University of Wuerzburg).*

4.2 Mechanism of CKs mediated resistance

As described above, it is evident from all transgenic and non-transgenic approaches that elevated levels of CKs enhance plant resistance against hemibiotrophic pathogen *P. syringae* pv. *tabaci.* Therefore, it was necessary to decipher the underlying mechanism of cytokinins mediated resistance. Though, all of the transgenic and pharmaceutical tools unanimously revealed the same conclusion: higher resistance with higher CKs contents against hemibiotrophic pathogen *P. syringae*, however, consistency in diseased phenotypes, reproducibility of results and expression of *IPT* gene coupled with synthesis of cytokinins at the host pathogen interface, gave distinction to transient 4xJERE::IPT expression system. We comprehensively sampled 4xJERE::IPT plant material such that to predict about mechanism in the early phase of *Pseudomonas* infection and subjected it for various types of analysis related to the current dynamics of plant immunity (Fig.10).

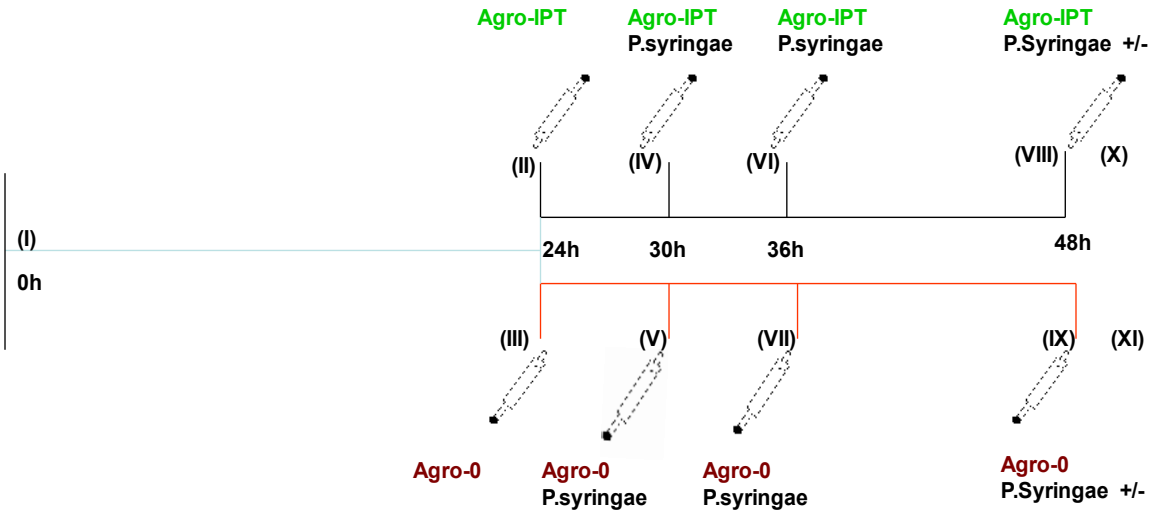


Fig.10 4xJERE::IPT transient expression in tobacco and sampling pattern.

(I) 0h at the beginning of the experiment (basic control) **0-0-0**

(II) and (III) 24 h after infiltration with *A. tumefaciens* containing pCambia-4xJERE-IPTon one site of the leaf and *A. tumefaciens* without pCambia-4xJERE-IPT on opposite site of the same leaf. **24-IPT-0 and 24-0-0**

(IV) and (V) 30 h after infiltration with *Agrobacterium* same as I and II and 6 h after infection with *P.syringae* pv. *tabaci* **30-IPT-Ps and 30-0-Ps**

(VI) and (VII) 36 h after infiltration with either *Agrobacterium* same as I and II strain and 12 h after infection with *P.syringae* pv. *tabaci.* **36-IPT-Ps and 36-0-0**

(VIII) and (IX) 48 h after infiltration with either *Agrobacterium* same as I and II and 24 h after infection with *P.syringae* pv. *tabaci.* **48-IPT-Ps and 48-0-Ps**

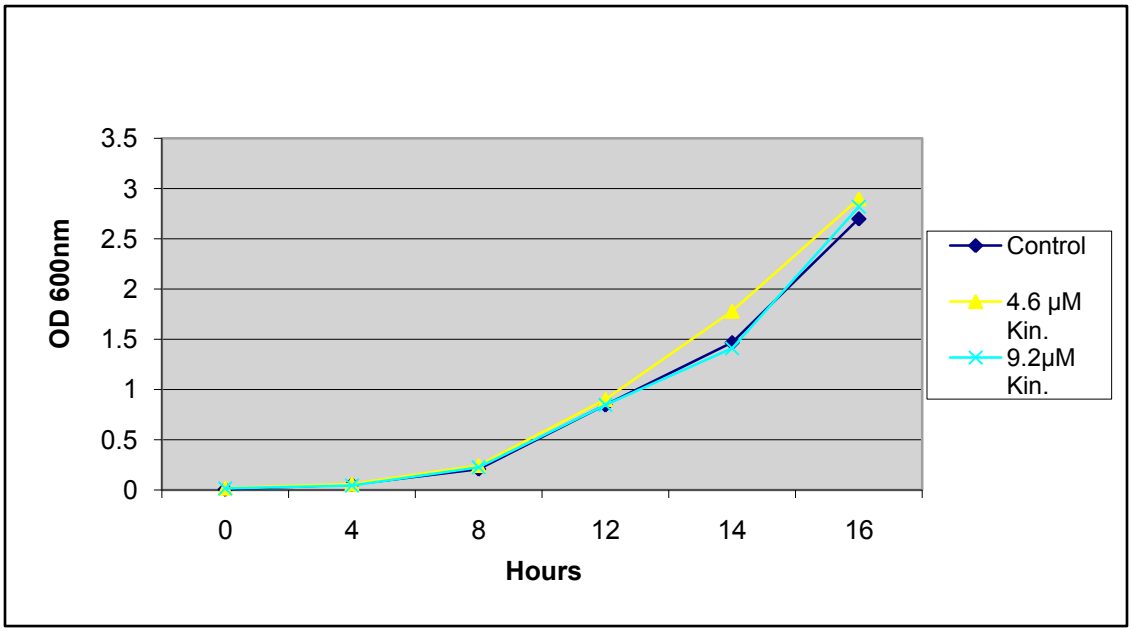
(X) and (XI) 48 h after infiltration with either *Agrobacterium* strain same as I and II. **48-IPT-0 and 48-0-0**

4.2.1 CKs have no inherent anti-microbial potential

Since elevated levels of CKs embark resistance on plants against the invading pathogen, it was logical to investigate the inherent antimicrobial potential of cytokinins. 9.2µM kinetin, proved to be an optimal concentration restricting *in planta* bacterial growth, was added as a source of elevated cytokinins into the growing liquid culture of *P. syringae* pv. *tabaci*. Besides, lower kinetin concentration of 4.6µM was also studied. No difference was observed in the growth of *P. syringae* pv. *tabaci* for both higher and lower kinetin concentrations. Moreover, the growth of the control culture was quite similar to those of kinetin added ones (Fig.11 A).

To further strengthen this evidence, in a disc diffusion assay approach filter disc was impregnated with 9.2 µM kinetin and placed on the growing lawn of *P. syringae* pv. *tabaci*. No visible growth inhibition zones were observed for kinetin absorbed discs as compared to that of water controls (Fig. 11 B) Lack of significant increase or decrease in the growing potential of *P. syrinage* pv. *tabaci* in the presence or absence of kinetin substantiates that *per se* it is inert in terms of bacterial multiplication and that there is no direct effect of kinetin (i.e. cytokinins) on the growth of the pathogen.

A



B

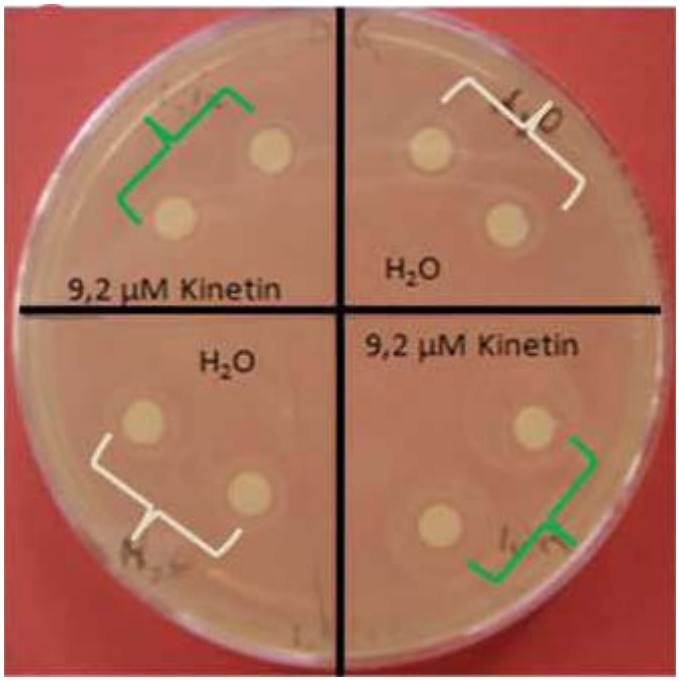


Fig. (11): CKs do not have inherent antimicrobial potential.
(A) Given in KB broth two concentrations (9.2µM and 4.6µM) of kinetin and control without addition of kinetin growth of *P.syringae* pv.tabaci shown in term of OD at 600nm on Y- axis for various time points on X-axis. (B) Filter disks impregnated with 9.2µM kinetin placed on bacterial lawn of *P.syringae* pv. *tabaci* along with water soaked discs as a control to compare zone of inhibitions against the growing pathogen. *(Performed and prepared by Dominik Grosskinsky, former Diploma student in the working group of Prof. Roitsch University of Wuerzburg)*
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4.2.2 Antimicrobial potential of plant extracts with elevated CKs

Enhanced levels of CKs in 4xJERE::IPT expression sites of tobacco leaves cut down the growth of *P. syringae* pv. *tabaci* in orders of magnitudes in comparison to their control counterparts. Similarly, exogenous feeding of kinetin also significantly diminished *in planta* bacterial multiplication (Fig. 8A&B). Moreover, cytokinins *per se* are lacking the innate antimicrobial potential against *P. syringae* pv. *tabaci* (Fig.11 A & B). The antimicrobial activity of tobacco crude extracts of 4xJERE::IPT as well as that of kinetin along with their corresponding controls was determined against wide range of the pathogens. Based on the disc diffusion assay, above

mentioned crude extracts resulted in differential inhibitory effects against the tested pathogens (Table 1). Growth of gram-positive bacteria was not influenced by the CKs status of the extracts. The same was true for the necrotrophic fungus *Sclerotinia sclerotiorum*, while *Botrytis cinerea* seemed to profit from higher CKs contents in the extract. On the contrary, growth of gram-negative bacteria such as *Pseudomonas* species and yeast *Candida maltosa* was inhibited (Table 1).

Table 1. Antimicrobial activity of extracts from *ipt* expressing vs. non-*ipt* expressing tissues before and after infection with *Pseudomonas syringae* pv. *tabaci-tet*. Values are means of two readings and refer to the zone of inhibition in mm. *B. sub.* = *Bacillus subtilis*, *E. coli* = *Escherichia coli*, *P. aer.* = *Pseudomonas aeruginosa*, *P. s.* pv. *phas.* = *Pseudomonas syringae* pv. *phaseolicola*, *P. s.* pv. *tab.* = *Pseudomonas syringae* pv. *tabaci-tet*, *C. mal.* = *Candida maltosa*, *B. cin.* = *Botrytis cinerea*, *S. aur.* = *Staphylococcus aureus*, *S. scl.* = *Sclerotinia sclerotiorum*. (*Assays on given 4xJERE-IPT samples was performed and data was prepared by Usama Ramadan a co-worker in the working group of Prof. Roitsch, University of Wuerzburg)*

	Gram-positive bacteria		**Gram-negative bacteria**				**Fungi**		
	B. sub.	***S. aur.***	***E. coli***	***P. aer.***	*P. s.* pv. p*has.*	*P. s.* pv. *tab.*	***C. mal.***	***B. cin.***	***S. scl.***
0-0-0	1	2	0	1	1	1	1	0	0
24-IPT-0	2	2	1	2	2	2	1	d.o.[2]	0
24-0-0	1	1	0	2	1	2	1	0	0
48-IPT-Ps	3	3	7	4	13	14	3	d.o.	0
48-0-Ps	2	2	1	2	8	8	2	0	0
Effect of elevated CK levels[1]	**0**	**0**	**-**	**-**	**-**	**-**	**0**	+	**0**

[1] 0 = no measurable effect compared with untreated control, - = inhibiting growth of pathogen, + = promoting growth of pathogen.
[2] d.o.= disk overgrown

4.2.3 Involvement of Antimicrobial peptides in CKs-mediated resistance

Bioautograpgy based experiments were performed to explore the presence of antimicrobial protein(s) in 4xJERE::IPT tobacco crude extracts. Fig.(12) Did not show any clear zone of antimicrobial activity against *P. syringae* pv. *tabaci* after covering a gel (12.5%) with low strength LB medium (20%) seeded with cells of the tester bacterial strain and incubation for overnight. This exclude the possibility of the involvement of antimicrobial peptide(s) in CKs mediated resistance against *P. syringae* pv. *tabaci.*

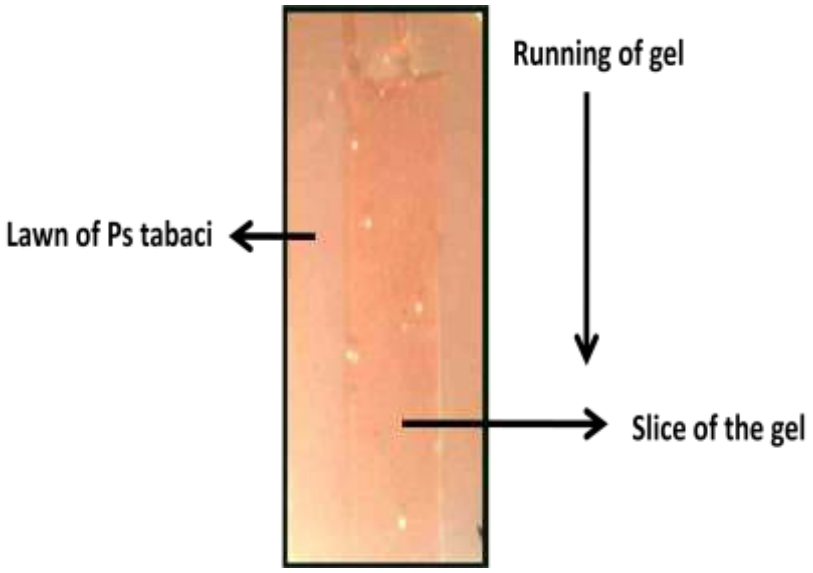


Fig.(12) 4xJERE::IPT tobacco extracts are devoid of antimicrobial peptides. Representative picture of the PAGE containing crude extract of the *IPT* expression areas of tobacco plant under the control of 4xJERE promoter which renders resistance against *P. syringae* pv. *tabaci*, placed on the bacterial lawn of *P. syringae* pv. *tabaci* (Bioautography) *(Running of perepared crude extract on PAGE was performed by Usama Ramadan a co-worker of working group of Prof. Roitsch, University of Wuerzburg)*

4.2.4 Status of ROS (reactive oxygen species) in CK-mediated resistance

Reactive oxygen species (ROS) also play an important role in stress response. In host plants they cause strengthening of the cell wall, may kill intruders and act as signalling molecules to activate defense pathways (Torres et al. 2006). We used DAB (diaminobenzidine) staining to compare H_2O_2 liberation in tobacco leaf areas expressing 4xJERE::IPT and in their corresponding controls. No difference in H_2O_2 accumulation was found in 1st (*before Pseudomonas infiltration*) and 2nd (*before development of the visible symptoms*) phases of the staining (Fig.13). However, a relatively high amount of H_2O_2 was accumulated in non-IPT expressing areas in the 3rd phase (*after symptoms development*) of staining (Fig.13). Accordingly, resistance against *P. syringae* pv. *tabaci* in IPT-expressing sites of the leaf is not due to the accumulation of ROS, rather latter is a consequence of pathogen spread after a prolong incubation of 1 week PPI and concomitant cell death.

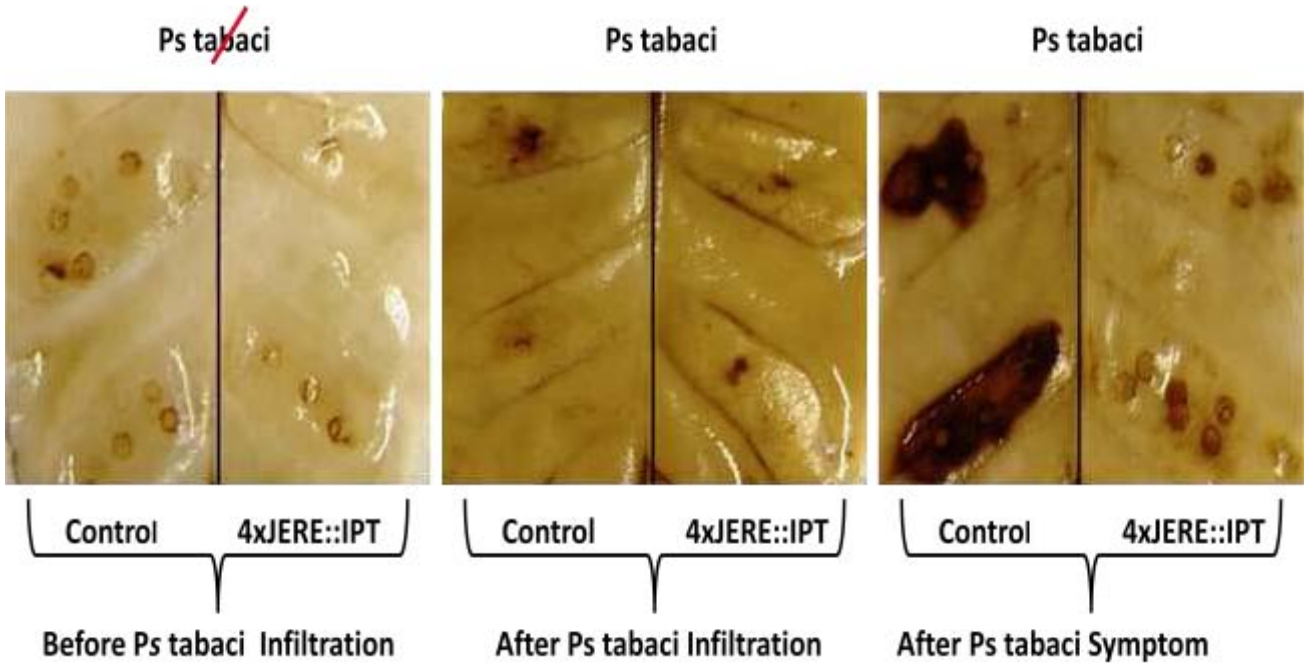
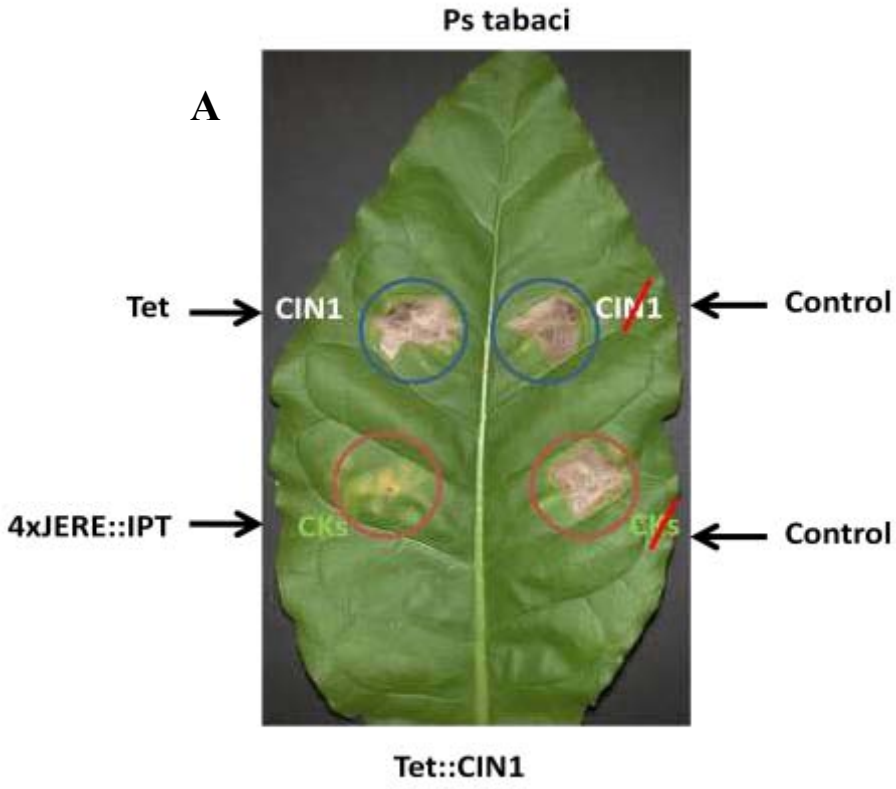


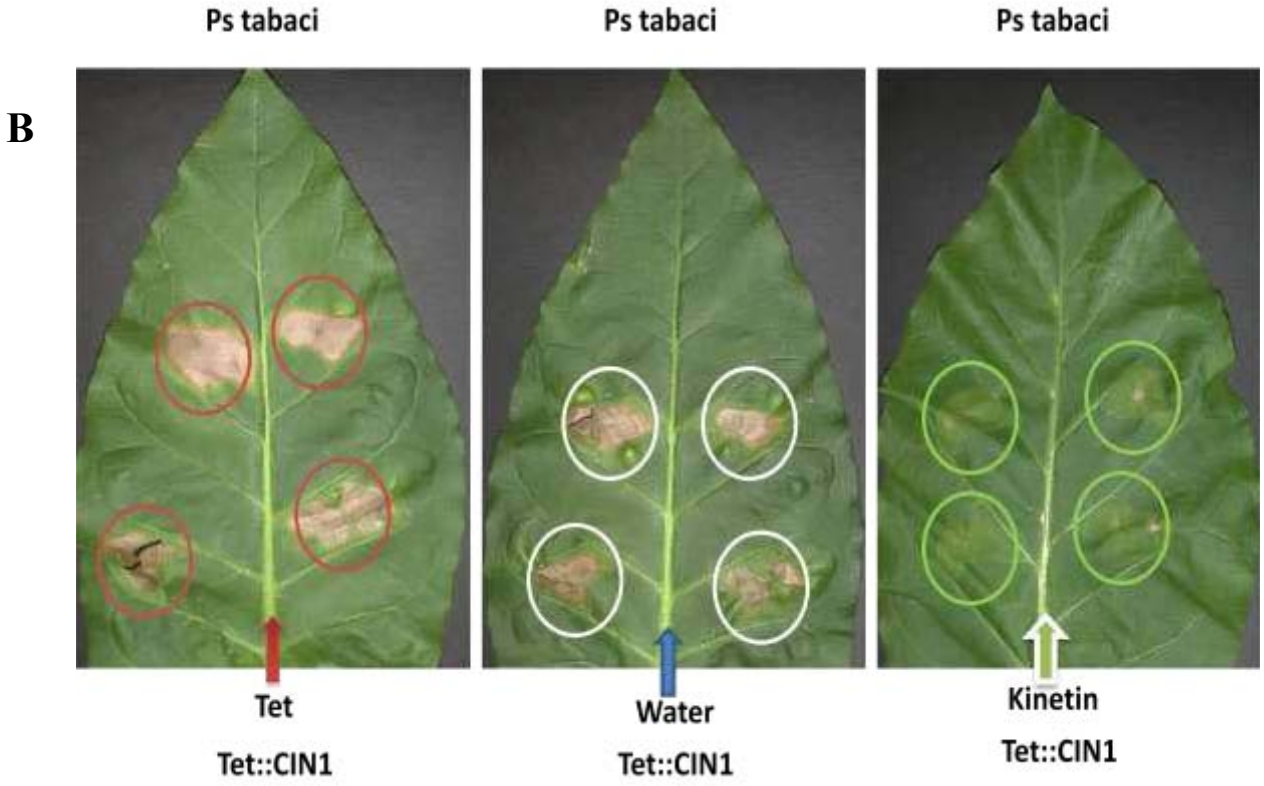
Fig. (13): ROS accumulation in higher cytokinins status during pathogen infection. Brown areas on leaves represent the extent of H_2O_2 accumulation. Right half of the leaf: *IPT*-expressing area; left half: *non-IPT* expressing area. Staining was performed shortly before, 24h after and one week after *P.syringae* pv. *tabaci* infiltration.

4.2.5 Cytokinins mediated resistance is independent of the function of cell wall invertase

To bridge a link between CKs and CWI in perspective of disease resistance, we conducted experiments on transgenically modulated extracellular invertase plants and that of cytokinins. To investigate the implication of increased invertase activity upon a pathogenic challenge, previously generated Tet::CIN1 (Lara et al., 2004) tobacco transgenic plants were used. 24h prior the inoculation of *P. syringae* pv. *tabaci* (10^6CFU/ml) leaves of Tet::CIN1 plants were treated with 1mg/L of Cl_2-Teracycline while for mock treatment 10mM MgCl2 was used. We did not observed any visible difference in symptom development on induced and non-induced halves of the same leaf. However, when generated 4xJERE::IPT and their corresponding non-IPT sites on same leaf of Tet::CIN1 plant, and challenged with similar bacterial suspension, more drastic disease symptoms were observed on non-IPT site whereas on 4xJERE::IPT site symptoms were very faint and less-pronounced Fig. (14 A).

To further elaborate these results we fed detached leaves of Tet::CIN1 plants with 1mg/L of Cl_2-tetracycline to induce CIN1 gene. Detached leaves of the same transgenic line were fed with 9.2µM kinetin solution. Water fed leaves of Tet::CIN1 was opted as a mock control for both tet and kinetin feeding. When challenged with same suspension of *P. syringae* pv. *tabaci* (10^6CFU/ml), no difference in symptom development was observed for tet-induced in comparison to that of water fed Tet::CIN1 leaves. However, kinetin fed Tet::CIN1 leaves manifested resistance phenotype by exhibiting less disease symptoms when rest of the combinations already entered into a stage of tissue necrosis (Fig.14B). To correlate CK mediated resistance with status of soluble sugars in extracts of 4xJERE::IPT plant material, various sugars such as glucose, fructose and sucrose were analyzed. Shown in Fig. (14 C), no consistent trend was observed in the accumulation of these three sugars for IPT-expressing and non-IPT expressing extracts. All these data pave way to the conclusion that CK-mediated resistance is independent of the plant sugar status and that extracellular invertase is not an essential component of CKs mediated resistance against *P. syringae* pv. *tabaci*.


A
Ps tabaci
Tet
CIN1
CIN1
Control
4xJERE::IPT
CKs
CKs
Control
Tet::CIN1


B
Ps tabaci
Ps tabaci
Ps tabaci
Tet
Tet::CIN1
Water
Tet::CIN1
Kinetin
Tet::CIN1

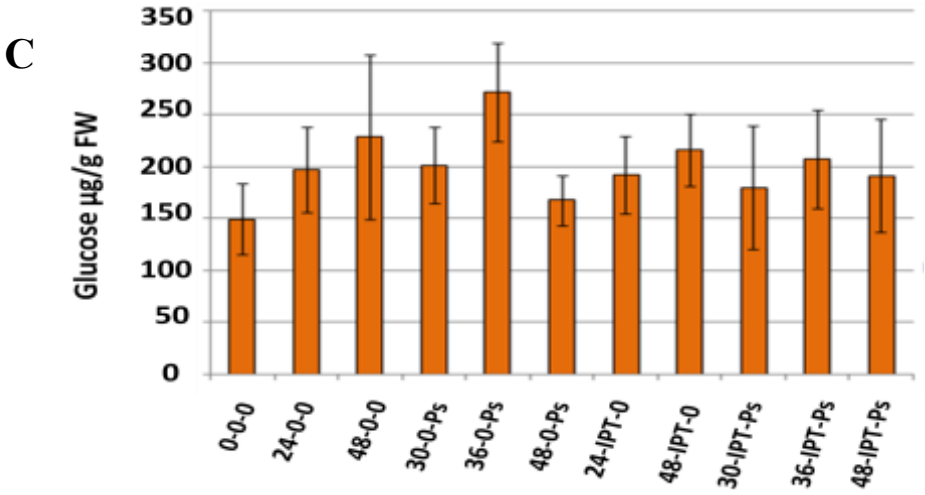


Fig. (14): CKs mediated resistance is independent of the sugar status of the plant.
(A) Representative picture of experiments where CIN1 gene has been expressed in tobacco plant under the control of Tet-inducible promoter. Upper left site is induced with 1mg/L Chloro-tetracycline corresponding right site is mock induced with 10mm MgCl2 Lower left site of the leaf contains *Agro*. strain ABL4404 expressing the plasmid pCambia 1380-4xJERE-IPT, corresponding right half of the leaf contains similar Agro. strain but devoid of pCambia 1380-4xJERE-IPT. 24h later all of the four sites are infiltrated with *P.syringae* pv. *tabaci* The pictures were taken 1 week PPI. (B) Detached leaves of NT35 Tet::CIN1 plants were petiole fed with 1mg/L Tet (left), water (middle) and 9.2µM of kinetin (right). 24h later all of the leaves were infiltrated with *P.syringae* pv. *tabaci*. Pictures were taken 10 days PPI. (C) Glucose measurements in term of µg/g of FW as a result of enzymatic cleavage of the starch present in the extracts of 4xJERE::IPT and corresponding controls of tobacco samples. Data bars represent mean values of 5 independent measurements ± s.d *4xJERE-IPT prepared samples were anlysed and graph was prepared by Dominik Grosskinsky, former Diploma student in the working group of Prof. Roitsch, University of Wuerzburg*)

4.2.6 Status of SA and JA in cytokinins mediated resistance

Definitely, it is an over simplification, but broadly speaking SA confers resistance against biotrophic pathogens while JA and ethylene are implicated in resistance to necrotrophic pathogens. Though, shift from one to other and prevalence of antagonism and synergism is also a well documented phenomenon in plant immunity (Robert et al. 2007). We determined levels of these compounds in transiently 4xJERE::IPT expressing and control areas of tobacco leaves at appropriate time intervals before and after *P. syringae* pv. *tabaci* inoculations.

We did not find any statistical difference in the accumulation of SA and JA in IPT expressing areas in comparison to that of non-IPT expressing counterparts (Fig.15). However, there occurred an increasing trend of SA accumulation in non-IPT expressing samples with gradual increase from earlier to later time points (24-0-0→48-0-Ps). Contrarily, gradual decrease was observed from earlier to later time points (24-IPT-0→36-IPT-Ps) for IPT-expressing samples. Nevertheless, a bit of increase was also observed in IPT-expressing areas for 48-IPT-Ps. No such detectable trend was found in the leaves JA-levels (Fig.15). It portrays that SA/JA may not be involved in CKs-mediated resistance.

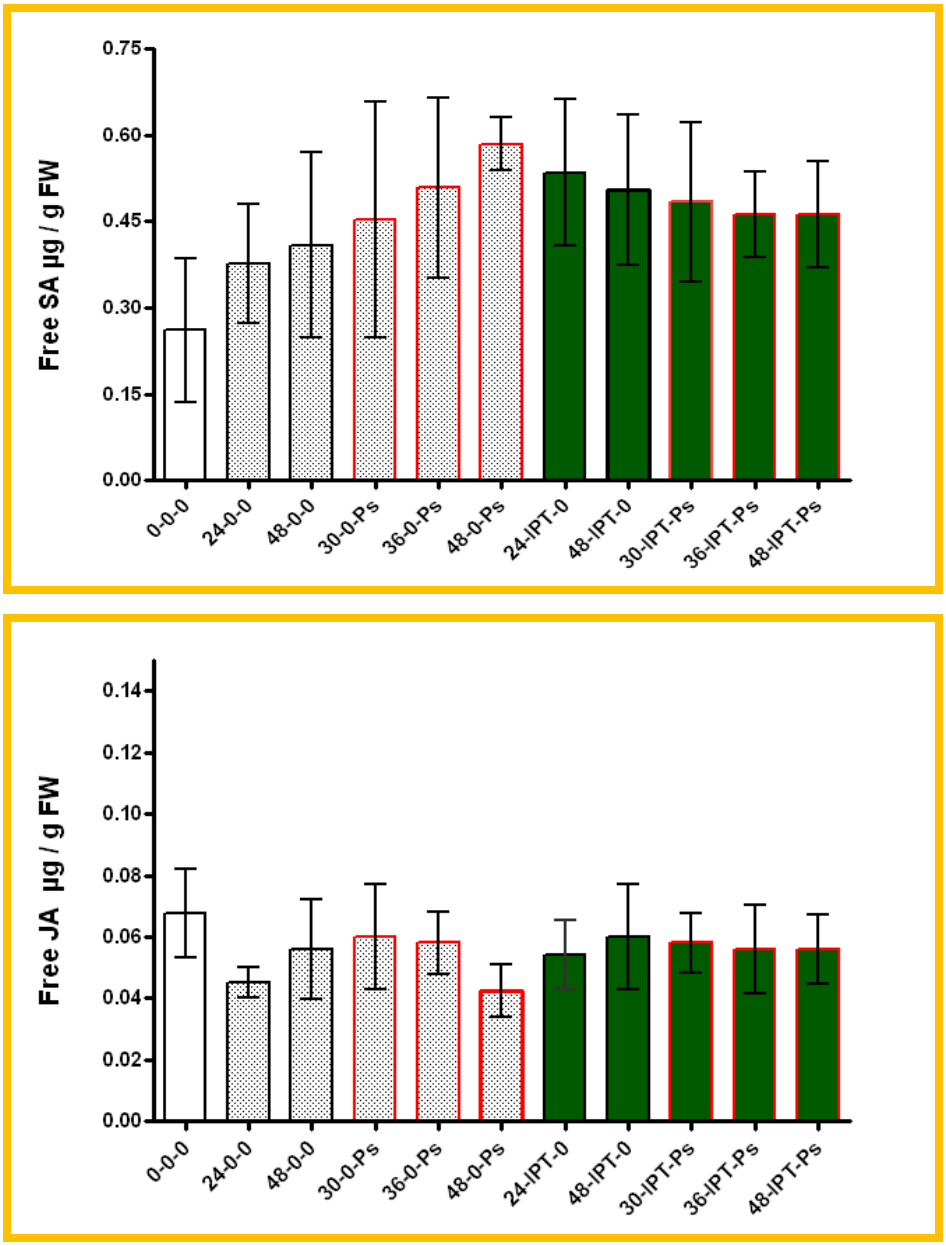


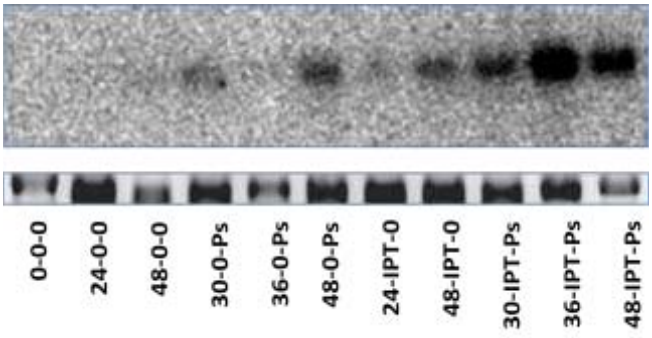
Fig. (15): CKs-mediated resistance is independent of the accumulation of SA and JA
Free SA levels in term of µg/g FW in 4xJERE-IPT (with and without *P.syringae* pv. tabaci) and corresponding control extracts are shown at y- axis for given time points on x-axis. Data bars represent mean values of five independent experiments while error bars represent s.d (n=5). Free JA levels in term of µg/g FW in 4xJERE-IPT (with and without *P.syringae* pv. *tabaci*) and corresponding control extracts are shown at y- axis for given time points on x-axis. Data bars represent mean values of five independent experiments while error bars represent s.d (n=5). *(Analysis of 4xJERE-prepared sampes was performed by Thomas Griebel, Phd student in Bot-II, University of Wuerzburg).*

4.2.7 CKs up-regulate the expression of *PR-1* gene

PR (Pathogenesis related proteins) genes expression is a disease marker and is often coupled with accumulation of SA being an endogenous signalling molecule in resistance response against invading pathogens (Malamy et al, 1990). To investigate the implication of *PR* genes in elevated CKs status of the plant and subsequent resistance response against *P. syringae* pv. *tabaci* we analyzed the expression levels of *NtPR1* gene in 4xJERE::IPT and corresponding control tobacco samples. Northern-Blotting analysis of *NtPR1* gene revealed a strong up-regulation in later time points (36-IPT-Ps & 48-IPT-Ps) when *IPT* gene expression was already up-regulated by *P. syringae* pv. *tabaci* which induces the 4xJERE-promoter in the front of IPT. However, we also noticed weaker expression levels for early time points where *Pseudomonas* and/or *Agrobacterium,* being plant pathogens, were also operational in the tissue (Fig.16A)

To get an unequivocal picture of the expression of *NtPR1* gene in CK mediated resistance we devised experiments to exclude the contribution of both *Pseudomonas* and *Agrobacterium* to the expression levels of PR1 gene. Therefore, we induced PR1::GUS promoter-reporter line with exogenous application of that concentration (9.2µM) of kinetin which already proved to be sufficient in rendering tobacco plants resistant against *P. syringae* pv. *tabaci*. For this purpose, left half of the leaf was infiltrated with kinetin and right half of the leaf was mock induced with H_2O and incubated for 24h. Clear GUS staining was observed on the left half of the leaf while right half did not show any such staining (Fig.16.B). Furthermore, when fed via petiole to the detached PR1::GUS leaves more stringent staining was observed for kinetin than for the corresponding water fed control. (Fig.16 C & D). It is, therefore, assumed that PR1 gene expression has a special relevance to the CKs mediated resistance response against *P. syringae* pv. *tabaci*.

A



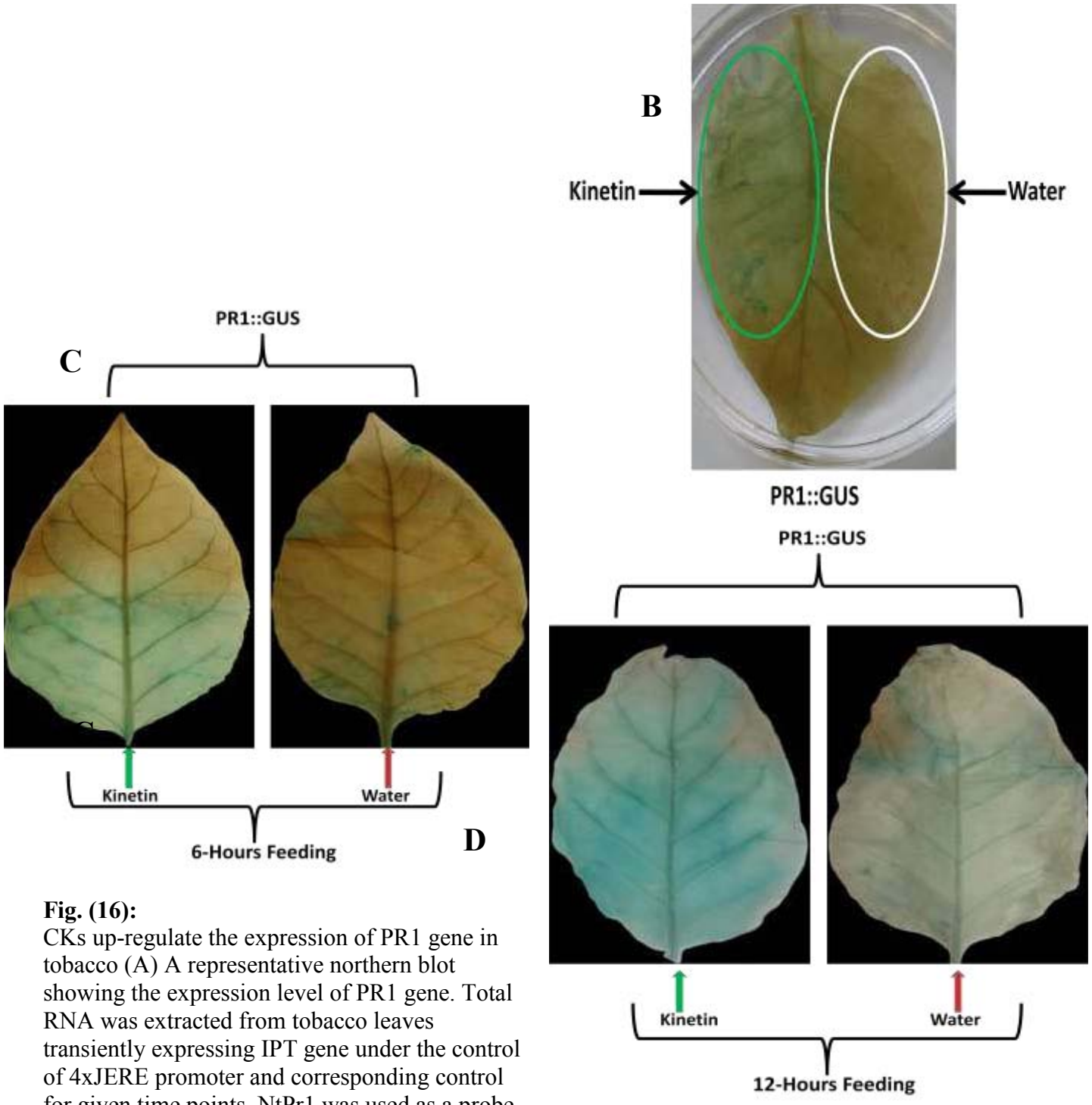
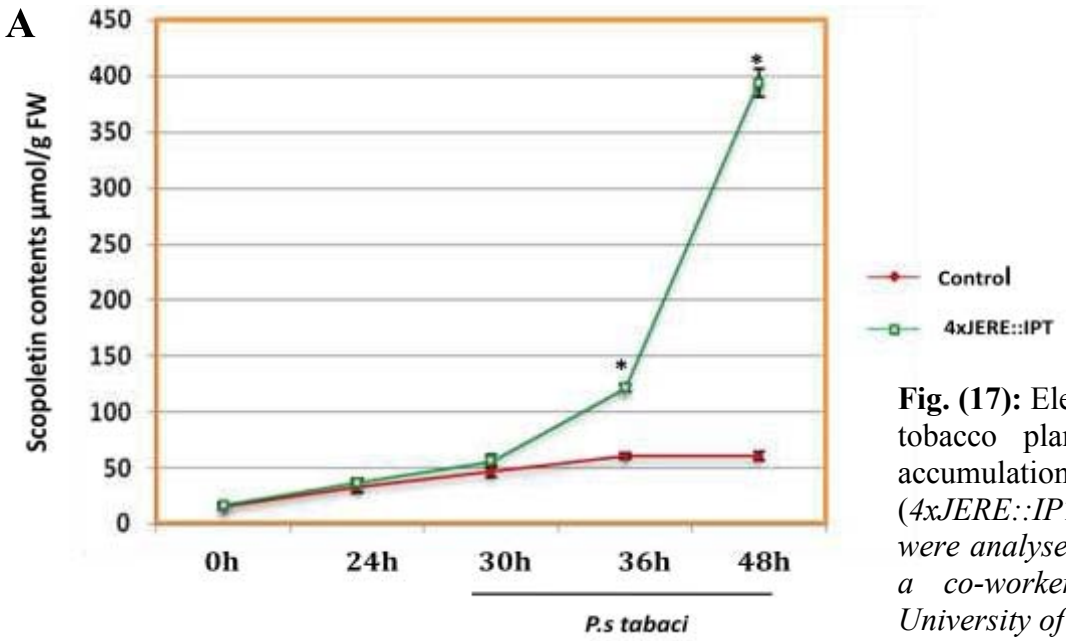


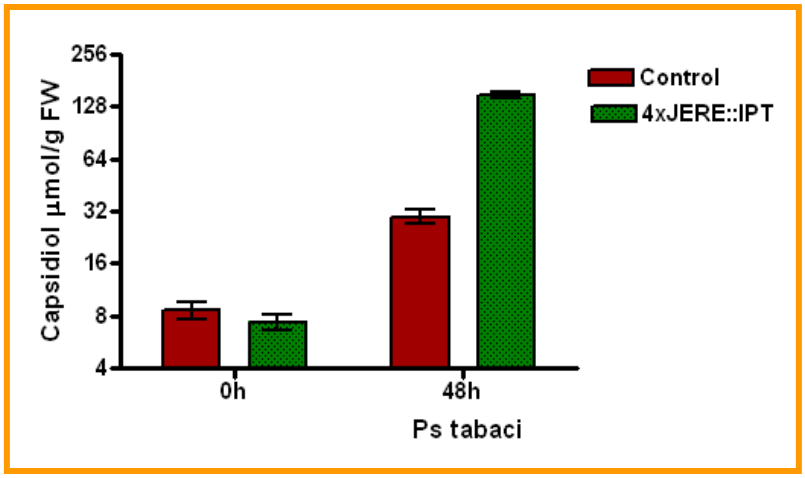
Fig. (16):
CKs up-regulate the expression of PR1 gene in tobacco (A) A representative northern blot showing the expression level of PR1 gene. Total RNA was extracted from tobacco leaves transiently expressing IPT gene under the control of 4xJERE promoter and corresponding control for given time points. NtPr1 was used as a probe. 28S rRNA is shown as loading control. *(RNA isolation of prepared samples and Hybridization was performed by Dominik Grosskinsky, former Diploma student in the working group of Prof. Roitsch, University of Wuerzburg).* (B) Promoter reporter PR1::GUS line induced with 9.2µM of kinetin on left half of the leaf while for mock induction water is used in the right half of corresponding site. Leaf is GUS stained 24 h post kinetin induction. (C) Kinetin and water feeding for 6h (D) Kinetin and water feeding for 12h.

4.2.8 Extracts of leaves with elevated CKs contain significantly increased levels of the phytoalexins Capsidiol and Scopoletin

To scrutinize specific attributes towards cytokinins mediated resistance against *Pseudomonas*, experiments were designed to evaluate the presence of phytoalexins in tobacco 4xJERE::IPT. The measurements were done as well on kinetin fed extracts, as their corresponding controls. Because, the sesquiterpene capsidiol and the hydroxycoumarin scopoletin are well-known antimicrobial compounds in Solanaceae (Stoessl et al. 1976, Al Goy et al. 1993, Dorey et al. 1997), we therefore quantified the concentration of these substances in the mentioned tobacco extracts. As shown in Fig. (17A) extracts of 4xJERE::IPT contained significantly higher amounts of scopoletin for late time points (36-IPT-Ps &48-IPT-Ps) than their corresponding controls. Similarly, there accumulated significantly higher amounts of capsidiol in 48-IPT-Ps (4xJERE::IPT) extracts than control (Fig.17.B).

For further validation, apart from transgenic approach we also determined the amounts of scopoletin and capsidiol in tobacco kinetin fed and control extracts. We found significantly higher amounts of scopoletin as well as capsidiol in the kinetin treated extracts compared to their water fed controls (Fig. 17.C). Accumulation of substantially higher amounts of phytoalexins in CKs enriched extracts prompted us that these could be the proposed antimicrobial agents in the underlying resistance mechanism.





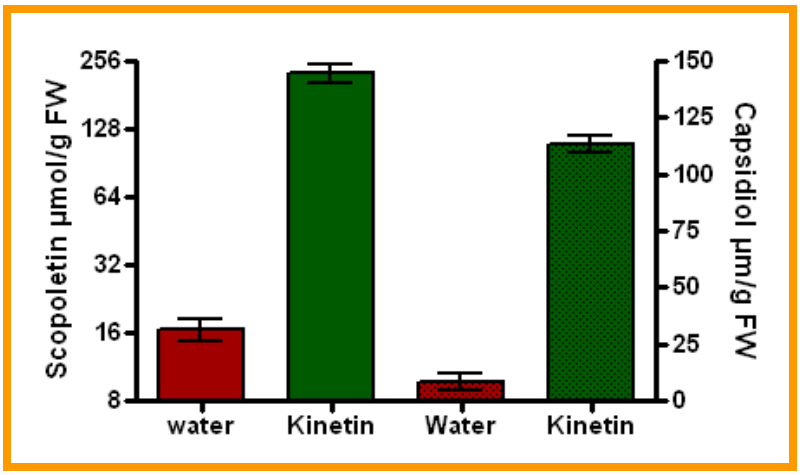


Fig. (17): Elevated levels of CKs in tobacco plants result in hyper-accumulation of phytoalexins. (*4xJERE::IPT prepared samples were analysed by Usama Ramadan a co-worker of Prof. Roitsch, University of Wuerzburg)*
(A) Accumulation of Scopoletin in IPT expressing areas of tobacco laves under the control of 4xJERE promoter and control samples devoid of IPT gene, in term of µmol/gFW on Y-axis of the graph for given time points on X-axis. Data points are mean values of three independent experiments ± s.d.. (B) Accumulation of capsidiol in IPT expressing areas of tobacco leaves under the control of 4xJERE promoter and control samples devoid of IPT gene, in term of µmol/gFW on Y-axis of the graph for given time points on X-axis. Data bars are mean values of three independent experiments ± s.d.. (C) Accumulation of scopoletin and capsidiol in SR1 tobacco leaves fed with 9.2µM kinetin solution for 24h, while mock feeding with water generated control samples for analysis. Shown on Left Y-axis are values for scopoletin accumulation in term of µmol/gFW, while that of capsidiol on Right Y-axis. X-axis depicts nature of petiole feeding in SR1 leaves.

4.2.9 Cytokinins up-regulate the expression of genes involved in the biosynthetic pathway of capsidiol and scopoletin.

Besides the contents of scopoletin and capsidiol in 4xJERE::IPT expressing samples, we also analyzed transcript levels of genes of key enzymes involved in the biosynthetic pathway leading to accumulation of these phytoalexins. Expression levels of the genes such as 5-epi-Aristolochen Synthase (NtEAS), cinnamic acid-4-hydroxylase (NtC4H) and Phenylpropanoid-Glucosyltransferase 1 (TOGT 1) were determined through Northern Blot analysis while probes were generated by PCR amplification of the identified sequences which were then cloned in pGEMT vector (Table 2).

*Nt*EAS is responsible for the synthesis of precursor of capsidiol (Bohlmann et al., 2002). *IPT* expressing samples exhibited stronger signals of *Nt*EAS than the non-IPT counterparts; nevertheless, transcript accumulation was higher for 24-IPT-0 than the others. Moreover, weaker signal were also detected for non-IPT expressing sample such as 24-0-0 and 48-0-0 (Fig18.A). Nt C4H is responsible for the conversion of cinnamic acid to coumaric acid hence play a role in biosynthetic pathway to scopoletin (Chong et al., 2002). Transcripts levels were detected in IPT-expressing samples though very faint signals for later time points and stronger for early ones. No signal appeared for non-IPT expressing samples and even did not for 48-IPT-Ps(Fig.18.B). Nt TOGT is an enzyme glycosylating scopoletin and also converting scopoletin to scopolin, though biosynthetic enzyme for scopoletin is not known thus far (Chong et al., 2002). No clear signals were detected for any of the samples, even if a faint signal appeared at the late time point for IPT- expressing sample of 48-IPT-Ps (Fig.18 C).

Table 2 Genes of Phenylpropanoid pathway *(PCR products as probes were generated by Dominik Grosskinsky, fromer Diploma student in the working group of Prof. Roitsch)*

Gene	Function	Template	PCR-Product
NtEAS	5-epi-Aristolochen-Synthase; synthesis of Capsidiol precursor.	gDNA	738 bp
NtC4H	Cinnamic acid-4-hydroxylase; synthesis of precursor for scopoletin	cDNA	555 bp
NtTOGT 1	Tobbaco-Phenylpropanoid-Glucosyltransferase 1; synthesis of scopolin from scopoletin	gDNA	570 bp

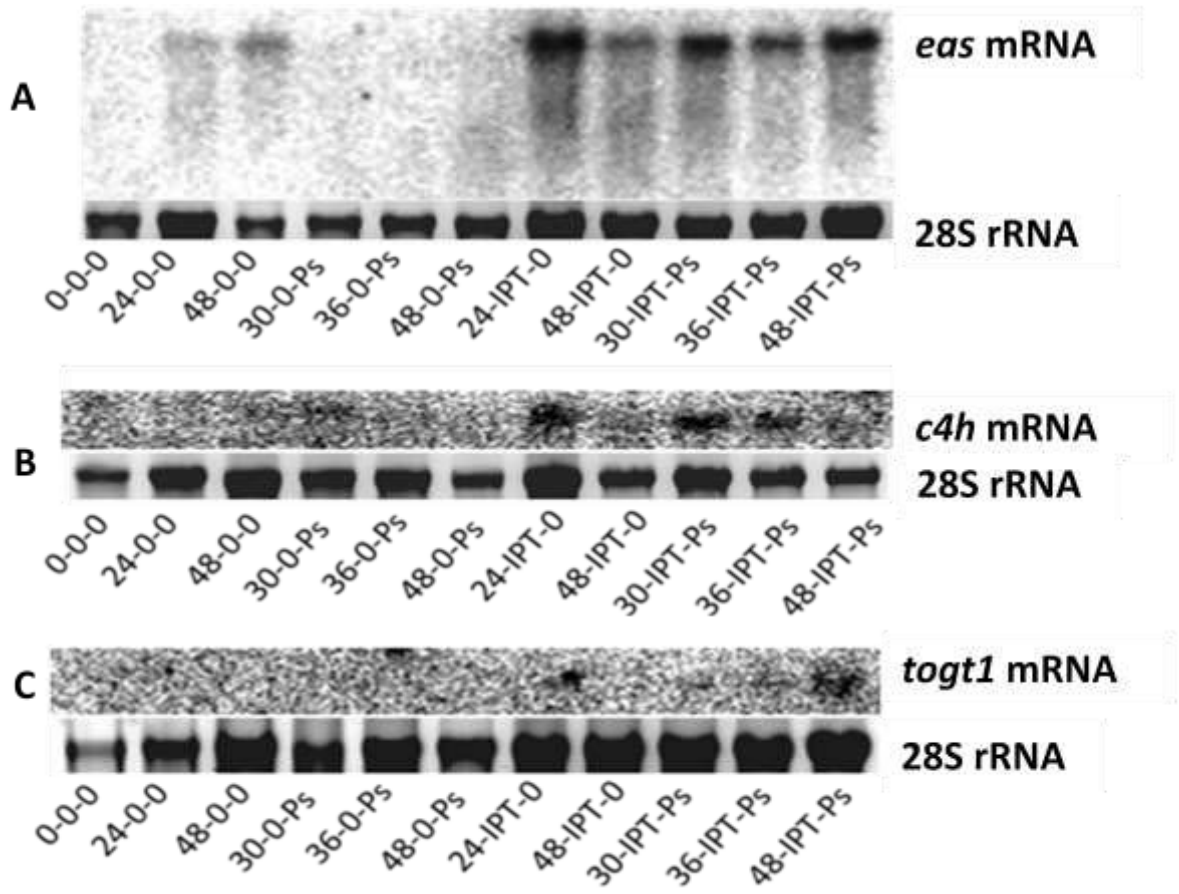


Fig.(18): Northern-Blot analysis of genes involved in the synthesis of phytoalexins. Total RNA was isolated from IPT and Non-IPT expressing areas of tobacco leaves, with and without infection of *P.s tabaci* and hybridized with probes of key enzymes of the synthesis of Capsidiol (A) and Scopoletin (B und C) while 28S rRNA has been shown as a loading control. (*RNA isolation from prepared 4xJERE-IPT samples and hybridization was performed by Dominik Grosskinsky, former Diploma student in the working group of Prof. Roitsch)*

4.2.10 Reconstitution of antimicrobial activity, a disclosure of cytokinins mediated resistance

As described above that higher CK contents in 4xJERE::IPT and kinetin fed extract accumulated significantly higher amounts of two phytoalexins capsidiol and scopoletin. To rectify their exact role in CKs mediated plant immunity a reconstitution of activity based assay was performed. 48-IPT-Ps is that time point of CKs modulation in which tobacco plants expressing 4xJERE::IPT manifested significantly lower bacterial growth counts, extracts shown higher zone of inhibition on *P. syringae* pv. *tabaci* lawn, and where accumulated significantly higher amounts of capsidiol and scopolitin in crude extracts. Therefore, the accumulated amounts of capsidiol and scopoletin in crude extract of 48-IPT-Ps were compared to that of untreated crude extracts of tobacco leaves. It is difference of both these compounds which otherwise entitled the former extracts potent in jeopardizing the growth of bacteria than later. When added from exogenous source equivalent amounts of capsidiol and scopoletin in control extracts, their antimicrobial potential also raised almost up to that of 48-IPT-Ps (Fig.19). This underpins the role of these two phytoalexins in CKs mediated resistance against *P.syringae* pv. *tabaci.* Furthermore, when scopoletin or capsidiol were given on individual basis into control crude extracts, they did not prove to be as efficient as in combination.

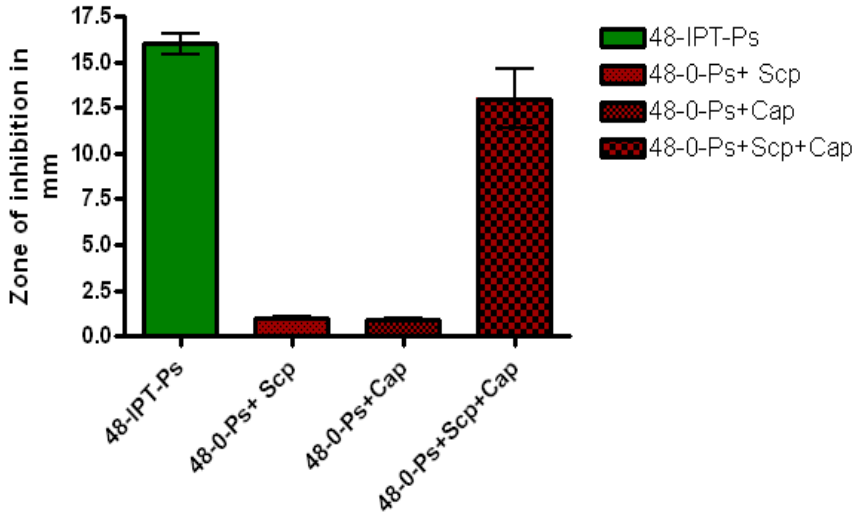


Fig. (19): Reconstitution of CKs mediated antimicrobial activity in disc diffusion assays
Zone of inhibition generated in lawn of *P.s tabaci* around dics provided with original leaf extracts and extracts supplemented with either scopoletin or capsidiol or both. Phytoalexins were added according to the difference between 48-IPT-Ps and 48-0-Ps (Fig.17). Values are means of 3 readings. +/- = standard deviation. *(Disc-diffusion assays were performed by Usama Ramadan with given 4xJERE-IPT samples, a co-worker in the working group of Prof. Roitsch, University of Wuerzburg)*

4.3 Host pathogen interaction in modulated carbohydrate status of the plant

Attack of a pathogen on plant generates an additional respiratory sink and thus molds plant metabolism at host pathogen interface in its favor. Plant enzyme extracellular invertase (CWI) has a pivotal role in source to sink transition and particularly implicated in pathogenesis by inducing PR gene expression (Roitsch 1999, Herbes et al., 1996, Berger et al., 2004 and Bonfig et al., 2006). To get an unequivocal picture of the host pathogen interaction in modulated carbohydrate status of the plant, previously generated tobacco Nt-35 Tet:: CIN1 (Lara et al., 2004) and Nt-68 Tet::*Nt*CIF (Bonfiq et al., 2007) and tomato To-19 Lin6::*Nt*CIF transgenic plants (Lara et al., 2004) were challenged with *P. syringae* pv. *tabaci* and *P. syringae* pv. *tomato DC3000* respectively. Moreover, To-19 Lin6::*Nt*CIF plants were also tested with *Botrytits cinerea*.

4.3.1 Interaction of *P. syringae* pv. *tabaci* with Tet:: CIN1 and Tet::*Nt*CIF transgenic plants

Preliminary experiments were conducted on wild type tobacco plants of accession W-38 by the infiltration of *P. syringae* pv. *tabaci* (10^6CFU/ml) and 10mM $MgCl_2$ as mock inoculation for comparison. Slight increase for early time points (24 & 48h) was observed in invertase activity for pathogen treated samples compared to corresponding controls. Furthermore, the difference in activity was highly significant for late time points of 72h PPI (Fig.20A). To investigate the implication of increased invertase activity upon a pathogenic challenge, previously generated Tet::CIN1 (Lara et al., 2004) tobacco transgenic plants were used. 24h prior the inoculation of *P. syringae* pv. *tabaci* (10^6CFU/ml) leaves of Tet::CIN1 plants were treated with 1mg/L of Cl_2-Teracycline while for mock treatment 10mM MgCl2 was used. We did not observed any visible difference in symptom development on induced and non-induced halves of the same leaf Fig. (20 B). Similarly, when leaves of Nt-68 Tet::*Nt*CIF were induced with 1mg/L of Cl_2-teracycline and 24h post tet induction challenged with *P. syringae* pv. *tabaci* (10^6CFU/ml), no visible difference was observed in symptom development for induced and non-induced sites Fig. (19C). Outcome of both these approaches substantiates that modulation in cell wall invertase do not affect the growth of *P. syringae* pv. *tabaci.*

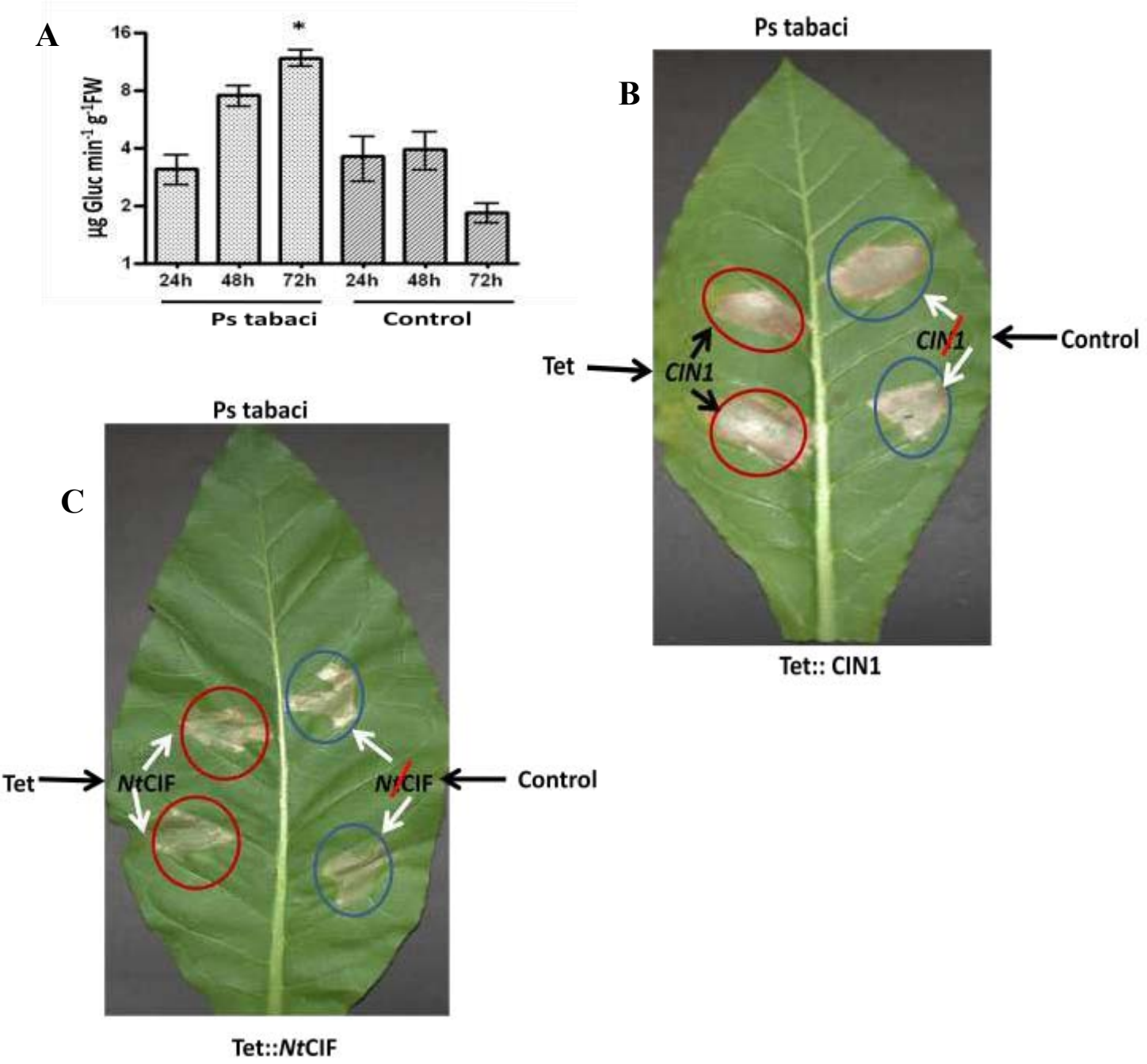
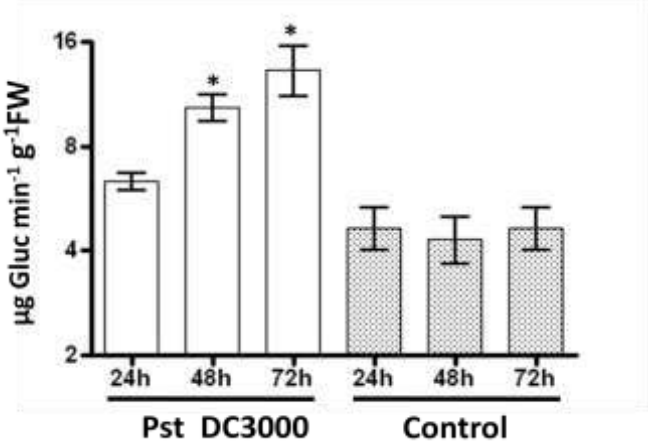


Fig. (20): Modulations in apoplastic invertase and growth of *P. syringae* pv. *tabaci* . (A) Represents the fate of tobacco cell wall invertase upon pathogen infection. Invertase activity in terms of µg Gluc./min/gFW on Y-axis for *P.syringae* pv. tabaci and mock inoculation with 10mM MgCl2 for denoted time points on X-axis. Data bar represents means values of three independent experiments ± s.d. (B) Representative picture of experiments where CIN1 gene has been expressed in tobacco plant under the control of Tet-inducible promoter. left site is induced with 1mg/L Chloro-tetracycline corresponding right sites are mock induced with 10mM MgCl2. 24h later all of the four sites are infiltrated with *P.syringae* pv. *Tabaci*. Pictures were taken 1 week PPI. (C) Representative picture of similar experiments with Tet::*Nt*CIF plants

4.3.2 Interaction of *P. syringae* pv. *tomato DC3000* and *Botrytits cinerea* with Lin6::*Nt*CIF tomato transgenic plants.

Necessarily, it would be an over simplification to extrapolate the behavior of one type of pathogen to another and hence resistance response of one host plant to another. In this context, when challenged wild type tomato plants of the accession Moneymaker with *P. syringae* pv. *tomato DC3000*, significantly higher invertase activity was observed for pathogen treated plants than control Fig. (21 A). When inoculated tomato transgenic Lin6::*Nt*CIF (invertase inhibitor gene under the control of tomato invertase gene promoter) plants with *P. syringae* pv. *tomato DC3000*, no difference in symptoms development was observed in comparison to that of their control counterparts (Fig 21 B). On the contrary, when challenged with necrotrophic pathogen *Botrytits cinerea*, severe disease symptoms were observed on leaves of To-19 Lin6::*Nt*CIF in comparison to wild type plants (Fig. 21C). Before conducting experiments on tomato transgenic plants, an extensive *in-situ* invertase activity based screening procedure was adopted to lower down the number of transgenic lines upto a manageable number.

A



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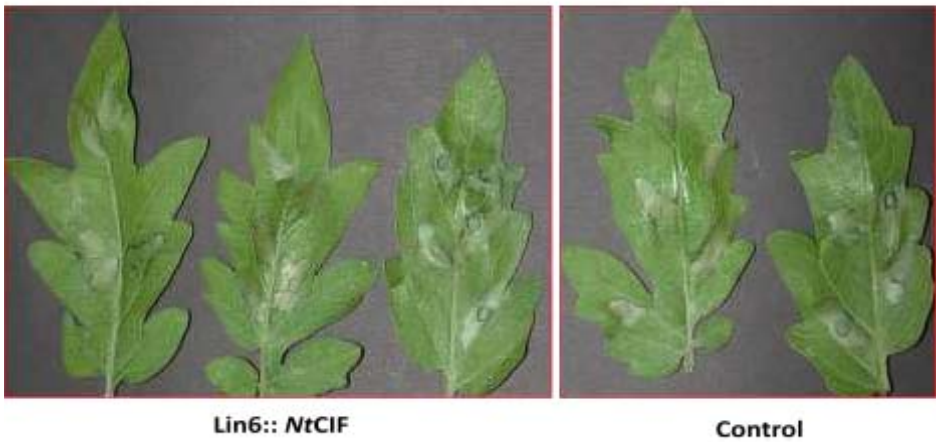

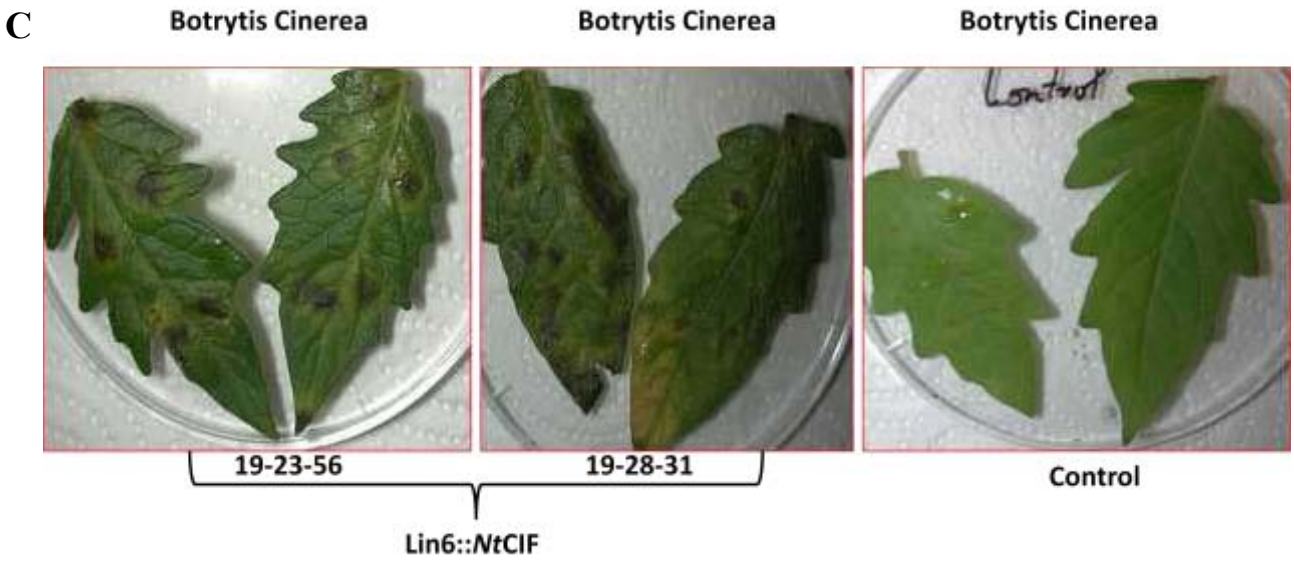

Fig. (21): Inhibition of cell wall bound invertase in tomato manifests differential responses to hemibiotrophic and necrotrophic pathogens. (A) Represents the fate of tomato cell wall bound invertase upon pathogen infection. Invertase activity in term of µg Gluc.min^{-1}gFW^{-1} on Y-axis for *P.syringae* pv. *tabaci* and mock inoculation with 10mM MgCl2 for denoted time points on x-axis. Data bar represents means values of three independent experiments ± s.d. (B) Transgenic tomato plants expressing invertase inhibitor under the control of invertase gene Lin6 promoter (Lin6::*Nt*CIF) and wild type Moneymaker infiltrated with *P.syringae* pv. *tomato DC3000*. Picture was taken three days PPI (C) Transgenic tomato plants expressing invertase inhibitor under the control of invertase gene Lin6 promoter. (Lin6::*Nt*CIF) and wild type Moneymaker, inoculated with spore suspension of *Botrytis cinerea*. Picture was taken 6 days PPI.

5. Discussion

Phytohormones are known for their pivotal roles in promoting normal plant growth and development and contributing to defense mechanisms (Robert et al., 2007). Cytokinins are active plant substances and predominantly implicated in physiological and biochemical processes related to growth and development. Apart from their role in *Agrobacterium* mediated tumorigeneses and generation of green island around the infected areas of fungal biotrophs (Walters et al., 2008) they hardly got consideration in plant immunity in the last few decades. To gain insight into the functional role of cytokinins in plant pathogen interaction, we exclusively examined three independent tobacco transgenic approaches whereby bacterial IPT (Iso-pentenyl transferase) gene has been expressed under the control of chemical, developmental and pathogen-inducible promoters. In order to complement the out-come of transgenic approaches and elevate the levels of cytokinins, we exogenously applied different types of cytokinins such as kinetin, zeatin and TDZ to the detached tobacco leaves. Upon infiltration of the pathogen *P. syringae* pv *tabaci,* in all of the above mentioned approaches, we unanimously observed more resistant phenotypes in plants with higher cytokinins status and vice versa. We, therefore, hypothesize that elevated levels of cytokinins impede the growth of *P. syringae* pv. *tabaci* in tobacco plants.

5.1 Cytokinins mediated resistance to plant pathogens; a novel concept with a novel tool and additional proofs.

Besides a decent range of plasticity, plants faithfully maintain innate physiological balance for their optimal well being. However, when microbial pathogens reach appropriate host plant species and if favorable conditions prevail, they develop rapidly resulting in derangement of physiological functions of the host plant, followed by characteristic macroscopic symptoms. Apart from other alterations, pathological invaders cause hormonal imbalance at the host pathogen interface and even transmit signals across the whole body of the plant (Robert et al. 2007). When challenged with *P. syringae* pv. *tomato DC3000*, *At*ARR5::GUS reporter line displayed hardly visible GUS-staining while mock inoculation portrayed residual staining on leaves (Fig.1). Decline in GUS-staining reflects decrease in cytokinins contents of the leaf in general and at host-pathogen interface in particular. *At*ARR5::GUS is promoter- reporter line of *At*ARR5 gene which is an early cytokinins type A response regulator, and have long been in use

as a marker for the presence of cytokinins in plant tissues (Hwang and Sheen 2001 and Spichal et al., 2008). Lack of GUS- staining at the site of infection and hence CKs, leads to the hypothesis that to cause infection *P. syringae* needs to lower the level of CKs down at the host-pathogen interface. It is, however, not very clear what happens to cytokinins upon pathogen infection. A likely explanation could be their degradation as does occur for auxins (Johan and Saskia. 2008) or *P. syringae* might be up regulating plant cytokinin oxidase genes to get ride of cytokinins. Nevertheless, their redistribution to other parts of the plant can also not be excluded. How incredible are the early response regulator genes in predicting cytokinins status of the plant with their limitations of cytokinins independent regulation (Muller and Sheen 2008), our data with *At*ARR5::GUS plants proved to be highly valuable when detailed analysis down these lines were performed.

To get an unequivocal picture of the pathogenecity of *P. syringae* in modulated levels of CKs we owned multiple approaches. Our results with *Arabidopsis* 4xJERE::GUS line and *P. syringae* pv. *tomato DC3000* interaction revealed the significance of 4xJERE being a magnificent tool for manipulating gene expression at the host-pathogen interface (Fig.2). Our findings regarding pathogen based inducibility and tightness in gene expression on the part of 4xJERE promoter are in line with those of Rushton et al. (2002). Expression of bacterial-IPT gene under the control of constitutive and inducible promoters in tobacco plants and their interaction with viruses (Sano et al., 1996) and herbivores (Smigocki et al. 1993) has already been reported. We, however, demonstrate that our devised novel pathogen inducible transient IPT expression system (4xJERE::IPT) in tobacco is more contextual where better view of the underlying host pathogen interface is evident. We show that IPT-expressing areas are clearly devoid of the symptoms of *P. syringae* pv. *tabaci* compared to non-IPT control that showed localized necrosis (Fig.3 A).

Practice of transient expression (Asai et al., 2002) is an efficient tool of plant transformation, which reduces time required in generating stable transgenics with aftermaths of undesirable phenotypes and gene silencing. Northern blot analysis for expression of IPT gene in transient 4xJERE::IPT system further supports the notion that 4xJERE promoter regulates IPT gene upon pathogen infection and that the prevailing modulation is in the right time and right space as (0-0-0, and 24,30,36,48-0-Ps) non-IPT expressing areas did not show signal for IPT transcripts (Fig.3.B). Moreover, accumulation of enhanced cytokinins contents in IPT-expressing area in pathogen dependent manner is an additional proof (Fig. 3 F, G, H and I). IPT-expressing areas

exhibited phenotypes reminiscent of plants with delayed senescence, which is a direct functional proof of the enhanced CKs contents. When kept even for 4 weeks under pathogen attack, 4xJERE::IPT expressing areas were still juvenile whereas the corresponding counterpart had already been macerated (Fig.3.E). Results regarding 4xJERE::IPT transient expression and lack of visible disease symptoms on *Arabidopsis* upon application of *P. syringae* pv. *tomato DC3000* further validate the utility of 4xJERE::IPT transient expression system and support the hypothesis of CKs mediated resistance even in a different plant-patho system (Fig.4).

Our data regarding resistant phenotypes on Tet- induced sites and disease on non-induced sites of the very same leaf is additional evidence supporting enhanced CKs based resistance in plants (Fig.5B). Tet::IPT tobacco transgenic plants work as per de-repression mechanism (Redig et al. 1996). Inoculated *P. syringae* pv. *tabaci* on induced and non-induced sites gave differential disease phenotypes. Contrary to 4xJERE system, upon prolonged incubation, visible but faint symptoms also appeared even on induced sites of the leaves (Fig 5.B). Dilution of the inducer (Tet) due to apoplastic fluid could be a likely explanation for the release of de-repression of IPT gene. One likely explanation could be that there occurred a dilution of inducer due to apoplastic fluid. Degradative factors may also cause tetracycline unable to keep the binding of suppressor away from operator for a longer time. The durable resistant phenotype of 4xJERE system and comparatively transient resistance in Tet::IPT may also be attributed to the levels of cytokinins synthesized. Upon comparison of one month senescence phenotype of 4xJERE::IPT (Fig.3E) and that of Tet::IPT (Fig.5A) clearly reflects the differences in tissue juvenility and so the state of resistance.

SAG12 is senescence associated gene and predominantly implicated in studies dealing with senescence in plants, hocked with IPT gene caused delay in senescence in tobacco plants (Gan and Amasino 1995 and Lara et al., 2004). Our results regarding induction of SAG12 promoter in SAG12::GUS line during early advent of senescence, necessitates the significance of SAG12 promoter in developmental modulations (Fig.6). When challenged with *P. syringae* pv. *tabaci*, SAG12::IPT and corresponding wild type W-38 tobacco plants displayed differential pathogenic responses, however one should keep in view that they got identical senescence induction conditions, prior to the inoculation of pathogen (Fig.6). Developmental induction of ectopic IPT gene pertains resistance to SAG12::IPT plants while susceptibility in W-38 is a consequence of the lack of such sort of transgenecity. Although driving the expression of the same IPT gene, yet

all these mutually exclusive expression systems yielded congruent results that elevated CKs impede the growth of *P. syringae* pv. *tabaci* in tobacco plants.

Above mentioned transgenic approaches unanimously end up with the same basic conclusion of cytokinins mediated resistance however, the magnitude of resistance varied from approach to approach by virtue of the reason that target IPT gene was driven by various nature of promoters. Transgenic approaches keep it on the disposal of their own and may not ideally permit to modulate CKs in defined doses and time. Therefore, although very simple in operation but enduring conclusions may be drawn from exogenous application of cytokinins and their analogues in plants. Interestingly, most apt to our work is the report in *Nature,* which dates back to 1963, about exogenous feeding of 10^{-5}M of kinetin and its protection effects against the culture filtrate of *P. syringae* pv. *tabaci* (Lovrekovich and Farkas 1963).

Our results of exogenous feeding of various types of cytokinins give rise to protection in tobacco leaves against the spread of *P. syringae* pv. *tabaci* when compared to mock feeding with water which showed residual disease symptoms (Fig.7). Exogenous petiole feeding of cytokinins into detached tobacco leaves support our cytokinins mediated resistance on one hand but on the other hand give opportunity to quantitatively monitor this protection effect in dose and time dependent manners. Exogenous petiole feeding of 10µM of *trans*-zeatin and 9.2µM of kinetin for 24h PPI, proved to be optimal in embarking resistance on tobacco leaves against *P. syringae* pv. *tabaci* (Fig.7) with-out compromising on leaf wilting, being a residual property of cytokinins (Biddington and Thomas 1978). Besides these optimal doses, lower kinetin concentration as low as 4.6µM also proved to be effective in preventing macroscopic disease symptoms of *P. syringae* pv. *tabaci* (Fig.7).

Like with adenine based cytokinins our results with phenyl urea derived cytokinins (TDZ) also proved to be equally effective in abrogating disease symptoms of *P. syringae* pv. *tabaci* (Fig.7). Interesting, TDZ is equally potent in preventing disease in nM (20nM-200nM) concentrations and thus posing superiority to adenine derived ones. The proposed, but indirect mechanism of TDZ being an entitled cytokinin is its cytokinin oxidase (CKX) inhibition potential and thus sustains available cytokinins in the tissue (Hare and Stadan 1994). More relevantly, TDZ, equally like adenine derived cytokinins, binds to the cytokinin-receptor CRE1 in *Arabidopsis* (Inoue et al., 2001). More specifically, 100nM of TDZ proved equal effectiveness in inducing *TCS::LUC* reporter in mesophyll protoplast assays (Muller and Sheen 2008). This further

validate our findings that TDZ concentration between 20 and 200nM is effective in terms of CKs signaling and hence protection against *P. syringae* pv. *tabaci*.

5.2 Mechanism of cytokinins mediated resistance

As discussed, all our cytokinins modulating, but mutually exclusive approaches resulted in the same basic conclusion of resistance against *P. syringae* pv. *tabaci* in higher cytokinins status of the plant and vice versa. Contrarily, necrotrophic fungal pathogen *Sclerotinia* spreaded on generated 4xJERE::IPT areas as good as on the control (Fig.9A). However, with exogenous kinetin feeding approach, CKs fed leaves even proved to be slightly more susceptible to *Sclerotinia* than their control counterparts (Fig.9B). Manifestation of differential responses substantiates that elevated cytokinins level of the plant differ in term of resistance for biotrophic and necrotrophic pathogens.

P. syringae being pathogenic bacteria, after their inoculation exist as epiphytes on plant surface until opportunity enables them to approach intercellular apoplastic space inside the leaf tissues. While incubating they express their virulence machinery (PAMPS and Effectors) to circumvent the host defense and elicit macroscopic symptoms after exponential multiplication for ultimate dispersal (Nomura, et al. 2005, Jones and Dangl 2006 and Mole et al., 2007). To decipher the underlying mechanism of CKs mediated resistance against *P. syringae* pv. *tabaci*, it is however, very important to quantify the *in planta* growth kinetics of invading bacteria. Our results of significantly diminished bacterial growth in transient 4xJERE::IPT and exogenous kinetin feeding samples Fig.(8A&B) with bacterial plate-counting (Whalen et al., 1991) experiments validate the diseased and resistance phenotypes of the above mentioned CKs modulating approaches. These results also signify the importance of the overexpression of IPT-gene and CKs accumulation in late time points (i.e. 48-IPT-Ps) Fig. (3B), where proposed cytokinins mediated defense of the plant is thought to be already activated. Because of their robust and highly reproducible disease resistance phenotypes (Fig.3) coupled with strong antimicrobial potential (Fig.8A), transient 4xJERE::IPT has been opted for detailed characterization of the underlying mechanism of CKs- mediated resistance. Moreover, outcome of plate counting experiments also proved valuable in terms of sampling time points in devising experiments for the mechanistic characterization of CKs-based resistance against *P. syringae* pv. *tabaci* (Fig.8).

Our hypothesis, that elevated cytokinins level enhance plant immunity against *P. syringae* pv. *tabaci* lacks a mechanistic explanation behind. Therefore, it was logical to investigate CKs innate antimicrobial potential. Our results regarding direct antimicrobial potential of cytokinins via bacterial culture fortification with (optimized *in planta* resistance provoking) kinetin concentration (9.2µM) and even below (6.4µM) did not entitle them to be directly involved in our proposed resistance phenomenon (Fig. 11). Nevertheless, exogenously added kinetin in the growing culture substantially enhanced the antibiotic production of streptomycetes (Yell et al., 2006). Whether enhanced level of cytokinins provoke *P. syringae* pv. *tabaci* to produce *in planta* antibiotic and cause growth cessation of its own, needs to be elucidated. However, our disc diffusion and cytokinins enriched growing culture experiments exclude this possibility because cultures grew equally well with and out cytokinins (Fig.11). Interestingly, when discs were impregnated with 48-IPT-Ps (4xJERE::IPT) crude extracts, a larger growth inhibition zone was observed for *P. syringae* pv. *tabaci* than with corresponding non-IPT expressing control. Similar was the case of exogenous kinetin fed extracts (Table.1). Bioautographgy Vigers et al (1991) of 48-IPT-Ps extracts excluded the possibility of the involvement of antimicrobial peptide(s) Fig. (12). Results of these experiments emphasize that CKs potentiate otherwise inactive crude extracts to manifest antimicrobial activity and thereby prompting their indirect and/or signalling role in the underlying immunity against *P. syringae* pv. *tabaci*.

To elaborate the mechanism of CK-mediated cessation of the growth of *P. syringae* pv *tabaci* we examined possible attributes of resistance in a given plant pathogen interaction. Reactive oxygen species (ROS) also play an important role in stress response. In host plants they cause strengthening of the cell wall, may kill intruders and act as signalling molecules to activate defence pathways (Torres et al. 2006). Our observations with DAB-staining that there is no substantial difference in H_2O_2 accumulation for IPT and non-IPT expressing areas before symptoms development (Fig.13) led us to the conclusion that ROS may not be directly involved in the process of CK-mediated resistance. It is generally believed that tobacco plants harboring bacterial IPT gene are incorporated with higher antioxidative potential concomitantly lower lipid peroxidation and better tolerance against HR-inducing agents and abiotic stresses (Rivero et al., 2007). However, very dense DAB staining at non-IPT expressing areas (Fig.13) is a consequence of pathogenic necrosis at well advanced stage of the disease. This supports the notion that *P.*

syringae pv. *tabaci* being a virulent pathogen avoids host recognition by inducing transient and low magnitude of ROS accumulation during early stage of infection (Toress et al., 2006).

P. syringae pv *tabaci* being a hemi-biotrophic pathogen is expected to enhance the levels of SA Robert et al., (2007) and consequently up-regulate the expression of PR-1 gene (Malamy et al., 1992). Contrary to this plant pathological conjecture, we did not find significant increase in the level of SA in 4xJERE::IPT samples with elevated CKs, contents of which otherwise, is a source of resistance in our case (Fig.15). Moreover, the levels of JA remained unaffected throughout the analysis (Fig.15). In spite of increase in the level of SA, statistically non-significant decrease was observed in cytokinins accumulating samples (48-IPT-Ps). On the other hand a gradual trend of non-significant increase was found for non-IPT expressing samples (Fig.15). No apparent increase in SA in the presence of elevated CKs status of the plant supports the previous finding of Schnablová et al., (2006). However, in our case treatment of *P. syringae* pv. *tabaci* being an attribute of the increase of SA, even failed to cause its elevation due the presence of more cytokinins. SA suppressing behavior of cytokinins might be having some relevance with the mechanism of cytokinins mediated resistance in plants, though SA promotes resistance by repressing auxin signaling (Wang et al., 2007). State of the plant in which it is resistant to a biotrophic pathogen despite no significant increase in SA, leads to the conclusion that CKs-based resistance is independent of SA and that there is possibility of the alternative mechanism.

In spite of no increase in the accumulation of SA, expression of PR1 gene in 4xJERE::IPT samples strongly upregulated in the later time points in cytokinins dependent manner (Fig. 16A). Increase in PR1 is an innate resistance response of plant to the invading pathogen. Therefore, to get an unequivocal picture of the PR1 gene expression, we treated tobacco PR1::GUS lines exclusively with kinetin. Visibly intense staining in leaves of PR1::GUS with the application of CKs and no such staining with mock induction gave an inescapable conclusion that cytokinins induce the expression of PR1 in pathogen independent manner (Fig.16B). Supporting the findings of Schnablová et al., (2006) and Memelink et al. (1987), these results suggest an in built increase in PR1 and hence a premeditated defense capability of the plant against the invading pathogen. Contrarily, auxins embark on susceptibility on plants against biotrophic pathogens by down regulating the expression of PR1. When treated with SA susceptibility turned into resistance and PR1 popped-up again (Navarro et al., 2006; Wang et al., 2007; Chen et al., 2007

and Paponov et al., 2008). This further necessitates that to cause resistance cytokinins provoke other factor(s) than SA to enhance the expression of PR1.

5.3 Cytokinin mediated resistance in perspective of sugar metabolism

In optimal physiological conditions of the plant, hemibiotrophic pathogenic bacteria *P. syringae* pv. *tabaci* multiply tens of folds higher levels compared to the beginning of infection. However, when cytokinins status of the plant is elevated, bacterial growth cut down significantly up to a number where they lose the potential of manifesting visible symptoms. To envision this state of resistance in sugar metabolism, pathogenic response in modulated invertase activity of the plants would be a valuable addition. Generally, metabolic fluxes and sugar concentrations alter dramatically both during development and in response to environmental signals such as diurnal changes and biotic and abiotic stresses (Blasing et al., 2005; Borisjuk et al., 2003; Wiese et al., 1999 and Roitsch, T 1999).

In addition to regulating carbon partitioning, plant development, and hormone responses, INVs (Invertases) have an important role in stress responses as central signal integrators and modulators (Roitsch and Gonzalez 2004). Among them, CW-INV is induced by both abiotic stress and pathogen infection to locally increase respiratory sink activity and can be regarded as a PR protein. However, PK inhibitor studies indicate that sugars and stress regulate source and sink metabolism and defense responses through different pathways (Roitsch 1999 and Ehness et al., 1997). Both exogenous sugars and overexpression of yeast INV in the plant vacuole or cell wall can induce *PR* gene expression (Xiao et al., 2000 and Thaibud et al., 2004). Cytokinins, on the other hand, can delay plant senescence, and studies with *gin2* show that sugars and cytokinins work antagonistically (Moore et al., 2003). Interestingly, cytokinin-induced CW-INV expression is an essential downstream component of cytokinin-mediated local delay (green islands) of leaf senescence (Lara et al., 2004). A part from the descriptions of Walters et al., (2008) and Walters and Roberts (2007) most of the report independently highlight the implication of either cytokinins or the sugar metabolism in pathogenesis. Therefore, it is rationally sound to bring them together, so that to get an unequivocal conclusion of their dependence on each other during the course of pathogen infection.

Our results that Tet::CIN1 tobacco transgenic plants expressing cell wall invertase gene under the control of a tetracycline inducible promoter did not show differential responses in induced

and non-induced state of the plant to the spread of the invading pathogen . Contrary to this, when generated IPT-expressing sites on the leaves of Tet::CIN1 plants, resistance was observed for *P. syringae* pv. *tabaci* as compared to the control treatment on opposite half of the leaf. (Fig.14). Apart from this, when fed with tetracycline-inducer and kinetin even more visible differences in symptoms development were observed (Fig.14). Moreover, when Tet::IPT tobacco lines were induced with tetracycline, clearly visible differences in symptom development were observed on induced and non-induced halves of the leaf which thereby legitimate the nature of tetracycline based inducible system (Fig.5 B & D).

Our data support two mutually exclusive hypotheses. Namely, cytokinins render plants resistant against *P. syringae* pv. *tabaci* and that this resistance is independent of the sugar status of the plant. Hence, later part of the hypothesis is even more evident regarding to our results of the determination of sugar status of the 4xJERE::IPT material. When analyzed for sucrose, glucose and fructose, no consistent trend was observed for the accumulation of mentioned sugars with elevated and non-elevated cytokinins samples (Fig.14 C). Lack of bacterial resistance in Tet::CIN1 plants and exhibition of resistance phenotype in modulating cytokinins levels on the very same plant is a direct evidence that extracellular invertase is not an essential part of cytokinins mediated resistance in plants. This contradicts the previous studies, where extracellular invertase is considered to be an essential component in cytokinins mediated delay of plant senescence (Lara et al., 2004). However, senescence is a developmental phenomenon and necessarily may not to be considered identical to pathogenesis as both of them are having their own dynamics in plants. Nevertheless, it remains to be elucidated if it matches the concept of sugar-cytokinins antagonism as suggested by Moore et al., (2003) for growth and development.

Second hypothesis regarding our data is that modulated invertase activity has no effect on the spread of hemibiotrophic pathogen *P. syringae* pv. *tabaci* in tobacco. When challenged wild type tobacco plants, significant increase in invertase activity was observed for *P. syringae* pv. *tabaci* compared to that of corresponding control (Fig.20 A). To get functional insights of this increased invertase activity upon pathogenic infection, Tet::CIN1 and Tet::*Nt*CIF plants were investigated. When challenged with *P. syringae* pv. *tabaci*, no visible difference in symptoms were observed for Tet-induced and non-induced sites (Fig. 20 B & C). Similarly, when wild type tomato plants were challenged with *P. syringae* pv. *tomato DC3000*, significantly higher invertase activity was

achieved for pathogen treated samples than corresponding controls (Fig.21 A). However, when tomato transgenic Lin6::*Nt*CIF plants were infiltrated with *P. syringae* pv. *tomato DC 3000* also no difference was observed in symptoms development in comparison to wild type control. Nevertheless, when similar transgenic were inoculated with *Botrytis cinerea*, drastic symptoms appeared on transgenic Lin6::*Nt*CIF plants as compared to the corresponding controls.

Results regarding increase in invertase activity upon pathogenic challenge are in line with those of (Berger et al., 2004). Lack of effects on the growth of invading hemibiotrophic pathogens *P. syringae* pv. *tabaci* in Tet::CIN1 and Tet::*Nt*CIF and that of *P. syringae* pv. *tomato DC3000* in Lin6::*Nt*CIF lines could be due to two reasons. First, as both of these are using leaf apoplast as a niche and that apoplastic fluid is well enriched and better than synthetic growth medium for *P. syrinage,* (Rico and Gail 2008). Alternative carbon sources may efficiently be metabolized in case a particular one is rare or missing completely. Therefore, increase or decrease in glucose may not be a growth limiting factor when alternative carbon source is readily available. Secondly, some of *P. syrinage* strains, including *P. syringae* pv. *tomato DC3000,* contain levansucrase, which converts sucrose into exopolysaccharide levan (Laue et al., 2006). When converted already into exopolysaccharide levan, invertase might not be getting optimal substrate concentration of sucrose to convert it to glucose and fructose. Nevertheless, it needs more focused efforts to dissect the complexity of apoplast and nutrient assimilation pathways which apoplastic fluids induce in invading bacteria. Results regarding enhanced sensitivity to necrotrophic pathogen *Botrytits cinerea* in tomato inhibitor lines (Lin6::*Nt*CIF) are more intriguing (Fig.21). Apart from hindrance in glucose signaling based resistance due to the expression of invertase inhibitor (Roitsch 1999 and Ehness et al., 1997), a proposed hypothesis could be that a low C/N ratio (more N than C) is shutting down the production of C-based defense molecules or compounds such as phytoalexins, which might be contributing to the enhance susceptibility of invertase inhibiting plants.

5.4 Role of cytokinins in plant immunity with reference to secondary metabolites and phytoalexins.

Plant is a terrific depository of over 100,000 small molecule compounds, many, if not most of them have antimicrobial activity (Lewis and Ausubel 2006). To scrutinize elite antimicrobial compounds which impart CKs-mediated resistance against *P. syringae* pv. *tabaci*, we performed

various antimicrobial assays. If cytokinins themselves are embodied with antimicrobial potential, we exclude it by getting identical growth curves of *P. syringae* pv. *tabaci* when grown in cultures with and without added CKs (Fig.11). An extensive screening of various pathogens with disc diffusion method was performed by using extracts of transient 4xJERE::IPT expression regions and those of kinetin fed tobacco leaves. Besides inhibition in the growth of *P. syringae* pv *tabaci*, some degree of inhibition for other strains was also observed, which underpins the *in-planta* effects of cytokinins. Ironically, gram positive bacteria exhibited no inhibition response (Table.1). Whether or not CK mediated resistance is due to antimicrobial peptides, our bioautography results exclude this possibility as well (Fig.12).

To decipher the mechanism of our proposed CK-mediated resistance, we focused on phenylpropanoid pathway which gives rise to a variety of metabolites and most of them participate in plant resistance responses (Nicholson et al., 1992). More specifically, we focused on phytoalexins that are plant defense compounds and are synthesized by the activation of this pathway (Smith et al., 1996). Scopoletin and capsidiol are the predominant forms of phytoalexins present in the *Solanaceae* family of the plants (Bholmann et al., 2002; Stoessl et al. 1976; Al Goy et al. 1993 and Dorey et al. 1997). Exploring the connection between CKs-mediated resistance and the accumulation of said phytoalexins, our gene expression data shown up-regulation for genes (TOGT and C_4H for scopoletin and EAS for capsidiol) encoding enzymes of their biosynthetic during late time points (Fig.18). It is worth mentioning that elevated *IPT* transcripts and accumulated CKs contents synchronized with expression of genes of enzymes for scopoletin and capsidiol. To further strengthen the evidence of accumulation of scopoletin and capsidiol in enhanced cytokinins status, the 4xJERE::IPT expressing plant extracts and kinetin fed samples along with their corresponding controls, were analyzed. As indicated in Fig. (17A&B), it is evident that accumulation of both scopoletin and capsidiol is significantly higher in IPT expressing samples than corresponding controls. Furthermore, exogenous kinetin fed samples also showed significantly higher amounts of scopoletin and capsidiol as compared to the control (Fig.17C), which further augments our findings with 4xJERE::IPT transient expression system. It has previously been described that overproduction of CKs causes the over accumulation of phenolics in general (Schnablová et al., 2006). Nevertheless, our finding with CKs-mediated over-accumulation of phytoalexins such as scopoletin and capsidiol in a pathogenic scenario is an innovative approach.

While conjugating the functional implication of CKs-oriented enrichment of plant tissue with both of these phytoalexins in cytokinins mediated immunity, we believe that there occurs a shift from SA-dependent to SA-independent resistance and that other phenolics are getting an urge of combating the spread of invading pathogen. This goes in concert with findings of Schnablová et al., (2006) for a general increase in phenolics without SA in CKs enhanced status of plant and indirectly supports the notion that enhanced SA accumulation suppresses those of other phenolics (Nugroho et al. 2002). It is quite evident that cytokinins replace SA with other secondary metabolites, such as capsidiol and scopoletin, to bring plant in resistance against the infection of *P. syringae* pv *tabaci*. Besides, enhancement of capsidiol and scopoletin has also been implicated in ROS scavenging and induction of PR1 (Al Goy et al. 1993 and Dorey et al. 1997), which support our data on ROS accumulation and PR gene expression in cytokinins dependent manners. Although, higher accumulation of mentioned phytoalexins coordinately support some of the resistance parameters, but did not provide a direct evidence of their involvement in CKs mediated bacterial growth cessation. Consequently, a dire need of an assay was felt to access the direct antimicrobial potential of the accumulated phytoalexins.

Our results of reconstituted antimicrobial activity based assay Fig.(19), provides categorical evidence to demonstrate the mechanism of cytokinins mediated resistance against *P. syringae* pv. *tabaci.* It is, therefore, substantiated that higher cytokinins status of the plant elicits the accumulation of phytoalexins such as capsidiol and scopoletin and these are the key elements which potentiate even a control extract to cause inhibition in the growth of *P. syringae* pv. *tabaci*. Surprisingly, when added these phtyoalexins on individual bases to control extracts, significantly lower antimicrobial potential has been observed in comparison to 48-IPT-Ps extracts and their combined effects (Fig.19). It further underscores another aspect of combinatorial efficacy of an individual antimicrobial compound and supports the notion that weakly active phytochemicals can be combined synergistically to achieve boosted effects (Lewis and Ausubel 2006). To encapsulate this discussion, we first hypothesized that enhanced cytokinins status of the plant concomitantly enhance the level of resistance against hemibiotrophic pathogen *P. syringae* pv. *tabaci*. We then substantiated a mechanism for this resistance and explored a link between cytokinins and secondary metabolism, whereby elevated cytokinins concurrently elevate the levels of phtyoalexins and these phtyoalexins are the core factors in abrogating the growth of invading pathogen.

5.5 Cytokinins mediated resistance; future out-look

A local increase in the amount of free CKs at the site of *P. syringae* pv. *tabaci* infection in our 4xJERE-IPT transient expression system could potentially change many aspects of plant signaling and physiology and thus jeopardize the virulence of the pathogen. Whether or not T3SS based effectors molecules in *P. syringae* pv. *tabaci* enhance pathogenecity via hormonal imbalance at the host pathogen interface, is still elusive. However, *P. syringae* pv. *tomato DC3000* is capable of using its effector AvrRpt2 as an agent for changing host auxins physiology at the site of infection as a tool for promoting disease in *A. thaliana* (Navaro et al., 2006 and Chin et al., 2008). The significant increase in CKs usually affects the balance with other hormones, particularly auxin. CKs overproduction decrease auxins contents either by decreasing rate of synthesis or transport (Schnablová et al., 2006). On the other hand, auxin signaling also influences cytokinins concentrations via CKX6 and consequently *At*RR (Arabidopsis Response Regulators) activity in *A. thaliana* (Chin et al., 2008). If this "hide and seek" between these two growths promoting hormones can be explained in term of plant immunity as an evolved version of gene-for-gene hypotheses, remains to be elucidated.

Cytokinins mediated immunity may also be characterized in terms of cytokinins signaling and response of type A and type B response regulators, which would further pave way to the exact nature of interaction of cytokinins with secondary metabolites. Cytokinins mediated resistance in SA independent manner may further be elaborated while taking advantage of *NahG or sid* mutants to experimentally substantiate exact nature of the interaction between these hormones and their after effects on plant immunity. Down these lines, experiments with other growth promoting hormones will also be helpful in the overall context of hormonal networking.

To further elaborate the proposed mechanism of cytokinins mediated resistance, mutants and transgenics of the enzymes of mentioned phytoalexins would decipher ahead the implication of these compounds in plant immunity. Moreover, it would also be very interesting to investigate the effect of mentioned phtyoalexins on the gene expression profile of invading bacteria. These genes might be causing repression or induction of vital life sustaining genes of bacterial metabolic pathways or particularly importantly the genes of effectors delivery system. Besides, how bacterial effectors modulate plant cytokinins and generate auxins to spread around in the apoplast should also be addressed.

6. Material and Methods

6.1 Buffers

Mini prep-Resuspension buffer

Glucose	50mM
Tris-HCL pH 8	25mM
EDTA	10mM

Lysis buffer *

NaOH	0.2N
SDS	1%* freshly prepared

Neutralization buffer (For 100ml)

5M KAc	60ml
Glacial AcOH	11.5ml
Water	28.5ml

Agarose gel electrophoresis 5X TBE-buffer

Tris HCl pH 8.0	890mM
H3BO4	890mM
EDTA	20mM

10X DNA loading buffer

Glycerin	50%
EDTA	100mM
Bromophenol blue	0.25%
Xylen Cyano	l0.15%

RNA Isolation RNA-denaturing solution For 50ml

Guanidium – thiocyanat	4M	23.66ml
(Tri)-Na-citrate	25mM	0.37ml
Sarcosyl (N-Lauryl Sarcosine)	(0.5%)	0.25ml

Add 7ul of β-ME freshly before use/ml

CIA Chloroform/Isoamylalcohol 24:1

5X RNA gel running buffer

MOPS	41.86g/L
NaAc	6.8g/L
NaOH	7 pellets/L
EDTA	10ml/L
DEPC	1ml/L

RNA Loading dye

5X RNA-loading buffer	76ul
Formaldehyde	126ul
Formamide	378ul
10X DNA-loading dye	76ul
Ethidium bromide (5mg/ml)	5ul

Formaldehyde gel

Agarose	1.2g
Water (heat to dissolve agarose)	62.2ml
5X RNA running buffer	20ml
37% Formaldehyde	17.8ml

DNA Isolation Extraction buffer

Tris-HCL pH 8	100mM
EDTA	50mM
NaCl	500mM
SDS	1.5%
β-ME freshly before use	1%

6.2 Instruments

Protein purification system: Äkta*p*rime, Amersham Pharmacia Biotech

Gel documentation system: LTF M/WL 312nm, camera: CCD XCST50 Mitsubishi video copy processor P67E

SDS-Minigel-Apparatus: Biometra

PCR-machine: Hybaid: PCR*Sprint* and PCR*Express*

pH-Meter: WTW pH525 with electrode Ingold 40557

Spectrophotometer: Kontron Uvikon 860 with Plotter 800Pharmacia Gene Quant II

Centrifuge: Eppendorf 5415-C and 5417R, Heraeus Megafuge 1.0, Sorvall centrifuge RC-2B

6.3 Chemicals and Enzymes

AppliChem, **Darmstadt:** Dithiothreitol (DTT)

Biomers, **Ulm:** Oligonucleotides

BioRad, Richmond: Ammoniun persulphate, TEMED

Biozym, Hameln: Agarose

Gibco BRL: Ribonuclease Inhibitor

Fermentas, Germany: Protein molecular weight marker

New England Biolabs: Restrictionenzymes

Promega, Madison: Reverse Transcriptase MMLV H(-)

Roth, Karlsruhe: EDTA, Glycerin, Glycin, Chloroform p.a.,Ethanol p.a., Phenol, RNAse, SDS, Tris

Serva, Heidelberg: Acrylamid, Bisacrylamid

Sigma, Deisenhofen: Ammonium-Persulfat, Ampicillin, DEPC, Formamid, HEPES, MOPS

USB, Cleveland: Ethidiumbromide

6.4 Work with pathogens

6.4.1 Cultivation and infiltration with *P. syringae* pv. *tabaci* and *P. syringae* pv *tomato DC3000*

P. syringae pv. tabaci and *P. syringae* pv. *tomato DC300* were grown on King's medium at 28°C. Overnight starter 3ml culture was grown in culture tubes at 28°C. From this a new 50ml culture was grown for 4-5h by adding 1ml from pre-culture at 28°C, 120 rpm, provided with appropriate antibiotic (*P. syringae pv. tabaci* tetracycline 20 mg L^{-1} and *P. syringae* pv. *tomato DC300* 50 mg L^{-1} Rifampicin). Bacterial cells were harvested at a speed of 3500rpm for 10 minutes and resuspended in 10mM $MgCl_2$. Optical density was adjusted at 0.2 at 600nm. However, for infiltration into plant leaves, needle less syringe was used after the bacterial load has been re-adjusted to a particular OD.

6.4.2 Quantification of bacterial growth by plate counting method

To determine bacterial growth in leaves of wild type tobacco plants treated or not with Agro-4xJERE-IPT as decribed above, 3 leaves per plant from 7 plants were infiltrated with 10 µl of *P.syringae* pv. *tabaci-tet* (10^6 cfu/ml). Bacteria were re-isolated by taking 2 leaf discs per leaf with 1 each from IPT and from non-IPT expressing areas using a cork borer (0.4 cm diameter), macerating the discs in 10 mM $MgCI_2$, and plating appropriate dilutions on fresh LB-agar containing 20 mg/l tetracycline. Re-isolation was done for 6, 9, 12, 15, 24, and 48 h after infection. Counting of colony number was repeated thrice for each disc.

6.4.3 Test for antimicrobial activity in differently treated plants

Ca. 500 mg of leaf material from different treatments were lyophilized, powdered and extracted three times with 75% ethanol. After centrifugation at 5000 rpm for 10 min, supernatants were pooled and filtrated. Extracts were tested in vitro using the paper–disk assay (modified from Murray et al. 1995) against a range of micro-organisms. Broth cultures of bacteria and *Candida* were freshly prepared for each assay by overnight incubation at 28 °C in LB broth medium. 1.5 ml of the culture were diluted to OD_{600} = 0.2 and spread on 100 mm petri dishes containing solid LB-agar medium. The suspension was allowed to be absorbed for 10 min. Then petri dishes were

inverted and incubated at 28 °C for 30 min until the bacterial overlay had completely dried. Subsequently, sterile 5 mm Whatman filter paper discs impregnated with the extract to be tested (20 µl /disc) were placed on the agar and gently tapped to ensure contact with the agar surface. Plates were stored at 28 °C for 24 h. After this period, it was possible to assess antimicrobial activity of different extracts qualitatively and quantitatively by the presence or absence of inhibition zones and zone diameter measurement. Each result was the average of two readings on one plate and each experiment was done twice. For control the same solvent was used as for extraction.

Extracts showing significant antimicrobial activity were further tested to determine the Minimal Inhibitory Concentration (MIC) against *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tabaci-tet* as described by the National Committee for Clinical Laboratory Standards (2000). Bacterial samples were grown in liquid LB medium for 6 h. Then, 100 µL of a bacterial suspension (10^6 cfu/ml) were transferred to tubes containing liquid LB medium supplemented with different concentrations (5 - 500 µl) of the extracts to be tested. After 24 h at 37 °C, the MIC of each sample was determined by measuring optical density and comparing the sample readout with the non-inoculated medium. Analyses were repeated thrice.

6.4.4 Antimicrobial activity assay

This assay was done according to a method modified from the that described by Vivanco *et al* (1999) as follows: The fungal and bacterial isolates were grown overnight at 30_oC (for the fungus) or at 37_oC (for the bacterial strains), with shaking, in LB broth medium. All the cultures were diluted to an optical density reading OD_{600}=0.2 and spread on 100mm-plates containing solid LB-agar medium. Sterile Whattman filter paper discs with a diameter of 0.5cm were saturated with different plant extracts and lied on the top of the inoculated plates. The plates were incubated for 24houres at 30_oC for the fungal culture and 37_oC for the bacterial ones, and inhibition was defined as bacteria- or fungus- free zone surrounding the filter discs after inoculation. Two replicates per treatment were included, and each experiment was done twice.

6.4.5 Generation of electro-competent *P. syringae* pv. *tabaci* cells

To generate electro competent cell of *P. syrinage* pv. *tabaci* , 500ml cultre was grown from a pre-culture upto an appropriate OD of 0.5-0.7. Cells were harvested at $4C^0$ at 3500rpm. Cells

were resuspended in 250ml of the 10% ice cold glycerol, again harvested at the above mentioned conditions and again resuspended in 125ml of 10% ice cold glycerol. Reharvested and stored at 80C^{o} as aliquots of 200 µl after being treated with liquid–nitrogen.

6.4.6 Electroporation

Electro-competent cells were first thawed on ice. To 60µl of the competent cells, 1-2µl of the plasmid DNA (appox. 20ng μl^{-1}) was given in cuvette for electroporator, such that air bubbles were completely avoided. After electroporation, 1ml of KB- broth was added to the transformed cells. Cells were then grown for 2 hours at 350 rpm and 28C^{o}. Bacterial cells were harvested at 4C^{o} and spread on LB-plates containing tetracycline as antibiotic. Single tet- growing colonies were re-grown on tet containing medium alongside their wild counterpart.

6.4.7 Inoculation of fungal strains.

S. sclerotiorum was growth on PDA plates at RT for 1 week under darkness condition. For inoculation, an agar blocks (0.4 cm in diameter) containing the advancing edge of growing mycelia was placed in the center of leaf. Infected plants were kept in a clear plastic box under saturating humidity at 22^{o}C under white fluorescent light. Water Suspension of ***Botrytits cinerea*** used to be applied on detached tomato leaves such that after application of spores plates provided with sufficient humidity by giving 2-3ml of distilled water to the wet tissue papers and were raped with parfilum and kept in semi dark condition.

6.5 Work with plant material

6.5.1 Cultivation of plants

Homozygous tobacco transgenic and wild type plants (Tet::IPT, Tet::CIN1, Tet:: Nt CIF, SAG12::IPT, PR1::GUS and control W-38) were grown in soil in the green house at approx. 23-25 C^{o} in long day light conditions. Experiments were performed on healthy leaves of 10-5 weeks post sowing of seed in the soil pots. However for Arabidopsis seed of AtARR5:.GUS and AtDR5:.GUS lines instead of green house growth chamber were used to grow plants. Plants of 6-8 weeks were used for experiments. Moreover, tomato transgenic plants were maintained through cuttings in the green house under the above mentioned conditions.

6.5.2 Transient expression of IPT

For transient transformation with the 4XJERE::IPT construct, *Agrobacterium tumefaciens* strain ABL 4404 was transformed with pCam-4xJERE-IPT (heat shock) and maintained as Agro-4xJERE-IPT for future experiments. Grown overnight as pre-culture in 5 ml of LB- liquid media, Agro-4xJERE-IPT was re-grown in 50 ml of LB-liquid media containing kanamycin (50 mg/l), 10 mM MES (pH 5.5) and acetosyringone (20µM). Cells were grown up to an OD = 0.8. Then, bacteria were harvested and resuspended in $MgCl_2$ (10mM), MES (10mM, pH 5.5) and acetosyringone (200 µM), and kept at room temperature (RT) for 2 h. To cause *ipt* expression in tobacco plants due to pathogen infection, ca. 350 µl of the suspension were infiltrated into three sites on one half of 9-10 wks old *N. tabacum* cv. W38 leaves. For control, non-transformed *Agrobacterium* strain ABL 4404 was infiltrated into the other half of the same leaf. Plants were kept in the green house for 24 hr before application of *P.syringae* pv. *tabaci-tet* to both halves of the leaf. 3 plants with 3 leaves each were used per experiment, and 9 independent experiments were performed. Thus, 81 leaves were studied.

6.5.3 Induction of promoter systems of the transgenic plant lines

Tet::IPT, Tet::CIN1 and Tet::*Nt*CIF

15 wks old Tet::IPT stable transgenic plants (Redig et al. 1996) were treated with 1 mg/l tetracycline (Tet) on one half of a leaf while the other half of the same leaf was mock induced with 10 mM $MgCl_2$. After 24 h *P. syringae* pv. *tabaci-tet* was infiltrated on both induced and mock-induced areas and symptom development was observed 48 h post inoculation. Each experiment comprised 5 plants, with 3 leaves per plant, and was repeated 5 times. Thus, overall 75 leaves were tested.

SAG12::IPT

Petioles of detached leaves of 7 wks old SAG12::IPT stable transgenic plants and *Nicotiana tabacum* cv. W38 wild type plants were placed in water heated to 49 ^{0}C for two minutes to speed up and synchronize the onset of senescence (Lara et al., 2004). They were then kept at conditions described above. *P.syringae* pv. *tabaci-tet* was infiltrated in both wild type and SAG12::IPT transgenic plants (displaying delayed senescence) once initiation of senescence in wild type plants had begun and symptom development was continuously recorded. Here, 10 plants were

used per treatment, and 3 leaves per plant. The experiment was repeated thrice. Consequently, 90 leaves were analysed in total with 45 leaves from SAG12::IPT and 45 leaves from wild type plants.

6.6 Molecular Biological methods

6.6.1 Polymerase Chain Reaction

PCR-Reaction Cocktail

DNA	100 ng
Primer-F (100 pmol μL^{-1})	1 µL
Primer-Rev (100 pmol μL^{-1})	1 µL
dNTP-mix (10 mM)	1 µL
$MgCl_2$ (25 mM)	1-3 µL
Taq (0.5u μL^{-1})	1 µL
10xPCR Buffer	2.5 µL
H_2O	25 µL

PCR running programme

Process	Temperature (^oC)	Time	Number of cycle
Denaturation	94	5 min	1
Denaturation	94	1 min	
Annealing	55-58	1 min	40
Elongation	72	1 min -2 min	
Extension	72	10 min	1

Primer for Isopentenyltransferase from *Agrobacterium tumefaciens* (IPT)
IPT fwd neu 5´-TTAC*CTCGAG*ATGATGGACCTGCATCTA-3´
IPT rev neu 5´-TAA*GAGCTC*CTAATACATTCCGAACGGATG-3´
Annealing temperature: 49 °C
Resitrction sites for: *Xho*I: CTCGAG; *Sac*I: GAGCTC
(Designed by Dr. Thomas Engelke and Nicole Plickert, co workers of the working group of Prof. Roitsch, University of Wuerzburg)

Primer for pathogen related 1a"-Gen (*Nt*PR 1a)
NtPR1 fwd 5´-TAGAGGCGAAAGTCCATACTAATT-3´
NtPR1 rev 5´-TCATCAACTCATATTTTTCCCCTTA-3´

Annealing temperature r: 50 °C
NtPR1-Primers were taken from Jürgen Zeier, Bot-II at JVS University of Wuerzburg . Gensequence: NCBI GenBank accession no. X12737 / Payne *et al.* (1988).

Primers for Phenylalaninammoniumlyase 1 (NtPAL 1)
NtPAL for 5′-CAACATTATACCCATCCC-3′
NtPAL rev 5′-GAAGTAGCACCAAAGCC-3′
Annealing temperature: 57 °C
(Designed by Dominink Grosskinsky, Diploma student of the working group of Prof. Roitsch, University of Wuerzburg)
Gensequence: NCBI GenBank accession no. D17467 / Nagai *et al.* (1994).

Degenerate primers for Phenylpropanoid-Glucosyltransferase 1 und 2 (TOGT 1 / 2)
NtTOGT/IS for 5′-CTTCTTTCCTGTGATGGC-3′
NtTOGT/IS rev 5′-ATAG(C/T)(C/T)GTCTCTTCCCC-3′
(Designed by Dominink Grosskinsky, Diploma student of the working group of Prof. Roitsch, University of Wuerzburg)
Annealing temperature: 57 °C
Gensequence: NCBI GenBank accession nos. AF346431 (TOGT 1) und AF346432 (TOGT 2) / Fraissinet *et al.* (1998).

Primers for Zimtsäure-4-Hydroxylase (*Nt*C4H)
NtC4H for 5′-GCAGCAATATAGAGGGGGG-3′
NtC4H rev2 5′-ATGTGAGGGTGGTTGACTAACTCGG-3′
Annealing temperature: 52 °C
(Designed by Dominink Grosskinsky, Diploma student of the working group of Prof. Roitsch, University of Wuerzburg)
Gensequence: NCBI GenBank accession nos. AJ937847 / Szatmari *et al.* (2006) und DQ350353 / Xu *et al.* (2006
Primers for 5-epi-Aristolochen-Synthase (NtEAS)
NtEAS for 5′-GAACGCCTTGGTATATCC-3′
NtEAS rev 5′-TCGATCTCTAGCATATGG-3′
Annealingtemperatur: 57 °C
Gensequennce: Facchini und Chappel (1992).
(Designed by Dominink Grosskinsky, Diploma student of the working group of Prof. Roitsch, University of Wuerzburg)

IPT fwd neu 5′-TTACCTCGAGATGATGGACCTGCATCTA-3′
IPT rev neu 5′- TAAGAGCTCCTAATACATTCCGAACGGATG-3′

(Designed by Dr. Thomas Engelke and Nicole Plickert, co workers of the working group of Prof. Roitsch, University of Wuerzburg)

6.6.2 DNA-Agarose gel electrophoresis

DNA was electrophorsed in 0.8% to 1.5% agarose gel made in TBE buffer with 3.5ug of ethidium bromide in 100ml total volume. The DNA sample was mixed with appropriate amount of loading buffer and subjected to electrophoresis performed in 1X TBE buffer at a voltage of 5 to 10 V/cm. As a DNA molecular weight marker either λDNA restricted with *Hind*III and *Eco*RI or PUC 19 restricted with HinfI was used.

6.6.3 Competent *E. coli* cells

500µl of overnight grown preculture of *E. coli* strain in LB (5ml) were used to inoculate 50ml LB medium. The cells were incubated until the absorbance at OD_{600} reached between 0.5-0.6 units. Cells were harvested at 2500rpm for 10min at 4C and then the pellets were resuspended in 15ml of cold $CaCl_2$ (100mM) and were incubated on ice for 40min. The cells were then harvested at 2500rpm for 5min at 4°C. The pellets were resuspended in 5ml of $CaCl_2$ (100mM) and incubated on ice for 1-3 hours. 1ml of ice cold glycerol (87%) was added to cells and after mixing 200ul of cells were added to ice-cold tube and tubes were snap frozen in liquid N_2. The competent cells are stored at -80Cº

6.6.4 Extraction of genomic DNA

100 mg of homogenized plant material were mixed with 1ml of extraction buffer with vortex till all the material was suspended in the solution. The suspension then is incubated at 65 °C for 10min, then 2ul of KAc (5M) is added to the mixture. Then tubes on ice for 10-60min. After centrifugation at 14000rpm for 10min at 4 °C the liquid phase in every tube is mixed with phenol, Chloroform and Isoamylalchol (400ul:400ul:7ul per sample) with mixing followed by centrifugation for 5min at room temperature. Upper phase is mixed with 500ul isopropanol and incubated at -20 °C for overnight. DNA is precipitated by centrifugation for 10min at 14000rpm at 4 °C. Pellets are washed with 70% ethanol and dried at room temperature. DNA is then suspended in 50ul Tris buffer containing RNase (100:1 v/v) and incubated at 37 °C for 1 hour to obtain RNA-free DNA.

6.6.5 Reverse Transcription-PCR

Reverse transcription was carried out using a kit for first strand cDNA synthesis from Fermentas (RevertAid™) on total RNA. 2ug RNA was used for reverse transcription. To the RNA, 2ul of antisense primer (1:10 diluted) was added and volume was adjusted up to 12ul with ddH2O. The mixture was incubated at 70 oC for 5min and immediately transferred on ice. To this, 4ul of 5x

1st strand buffer, 2ul of 10mM dNTPs and 1ul of Ribonuclease inhibitor was added and incubated after mixing for 5min at 37oC. The reverse transcription reaction was then carried out at 42oC for 60min after adding 1ul of reverse transcriptase (200U/ul). Then the reaction was incubated at 70 oC for 10min then immediately on ice to stop the reaction. cDNA formed was used for regular PCR using 5ul of the product.

6.6.6 Elution and purification of DNA fragments

The DNA band from 1% agarose gel was cut out under UV light and DNA was eluted and purified using a NucleoSpin Extraction Kit (Clontech). 500ul of NT buffer was added to the cut piece and agarose was melted at 65oC for 10min. Solution was added to purification column and column was centrifuged at 11000g for 1min, and the eluted solution was discarded. 600ul of NT3 buffer was added to the column to wash the membrane followed by centrifugation of the column at 11000g for 1min. The membrane was dried by centrifugation of empty column for 2min at 11000g. For elution of the purified DNA from the column, 20-25µl of NE buffer was added to the membrane and after incubation for 1min at room temperature, the column was centrifuged for 1min at 11000g, the eluted buffer contains the purified DNA. 5µl of purified DNA was loaded on agarose gel for estimating DNA amount.

6.6.7 Ligation

Ligation was carried out in pGEM-T Easy Vector using pGEM-T Easy Vector kit from Promega. Ligation mixture consisted of 1ul (50ng) of vector DNA, 1ul of purified DNA, 5ul of 2x ligation buffer, 1ul of T4 ligase and 2ul of water, in total volume of 10ul. Ligation reaction was run at 21^{o}C for 2-3houres or overnight at 14^{o}C.

6.6.8 Transformation of *E. coli* cells

The competent cells were thawed on ice; one vial (200 ul) for each transformation was taken. 4ul of ligation mixture were added and cells incubated on ice for 15-20 min. The cells were given heat shock at 24^{o}C for 90min then were chilled on ice for another 90 min. 800ul of LB medium was added to cells and incubated them at 37^{o}C for 1hour. After centrifugation for 3min at 12000rpm, 870ul of cell suspension were plated on LB medium with specific selection marker and incubated at 37^{o}C for overnight.

6.6.9 Mini Prep and restriction analysis

The bacteria were grown in 2-3ml of LB medium with antibiotic (Amp. 100ug/ml) overnight at 37^{o}C. *E. coli* cells were harvested by centrifugation at 12000*g* for 3 min. The residual medium was removed and cells were resuspended in 100ul of ice-cold resuspension buffer by vortexing. 200ul of lysis buffer was added on the resuspended cells and incubated on ice for 3 min. 150ul of neutralizing buffer was added and again incubated on ice for 3 min. Following this the cells were centrifuged at 12000*g* for 5 min at 4^{o}C. The supernatant was transferred into a new tube and an equal volume of Phenol: Chloroform was added followed by vortexing. After centrifugation at 12000g for 8 min at 4^{o}C, the supernatant was transferred into a fresh tube and double-stranded DNA was precipitated with 2 volumes (800ul) of ice cold ethanol (100%). Tubes were kept at -20^{o}C for 10-15 min, then centrifugation at 12000g for 10 min at 4^{o}C. Supernatant was discarded and pellets were washed with 70% ethanol (500ul) and after vortex and centrifugation, ethanol was removed and pellets were dried at 37^{o}C and resuspended in 25ul sterile water. For restriction analysis, 4ul of miniprep DNA was restricted in an end volume of 18ul using 2ul of appropriate buffer, 1ul RNase and 1ul of enzyme (10U). The reaction was incubated at 37^{o}C for 3houres or overnight. The restriction digest was then analyzed by agarose gel electrophoresis.

6.6.10 Transformation of Agrobacterium pCambi1380-4xJERE-IPT

Take fresh *Agrobacterium tumefaciens* strain ABL 4404 from plate in 3 ml LB medium with streptomycin (100µg) and keep overnight at 28^{0} C. Take 1 ml of this overnight culture in 100ml in YEB medium with streptomycin (100µg) and keep for 3-4 hours at 29^{0}C . Centrifuge at 5000 rpm for 20 Minutes at 4 ^{0}C. Wasch with 5ml cold TE buffer (Tris-Cl &EDTA PH 8). Resuspend in 10ml (Tris-Cl &EDTA PH 8). Resuspend in 10ml YEB medium with 2ml glycrin. Make aliquots of 500µl in Eppendorf Freeze in liquid nitrogen & store at -80^{0}C.

Agrobacterium tumefaciens strain ABL 4404 competent cells were thawed on ice, 1-2 µl of pCambi1380-4Xjere-IPT plasmid was added and kept at ice for 5 min, kept on 37° C for some time and then added 1ml of YEB broth. Kept on feeding for 2-3 hours and cells were recollected and spreaded on LB plates, grown overnight at28°C.

6.6.11 Northern Blot Analysis

Total RNA (15 µg) was electrophoresed on a 1% formaldehyde agarose gel, transferred to a positively charged nylon membrane, and fixed by baking at 80°C for 2 h. The probe was prepared using DNA labeling kit (Fermentas, St. Leon-Roth). 150 ng of DNA was incubated with 10 µL of hexanucleotide buffer at 95°C. 10 min after incubation, sample was put on ice for 2 min, and then incubated with 3 µl Mix A, 5 µL α^{32}P-ATP, and 1 µl Klenow enzyme at 37 °C for 10 min. After incubation, 4 µL dNTP mix was added into probe sample, and incubated at 37 °C for 10 min. The labelled probe was purified by microspin column, and denatured by incubation at 95 °C for 5 min. The membrane was incubated in hybridization buffer at 42 °C with shaking. 2 hours after incubation, the denatured probe was added to hybridization buffer, and incubated again for overnight at 42 °C with shaking. The non-binding probe was removed by washing with wash buffer I and II.

6.7 Biochemical Methods

6.7.1 Invertase activity

To assay invertase activity in *Arabidopsis* leaves 0.5 g plant material was homogenized in liquid nitrogen in a mortar-pestle and resuspended in 1 ml homogenization buffer (200 mM Hepes, 3 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 0.1 mM PMSF, 1 mM benzamidine). The homogenate was mixed for 20 min at 4°C and centrifuged for 15 min at 10500 *g* and 4°C. The supernatant was removed and used for soluble enzyme preparation. The pellet (cell-wall fraction) was washed three times with distilled water and resuspended in 200 mM Hepes, 3 mM $MgCl_2$, 15 mM EDTA, 2% glycerol, 0.1 mM PMSF, 1 mM benzamidine, 1 M NaCl. Both enzyme preparations were dialysed against 12.5 mM potassium phosphate buffer, pH 7.4 for 2 to 10 h at 4°C. Invertase activity at pH 4.5 and 6.8 was analysed according to the method of Sung et al. (1989) except that samples (with or without 10mM acarbose) were incubated for 30 min at 26°C and the concentration of sucrose was 1 mM. The amount of liberated glucose was determined by addition of fivefold excess of GOD reagent (0.1 M potassium phosphate buffer, pH 7, 0.8 U ml^{-1} horseradish peroxidase, 10 U ml^{-1} glucose oxidase from *Aspergillus niger*, 0.8 mg ml^{-1} ABTS) and measurement of the absorbance at 595 nm.

High salt buffer

200 mM Hepes, 3 mM $MgCl_2$, 15 mM EDTA, 2% Glycerol, 0,1 mM PMSF,
1 mM Benzamidin, 1 M NaCl
Homogenization buffer
200 mM Hepes, 3 mM $MgCl_2$, 1 mM EDTA, 2% Glycerol, 0,1 mM PMSF, 1 mM Benzamidin
GOD-Reagent
0,1 M KPO4-Puffer (pH 7,0), 0,8 U/ml Meerrettich Peroxidase,
10 U/ml Glucoseoxidase aus *Aspergillus niger*, 0,8 mg/ml ABTS

6.7.2 Test for presence of antimicrobial proteins in plant extracts

Proteins were extracted and precipitated using ammonium sulfate as described by Cammue et al. (1995). Antimicrobial potential of the protein fraction was tested using bioautography (Vigers et al. 1991). 10 g of plant material were extracted with Na-phosphate buffer (10 mM Na_2HPO_4), 15 mM NaH_2PO, pH=7) containing 2 mM PMSF and 1 mM benzamidine hydrochloride overnight at 4°C while shaking. After low speed centrifugation, the supernatant was treated with ammonium sulfate (85 % saturation;. Ammonium sulfate was added gradually under continuous stirring and the solution finally stored over night at 4°C. The precipitate was collected by centrifugation at 10,000 rpm, resuspended in distilled water, and dialyzed extensively against distilled water. The dialyzed fraction was used for testing antimicrobial activity on SDS-PAGE using bioautography. SDS-PAGE was performed following the method of Laemmli (1970) using 12% polacrylamide gel with a 5% stacking gel. Samples were boiled for 5 min in sample buffer (15 % (w/v) sucrose, 2.5 % (w/v) SDS, 125 mM Tris, pH=6.8). Electrophoresis was run for 90 min at RT at 35 mA. The resulting gel was incubated in 250 ml water containing 1 % (v/v) Triton X-100 for 20 min on a shaker at RT. After brief washing in water, the gel was incubated in 100 ml of 20 % LB broth medium and gently shaken for 30 min at RT. The gel was then placed in a 100 mm petri dish. On top of the gel 20 ml of warm LB medium (20 %) containing agar (1.5 % (w/v)) and 100 µl of bacterial suspension (*P. syringae* pv. *tabaci*, 4-6 x 10^5 cfu/ml) were loaded, allowed to solidify and incubated at 30 °C overnight.

6.8 Analytical methods

6.8.1 Determination of SA and JA

SA and SAG were determined as described in Zeier et al. (2004). Frozen leave tissue (0.25 g) was homogenized in 1 ml of 90% methanol and extracted for 10 min at 40°C. The mixture was

centrifuged for 5 min at 14 000 g, and the pellet was extracted for another 10 min at 40°C with 100% methanol. Supernatants from both extractions were combined and dried under a gentle stream of N_2 at 40°C. The residue was resuspended in 1.5 ml of 0.1 M HCl and 100 ng p-hydroxybenzoic acid (HB) was added as an internal standard. After centrifugation for 10 min at 14 000 g, the aqueous solution was extracted with 3 × 2 ml of cyclopentane/ ethylacetate (1 : 1). The extracts were combined and the solvent removed under N_2 at 40°C. The residue was dissolved in 200 µl $CHCl_3$ and derivatized for gas chromatography by addition of 20 µl pyridin und BSTFA (N,N-bis-trimethylsilyltrifluoroacetamide, Macherey-Nagel, Düren, Germany). For detection of SAG, the aqueous, acidic phase from the first extraction step was heated to 100°C for 30 min to convert the SAG to free SA. Again, 100 ng of HB was added for standardization, and the above protocol was repeated starting with solvent extraction with cyclopentane/ethylacetate. Sample separation was carried out on a gas chromatograph (GC 8000 Top series, CE Instruments, Milan, Italy) equipped with a fused silica capillary column having an internal diameter of 0.32 mm and a film thickness of 0.1 mm (DB-1; Fisons, Folsom, CA). In combination with an MD1000 mass spectrometric detector (CE Instruments), quantitative determination of SA was realized using electron impact ionization (70 eV) by integration of the m/z 267 ions of SA and the internal standard, respectively, and by considering an experimentally determined correction factor using standard solutions of SA and HB.

6.8.2 Determination of free soluble sugars

The levels of glucose, fructose and sucrose in plant tissues were determined for experiments based on the 4xJERE::IPT construct (for time points)Sugar analysis was done according to Bonfig et al. (2006) with the following modifications: Heating steps (105 °C) were reduced to 3 minutes and centrifugations took place at 13,000 rpm. In detail, 100 mg of ground material was mixed with 900 µl water. After centrifugation the supernatant was incubated at 105 °C for 3 minutes. Following another centrifugation step the sugar containing supernatant was diluted 1:10 and 100 mg per ml mixed-bed ion exchanger (Serdolit MB-1; Serva, Heidelberg, Germany) added. After removal of resin the solution was analyzed by High Performance Anionic Exchange Chromatography - Pulsed Amperometric Detection on a Dionex 4500i (Dionex, Idstein, Germany). To study if starch reservoirs were remobilized, the remaining pellet of ground material was washed with water and treated with 1.5 U/ml amyloglucosidase solution from *Aspergillus niger* (Fluka, Buchs, Switzerland) for 2 hours at 37 °C. After centrifugation the

supernatant was incubated for 2 minutes at 105°C and analyzed by HPLC. As external standard a Serdolit MB-1 treated mixture of 0.05 mM mannitol, arabinose, glucose, fructose, sucrose and maltose was used. Results are based on 5 replicates for experiments with the 4XJERE::IPT construct, and on 3 replicates for exogenous kinetin supply with each analysis done twice.

6.8.3 Determination of Capsidiol and Scopoletin

Leaves were frozen and ground in liquid nitrogen. Ground material was mixed three times with 75% ethanol (1:4) for 1 h and centrifuged at 5000 rpm for 10 min. Supernatants from all the three extractions were combined and dried using a SpeedVac concentrator. After drying, the residue was dissolved in 1 ml methanol for further analysis. The amount of scopoletin was determined using HPLC on a RP- 18 e Purospher star column (particle size, 5 µm, 4 x 250 mm; Waters using a gradient of CH_3CN at a flow rate of 1 ml/min. The gradient was 20 – 60 % for 35 min. Scopoletin (retention time: 7 min) was detected by fluorescence (excitation, 350 nm; emission, 430 nm). The analysis of capsidiol was done using GC-MS

6.8.4 Cytokinins Determination

Previously ground samples of tobacco leaves (500mg FW) was placed individually in 1000 µl of Bieleski buffer (60% methanol, 25% CHCl3, 10% HCOOH and 5% H2O) and re-extracted in. Stable isotope-labeled CK internal standards were added to each of the samples (1 pmol of each compound per sample) to check the recovery during purification and to validate the quantification. The tube contents were ultrasonicated for 3 min and then stirred for 30 min at 4 . After centrifugation (8 min, 15,000 rpm, ºC) the supernatants were transferred to glass tubes and stored at 4 ºC. The pellets were extracted in the same way for 30 min at 4 ºC and after centrifugation (8 min, 15,000 rpm, ºC) both supernatants were combined and immediately purified. Samples were dissolved in 20 µl of mobile phase (initial conditions), and 10 µl of each sample was injected onto a reversed phase column (BEH C18, 2.1×50 mm, 1.7µm; Waters). The samples were eluted with an 8-min linear gradient of 90:10 A:B to 50:50 A:B (v/v) with 15 mM ammonium formate (pH4.0). At the end of the gradient the column was washed with 100% B (1 min) and equilibrated to initial conditions for 3 min. Under these conditions, retention times for the monitored compounds ranged from 2.5 to 7.5 min. The effluent was passed through an ultraviolet-diode array detector (scanning range 210–400 nm, resolution 1.2 nm, sampling rate 10 spectras_1) and the tandem mass spectrometer without post-column splitting. Analytes were quantified by multiple reaction monitoring of [M+H]+ and the appropriate product ion.

Conditions for MRM were as follows: capillary voltage, 0.6 kV; source/desolvation gas temperature, 100/350ºC; cone/ desolvation gas flow rates, 2.0/550 l h^{-1}; LM/HM resolution, 12.5; ion energy 1, 0.3 V; ion energy 2, 1.5 V; entrance, exit and multiplier voltages, 2.0 V, 2.0 V and 650 eV, respectively.

6.9 Histochemical Methods

6.9.1 ROS- detection

Dissolve DAB reagent in water @ 1mg/ml and adjust PH at 3.8. Submerge samples in DAB solution. Put on shaker for 8h with gentle shaking. Remove DAB reagent and extract chlorophyll for clear. Localization of H2O2 as dark brown coloured stain. Leaves were detached from seedlings. Vacuum-infiltrated with 10 mM NaN3 in 10 mM potassium phosphate buffer, PH 7.8, for 1 min, and incubated in 0.1% Nitroblue tetrazolium (NBT) (in 10 mM potassium phosphate buffer, pH 7.8) for 30 min at room temperature.

6.9.2 *In situ* Invertase Activity Assay

In order to analyze the expression of lin6::InvInh gene in transgenic Tomato plants, an In-situ invertase assay was established. Leaf sections of the transgenic tomato plants were sliced such that a rectangular area of 60cm^2 was obtained. Sliced sections were wounded with a scalpel from lower side of the leaf. Sections from the same plant were pooled in the same well of 6-welled Micro Titer Plate. Besides wounding as a source of stimulus for the induction of Lin6 promoter, Methyl Jasmonate and Sucrose were also added to 5ml MS 0221 medium in separate plates. Microtiter plate was adjusted on a shaker for overnight shaking at 4ºC.

Invertase activity was assayed same as determined by McLaughlin and Boyer (2004). To remove exogenous sugar and other soluble molecules from the overnight incubated samples, these were washed three times in phosphate buffer with constant stirring. Staining of the samples was accomplished by exposing washed sections to a reaction medium containing phenazine methosulphate (PMS), nitro blue tetrazolium (NBT) and glucose oxidase (GOD) in Sodium Phosphate buffer. Samples were incubated in the dark and observations were recorded for 1st half hour and then for overnight.

6.9.3 Gus staining

GUS-reporter lines which are transformed with *uidA* gene, encodes β-glucuronidase, and gives colour reaction upon addition of the substrate X-gluc. Intensity of the colour development depicts the extent of gene expression in a promoter-reporter combination. To activate promoter, plants are treated with stimuli of interest and then desired plant material is overnight incubated in gus-solution at 37 °C in dark. In order to remove chlorophyll and preserve the tissue, it is repeatedly washed with 70% Et-OH until the complete removal of pigment. Leaves are then appropriately photographed and presented.

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Muhammad Naseem

Curriculum Vitae

Name: Muhammad Naseem

Nationality: Pakistani

Date of Birth: 15-06-1977

Marital Status: Married.

Education

October 2005-To date ***PhD (Biology)***

Research Topic: **Role of cytokinins in plant immunity**

Department of Pharmaceutical Biology
University of Wuerzburg, Germany

2002-2004 ***M.Phil. (Molecular Biology)***

.***Research Topic*****: Expression of *At*DREB1A gene in Indica rice super-basmati**

National Centre of Excellence in Molecular Biology,
University of the Punjab Lahore, Pakistan.

1998-2002 ***B. Sc (Hons) Plant Breeding & Genetics***

Research Topic: **Compilation of literature on the use of Antisense-Technology for crop improvement**

Department of Plant Breeding and Genetics NWFP
Agricultural University Peshawar, Pakistan

Working Experience:

1. Worked as ***Research Officer*** (29-12-2003 to 10-11-2004), at National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore-Pakistan

2. Worked as ***Lecturer*** (11-11-2004 to 27-2-2005) at the Department of Biotechnology and Informatics in Baluchistan University of Information Technology and Management Sciences, Quetta-Pakistan

Ehrenwörtliche Erklärung

gemäß § 4 Abs. 3 Ziff 3, 5 und 8
der Promotionsordnung der Fakultät für Biologie
der Bayerischen Julius-Maximilians-Universität Würzburg

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.
Diese Arbeit hat weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen.
Ich habe bisher noch keinen akademischen Grad erworben Oder zu erwerben versucht.

Würzburg, den.....

……
Muhammad Naseem