Effects of overexpression of *NIMIN* genes on Salicylic acid-mediated *PR-1* gene activation and phenotype in *Nicotiana benthamiana* (Domin)

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I can do all things through Christ who gives me strength.

Philippians 4:3

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Abbreviations

A	Ampere
aa	amino acid
acd	*accelerated cell death*
AD	activation domain
appr.	approximately
A. tumefaciens	*Agrobacterium tumefaciens*
amp^r	ampicillin resistance
as-1	*activation sequence-1*
ASF-1	*as-1*-binding factors
AUX/IAA	auxin/indole-3-acetic acid
Avr	avirulence
BD	binding domain
BTB/POZ domain	broad-complex, tramtrack, and bric-a-brac/poxvirus, zinc finger domain
BOP	blade-on-petiole
bp	base pairs
BTH	benzothiadiazole S-methyl ester
bZIP	basic leucine zipper
°C	degrees Celsius
CaMV	cauliflower mosaic virus
CIAP	calf intestine alkaline phosphatase
cim	constitutive immunity
C-terminal	carboxyterminus
COR	coronatine
cpr	constitutive expressor of *PR* genes
cys	cysteine
Da	Dalton
DAB	3,3-diaminobenzidin
dT	deoxythymidine nucleotides
DMSO	dimethyl sulfoxide
DTT	1,4-dithiothreitol
dNTP	2'-deoxynucleotide-5'-triphosphate (dATP, dCTP, dGTP, dTTP)
ddTTP	2',3'-dideoxythymidine-5'-triphosphate
dpi	days post inoculation
dsDNA	double stranded DNA
EAR	ERF-associated amphiphilic repression
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ERF	ethylene responsive element binding factor
et *al.*	*et alia* (and others)
EtBr	ethidiumbromide
ET	ethylene
etc.	et cetera
e.g.	*exempli gratia*
ETI	effector-triggered immunity
EtOH	ethanol
ECL	enhanced chemiluminescence
EF-Tu	elongation factor Tu

Fig.	figure
FLS2	flagellin sensing2
g	gram
GFP	green fluorescent protein
GER	Germany
gent	gentamycin
GRX	glutaredoxins
GSNO	S-nitrosoglutathione
GUS	ß-glucuronidase
HA epitope	human influenza hemagglutinin epitope
HAc	glacial acetic acid
hr(s)	hour(s)
HR	hypersensitive response
HRP	horse radish peroxidase
IgG	Immunoglobulin G
i.e.	*id est* (that is)
INA	2,6 dichloro-isonicotinic acid
IPTG	isopropyl β-D -1- thiogalactopyranoside
JA	jasmonic acid
kbp	kilo base pairs
KOAc	potassium acetate
kDa	kilo Dalton
Lab. St.	laboratory stock
lacZ	β-galactosidase gene
l	liter
LB	Luria Bertani medium
lsd	lesions simulating disease
LS7	linker scan7
M	molar
MAMPs	microbial associated molecular patterns
MCS	multiple cloning site
mg	milligram
min(s)	minute(s)
sec	second
ml	milliliter
mM	mili molar
mol	mol
4-MUG	4-methylumbelliferyl-β-D-glucuronide
MeSA	methylsalicylic acid
MU	4-methylumbelliferone
µg	microgram
ng	nanogram
µm	micrometer
nm	nanometer
N. benthamiana	*Nicotiana benthamiana*
N. tabacum	*Nicotiana tabacum*
NaOAc	sodium acetate
Na-MOPS	sodium 3-(N-morpholino) propanesulfonic acid
NB-LRR	nucleotide-binding/leucine-rich repeat
NBS	nucleotide binding site

NEB	New England Biolabs
NH	NPR1 homologue
nim1	non inducible immunity
NIMIN	*nim1*-interacting
NLS	nuclear localization signal
npr1	non-expressor of pathogenesis related gene 1
NRR	negative regulator of resistance
N-terminal	aminoterminus
OD_x	optical density at x nm
ori	origin
ONPG	o-nitrophenyl-beta-D-galactopyranoside
o/n	overnight
Os	*Oryza sativa*
P. syringae	*Pseudomonas syringae*
PAMPs	pathogen-associated molecular patterns
PAGE	polyacrylamide gel electrophoresis
PCD	programmed cell death
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PR	pathogenesis related
PRRs	pattern recognition receptors
PTI	PAMPs-triggered immunity
pv.	pathovar
R genes	resistance genes
ROS	reactive oxygen species
XX_{pro}	$XX_{promoter}$
XX_{ter}	$XX_{terminator}$
pH	decimal logarithm of the reciprocal of the hydrogen ion activity in a solution
rif	rifampicine
rpm	revolution/round per minute
SA	salicylic acid
sai1	salicylic acid insensitive
SAR	systemic acquired resistance
S. cerevisiae	*Saccharomyces cerevisiae*
SD	synthetic dropout medium
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sec.	section
Ser	serine
SNI	*suppressor of NPR1, inducible*
ssi	*suppressor of SA insensitivity*
TAE	Tris-acetate-EDTA buffer
Taq	*Thermophilus aquaticus* DNA polymerase
TB	Tris-borate buffer
TBS	tris buffered saline
T-DNA	tumor-DNA
Ti-plasmid	tumor inducing-plasmid
TTBS	Tris-buffered saline with Tween-20
TGES	transient gene expression system

TE	Tris- EDTA buffer
TEMED	tetramethylethylenediamine
TIR	Toll-Interleukin receptor
TMV	tobacco mosaic virus
TPM	tri-parental mating
Tris	2-amino-2- (hydroxymethyl) propane -1,3 - diol
TTBS	Tris-buffered saline with Tween 20
TTSS	type III secretion system
TDT	deoxynucleotidyl transferase
TRXs	thioredoxins
US	United States (of America)
UV	ultraviolet
V	Volt
vir	*virulent*
wt	wild type
X -Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
XX®	XX^{registered}
YPAD	yeast extract- peptone- adenine -dextrose medium
Y1H	yeast one hybrid
Ø	diameter
w/v	weight per volume
v/v	volume per volume
λ	wavelength
∞	infinity

1 Introduction

1.1 Plants and their surrounding

During their lifetime, plants encounter many adverse conditions that have significant effects on their physiology and development. In the agricultural sector, huge losses are recorded every year because of biotic and abiotic stress factors. The abiotic factors that affect plant development and growth include topography, soil and climatic factors. Abiotic stresses like drought, salinity and extreme temperature conditions have negative effects on crop growth and productivity (Boyer, 1982). For example, under dehydrating conditions plant physiological processes are inhibited, thus plants have to initiate adaptive mechanisms (Lauan, 2002; Kwak et al., 2008). Conversely, biotic factors are living, both macro and micro-organisms. Macro-organisms include birds, insects, molluscs, arachnids, plants and mammals including humans. Micro-organisms on the other hand refer to viruses, fungi, bacteria and nematodes. These are the components of the environment which influence the manifestation of genetic factors on growth and development. The effects of these biotic factors on plant gene expression could be advantageous or disadvantageous, depending on the type of relationship they develop with plants. These interactions include mutualism, herbivory, parasitism and allelopathy.

Parasitism is an interaction between two organisms in which one organism, called a parasite, is benefited by causing harm to another, called a host. The parasite obtains its food from the host. Micro-organisms such as fungi, bacteria and viruses that injure crops by causing diseases are called pathogens. Plants are rich sources of nutrient and shelter, therefore they are attacked by different pathogens. The plant disease emergence is an important concern throughout the world (Anderson et al., 2004; Harvel et al., 2002). Pathogens use three main strategies of attack upon invasion: necrotrophy, biotrophy and hemibiotrophy (Hammond-kosack and Jones, 1997). Necrotrophic pathogens are characterized by killing their host plant, before they start feeding on them. They have a broad host range, while biotrophic pathogens have a narrow host range. Biotrophs invade and exploit the metabolism without killing the plant. Hemibiotrophic pathogens act somewhat in similar fashion as biotrophic pathogens, but finally invasion leads to host cell death.

1.2 Plant-pathogen interactions

Pathogen conversation with a plant starts after developing a physical contact with the plant However, pathogens get to the plants using diverse strategies. Plant pathogenic bacteria proliferate in intercellular spaces after entering the tissues through wounds, injuries, vectors or natural openings, like hydathodes and stomata. Symbiotic and pathogenic fungi, on the other hand invade tissues by extending hyphae into the intercellular spaces which in close contact to the plant plasma membrane develop special feeding structures called ‘‘haustoria’’. Nematodes use their stylet to gain access to plant cells for their nutritional requirements. Viruses get into the plant cells mainly through wounds, injuries and by the help of carriers called vectors which could be another pathogen. Successful penetration and establishment of a pathogen inside the plant without being detected means that the plant is not able to recognize the pathogen. Alternatively, it would mean that the plant defense is ineffective or the pathogen is able to suppress the host defense (Hammond-Kosack and Jones, 1996). If a pathogen’s invasion is successful and it causes disease, the pathogen is virulent; the host is susceptible and the interaction is compatible (Glazebrook et al., 1997a). On the contrary, if the host elicits a defense response to suppress colonization by the pathogen, then the pathogen is called avirulent; the host is resistant and the plant-pathogen interaction is said to be incompatible.

The incompatible interaction between pathogens and plants triggers defense responses at the site of infection. These include production of reactive oxygen species (ROS), programmed cell death (PCD) and synthesis of antimicrobial compounds, like phytolaxins and pathogenesis-related (PR) proteins (Abel and Hirth, 2004; Lamb and Dixon, 1997). In addition, cell wall fortification, activation of protein kinases, ion fluxes and accumulation of signaling molecules like salicylic acid (SA), nitric oxide, jasmonic acid (JA) and ethylene (ET) have also been observed in case of incompatible plant-pathogen interactions (Cohn et al., 2001; Hammond-Kosack and Jones, 1996).

1.3 Plant resistance

Although in nature plant species are exposed to a number of potential pathogens, they are susceptible to only a few. Mostly, plants are resistant to pathogens. Plants are well equipped to sense the presence of a pathogen on their surface by perceiving chemical and physical

signals of the pathogen, and react quickly to potential infection (Hardham et al., 2010; Hardham et al., 2007). A type of resistance, in which a certain plant species shows resistance to the majority of potential plant pathogens, is known as non-host resistance (Heath, 2000). Non-host resistance is of great interest in agriculture, as it is durable and the most common form of plant disease resistance (Ellis, 2006). This protective mechanism against pathogen invasion in a nonspecific manner is the constitutive armory, and a powerful weapon of the plants (Ryals et al., 1996).

Mammals have mobile defender cells and a somatic adaptive immune system, whereas plants lack this specialized system. However, plants have developed different strategies to combat potential pathogens. Plant passive defense mechanisms such as physical and chemical barriers are the first line of protection from pathogen infection. In order to access nutrients from the plant, pathogens must first overcome physical barriers, like the cell wall and the plant epidermis which is covered by a waxy cuticle. However, in order to break through these barriers, pathogens produce virulence factors which include cuticle and cell wall degrading enzymes. In nonhost resistance, two major components are involved (Mysore and Ryu, 2004; Ellis, 2006). The first one is preformed defense, in which pathogens are not able to overcome obstacles like the cytoskeleton, antimicrobial compounds and secondary metabolites pre-existing in plants. Another mechanism in which plants defend themselves is innate immunity (Hofius et al., 2007).

1.4 Innate Immunity

As plants lack a specialized immune system, they rely on innate immunity of individual cells and on systemic signals being emitted from the site of infection (Dangl et al., 2001; Ausubel, 2005; Chisholm et al., 2006). If a pathogen is able to cause infection, then as a counter-attack, plants have evolved two lines of defense: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI; Jones and Dangl, 2006; Chisholm et al., 2006; Hofius et al., 2007; De Wit, 2007). Pattern recognition receptors (PRRs) present on the cell surface are the first line of basal defense that sense generic microbial associated molecular patterns (MAMPs) or PAMPs and trigger immunity which is referred as PTI (Jones and Dangl 2006; Zipfel, 2009). The perception of PAMPs activates signal transduction cascades and turns on basal defense. The basal defense response includes deposition of callose and silicon to strengthen the cell wall, production of ethylene and

reactive oxygen species, induction of defense genes like *PR* genes. PRRs respond to slow evolving MAMPs or PAMPs, like flagellin (Zipfel and Felix, 2005), lipopolysaccharides, oligomer or other specific elicitors/effectors (Nürnberger and Lipka, 2005). In plants, flagellum-based motility is important for bacterial pathogenicity (Zipfel and Felix, 2005). Flagellin is a typical elicitor of PTI, which triggers defense responses in many plants (Gomez-Gomez and Boller, 2002). A 22 amino acid peptide conserved domain (flg22) of flagellin is enough to induce many cellular responses including induction of around 1100 *Arabidopsis thaliana* (Arabidopsis) genes in less than an hour (Felix et al., 1999; Zipfel et al., 2004). In Arabidopsis, PRR flagellin sensing2 (FLS2), a transmembrane receptor kinase, is able to detect flagellin and flg22. Another example of a pathogen protein that can induce basal defense in plants is the elongation factor Tu (EFTu) which is the most abundant protein in growing bacterial cells (Kunze et al., 2004). PTI is generally enough to prevent the microbial colonization in plant tissues, but some pathogens use another strategy by evolving effectors that are mostly secreted inside the host cells to repress the PTI, in order to facilitate host invasion. To overcome PTI, some pathogenic bacteria secrete virulence factors, like the small molecule coronatine (COR) that actually mimics the plant hormone JA (Melotto et al., 2006). COR suppresses stomata closure and therefore helps the pathogen gain access to the apoplast (Melotto et al., 2006). Some pathogens can even hide their PAMPs, for example *Agrobacterium tumefaciens* (Agrobacterium) with a modified flagellin are not efficiently detected by the Arabidopsis flagellin receptor FLS2 (Gomez-Gomez et al., 1999). *Xanthomonas campestris* (pathovar) pv. *campestris* strain is also able to avoid its modified flagellin detection by FLS2 (Sun et al., 2006). On the other hand, some pathogens use specialized effectors which are directly translocated from the bacterial cell into the host cell cytoplasm using the type III secretion system (TTSS). *Pseudomonas* (*P.*) *syringae* pv. *tomato* effector protein AvrPto has been shown to promote pathogen virulence by suppressing immune-related proteins (Jones and Dangl, 2006; Zipfel and Rathjen, 2008). Other examples in *Arabidopsis* include AvrRpt2 and AvrRpm1 effector proteins from *P. syringae* pv. *tomato* and *P. syringae* pv. *maculicola* respectively, that can suppress basal defense by inhibiting defense signaling induced by PAMP receptors (Kim et al., 2005). Bacterial effectors are able to abolish basal defense signaling and therefore plants have developed another defense that uses intracellular R proteins to detect the presence of TTSS effectors inside the plant cell. R proteins recognize specific microbial effectors and trigger a defense response termed ETI. Plant cells deploy this second layer of immunity using

polymorphic nucleotide-binding/leucine-rich-repeat (NB-LRR) proteins encoded by resistance genes (*R* genes; Dangl and Jones, 2001).

The effector receptors are related to *R* genes in agreement with Flor's gene-for-gene hypothesis (Flor, 1947). Harold Henry Flor proposed a genetic definition for plant pathogen interaction while working with flax and the flax rust fungus *Melampsora lini*, known as gene-for-gene hypothesis (Flor, 1971). According to the hypothesis of Flor, resistance occurs when a particular *R* gene product recognizes an avirulent (*Avr*) gene product carried by the pathogen. Based on the presence of specific motifs, *R* genes have been functionally grouped into five classes (Büschges et al., 1997; Van Ooijen et al., 2007). Class 1 is an intracellular protein that includes resistance genes encoding proteins with a coiled-coil (CC) domain, a nucleotide binding site (NBS) and a LRR. Class II includes those with a Toll-Interleukin receptor (TIR)-like domain, a NBS and a LRR. Class III R proteins are intracellular protein kinases, class IV are proteins with an LRR domain that encodes membrane bound extracellular proteins, and class V are receptor-like kinases with a extracellular LRR domain (Dangl and Jones, 2001; Martin, 1999; Martin et al., 2003; Mcdowell and Woffenden, 2003). The genome of the model plant Arabidopsis contains around 200 *R* genes that encode proteins with similarity to the NBS and the other domains of plant resistance proteins (Meyers et al., 2003). Intracellular *R* gene receptors can detect a pathogen effector either directly or indirectly (Dangl and Jones, 2001; 2006; Dodds and Rathjen 2010). RPM1, RPS2 and RPS5 are well-known examples of R proteins in Arabidopsis (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Waren et al., 1998). Indirect recognition of effectors by R proteins is explained by the guard hypothesis, stating that R proteins (the guard) monitor host proteins, the guardee that are targeted by pathogen effector molecules (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). RIN4 which is one of the well-known guardees, is guarded by two R proteins RPM1 and RPS2 and is targeted by three bacterial effector proteins namely AvrRpm1, AvrB and AvrRPt2 (Axtell and Staskawicz, 2003; Mackey et al., 2002, 2003). PBS1 is another example of a guardee, which is targeted by bacterial effector protein AvrPphB and guarded by RPS5 (Shao et al., 2003).

Defense responses in ETI happen quickly and last longer than those in PTI (Tao et al., 2003; Trauman et al., 2006; Tsuda and Katagiri, 2010). According to the zig-zag model, PTI is generally triggered by most pathogens, because PAMPs are conserved throughout many pathogens, whereas ETI is typically pathogen strain-specific (Jones and Dangl, 2006). ETI typically ends in programmed cell death at the site of infection as part of the hypersensitive

response (HR) to restrict pathogen growth (Greenberg and Yao, 2004; Jones and Dangl, 2006). It is assumed that HR, which is a form of PCD, enables plants to gain resistance against biotrophic pathogens (Greenberg and Yao, 2004; Heath, 2000). The local defense reaction triggers a systemic plant response which makes uninfected parts of plants less sensitive to attack of virulent or avr pathogens. Thus, this resistant state in plants is called systemic acquired resistance (SAR; Ross, 1961).

1.5 Systemic Acquired Resistance (SAR)

SAR is typically induced following ETI and is effective against many biotrophic pathogens (Durrant and Dong, 2004). However, induction of SAR by PTI has also been reported (Mishina and Zeier, 2007). SAR is a long lasting state of induced immunity against a broad range of pathogens (Ryals et al., 1996) which is characterized by reduction in disease symptoms after subsequent pathogen infection (Durrant and Dong 2004; Ryals et al., 1996; Sticher et al., 1997). It was postulated that in SAR, systemic signals produced at the infection site are translocated to uninfected parts of the plant (Ross, 1961). The establishment of SAR is accompanied by an increase in the level of SA in the phloem (Yalpani et al., 1991). It has been shown that tobacco treatment with acetylsalicylic acid (aspirin) results in accumulation of PR proteins and lesser disease symptoms (White, 1979). 2,6-dihydroxybenzoic acid which mimics the action of SA, can also induce SAR (Van Loon, 1983). In addition to that increased levels of SA were found in phloem of tobacco plants, which had been infected with *tobacco mosaic virus* (TMV; Malamy et al., 1990). Figure (1) depicts emergence of HR and SAR in a TMV-infected *Nicotiana (N.) tabacum* cv. Samsun NN plant. Increasing levels of SA are observed in local as well as systemic tissues. Moreover, PR-1 protein accumulates in tissues surrounding the HR eliciting part of the plant as early as at 7 dpi (days post inoculation). However, SAR exhibiting distal parts of the plant only show gradual build-up of PR-1 protein after 14 dpi.

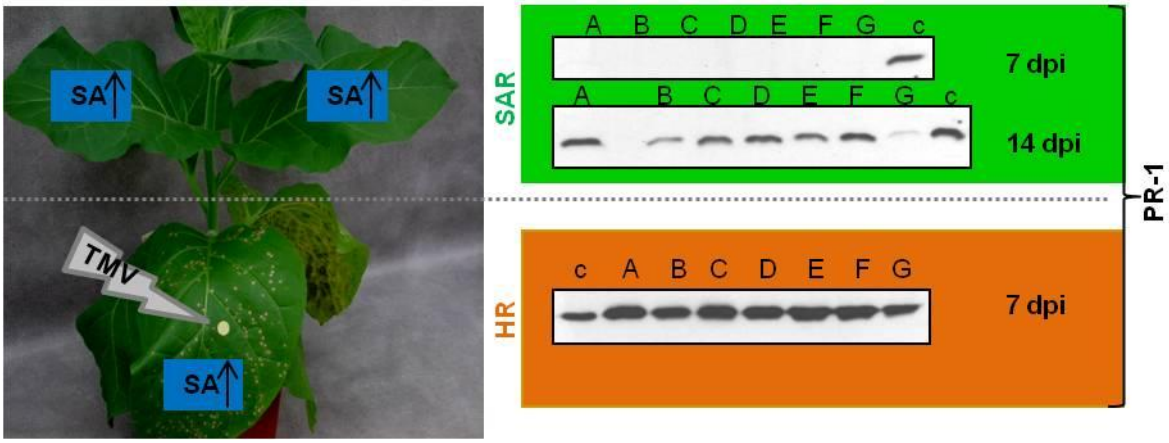


Fig. 1: Phenotypic and molecular characteristics of TMV infected *N. tabacum* cv. Samsun NN plant. Horizontal doted line running through the Figure separates the HR and SAR exhibiting parts of the plant at phenotypic as well as molecular level (photograph and western data kindly provided by U.M Pfitzner).

Additionally, the role of SA in SAR was further proven by using transgenic Arabidopsis and tobacco plants expressing the *NahG* gene. *NahG* encodes salicylate hydroxylase which converts SA to catehol (Gaffney et al., 1993; Delaney et al., 1994). Removal of SA by salicylate hydroxylase abolishes SAR (Gaffney et al., 1993). Some studies have shown that SA does not act as a primary mobile signal for SAR. The grafting experiments on *NahG* transgenic tobacco have shown that the *NahG* tobacco rootstocks were still capable of producing signals that can make non-transgenic scions resistant to further pathogen infection (Vemooij et al., 1994). Furthermore, some grafting experiments on tobacco plants showed that salicylic acid methyltransferase activity that converts SA to methylsalicylic acid (MeSA) is required in the tissue that generates the immune signal. On the other hand, conversion of MeSA to SA is dependent on MeSA esterase activity which is required in systemic tissues for signal perception. These results support that MeSA may be the transported immune signal (Perk et al., 2007). However, experiments using Arabidopsis mutants that lack salicylic acid methyltransfease showed that the plants are still able to accumulate salicylic acid and activate SAR (Attaran et al., 2009). So according to the above mentioned findings, in contrary to tobacco, MeSA has been proven dispensable in Arabidopsis. It seems that the nature of the mobile signal may depend on the plant species and type of plant-pathogen interaction. Yet, the local signal to induce SAR and PR proteins in uninfected tissue is SA.

1.5.1 Pathogenesis-related proteins: Markers for SAR

SAR has been well characterized in different plant species, as being associated with accumulation of PR proteins (Sticher et al., 1997). PR proteins were first discovered in tobacco leaves showing HR to TMV by two different groups working independently (Van Loon and Van Kammen, 1970; Gianinazzi et al., 1970). As their name indicates, PR proteins are induced in plants in response to pathogen attack (Van Loon et al., 1994). PR proteins are established in all plant organs, i.e., stems, roots, flowers and leaves, but the maximum amount of PR proteins is present in leaves which amount 5-10% of total leaf proteins (Van Loon, 1999). Currently there are 17 families of PR proteins (Van Loon et al., 2006). Among these, the PR-1 group serves as a marker for the SAR response, because it is the most abundant family of PR proteins. Although the functions of PR proteins are not clear in each case, they are believed to mediate the pathogen resistance. For example, *in vitro* and *in vivo* experiments have shown that PR-1 family members show direct inhibitory effects against the fungal pathogens *Phytopthora infestans* and *Uromyces fabae* in tomato and broad bean, respectively (Niderman et al., 1995; Rauscher et al., 1999). But the mode of action, cellular and molecular targets of PR-1 protein are not known.

Nevertheless, since PR proteins have been discovered, the regulation of PR proteins has been an active research topic. It is known that upon HR the level of SA, JA and ET production is enhanced (Pieterse et al., 2000; Seo et al., 2001). As a result, in Arabidopsis, SA inducible defense genes, e.g., *PR-1*, *PR-2* and *PR-5* together with JA and ET inducible genes like *PR-3*-type genes encoding basic chitinas, *PR-4* type genes encoding hevein-like protein and *PR-12* encoding defensin PDF1.2 have been reported to be activated (Thomma et al., 2001). However, in between these three pathways, complex cross talks have been reported (Thatcher et al., 2005). SA-independent/JA-dependent and JA-independent/SA-dependent pathways for PR protein induction have been observed (Niki et al., 1998; Fidantsef et al., 1999). Generally, JA and ET are considered to be involved in defense against necrotrophic pathogens. For example, Arabidopsis plants impaired in JA or ET signaling were found to be more susceptible to necrotrophic pathogens (Geraats et al., 2003; Thomma et al., 2001; Vijayan et al., 1998). On the other hand, increased susceptibility to *Hyaloperonospora parastica* (biotrophic pathogen) in Arabidopsis SA synthesis mutants indicates that SA-dependent defense is needed against biotrophic pathogens (Thomma et al., 2001; Glazebrook, 2005).

Hence, it shows that a complex signaling network exists in plants, which finally results in induction of specific PR proteins in plants in response to particular stimuli.

As discussed in the previous section, SA is important for establishment of SAR and PR protein induction in plants. Therefore it would be right to take SA as an endogenous regulator of SAR and stimulus for induction of PR proteins. It has been proven in many studies that not only naturally occurring SA but also some synthetic chemicals, such as aspirin, 2,6 dichloro-isonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH) can activate the expression of *PR* genes when applied exogenously thus acting as functional analogues of SA (Malamy and Klessig, 1992; Ryals et al., 1996; Shah and Klessig, 1999; Vernooij et al., 1995; Görlach et al., 1996; Wasternack et al., 1994; Morris et al., 1998; Pasquer et al., 2005; Makander et al., 2006).

1.5.2 Components of SAR

Using SA inducible *PR* genes, the signaling pathway leading to SAR activation has been described in Arabidopsis (Cao et al., 1994; Lebel et al., 1998; Zhang et al., 2003; Rochon et al., 2006; Kesarwani et al., 2007). In order to isolate and characterize genes that are involved in defense responses against pathogens, Arabidopsis was used extensively as a model plant (Glazebrook et al., 1997a), because of its unique features for research work, such as short stature, self-pollination capabilities, short life cycle, small genome size and feasibility for genetic and molecular methods (Meyerowitz, 1987). It was only then that screening was carried out for mutants in Arabidopsis that failed to activate a *BGL2* (*PR-2*): *ß-glucuronidase* (*GUS*) reporter construct in response to SA and INA (Cao et al., 1994; 1997). The mutants obtained can be divided into two distinct groups. The first group includes plants that showed constitutive activation of SAR, accompanied with elevated levels of SA and *PR* gene expression. These plants showed spontaneous necrosis and increased resistance to virulent pathogens, without involving HR (Dietrich et al., 1994). According to their phenotypes, mutants can be further classified as *cim* (constitutive immunity), *cpr* (constitutive expressor of *PR* genes), *lsd* (lesion simulating disease) and *acd* (accelerated cell death). The second group includes a mutant that was neither able to activate the SAR response upon pathogen attack or chemical signals nor to induce increased PR protein levels. The mutant is called *npr* (non-expressor of pathogenesis related gene) 1 (Cao et al., 1994). After that, several groups worked on mutant screens based on enhanced disease susceptibility (Glazebrook et al., 1996),

impaired SAR expression (Delaney et al., 1995) or reduced SA-induced *PR* gene induction (Shah et al., 1997). Several mutants allelic to *npr1* were identified and were termed non inducible immunity (*nim1*; Delaney et al., 1995) and salicylic acid insensitive (*sai1*; Shah et al., 1997). The findings show the involvement and importance of NPR1 in the plant defense response.

1.5.2.1 NPR1 master regulator of SAR

The *NPR1* gene has been isolated and cloned from Arabidopsis (Cao et al., 1997; Ryals et al., 1997). The *NPR1* gene encodes a novel 66kDa protein (Bork et al., 1993). With no apparent biochemical function, NPR1 has a nuclear localization signal (NLS) at its C-terminus. It also contains two domains involved in protein-protein interaction, an ankyrin repeat domain located in the central region and a BTB/POZ (broad-complex, tramtrack, and bric-a-brac/poxvirus, zinc finger) domain at the N-terminal end (Cao et al., 1997; Ryals et al., 1997). The presence of protein-protein interaction domains in NPR1 suggests its function as a regulatory protein, possibly interacting with other proteins, e.g., transcription factors (Cao et al., 1997; Ryals et al., 1997). Therefore, molecular and genetic approaches have been applied to analyze NPR1 function as a regulatory protein. It has been found that *NPR1* gene expression is constitutive under normal conditions, but upon SAR chemical inducers and *Pernospora parastica* infection, the expression level is slightly enhanced (Cao et al., 1997; Ryals et al., 1997; Weigel et al., 2001). In Arabidopsis and rice, overexpression of the *NPR1* gene resulted in enhanced disease resistance to bacterial and fungal pathogens after treatment with SAR inducers. However, overexpression of *NPR1* was not accompanied with constitutive *PR* gene expression, making it clear that NPR1 needs an activation step regulated by SA either directly or indirectly, which ultimately leads to the activation of *PR* genes (Cao et al., 1998; Chern et al., 2001; Friedrich et al., 2001). The promoter region of the *NPR1* gene contains W-box sequences, which are the binding regions for WRKY proteins. Adverse effects were noted in *NPR1* expression when a mutation was brought in at the W-box, suggesting an important role of WRKY transcription factors in mediating signaling between SA and NPR1 (Yu et al., 2001). It has been mentioned before that NPR1 has a clear NLS at its C-terminus. Consistently, *in planta* experiments showed that functional NPR1-green fluorescent protein (GFP) accumulates in the nucleus in response to SAR inducers (Kinkema et al., 2000). Hence, it seems that nuclear localization of NPR1 is needed for activation of *PR* gene expression. In the absence of SA or pathogen challenge, NPR1 resides as an oligomer in

the cytoplasm, because of redox sensitive intermolecular disulphide bonds. However, upon induction, NPR1 undergoes conformational changes from inactive oligomers to active monomers which enter the nucleus to activate defense gene transcription (Mou et al., 2003). This concludes SA as an important signal for NPR1 activity, not only in activating *NPR1* expression, but also for NPR1 translocation to the nucleus. SA induced redox state leads to reduction of residue cysteine (Cys)-156 by (thioredoxins) TRX-H5 and TRX-H3 (Tada et al., 2008) and it has been reported that S-nitrosoglutathione (GSNO) and TRX with their opposing action at Cys-156 regulate NPR1 by facilitating oligomerization and monomerization, respectively (Tada et al., 2008). Therefore, it has been concluded that conformational changes of NPR1 from oligomeric to monomeric form regulate its activity and nuclear localization (Mou et al., 2003; Tada et al., 2008). Furthermore it has been demonstrated that proteasome-mediated degradation of NPR1 promotes or prevents expression of the defense response, following infection or in absence of infection, respectively. In challenged plants, phosophorylation at serine (Ser)-11 and Ser-15 leads to NPR1 turnover, which is required for induction of defense genes. On the contrary, NPR1 is continuously degraded in unchallenged plants in order to prevent *PR* gene activation (Spoel et al., 2009). A recent report shows that NPR1 paralogs NPR3 and NPR4 are SA receptors and are involved in regulating NPR1 stability and activity. NPR3 and NPR4 degrade NPR1 in SA dependent manner and result in no *PR-1* expression in unchallenged and *PR-1* induction in SAR tissues (Fu et al., 2012). In the same study, it has been postulated that NPR1 suppresses cell death in SAR tissues and allows cell death in ETI tissues. It was also observed that NPR1 was not only detected in cytoplasm and nucleus but even has been found to be associated with the $PR\text{-}1_{pro}$ in unchallenged Arabidopsis plants (Despres, et al., 2000, Rochon et al., 2006). Binding of SA to NPR1 occurs in the C-terminus at Cys-521 and Cys-529 via the transition metal copper and thus acts as a SA receptor. By SA binding a C-terminal transactivation domain is released from the N-terminal auto inhibitory BTB/POZ domain (Wu et al., 2012).

In another work, based on biochemical evidence obtained from heterologous yeast system, it has indeed been shown that NPR1 is able to sense SA (Maier et al., 2011). Tobacco NPR1 gains transcription activity after SA supplementation to the culture medium in yeast one hybrid (Y1H) assays. This model is corroborated by an *en masse in planta* screen for Arabidopsis mutants insensitive to the functional SA analog BTH demonstrating that the mutation clusters in two regions in the NPR1 C-terminus (Canet et al., 2010). This shows that

SA signal is transmitted through two conserved domains in the C-terminal third of NPR1. One of these domains is the binding site for SA-induced (NIM1-interacting) NIMIN1 and NIMIN2-type proteins and the other one is the penta-amino acid motif LENRV (Maier et al., 2011; Canet et al., 2010; For detail see NIMIN section). Thus, three different models have been proposed how SA may signal through the NPR1 protein.

Much evidence, however, has shown that certain aspects of defense are controlled by SA-dependent but NPR1-independent signaling pathways (Clarke et al., 1998, 2000; Kachroo et al., 2000; Shah et al., 2001; Murray et al., 2002). A putative negative regulator of SAR, *suppressor of NPR1 inducible* (*SNI*) was identified in a genetic screen for mutants that suppress the *npr1* mutant background (Li et al., 1999). It was shown that *npr1-sni1* double mutation in plants restores the induction from the *PR* promoter after INA application. Consequently, unrevealing NPR1-independent pathway by activating *PR* promoter in the absence of functional SNI. Meanwhile, screening of *npr1-5* background mutation for constitutive *PR* gene expression brought forward *suppressor of SA insensitivity* (*ssi*) mutants, *ssi1*, *ssi2* and *ssi3* that show constitutive accumulation of SA and resistance to various pathogens (Shah et al., 1999, 2001; Shirano et al., 2002). It was shown that *npr1-ssi1* and *ssi2-npr1* double mutation accumulate less *PR* gene transcript levels as compared to *ssi1* and *ssi2* mutation in *NPR1* background plants (Shah et al., 1999, 2001). The finding therefore suggests that *NPR1*-dependent pathway actually gears the *NPR1*-independent pathway, by elevating *PR* gene transcripts.

In Arabidopsis genome, there are five paralogs of *NPR1* namely *NPR2, NPR3, NPR4,* (*blade on petiole1*) *BOP1* and *BOP2*. Phylogenetic studies revealed that NPR2 and NPR1 are the most closest, NPR3 and NPR4 form another subgroup and BOP1 and BOP2 form a third group furtherest from NPR1(Hepworth et al., 2005; Liu et al., 2005). It was shown that *bop1-bop2* double mutation brings loss of abscission of floral organs, leafy petioles and asymmetric flowers. Hence, it shows the role of BOP1 and BOP2 in controlling growth symmetry (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). Whereas, *npr3npr4* knockout-mutant studies have revealed NPR3 and NPR4 as a negative regulators of *PR* gene expression (Zhang et al., 2006). Homologs of At NPR1 have been cloned and characterized in many plants including rice (Chern et al., 2005), apple (Malnoy et al., 2007), cotton (Zhang et al., 2008), rosaceous tree (Pilotti et al., 2008), banana (Endah et al., 2008) grapevine (Henanff et al., 2009) and sugarcane (Chen et al., 2012).

1.5.2.2 *Activation sequence-1* (*as-1*) like elements

The induction of *PR* genes in the course of SAR leads to the hypothesis that *PR* gene expression is regulated by SA (Malamy et al., 1990; Yalpani et al., 1993). Initially, investigation of the 35S RNA promoter ($_{pro}$) of cauliflower mosaic virus (CaMV) revealed the presence of a *cis*-acting element, which is responsible for SA-dependent inducibility of the promoter (Qin et al., 1994; Lam et al., 1989). This *cis*-acting element is named *as-1* and contains a tandem repeat of the sequence TGACG. Later on, to better understand the SA-dependent defense responses in plants, regulation of *PR* gene promoters has been characterized and studied in more detail. It has been found out that related *as-1* elements also exist in the promoter regions of the tobacco *PR-1a* and Arabidopsis *PR-1* gene, termed *as-1* like elements (Strompen et al., 1998; Lebel et al., 1998). Expression studies with *PR-1a$_{pro}$:GUS* reporter gene constructs show that mutations in the *as-1 like* element of the *PR-1a$_{pro}$* result in decreased reporter gene induction after pathogen infection and SA application (Strompen et al., 1998).

Transcription regulation of gene expression depends on recognition of specific *cis*-acting promoter elements, by *trans*-acting specific DNA-binding factors. Several families of transcription factors are known in Arabidopsis like, ERF, Myb, WRKY and TGAs, each having a distinct type of DNA-binding domain (BD; Singh et al., 2002). Strompen et al., (1998) were the first one to show by experiment that the *as-1*-like element in the *PR-1a* promoter binds a TGA factor. Later on, investigation of the Arabidopsis *PR-1$_{pro}$* revealed that it contains two putative TGA factors binding targets termed as *linker scan7* (*LS7*) and *LS5* (Lebel et al., 1998). In term of *PR-1* expression in response to INA or SA treatment, *LS7* and *LS5* elements act as positive and negative regulators, respectively (For TGA see next section).

1.5.2.3 TGA transcription factors

NPR1 is the master regulator of SAR and it is essential for SA-dependent expression of *PR* genes, but it does not show any recognizable DNA-BD. This means NPR1 cannot be directly responsible for transactivation of *PR* promoters. Therefore, the presence of protein-protein interaction domains in NPR1 strongly suggests that NPR1 may associate with other proteins, e.g., transcription factors (Cao et al., 1997: Ryals et al., 1997). Consequently, NPR1 has been found to interact with TGA factors in Y2H screen experiments (Despres et al., 2000; Zhou et al., 2000; Zhang et al., 1999). In fact, interaction of NPR1 with TGA factors is required for

PR gene expression and SAR (Fan and Dong, 2002; Despres et al., 2000; Zhou et al., 2000; Zhang et al., 1999).

TGA family is a class of basic leucine zipper (bZIP) transcription factors. bZIP is composed of about 16 basic amino acids and a spacer of nine amino acids followed by a heptad repeat of leucine or any bulky hydrophobic amino acid (Jakoby et al., 2002). These structures on an α-helix allow interaction between proteins via the hydrophobic side chains, creating a coiled-coil structure called zipper. bZIP proteins interact with DNA through the basic region preferring sequences with ACGT core. Transcription factors containing a bZIP have been identified in both mammals and plants (Riechmann et al., 2000). In plants, they participate in various tasks from plant pathogen defense response (Kim and Delaney, 2002) to floral development (Wigge et al., 2005). There are several members of bZIP proteins in Arabidopsis and they have been divided into ten groups, TGA factor makes one group (Jacoby et al., 2002). Members of the TGA family bind to *as-1* elements and regulate the expression of many stress-responsive genes like *PR-1* (Strompen et al., 1998; Chen and Singh, 1999; Lebel et al., 1998). TGA1a is the first *as-1* binding protein that has been isolated from a tobacco cDNA library screen using labeled TGACG-containing DNA probe (Katagiri et al., 1989). TGA1a sequence analysis revealed that it contains a DNA-BD, a leucine zipper region (important for dimer formation), an acidic N-terminal domain (for transactivation) and a glutamine-rich (transcriptional activation potential) C-terminal region mediating dimer stabilisation (Katagiri et al., 1992; Neuhaus et al., 1994; Pascuzzi et al., 1998). Thereafter, many TGA1a homologous proteins have been isolated from different plants species.

In Arabidopsis ten members of this group have been described as TGA1 (Schindler et al., 1992), TGA2 (Kawata et al., 1992), TGA3 (Miao et al., 1994), TGA4 and TGA5 (Zhang et al., 1993), TGA6 (Xiang et al., 1997), TGA7 (Despres et al., 2000), PERIANTHIA (PAN; Chuang et al., 1999), TGA9 and TGA10 (Murmu et al., 2010). Interest in TGA factors gained further attention, as they were identified to interact with NPR1 in Y2H screens (Despres et al., 2000; Zhou et al., 2000). It was found that five TGA factors, i.e., TGA2, TGA3, TGA5, TGA6 and TGA7, interact with NPR1 in Y2H system, while TGA1, TGA4 and PAN did not interact or interacted only weekly (Hepworth et al., 2005; Despres et al., 2000; Zhou et al., 2000). PAN has a developmental role as it is a key regulator of floral patterning. Flowers lacking PAN typically contain an extra sepal or petal and fewer stamens, resulting in pentamerous arrangement of floral organs (Running and Meyerowitz, 1996; Chuang et al., 1999). It has been found that PAN does interact with glutaredoxins (GRXs). GRXs are

glutathione-dependent disulphide oxidoreductases that have been found to be involved in oxidative stress responses in plants (Rouhier et al., 2008). In addition to PAN, GRX also interacts with remaining two TGA factors TGA9 and TGA10. However it has been shown that TGA9 and TGA10 also interact with floral GRXs, i.e., ROXY1 and ROXY2, and that both TGA factors are redundantly required for anther development (Murmu et al., 2010). ROXY1 and ROXY2 are the land plant specific CC-type floral GRXs (containing a CC (M/L)(C/S) active site) with redundant function in Arabidopsis anther development.

It has been confirmed via in vitro binding assay that TGA2 and TGA5 factors interact with NPR1 (Despres et al., 2000; Zhang et al., 1999). TGA2 has also been shown to interact with NPR1 *in planta* and the interaction between the two proteins was found stimulated by treatment with SA (Fan and Dong 2002; Subramaniam et al., 2001). It was confirmed via Chromatin immunoprecipitation (ChIP) studies that TGA2 and TGA3 bind to the $PR\text{-}1_{pro}$ after SA treatment and the binding is abolished in *npr1* plants (Johnson et al., 2003). Therefore, it was concluded that TGA factors have a role in the SAR pathway, but, on the other hand TGA needs a functional NPR1. It has been reported that TGA1 and TGA4 are not able to interact with NPR1 although both possess all structural elements necessary for interaction with NPR1 (Despres et al., 2003). TGA1 and TGA4 contain two additional cysteine residues (Cys-260 and Cys-266) which lead to formation of intramolecular disulphide bridges. However, SA treatment leads to the reduction of disulphide bonds which possibly enables the interaction with NPR1 and expression of *PR* genes (Despres et al., 2003; Dong, 2004). Mutation in the responsible cysteines brought TGA factor to interaction with NPR1 in yeast and in untreated leaves of Arabidopsis. The regulation of the redox state was suggested to be carried out by TRXs and GRXs (Rouhier et al., 2004; Gelhaye et al., 2005). Since the mutation in a single TGA factor was not able to bring any phenotypic change, analysis of biochemical and regulatory functions of TGAs are comparatively difficult. But the most compelling evidence for the involvement of TGA factor in SAR became clear through the study done on the triple knockout-mutation in *tga2/tga5/tga6* (Zhang et al., 2003). Hence, it could be concluded that TGA2, TGA5 and TGA6 might be functioning as positive regulators in the SAR response.

In tobacco, the TGA factor family encompasses six members: TGA1a (Katagiri et al., 1989), PG13 (Fromm et al., 1991), TGA2.1 and TGA2.2 (Niggeweg et al., 2000a), TGA8 (Stos, 2002) and TGA10 (Schiermeyer et al., 2003). All TGA factors from tobacco show similarities to Arabidopsis TGA factors, but specially TGA2.2 from tobacco exhibits great

similarity to Arabidopsis TGA2, 5 and 6 (Zhang et al., 2003). It has been proven that TGA 2.2 and TGA2.1 are part of a protein complex, ASF-1 (*as-1*-binding factors) that functions as a transcription complex (Lam et al., 1989, Niggeweg et al., 2000b). *TGA1a* could only be detected at mRNA levels in leaves (Niggeweg et al., 2000a). However, *TGA1a* was represented in meristematic cells of roots (Katagiri et al., 1989) where it was co-expressed with *as-1* regulated genes (Klinedinst et al., 2000). Detailed studies had been conducted to characterize TGA8 (Stos, 2007). The result from *NtTGA8*-RNAi transgenic plants brought spontaneous necrosis and leaf deformation in adult plant leaves. However the *PR* gene expression remained unchanged after SA application. It has also been shown that TGA8, unlike TGA1, can interact with tobacco NPR1 (Stos, 2007). *TGA10* mRNA on the other hand was only detected in roots where the protein binds to *as-1* elements (Schiermeyer et al., 2003).

1.5.2.4 NIM1-Interacting (NIMIN)

Using the Y2H system, NPR1 was not only found to interact physically with TGA factors, but also with three novel proteins, i.e. NIMIN1, NIMIN2 and NIMIN3, encoding structurally related proteins (Weigel et al., 2001). The discovery of another gene of this family was based on database search and because of its homology to *NIMIN1*. The gene was named *NIMIN1b*. NIMIN proteins interact differently with NPR1. NIMIN1b, NIMIN1 and NIMIN2 have a binding motif in the N-terminal half, with help of which they bind to the C-terminus of NPR1. However, NIMIN3 binds to the N-terminus of NPR1. NIMIN3 also contains a NPR1 binding motif but this motif is different from the motif present in the other NIMIN proteins. NIMINs have been found to contain an (ethylene responsive element binding factor) ERF-associated amphiphilic repression (EAR) motif, very similar NPR1-BD (FFK domain) and a NLS in their sequence. In addition to that some members of NIMIN proteins also contain an EDF motif and stretches of poly E (glutamic acid) or D (aspartic acid) rich repeats in their amino acid sequence (Fig. 2A). It has been reported that except for NINIM3, which does not interact with NtNPR1, all other NIMIN proteins interact with NtNPR1 and AtNPR1 equally (Weigel et al., 2001; Zwicker et al., 2007). Expression of *NIMIN-GUS* fusion proteins showed NIMIN proteins to accumulate in the nucleus (Weigel et al., 2001).

Homology comparison led to the discovery that previously uncharacterized G8-1 protein from tobacco which had been discovered as SA induced gene (Horvath et al., 1998), actually falls into NIMIN protein family. Tobacco G8-1 protein showed high homology to

Arabidopsis NIMIN2, so therefore it is called NIMIN2a. Afterwards, two other NIMIN2 homologues were identified in tobacco and were named NIMIN2b and NIMIN2c (Zwicker et al., 2007). All members of the NIMIN protein family in tobacco contain NPR1-BD, NLS and EAR motif (Fig. 2b). All of them bind to the C-terminus of Arabidopsis and tobacco NPR1 (Weigel et al., 2001; Zwicker et al., 2007). NIMIN2 homologues have also been identified in other plants. For example, in rice, a *NIMIN2* like gene *NRR* (*negative regulator of resistance*), has been identified which interacts with (*Oryza sativa)* OsNPR1 (Chern et al., 2005a). Overexpression of NRR leads to susceptibility to *Xanthomonas oryzae* pv. *oryzae* in rice (Chern et al., 2005a) and it has been found out that *NRR* overexpression in Arabidopsis compromises SAR (Chern et al., 2008). Recently three other *NRR* homologous genes have been identified in rice, namely (Rice NRR homologue) RH1, RH2 and RH3 (Chern et al., 2012). All rice NRR homologues including NRR contain an eight amino acid long stretch, which is also shared by NIMIN1 and NIMIN3 and it has been shown that this motif is the main OsNPR1/NH1 (NPR1 homologue 1) interacting motif in rice NIMIN proteins (Chern et al., 2012).

Many biochemical differences between NIMIN proteins have been found in Arabidopsis, tobacco and rice (Zwicker et al., 2007; Maier et al., 2011, Chern et al., 2005a). In Arabidopsis, *NIMIN* gene expression analyzed by RT-PCR revealed that *NIMIN3* is expressed constitutively at low level (Hermann, et al., 2013) and the $NIMIN3_{pro}$ does not respond to SA in transgenic tobacco seedlings. In contrast *NIMIN1* and *NIMIN2* are clearly SA-induced genes (Glocova et al., 2005).

An interaction between NPR1 and NIMIN1 has also been confirmed in plant extracts (Weigel et al., 2005). In Y3H system, it has been shown that TGA factors, NIMIN1 and NPR1 form a ternary complex, which can bind to the *as-1* element (Weigel et al., 2005). Overexpression of *NIMIN1* suppresses SA-dependent *PR-1* gene induction and results in reduced SAR resistance in Arabidopsis (Weigel et al., 2005). On the other hand, *NIMIN1* RNAi lines showed only slightly enhanced *PR-1* gene expression after SA treatment (Weigel et al., 2005). So in accordance with the results discussed previously, it could be said that NIMIN1 and NRR are working in the SAR pathway as repressors of *PR-1*. But overexpression of tobacco *NIMIN2a* resulted in just a slight decrease of *PR-1* expression (Zwicker et al., 2007). This shows that in order to activate SAR, the repressors must be suppressed. Recently, it has been shown that interaction between NIMIN1 or NIMIN2 and NPR1 is negatively regulated

by SA (Maier et al., 2011) indicating that SA targets NPR1 which ends up in loss of NIMIN1 and NIMIN2 binding.

Interestingly, SA treatment does not impair the binding of NIMIN3 or TGA factor with NPR1 in Y2H assays (Maier et al., 2011), indicating that SA only targets the C-terminus of NPR1, leaving the other binding domains undisturbed. NIMIN proteins and TGA factor have independent binding sites on NPR1. However, the binding of NIMIN1 or NIMIN2 simultaneously with NIMIN3 is not possible (Hermann et al., 2013). Figure (2C) gives an overview on the current model for different Arabidopsis NIMIN proteins regarding their differential interaction with NPR1.

Based on previous observations NIMIN proteins seem to be very important in executing plant defense responses against invading biotrophic pathogens. Arabidopsis *NIMIN* genes have recently been classified as LSGs (lineage-specific genes), which are considered as fast evolving genes that are important for adaption of plants in response to various stress conditions (Donoghue et al., 2011). Unfortunately, not much is known about the NIMIN proteins. Therefore, the goal of this work was to further characterize the action of different NIMIN proteins on NPR1 with respect to activation of *PR-1* genes. To this end a transient gene expression system was established in *N. benthamiana* in order to assign role of multiple *NIMIN* genes in Arabidopsis and tobacco. It is known that Arabidopsis contains four *NIMIN* genes, however, tobacco contains some novel *NIMIN* genes based on Genbank data base. Therefore, another focus of this study was to clone and characterize novel *NIMIN* genes present in tobacco.

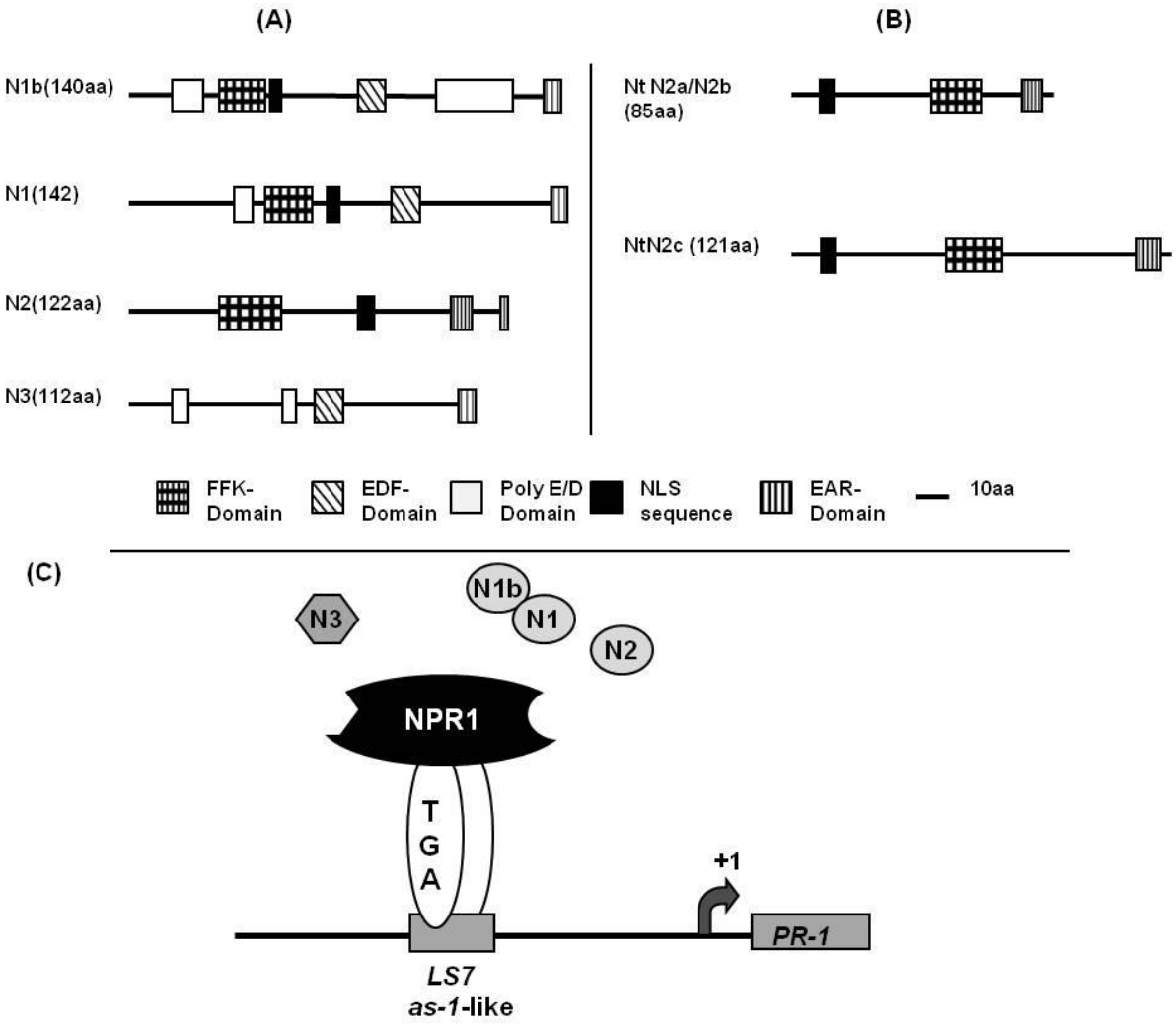


Fig. 2: Different NIMIN proteins in Arabidopsis and tobacco. Schematic representation of NIMIN proteins showing their characteristic domains **A)** Arabidopsis NIMIN proteins **B)** tobacco NIMIN proteins. **C)** Model for differential interaction of Arabidopsis NIMIN protein with NPR1 and establishment of ternary complex between NIMINs-NPR1 and TGA factor.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Standard chemicals were obtained from the companies Roth (Karlsruhe, GER), Merck (Darmstadt, GER), Sigma (Deisenhofen, GER) and ICN (Eschwege, GER). Deviations from these sources are mentioned in the text.

2.1.2 Organisms

2.1.2.1 Bacteria

Escherichia (E.) coli DH5α: Laboratory stock (Lab. St.; Hanahan, 1983).

Genotype: F'ϕ*80*d*lac*ZΔ*M15* Δ (*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44λ^- *thi*-1, *rec*A1, *gyr*A96 *rel*A1

Agrobacterium (A.) tumefaciens LBA4404: Lab. St. (Hoekema et al., 1983)

A. tumefaciens GV3101: Lab. St. Prof. Dr. A. Schaller (University of Hohenheim, GER)

2.1.2.2 Yeast

Saccharomyces (S.) cerevisiae HF7c: Lab. St. (Bartel et al., 1993)

Genotype: MATa, *ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ*

The yeast strain HF7c contains the *HIS3* and *lacZ* reporter genes. The yeast strain also contains *trp1* and *leu2* transformation markers.

S. cerevisiae Cen. PK2: Lab. St. (Niedenthal et al., 1996)

Genotype: MATa/α *ura3-52/ura3-52, trp1-289/trp1-289, leu2-3, 112/leu2-3, 112, his3-Δ1/his3-Δ1*

The yeast strain Cen. PK2 contains *trp1, leu2 and ura3* transformation markers.

2.1.2.3 Plants

Nicotiana (N.) tabacum (L.) cv. Samsun NN and N. benthamiana (Domin)

Origin: Tobacco Institute (North Carolina, USA) provided by Prof. L.-W. Mundry (University of Stuttgart, GER).

-1533PR-1a$_{pro}$:GUS N. benthamiana
Origin: Lab. generated transgenic reporter line

2.1.3 Nucleic Acids

2.1.3.1 Plasmids

2.1.3.1.1 pBluescriptII KS+ (PBS II)/TA-Vector

The pBluescript vector (Stratagene, USA) has a size of 2,961 bp. The pBluescript phagemid was designed for cloning, sequencing, *in vitro* mutagenesis and *in vitro* transcription of introduced genes. It contains a multiple cloning site (MCS) in the coding region of the β-galactosidase (*lacZ*) gene that helps in identifying recombinant clones via the blue-white-detection. This is achieved through intra-allelic complementation with the defective form of *Lac*Z encoded by the host, e.g. in *E. coli* DH5α. Integration of a DNA fragment into the MCS results in disruption of the reading frame and abolishment of α-complementation. Therefore, the recombinant clones remain white. As another selection marker, plasmid contains an ampicillin resistance (*amp^r*) gene for selection in *E.coli.* For the cloning of PCR products, the vector was modified in collaboration with Dr. Bernhard Roth (Novartis, Marburg). For this, the plasmid pBluescript II KS+ was linearized by restriction with *Eco*RV. The linearized vector DNA was treated with terminal deoxynucleotidal transferase (TDT) to add 2',3'-Dideoxythymidine-5'-Triphosphate (ddTTP) at the ends. Since many *Thermus aquaticus* (*Taq*) polymerases tend to add an extra adenosine, modified vector (i.e. TA vector) is useful for the cloning of PCR products amplified with *Taq* DNA polymerases enzyme.

2.1.3.1.2 pBin19

The binary plant vector pBin19 (Bevan, 1984) is used for the transfer of DNA sequences by means of *A.tumefaciens* in plants. The plasmid is 11,777 bp in size and contains a modified T-DNA that carries a MCS and the kanamycin resistance (kan^r) gene *NPTII* (neomycin-phosphotransferase) between its right and left border. The kan^r serves for selection in plants. Outside T-DNA the plasmid contains a second gene for neomycin phosphotransferase for selection in bacteria. Since the vector has two different origins of replication (ori), it can replicate both in *E. coli* and in *A.tumefaciens*.

For transferring the T-DNA, i.e., excision from the binary vector and the following integration into the plant genome, yet another plasmid is needed. This is possible by using *Agrobacterium* strain LBA4404 cells that contain the disarmed Ti plasmid, which has only the *vir* (necessary for the transfer of T-DNA) and *ori* region of the Ti plasmid.

2.1.3.1.3 pRK2013

The helper plasmid pRK2013 is required for bacterial conjugation during the tri-parental mating (TPM; Figurski and Helinski, 1979). It provides the necessary gene products and allows the transfer of the binary vector from the *E. coli* strain DH5α in the Agrobacteria.

2.1.3.1.4 pUC18 and pUC19

The vectors pUC18 and pUC19 were developed by Yanisch-Perron and coworkers (Yanisch-Perron et al., 1985). These are small (2686 bp), high copy replicating *E. coli* plasmid vectors sharing most of the features of pBSII. The plasmid carries an amp^r gene and allows the identification of recombinant bacterial clones via blue-white-detecting i.e., possible by α-complementation of the *lacZ* gene in *E. coli* DH5α. The two plasmids differ only in the orientation of the MCS.

2.1.3.1.5 pGAD424

The vector pGAD424 (Bartel et al., 1993) is used for studies in the yeast two hybrid system (Y2HS). pGAD424 has a size of 6.6 kbp, it replicates autonomously in both *E. coli* and *S. cerevisiae*. Selection in bacteria is possible by means of amp^r gene. In addition to that the vector has one *LEU2*-marker, which allows yeast cells containing pGAD424 to grow on minimal medium without leucine. Vector contains a constitutive *ADH1* promoter ($_{pro}$) for

expression of a fusion protein and a MCS at the 3' end of open reading frame (ORF) of the GAL4 transcription activation domain (AD). The gene for the protein to be examined is cloned into the MCS. For the expression of desired fusion protein cloning at the correct reading frame of GAL4-AD is necessary.

2.1.3.1.6 pGBT9

The vector pGBT9 (Bartel et. al., 1993) is used for Y2HS studies. It replicates autonomously in both *E. coli* and *S. cerevisiae* and carries amp^r gene which enables the selection in bacteria. Furthermore the vector has one *TRP1* marker, which allows yeast cells containing pGBT9, to grow on minimal medium without tryptophan. Additionally, the vector contains a constitutive $ADH1_{pro}$ for expression of a fusion protein, as well as a multiple cloning site at the 3 'end of the GAL4 DNA binding domain (BD). The gene for the protein to be examined is cloned into the MCS. For the successful expression of a GAL4-BD fusion protein, it is necessary that the cloning is done in the correct reading frame. The size of the vector is 5.4 kbp.

2.1.3.1.7 pBridge (pBD-/-)

Vector pBridge (Tirode et al., 1997) is used in Y3HS studies and is capable for the expression of two proteins. It also replicates autonomously in both *E. coli* and *S. cerevisiae*. Selection in bacteria is by means of amp^r. Furthermore it has the one *TRP1* marker, which allows yeast cells containing pBridge to grow on minimal medium without tryptophan. Additionally, the vector contains a constitutive $ADH1_{\text{pro}}$ for expression of a fusion protein, as well as a MCS at the 3' end of the ORF for the GAL4-BD. The gene for the protein to be examined is cloned into the MCS-I. For the fusion of a GAL4-BD and the desired protein, it is necessary that the cloning is in the correct reading frame. For the expression of a second protein, the vector contains an additional expression cassette (MCS-II) controlled by the $MET25_{\text{pro}}$. In the presence of methionine, this promoter is repressed and suppresses the expression of the cloned gene and *vice versa*. The vector has a size of 6.5 kbp, and may be used in combination with other GAL4-based vectors of the Y2HS for the investigation of interaction of three proteins. pBridge vector was slightly modified and was designated pBD-/ - (Weigel, 2000). The pBD-/ - enables in frame fusion of the gene with sequence for nuclear localization signal (NLS) and a human influenza hemagglutinin (HA) epitope tag, at MCS-II (in $MET25_{\text{pro}}$). This fusion ensures protein transportation to nucleus and also enables protein detection by using an antiserum raised against HA-epitope.

2.1.3.1.8 pEGFP C-FUS

For sub-cellular localization of desired proteins modified pGFP C-FUS vector (Niedenthal et al.,1996) was used. pGFP C-FUS vector from which wild type (wt) GFP had been replaced with codon-optimized yeast enhanced-GFP (yEGFP), this exchange result in higher GFP expression levels. In addition to that yEGFP harbors two previously identified mutations in the chromophore (serines at position 65 and 72 are changed to glycine and alanine, respectively) that enhances GFP fluorescence by factor 75 (Cormack et al., 1996). The modified plasmid has been named pEGFP C-FUS vector (Cormack et al., 1997). The vector has a size of 5992bp. The vector carries a centromere DNA *(CEN6),* a replication origin *(ARSH4),* an amp^r and the *URA3* marker. Transcription of the fusion constructs is under the control of $MET25_{pro}$ and is terminated by the $CYC1_{ter}$. The expression of GFP-fusion is controlled by $MET25_{pro}$ that is strongly expressed in the absence of methionine in yeast medium. pEGFP-C-FUS has unique cleavage sites between the $MET25_{pro}$ and the GFP coding region, that enables fusion of GFP to the C-terminal end of coding regions. In frame fusion of the uncharacterized proteins to GFP was done by using MCS of pEGFP-C-FUS vector.

2.1.3.2 Primers

For the sequencing of the cloned gene fragments, the plasmid DNA was sent to the company MWG Biotech (Ebersberg, GER). The sequencing was done with the help of following primers.

Primer	**sequence 5'-3'**
M13 rev (-29)	CAG GAA ACA GCT ATG ACC
M13 uni (-21)	TGT AAA ACG ACG GCC AGT

2.1.3.3 Oligonucleotides and DNA Fragments

During this research, the oligonucleotides were obtained from the company Invitrogen™ life technology. Primer sequence contains different restriction sites for the cloning of the PCR products in different vectors.

Primer	**sequence 5'-3'**
N1 fwd	CGG GAT CCA TAT GTA TCC TAA ACA ATT TAG

N1-7	TTG GAT CCC AAT GCA GCA TTA GCA TCT AAA GCC TTG TC
N1-4	TTG GAT CCC AAT GCA AGA TTA AGA TC
N1-5	GCG TTT CAG CCG GCG GTT TTC TCT CAG TG
N1-6	CAC TGA GAG AAA ACC GCC GGC TGA AAC GC
N1b fwd	GGA TCA ACG AGA TCG ATG
N1b bck2	CTC ATC ACC TTC TCA ACT TC
N2 fwd	ACG CGT AGA AGA AGA TAA CGG
N2 bck	CTA ACG CTG TCT GGT TCC GGT
N3fwd	GGG GAT CCA TAT GGA CAG AGA CAG AAA GAG
N3bck	TTC CCG GGC TAC AGA GAA AGA TTC AAG TC
N3-5	CCA GAT CTG TCT AAA CAA ACG TTA GTC TCA GAT CC
N3-3	CAT GAA AAT GAA AAC TGC TGG CTG AAA CG
N3-2	CGT TTC AGC CAG CAG TTT TCA TTT TCA TG
N3-1	TTG GAT CCC AGA GAA AGA TTC AAG TC
N3-4	TTG GAT CCC AGA GAA GCA TTC GCG TCT AAA CAA ACG TTA GTC TC
G8-1/1	CCG GAT CCA TAT GGA CGG AGA GAA GAA GAG G
G8-1/2	AAG GAT CCG TCT CCG TTT CCT GGT AG
BP-1	TTG GAT CCA TAT GGA AGT GCA GAA ACG
BP-2	GGG GAT CCT TCG CAT ATC TTC TTT TCA TG
BP-3	GGG AGC TCC TAT TCG CAT ATC TTC TTT TCA TG
FS-1	TTG GAT CCA TAT GCC GCT AAT GGA GGG TG
FS-2	AAG GAT CCA ACG CCG TTA GTC TCT GG
AD-10-2	CCG GAT CCA TAT GCT ACT TAC TAT GGA CG
N2c-5	AAG AGC TCT TAG TCT CCG CCT TCT GG
NOS	CAT CGC AAG ACC GGC AAC AGG
35S	TCC TTC GCA AGA CCC TTC CT

2.1.3.4 DNA Standard

Lambda DNA digested with *Eco*RI and *Hind*III was used as a DNA size standard in the separation of DNA on 1% agarose gels. This lambda DNA comprises bands the following lengths:

21226 bp / 5148 bp / 4973 bp / 4268 bp / 3530 bp / 2027 bp / 1904 bp / 1584 bp / 1375 bp / 947 bp / 831 bp / 564 bp / 125 bp

A 100 bp marker from MBI Fermentas (# SM0241) was used as a size standard in the separation of small DNA fragments on a 1.5% agarose gel. 100 bp marker has the following lengths of DNA standards:

1031 bp / 900 bp / 800 bp / 700 bp / 600 bp / 500 bp / 400 bp / 300 bp / 200 bp / 100 bp / 80 bp

2.1.4 Proteins

2.1.4.1 Antibodies

2.1.4.1.1 At NIMIN3-Antiserum

For detection of the AtNIMIN3 protein, a polyclonal NIMIN3 antiserum (dilution 1:1000) was used. The antiserum was produced by immunization of rabbits with *E. coli* over-expressed and purified protein.

2.1.4.1.2 Nt NIMIN2a:MBP-Antiserum

For the detection of NIMIN2 and NIMIN2-related proteins a polyclonal antiserum was used at a dilution of 1:1000. The antiserum was produced by immunization of rabbits with *E. coli* over-expressed and purified protein.

2.1.4.1.3 Anti-rabbit IgG-HRP conjugate

For the detection of primary antibodies, a secondary anti-rabbit IgG antiserum, which is coupled to horseradish peroxidase, was used at a dilution of 1:10,000 in the blocking solution (Rockland, USA).

2.1.4.2 Enzymes

Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (NEB) and MBI Fermentas (St. Leon-Rot). Deviations from these sources are highlighted in the text. *Taq* DNA polymerase was supplied from MBI Fermentas and Genaxon (Ulm, GER).

2.1.4.3 Protein Standard

The pre-stained protein molecular weight marker (#SM0671, MBI Fermentas).

180 kDa / 130 kDa / 100 kDa / 70 kDa / 55 kDa / 43 kDa / 34 kDa / 26 kDa /
16 kDa / 10 kDa

2.1.5 Kits

2.1.5.1 Plasmid Preparation

For isolation of high-quality plasmid DNA, the GeneJET™ Plasmid Miniprep Kit (#K0503) purchased from MBI Fermentas was used. The kit was used for purification of plasmid DNA from o/n *E. coli* cultures on columns and obtained plasmid DNA was used for sequencing. Plasmid isolations were performed according to attached protocol.

2.1.5.2 DNA Elution from Agarose Gels

To elude embedded DNA fragments from agarose gels and from respective reagents and salts, the E.Z.N.A.^{TM} Gel Extraction Kit (Omega Bio-tek, USA) was used. Elutions were carried out according to attached protocol.

2.2 Methods

2.2.1 Preparation of Solutions

All solutions pH value were adjusted with diluted hydrochloric acid (HCl) or dilute sodium hydroxide (NaOH), otherwise indicated separately.

2.2.2 Standard Molecular Biology Methods

Standard methods in molecular biology were used according to the instructions of the supplier (e.g. kits and enzymatic reactions) and according to Sambrook and co-workers (Sambrook et al., 1989). Modified and new methods are mentioned in the text.

2.2.3 Bacterial Growth Medium

All culture mediums and stock solutions for bacterial growth were prepared as described by Sambrook and colleagues (1989).

Luria Bertani (LB) Medium

Recipe for 1 Liter
10 g Peptone
5 g Yeast extract
10 g NaCl
pH 7.5 with 1 M NaOH
ad 1 l H_2O

Agar Plates: 15g of micro-agar (Duchefa Biochemie, Haarlem, Netherlands) was added to one liter LB medium (before autoclaving). Thereafter, when the broth medium temperature dropped down to 50°C, selective LB medium plates were prepared by addition of respective amount of antibiotics.

Antibiotics concentration in liquid and Agar plates

Ampicillin (amp)	:	50µg/ml
Kanamycin (kan)	:	50µg/ml
Rifampicin (rif)	:	50µg/ml
Gentamycin (gent)	:	50µg/ml

Ampicillin

To prepare 50mg/ml stock solution, 2.5g of amp was mixed in 50ml of de-ionized H_2O. The solution was filtered using 0.2µm sterile filter (Nalgene, Hamburg, GER). Subsequently, one ml aliquots of stock solutions contained in 1.5ml eppendorf tubes were stored at minus 20°C.

Kanamycin

To prepare 25mg/ml stock solution, 1.25g of kanamycin was mixed in 50ml of de-ionized H_2O. The solution was filtered using 0.2µm sterile filter. Subsequently, one ml aliquots of stock solutions contained in 1.5ml vials were stored at minus 20°C.

Rifampicin

To prepare 50mg/ml stock solution, 2.5g of rifampicin was dissolved in 50ml of DMSO. The solution was filtered using 0.2µm sterile filter. Subsequently, one ml aliquots of stock solutions contained in 1.5ml reaction tubes were stored at minus 20°C.

Gentamycin

To prepare 50mg/ml stock solution, 2.5g of gentamycin was mixed in 50ml of de-ionized H_2O. The solution was filtered using 0.2µm sterile filter. Subsequently, one ml aliquots of stock solutions contained in 1.5ml tubes were stored at minus 20°C.

IPTG/X-Gal

IPTG (Isopropylthiogalactosid)
100mM (23.83mg/ml in water) stock solution (50 µl for 125ml medium)

X-Gal (5-Brom-4-Chlor-3-Indolyl-β-D-Galactopyranosid)
20mg/ml (2%) prepared in DMFA (250µl for 125ml)

IPTG and X-Gal are used in the blue-white selection of recombinant bacterial colonies. IPTG functions by binding to the *lacI* repressor and altering its conformation, which prevents the repression of the *lacZ* gene that codes for β-galactosidase. As a consequence X-Gal is spilt by β-galactosidase into blue dye plus galactose (see Sec. 2.1.3.1.1 for application).

Culturing of bacteria in LB liquid medium containing corresponding antibiotics was carried out at 37°C and 250 rpm overnight (o/n). The cultivation on solid medium with the appropriate antibiotics or additional X-Gal and IPTG was carried out o/n in the incubator at 37°C.

2.2.4 Transformation of *E. coli*

The *E. coli* has no competence nature, i.e., they are not able to accept naked DNA molecules from the environment. To enable the bacterial cells to take up circular vector DNA they have to be made competent using special treatments. Transformations of *E. coli* DH5α (strain) were performed as proposed by Inoue et al., (1990).

2.2.4.1 Preparation of Competent Bacterial Cells (DH5α)

DH5α cells from a frozen culture were streaked on an LB plate and incubated o/n at 37°C. A single colony was picked from the plate and cultured in 5 ml LB with 10mM $MgSO_4$ for two hrs at 37°C and 250 rpm. This culture was used to inoculate an Erlenmeyer flask containing 100 ml of LB medium with 10mM $MgSO_4$, and incubated for an additional two to three hrs at 37°C and 250 rpm. At a cell density of 0.5 at OD_{550}, the flask was placed on ice and the cells were spun down at 6000 rpm for 10 min at 4°C in cool centrifuge (Sigma 3K15, SIGMA Laborzentrifugen GmbH, GER). The supernatant was discarded and the cell pellet was re-suspended in 2 x 40 ml TfbI. After 5 mins incubation on ice, the centrifugation was repeated, the TfbI medium was discarded and the cells re-suspended in a total of 8 ml TfbII. 200 µl aliquots of the cells were dispended in pre-cooled reaction vials and frozen in liquid nitrogen. The cells could be stored (for up to six months) at a temperature of minus 70°C. All solutions and equipment used were sterile and cooled.

TfbI (sterile filtered)

30mM	KOAc
50mM	$MnCl_2$
100mM	KCl
10mM	$CaCl_2$
15%	Glycerin (w/v)

TfbII (autoclaved)

10mM	Na-MOPS pH 7
75mM	$CaCl_2$
10mM	KCl
15%	Glycerin (w/v)

2.2.4.2 Transformation of competent bacterial cells (DH5α)

100µl of ice thawed competent bacteria cells were mixed with 10 µl of ligation mixture and incubated for 30 min on ice. Subsequently, the transformation mixture was subjected to a heat shock (37°C) in a water bath for 5 mins and then immediately incubated for 2 mins on ice. After addition of 800 µl (37°C) warm LB liquid medium, the cells were incubated at 37°C and 250 rpm for 1 hr. 50 µl of the transformation mixture was plated out on an LB plate with

the appropriate selection antibiotic. The rest of transformed bacterial cells were subjected to sedimentation in a tabletop centrifuge (Sigma 112, SIGMA Laborzentrifugen GmbH, GER). Thereafter, the supernatant was discarded leaving behind approximately (appr.) 100 µl of residual supernatant. The sediment was re-suspended and plated on another selective plate. The incubation was practiced o/n at 37°C. Grown colonies were picked with sterile tips and incubated in 5 ml of LB medium for plasmid preparation (Sec. 2.2.5).

2.2.5 Plasmid Preparation from Bacterial Cells

For plasmid DNA isolation, 1.5 ml of each o/n culture was transferred to a 1.5 ml reaction tube and centrifuged (in a table top centrifuge for 2 mins) to pellet the cells. The supernatant was removed and the cells were re-suspended in 100 µl of solution 1 (ice cold). The cell suspension was incubated for 5 mins on ice. Thereafter, cells were lysed using 200 µl of solution 2. The suspension was neutralized with 150 µl of solution 3 (ice cold), mixed gently by inverting the tube several times. The vials were left on ice for 5 mins to precipitate proteins and genomic DNA of the bacterium and then centrifuged (15,300 rpm at 4°C for 10 mins). The supernatant was carefully transferred into a new reaction tube containing 1 ml of 96% (v/v) cold ethanol. The DNA was left to precipitate for at least 30 mins at minus 20°C. Plasmid DNA was collected by centrifugation for 3 mins at 15,300 rpm and 4°C. The supernatant was discarded and the sediment was re-suspended in 100 µl 1 x TE. The DNA was precipitated by adding 50 µl of 7.5 M ammonium acetate and 200 µl of 96% ethanol for at least 30 mins at minus 20°C. Then the reaction tube was again centrifuged as before. The pellet was washed with 200 µl of 70% (v/v) cold ethanol and dried for 10 mins using a vacuum centrifuge (speedvac; Bachhofer Laboratoriumsgeräte, Reutlingen, GER). The DNA was dissolved in 100 µl of 1xTE buffer. The storage of the plasmid DNA was carried out at minus 20°C.

A. tumefaciens cells are used to transfer the gene of interest embedded in a binary plasmid (pBin19). As plasmid preparation from *A. tumefaciens* cells is quite difficult compared with the preparation of *E. coli* cells due to the low plasmid copy number in *A. tumefaciens* and the defiance of the bacterial strain to cell lysis (Chen et al., 2003). To address these problems, the o/n grown culture was spun down 3 x 1.5ml in same vial to collect more cells and then procedure was followed as mentioned above for *E.coli*.

Solution 1		**Solution 2**
50 mM	Glucose	0.2 N NaOH
25 mM	Tris/HCl, pH 8.0	1% SDS
10 mM	EDTA, pH 8.0	
100 µg/ml	RNase A	

Solution 3		**1 x TE**
3 M	sodium acetate (NaOAc)	10 mM Tris/HCl, pH 8.0
pH 4.8 adjusted with *glacial acetic acid* (HAc)		1 mM EDTA, pH 8.0

2.2.6 Preparation of Stock Cultures

To store bacteria cultures for a long time, 850 µl of freshly grown o/n bacteria liquid culture was mixed with 150 µl of sterile glycerol and stored in a screwable 2 ml micro tube (SARSTED Aktiengesellschaft and Co., Nümbrecht, GER) at minus 70°C.

2.2.7 Sequence Specific Restriction of DNA

Plasmid DNA can be restricted with specific restriction enzymes, according to the instructions from the company using recommended restriction buffers. 2 µl of plasmid DNA were restricted in a final volume of 20µl. The DNA was digested for at least 2 hrs at the optimal temperature for the enzyme. If a double digestion was performed, NEB buffer activity chart for endonuclease was used to choose the best buffer for both enzymes. Double digestions were mostly incubated o/n.

2.2.8 Ligation of DNA Fragments

Ligation of DNA fragments into a selected plasmid was performed using the enzyme T4 DNA ligase, which catalyzes the formation of phosphodiester bonds between the free 5'-phosphate and 3'-hydroxyl of double-stranded DNA fragments and vectors. The insert DNA fragment (5x excess to the vector) was incubated with the vector DNA, 2 µl of ligation buffer and 1 µl of T4-DNA ligase for at least 5 hrs at 4 ˚C. The ligation mixture was then used directly for transformation into competent *E. coli* DH5α cells.

Reaction:
x µl Vector-DNA
y µl Insert-DNA
2 µl T4 10 x DNA-Ligase buffer
1 µl T4 DNA-Ligase 400 E/µl
ad 20 µl H_2O

2.2.9 Separation of DNA Fragments

To determine the size of DNA fragments, plasmid agarose gel electrophoresis was performed. The gel was prepared by dissolving agarose in 1x TAE. The concentration of the gel ranged between 1-2% depending on the size of the expected DNA fragments i.e., the smaller the fragment the higher the agarose concentration. To detect the DNA fragments, ethidiumbromide (EtBr: 10 µg/ml) was added to the agarose with TAE solution (EtBr final concentration of 0.3µl/ml) at gel temperatures below 55°C. The fractionating samples were mixed with 5 x loading buffer and applied to the gel. Electrophoretic separation was carried out at 80 to 100 Volts (V). In order to determine the fragment sizes, a suitable DNA ladder was loaded onto the gel as well. The visualization of the DNA was carried out under ultra violet (UV) light.

50 x TAE buffer	
242 g	Tris
100 ml	0,5 M EDTA, pH 8,0
57.1 ml	Acetic acid
ad 1 l	H_2O

5 x Loading buffer	
50 %	Glycerin
1 mM	EDTA
0.25 %	Bromphenol blue
0.25 %	Xylene cyanol

2.2.10 Elution of DNA Fragments from Agarose Gel

Gel-embedded DNA fragments were isolated using the E.Z.N.A™ Gel Extraction Kit (Sec. 2.1.5.2). For cloning procedures, first 40 µl of plasmid DNA were digested and then the resulting DNA fragments were separated by electrophoresis. Finally the DNA fragments were isolated with the aforementioned kit following the provided protocol and recommendations.

2.2.11 Estimation of Nucleic Acids Concentration

In order to use the purified DNA for sequencing, first the concentrations of nucleic acids had to be determined by measuring the absorbance of ultraviolet light by the samples. A 1:25 dilution of each sample was prepared and added to a quartz cuvette (Eppendorf UVette®,

GER), i.e., 2 µl of DNA solution (in 1xTE) was diluted with 48 µl of water. A cuvette containing 50 µl of water was used as a reference. Measurements were executed in the Ultrospec™ 3000 spectrophotometer (Pharmacia Biotech, Cambridge, UK).

The concentration of a nucleic acid in solutions is calculated according to the following formula:

Concentration = [(absor. λ 260 nm)-(absor. λ 320 nm)] x 50µg/ml x dilution factor

λ 320 nm = background absor. (Wavelength)
50µg /ml = Factor for dsDNA
Dilution factor= 1:25
absor. = absorbance at

2.2.12 Dephosphorylation of DNA Fragments

When a plasmid DNA (i.e. vector for cloning an insert DNA fragment) was linearized using a single restriction enzyme, a hydrolization of the 5´-phosphate residue (dephosphorylation) was performed using calf intestine alkaline phosphatase (CIAP) enzyme. CIAP enzyme prevents self-enclosure of the vector in a subsequent ligation reaction. The linearized vector was incubated with the CIAP enzyme for 15 mins at 37°C before DNA separation on agarose gel.

2.2.13 DNA Sequencing

To confirm the DNA sequence of a positive clone, plasmid DNA was isolated using GeneJET™ Plasmid Miniprep Kit (Sec. 2.1.5.1). Sequencing was carried out by the company Eurofins MWG Operon, Ebersberg, GER.

2.2.14 Polymerase Chain Reaction (PCR) amplification

Specific DNA fragments were amplified using PCR (Saiki et al., 1988; Bej et al., 1991) by means of thermally stable DNA polymerases (*Taq* polymerase) and synthetic oligonucleotides. Using PCR technique DNA fragments can be produced harboring e.g., mutations or introduced restriction sites. The reaction starts with the denaturation of two strands of a DNA template. The 5´ complementary strands of the denatured DNA were

recognized and hybridized with specific primers (annealing). By changing the annealing temperature and ionic concentration of Mg^{++}, specificity of the reaction can be increased. A *Taq* polymerase enzyme catalyzes elongation and the synthesis of new strands of DNA using the free 3´-OH end of the primer as a start point. All reaction mixtures (see below) were prepared on ice in 0.5 ml thin-walled PCR tubes (Thermowell™ tubes; Corning Incorporated, UK). The reactions were carried out in the programmable PCR cycler (Mastercycler® personal Eppendorf AG, Hamburg, GER) featuring temperature control by Peltier elements and heated lid. The cycler was programmed to suit the best conditions for each PCR reaction. PCR products were verified by agarose gels electrophoresis (Sec. 2.2.9). For cloning purposes, the electrophoretically separated PCR products were eluted from the gel matrix (Sec. 2.2.10).

Standard reaction mixture for PCR reactions:

Template DNA:	1 µl
10x PCR buffer:	2 µl
Forward primer (10 µM):	2 µl
Reverse primer (10 µM):	2 µl
dNTPs [10 mM]:	2 µl
$MgCl_2$ [25 mM]:	2 µl
Taq polymerase:	0.2 µl
ad 20 µl H_2O, nuclease-free:	8.8 µl

Program used for standard PCR reactions:

1 min	94°C	Denaturation
10 x Cycle:		
30 sec	94°C	Denaturation
30 sec	55-60°C	Annealing
1 Min. 30 sec	72°C	Elongation
20 x Cycle:		
30 sec	94°C	Denaturation
30 sec	55-60°C	Annealing
90 sec +10 sec/Cycle	72°C	Elongation
7 min	72°C	Final Elongation
∞	08°C	Hold

2.2.15 PCR Site Directed Mutagenesis

In the PCR site-directed mutagenesis a mismatched oligonucleotide is extended to incorporate a mutation into DNA which can be cloned afterwards (Ho et al., 1989). In this

study, the PCR site-directed mutagenesis was used to create point mutations to define the role of different amino acid residues or domains in a protein. The point mutations were introduced using the PCR overlap extension method. In the overlap extension method two separate PCRs were conducted to amplify two PCR fragments (PCR1 and PCR2). Each PCR reaction uses one flanking primer that hybridizes at one end of the target sequence and one internal primer that hybridizes at the site of the mutation and contains the mismatched nucleotide bases. Finally, a third PCR (PCR3) was conducted using the two flanking primers that hybridize at each end of the target sequence and generate DNA fragments from the products of PCR1 and PCR2.

2.2.16 Screening Bacterial Colonies Using PCR

To verify the presence of positive clones among number of bacterial colonies (*E. coli* or *A. tumefaciens*) on growth plate, a PCR reaction was conducted using the cell lysate as a DNA template. Bacteria cells were picked up with yellow tips from a single colony and were streaked onto a labeled sector on a new plate with selection antibiotics. The rest of the bacteria on the tip were suspended in PCR tube containing 10µl of sterile water. Three colonies were re-suspended into the same PCR tube but streaked in different sectors on the selection plate (using new and sterile tip for each). The plate was incubated o/n at 37°C (*E. coli*) or 30°C (*A. tumefaciens*). In a PCR cycler the bacterial cell walls were broken and DNase molecules were inactivated, which otherwise would clip the DNA template. The bacterial cell walls were broken by subjecting them to the conditions written below.

5 min	96 °C
90 sec	50 °C
90 sec	96 °C
1 min	45 °C
1 min	96 °C
1 min	40 °C
∞	08 °C

Subsequently, a PCR mixture was added to the cell lysate and a PCR reaction was carried out (Sec. 2.2.14). In order to confirm the positive clone primer pairs, based on the flanking region of the insert or the vector were used. PCR reactions were loaded on agarose gel in order to check the amplified fragments. Samples with desired fragment size were grown o/n in liquid LB medium for DNA isolation to ensure the right clone.

2.2.17 Cloning of PCR Products

The DNA molecule amplified using the *Taq* Polymerase is characterized by the presence of additional dA at the 3´-end of the PCR product, which is due to non-template dependent terminal transferase activity of the *Taq* polymerase enzyme. PCR product with the 3´-dA overhangs can be cloned to a vector having complementary 3´-dT, e.g., TA vector (Sec. 2.1.3.1.1).

2.2.18 Standard Protein Biochemical Methods

2.2.18.1 Protein Concentrations Determination

Protein concentration was estimated by a colorimetric assay (Bradford, 1976). Protein determination was carried out as mentioned below.

798µl	H_2O
2µl	protein solution
200µl	Bradford reagent

The solution was mixed well, incubated for 5 mins at room temperature, and then the absorbance was measured at OD_{595} in a spectrophotometer. 200 µl Bradford reagent in 800 ml water was used as reference.

2.2.18.2 Denaturing SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In SDS-PAGE, proteins are separated largely on the basis of polypeptide length. The electrophoresis of the protein was done using a discontinuous buffer system, in which a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel (Laemmli, 1970). Separation gel makes up two thirds of the gel. The gel was poured between a glass plate and an aluminum plate, separated by two 1mm thick spacers. The construct was held together by several metal brackets. The gel cassette was sealed with 1 % agarose at the bottom and sides. The separating gel was first prepared. This consists of a volume of solution 1, together with two volumes of solution 2 and a volume of solution 3. As soon as the solution 3 was added, the gel needs to be poured rapidly because of the polymerization activity of solution 3. After casting separating gel, the gel was immediately overlaid with 1000µl of water in order to obtain a flat end surface of the separation gel. After

complete polymerization, the water was sucked off with a filter paper and resolving gel was poured. This is composed of a volume of solution 4, solution 5, de-ionized water and solution 3. After addition of solution 3, the gel needs to be poured rapidly and a 10-well teflon comb is inserting that molds the wells as the gel polymerizes. After the gel was polymerized, the gel wells were marked on the glass plate and the teflon comb was removed. The gel was clamped in the protein gel electrophoresis apparatus (Mighty Small II SE250/SE260, Hoefer Scientific Instruments, San Francisco, USA) and the apparatus was filled with 1x electrode buffer.

20µl of protein samples from plant extracts were mixed with 4µl of 5 x loading buffer and 1µl 20 x reducing agent (2M DTT). Proteins were denatured for 5 mins at 95 °C and then loaded on the gel. The pre-treatment of yeast extracts for SDS-PAGE is listed under Sec.2.2.21.6. To estimate the molecular weight of the protein via immunodetection, the standard size prestained protein marker (Sec. 2.1.4.3) was loaded in parallel with the protein samples. The protein samples were separated at 40mA/gel. After the gel electrophoresis the separated proteins were analyzed by immunodetection (Sec. 2.2.18.4).

1 x SDS Loading buffer

20 µl	5 x SDS Loading buffer
75 µl	H_2O
5 µl	20 x DTT (2M reducing agent)

5 x SDS Loading buffer (MBI Fermentas, St.Leon-Rot)

313 mM	Tris / HCl pH 6.8
10 %	SDS
50 %	Glycerin (w/v)
0.05 %	Bromphenol blue

20 x DTT 2M (MBI Fermentas, St.Leon-Rot)

2.2.18.3 Western Blot Analysis: Wet Transfer of Proteins onto a Nitrocellulose Membrane

After SDS-PAGE the proteins could be either stained by coomassie staining solution or transferred to a nitrocellulose membrane (porablot NCP, Macherey-Nagel GmbH, GER). For the specific detection, proteins were transferred to a nitrocellulose membrane and detected with specific antibodies (primary antibody). Another incubation of nitrocellulose membrane with secondary antibody coupled with the enzyme horseradish peroxidase (HRP) enables the protein antibody complex detection due to the peroxidase activity of HRP in a

chemiluminescence reaction. The transfer of proteins from the SDS-PAGE gel onto nitrocellulose membranes was performed using the tank electro blotting method. After the electrophoresis, the stacking gel was removed and the resolving gel was wetted with the transfer buffer.

A blotting sandwich was constructed, in which the gel was placed on two wetted Whatman filter papers (7x10 cm). Then a wet nitrocellulose membrane (6x9 cm) was placed above the gel. A second sheet of two moistened Whatman filter papers were placed on top of the Nitrocellulose membrane and any trapped air bubbles were removed by rolling a glass tube on the sandwich. The sandwich was placed between two layers of sponges on each side. The construct was placed between a western transfer cassette and compressed with a cassette locker (clip) to ensure uniform (continues) membrane contact with the gel. The assembled western transfer cassette was inserted into the blotting apparatus that was filled with transfer buffer. Proteins were transferred from the SDS-PAGE gel to the nitrocellulose membrane for at least 4 hrs or o/n using a constant voltage (50 V).

Solution 1
18.3 g Tris
115 µl TEMED
ad 100 ml H_2O
pH 8.9 (Storage at 4°C)

Solution 2 for 10% Gel
20 g Acrylamid
0.5 g Methylenbisacrylamid
0.2 g SDS
ad 100 ml H_2O (Storage at 4°C)

Solution 2 for 15% Gel
30 g Acrylamid
0.75 g Methylenbisacrylamid
0.2 g SDS
ad 100 ml H_2O (Storage at 4°C)

Solution 3
0.6 g Ammonium persulfat
ad 100 ml H_2O (Storage at 4°C)

Solution 4
6.1 g Tris
230 µl TEMED
ad 100 ml H_2O
pH 6.8 (Storage at 4°C)

Solution 5
12 g Acrylamid
0.3 g Methylenbisacrylamid
0.4 g SDS
ad 100 ml H_2O (Storage at 4°C)

10 x Electrode buffer
30 g Tris
144 g Glycin
ad 1 l H_2O

1 x Electrode buffer
100 ml 10 x Electrode buffer
900 ml H_2O
1 g SDS

Transfer buffer

4.9 g Tris	20 mM Tris (final concentration in 2 l)
22.6 g Glycin	150 mM Glycin (final concentration in 2 l)
400 ml	20 % Methanol
ad 2 l	H_2O

2.2.18.4 Immuno-detection of Proteins with Specific Antibodies

After transfer of the proteins onto the nitrocellulose membrane (Sec. 2.2.18.3), the membrane was taken from the cassette and briefly air dried. The non-specific protein binding capacity of the nitrocellulose membrane was blocked by incubating in blocking solution (appr. 25ml) at 4°C for 2 hrs or o/n under continuous shaking. Then, the membrane was washed with TTBS and incubated with primary antibody solution for 2 hrs at room temperature under continues (gentle) agitation. After hybridization, the membrane was washed three times for 10 mins with TTBS and one time with TBS. The membrane was incubated for two hr with the secondary antibody (linked with HRP conjugate) at room temperature under gentle agitation. After the second hybridization the membrane was washed again three times with TTBS and one time with TBS before Enhanced Chemiluminescence (ECL) solution could be applied. For ECL the membrane was placed on a flat surface, covered with 2ml of ECL substrate solution for two minutes, mixed according to the manufacturer's instruction. Afterwards, the membrane was wrapped in plastic foil, an x-ray film was laid on top and was exposed for 2 mins to a few hours (depending on the strength of the reaction) and finally developed.

TBS

100 ml	5M NaCl
20 ml	1M Tris/HCl, pH7.5
ad 1 l	H_2O

TTBS

0.5 ml	Tween 20
ad 1 l	TBS

Blocking solution

5 % skim milk powder
Dissolved in TTBS

Antibody solution:

Diluted 1:500 – 1:10,000 fold (depending on the antibody) in blocking solution
Diluted IgG-HRP conjugate 1:10,000 fold in blocking solution

ECL – Solution

Solution A
200 ml 0.1 M Tris / HCl pH 8.6
50 mg luminal

Solution B
11 mg p-coumaric acid
ad 10 ml DMSO

H_2O_2 (30 %)

2.2.19 Transformation of *A. tumeficians* via TPM

Triparental mating was used to transfer binary vector DNA construct from *E.coli* to Agrobacterium (Bevan, 1984). This conjugation is possible by the use of helper plasmid pRK2013 (Sec. 2.1.3.1.3). From the stock culture, Agrobacterium strain LBA4404 (Hoekema et al., 1983) was grown in 5 ml MinA medium (50 µg/ml rif) and incubated for 2 days at 30 °C and 250 rpm. One day after inoculation of Agrobacteria the *E. coli* strain MM294 with helper plasmid pRK2013 and *E. coli* strains with respective binary vector constructs were inoculated for o/n cultivation in 5 ml of LB medium (50 µg/ml kan) at 37 °C in the shaker. Every culture was centrifuged for 5 mins in tabletop centrifuge to pellet the cells. After centrifugation, the *E.coli* strains were suspended in 5 ml of sterile 10 mM $MgSO_4$ and the Agrobacterium cells in 1 ml of sterile 10 mM $MgSO_4$. 50 µl of each bacterial suspension was pipetted in the middle of a LB plate (without antibiotics), mixed together and spread carefully. As negative control, Agrobacteria were mixed with helper strain only. The LB plates were incubated o/n at 30 °C. Next day the bacterial cells were harvested from the plate by using 1 ml of the LB medium. The suspension was diluted to 1:1000 and 50 µl were spread onto selection medium. The plates were incubated at 30 °C for 3-5 days and then the single colonies were picked for liquid cultures. The negative control yields very weak growth of bacteria only. After binary plasmid trabsfer, Agrobacterium could be used directly either for stable integration of genes into the plant genome or for transient gene expression system (TGES; Sec. 2.2.20.3). For restriction analysis Agrobacterium clones were grown in 5 ml of MinA medium (with kan and rif or kan, rif and gent for strain LBA4404 or GV3101 respectively) at 30 °C and 250 rpm for 2-3 days. 3-4 ml of culture were used accordingly to obtain plasmid DNA (Sec. 2.2.5).

5 x MinA salts

52.5 g	K_2HPO_4
22.5 g	KH_2PO_4
5.0 g	$(NH_4)_2SO_4$
2.5 g	Sodium citrate x 2 H_2O
ad 1 l	H_2O

MinA medium

1 ml	5 x MinA-Salts
4 ml	H_2O (steril)
5 µl	20 % $MgSO_4$ x 7 H_2O (steril filtrate)
50 µl	20 % Glucose (steril filtrate)

MinA plates

MinA medium with1.5 % Agar
$MgSO_4$, glucose and antibiotics were added after autoclaving.

2.2.20 Plant Work

2.2.20.1 Plant production and Growth in Greenhouse

Production of *N. benthamiana* and *N. tabacum* plants were carried out under green house conditions i.e. with 16hr/day natural or artificial light and the temperature between 22-28 °C. The seeds were sown in small trays containing moist soil (Plantaflor® 701 Aussaat-und-Anzuchtsubstrate, GER). After germination, the small seedlings were transferred in pots containing autoclaved soil.

2.2.20.2 Isolation of Plant Genomic DNA

The extraction of genomic DNA from plant leaf tissue was performed as reported by Fulton (Fulton et al., 1995). Two discs of about 1 cm diameter were finely grinded (50-100 mg) with 200 µl freshly prepared microprep buffer in a reaction tube. Thereafter, another 550 µl microprep buffer was added and mixed gently. The suspension was incubated for 30-120 mins at 65 °C in water bath with occasional inversion of tubes. Subsequently, the reaction tubes were filled with 750 µl chloroform:iso amyl alcohol (24:1), mixed well and centrifuged at in tabletop centrifuge for 5 mins. Upper water phase (0.5ml) was transferred in a new reaction vial and was mixed with equal volume of cold isopropanol, until the DNA precipitate became visible. Once the DNA is precipitated, the vials were immediately centrifuged for maximum 5 mins in tabletop centrifuge. DNA pellet was washed with 70 % Ethanol and air dried. The dried DNA pellet was dissolved in 50 µl of 1x TE and incubated at

65 °C for 15 mins for better resuspension. Plant DNA was stored at minus 20 °C and 1 µl of the genomic DNA was used in a standard PCR reaction (Sec. 2.2.14).

DNA- Extraction buffer

0.35 M	Sorbitol
0.1 M	Tris
5 mM	EDTA pH 7.5

Cell Lysis buffer

0.2 M	Tris
50 mM	EDTA pH 7,5
2 M	NaCl
2 % (w/v)	CTAB (Hexadecyl-trimethylammoniumbromid)

Sarkosyl solution

5 % (w/v)	Natrium-N-Lauroylsarcosin

Microprep buffer

2.5 Volume	DNA-Extraktionspuffer
2.5 Volume	Lysepuffer
1 Volume	5% Sarkosyl
0.3 – 0.5 g	Natriumbisulfite ($NaHSO_3$) /100 ml

2.2.20.3 Agrobacterium Mediated Transient Assay System in *N.benthamiana*

The -*1533PR-1a*$_{pro}$:*GUS* reporter gene construct (Grüner and Pfitzner, 1994) was stably integrated into the genome of *N. benthamiana*. Primary transformants exhibiting strong induction of the *GUS* reporter gene and endogenous PR-1 protein induction in response to SA were selected. One typical line (3 GUS units uninduced and 1100 GUS units after SA treatment) was propagated (U.M. Pfitzner personal communication). Four to six week old T^2 generation plants were used in TGES. Agro-infiltrations in reporter line plants were carried out according to the protocol modified from Morilla et al., (2006). Agrobacterium strains LBA4404 or GV3101 containing a binary vector (pBin19) with the desired gene for expression were incubated in 5 ml MinA medium containing required antibiotics. Agrobacteria were grown at 30 °C for two to three days at 250 rpm and then OD_{600} was determined using spectrophotometer. The cells were centrifuged for 3 mins and pelleted cells were resuspended in 10 mM $MgCl_2$ and 150 µM of acetosyringone to an OD_{600}=0.5. Thereafter, cell suspensions were incubated for at least 2 hrs at room temperature. The cell suspensions were infiltrated on the lower side of 4-6 week old *N.benthamiana* plant leaves with the help of 1 ml needleless syringe (Omnifix®-F Braun, Melsungen, GER). To suppress

the post-transcriptional gene silencing, the bacterial suspensions were mixed with an equal volume of a strain carrying the CarMV CP (coat protein) or p19 silencing suppressors from *Carnation mottle virus* and *Tomato bushy stunt virus* adjusted to OD_{600}=0.5, respectively (Voinnet et al., 2003). Leaf samples were collected at 4-5 dpi for the reporter gene induction.

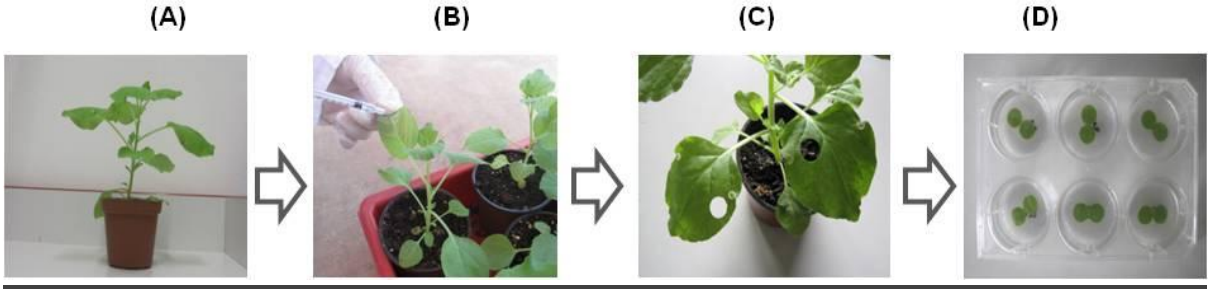


Fig. 3: Illustration of transient expression assay. **A)** *-1533PR-1a$_{pro}$:GUS N. benthamiana* reporter line plants. **B)** Infiltration of Agrobacterium strains harboring *35S$_{pro}$:NIMIN* constructs. **C)** Harvest of leaf disks. **D)** Floating of leaf disks.

2.2.20.4 Induction of Reporter Gene Expression by SA Stimuli

Two to four leaf discs were harvested from the agroinfiltrated areas of *N. benthamiana* reporter line plants with a cork borer (Ø 0.5 cm). Leave discs were floated in petri dishes on H_2O and 1 mM SA for two days. The induction of *-1533PR-1a$_{pro}$:GUS* reporter gene was carried out on 1 mM SA solution. Incubation on water serves as a negative control. After reporter gene induction, leaf discs were air dried (for few mins) on multiple layers of blotting papers and protein extraction was conducted (Sec. 2.2.20.5).

2.2.20.5 Protein Extraction from *N.benthamiana* Plants

For protein extraction from *N. benthamiana*, air dried leaf discs were macerated in 150µl of GUS lysis buffer with plastic pestle loaded hand drill (Robert Bosch, GmbH, Echterdingen, GER). Cell lysate was centrifuged for 10 mins at 4 °C and 15,300 rpm. The protein containing supernatant was transferred to a new reaction tube and centrifuged again for 10 mins under the same conditions. Thereafter, the supernatant was again transferred to a new tube and used for the GUS enzyme assay (Sec. 2.2.20.7) or for immunodetection of proteins (Sec. 2.2.18.3). The protein extracts were stored at minus 70 °C.

GUS-Lysepuffer

50 mM	Na-Phosphate buffer pH 7.0
10 mM	EDTA
0.1 %	Triton-X 100 (w/v)

0.1 %	Lauryl sarcosin (w/v)
10 mM	β-Mercaptoethanol

Na-Phosphate buffer, 1 M Stock solution pH 7.0

57.5 ml	1 M disodium hydrogen phosphate
42.3 ml	1 M sodium-dihydrogen phosphate4

2.2.20.6 DAB (3,3- Diaminobenzidin) staining

N. benthamiana leaves were cut from the stem with a sharp sterilized blade and photographed under bright light using Canon, Digital IXUS 95 IS camera. Thereafter, leaves were treated with 3,3- diaminobenzidin (20 mg DAB in 20 ml MES-buffer, pH 6.5, freshly prepared) in a 50 ml syringe. Vacuum was created three times with the help of syringe pestle without damaging the leaf tissues to facilitate the infiltration of the staining solution into the leaf. The leaves were incubated for either 30 min in sunlight or 4 hrs to o/n in dark. The DAB stained leaves were de-stained with a 4:1 solution of ethanol and acetone, under continuous agitation. Finally, de-stained leaves were photographed to record H_2O_2 accumulation.

2.2.20.7 *GUS* Reporter Gene Assay

To determine the GUS reporter gene activity in plant extracts (from *N.benthamiana* reporter line) a modified GUS assay was utilized (Jefferson et al., 1987). The GUS assay allows the quantification of β-glucuronidase enzyme activity using 4-methylumbelliferyl-ß-glucuronid (4-MUG) as a substrate. The catalytic activity of GUS enzyme will convert the 4-MUG substrate into 4-methylumbelliferon (MU) and glucuronic acid. The fluorescent MU can be estimated using a fluorometer (Spectraflour Tecan, Crailsheim, GER) in microtiter plate (96 wells; Costar Bodenheim, GER). Enzyme assays were prepared as follows:

20 µl	protein extract
70 µl	GUS lysis buffer
10 µl	10 mM MUG solution

Samples were mixed carefully in mentioned order and incubated at 37°C for 30-60 mins. Substrate conversion was checked intervally after every 15 mins under UV light to visually evaluate fluorescence intensity. The reaction was stopped by adding 400 µl of 0.2M Na_2CO_3 solution and assays were kept cool afterwards. The quantification of MU fluorescence was determined using fluorometer. MU has an excitation maximum at a wavelength of 365 nm and an emission maximum at a wavelength of 455 nm. To quantify obtained data a

calibration curve was created. The calibration curve was established by using increasing concentration of MU in 0.2M Na_2CO_3 (from 0 to 10,000 pmole MU). 250µl of protein or calibration samples were applied on microtiter plates for recording the readings. Measurement parameters are detailed below:

Excitation filter:	360 nm
Emission filter:	455 nm
Shaking:	5 seconds
Gain:	60
Number of flashes:	3
Start of integration:	0 µs
Time of integration:	40 µs

The fluorescence values with the help of calibration curve, the incubation time of the enzyme assays and the protein determination (Sec. 2.2.18.1) were converted in GUS enzyme activity (pmol MU/ug protein x hr).

Stop solution
0.2 M disodium carbonate (Na_2CO_3)

10 mM MUG stock solution
0.35 g 4-MUG in 100 ml GUS-Lysis buffer

MU stock solution
100 µM MU in 96 % ethanol
Stored in dark at minus 20°C

2.2.21 Working with Yeast (*S. cerevisiae*)

2.2.21.1 Molecular Biological Methods for Yeast (*S. cerevisiae*)

Most of the listed molecular methods are described in the yeast protocol handbook from Clontech, Heidelberg, GER.

2.2.21.2 Yeast Media

Yeast extract- peptone- adenine -dextrose (YPAD) medium

19 g	peptone
4.7 g	yeast extract
37.9 mg	adenine sulfate
ad 900 ml	H_2O

pH adjusted to 5.8 with 1M KOH and autoclaved

After autoclaving 100 ml of a sterile 20% glucose solution was added. For agar plates, 1.5 % agar was added before autoclaving (3.75g in 250 ml)

SD medium

0.4 g yeast nitrogen base without amino acids
1.2 g $(NH_4)_2SO_4$ (ammonium sulfate)
ad 200 ml H_2O
pH adjusted to 5.8 with 1M KOH and autoclaved

After autoclaving, 25 ml of the corresponding drop-in solution and 25 ml of sterile 20% glucose solution were added. For solid medium, 3.75 g of agar was added to 250 ml of medium. This minimal medium was used to select for plasmids in yeast.

20 % glucose solution
20 g D-(+)-glucose
ad 100 ml H_2O
The solution was sterile filtrated with a 0.2 µm syringe filter.

100 x Drop-in-solutions

L-adenine	100 mg/50ml
L-histidine / HCl monohydrate	100 mg/50ml
L-leucine	500 mg/50ml
L-lysine / HCl	150 mg/50ml
L-methionine	100 mg/50ml
L-tryptophan	100 mg/50ml
L-uracil	100 mg/50ml

The stock solutions were prepared in de-ionized water. All of amino acids are readily dissolved by stirring except uracil which needs heating. For 10 x Drop-in-solution the above mentioned stocks were diluted 1:10. For selection of the desired plasmid and the genotype, 10 x drop-in solution containing only the essential amino acids was added to the autoclaved SD medium.

2.2.21.3 Cultivation of Yeast Cultures

Yeast cells were cultured at 30 °C and liquid cultures were shaken at 250 rpm. The yeast strains used (Sec. 2.1.2.2) are auxotrophic for certain components essential for their metabolism. That is why they cannot grow on minimal medium lacking adenine, histidine, lysine, leucine, tryptophan or uracil. Through the transformation of plasmids which complement the auxotrophy, foreign genes cloned in the same plasmids can be simultaneously introduced into the yeast cells. The complementation of auxotrophy in yeast works like the antibiotic resistance selection markers in *E. coli*.

2.2.21.4 Preparation of Competent Yeast Cells (HF7c)

For the preparation of competent yeast cells a 5 ml culture was grown o/n in YPAD medium. The culture was centrifuged in tabletop centrifuge for 10 mins and the cell pellet was resuspended in 500µl of the supernatant. The cell suspension was used to inoculate 100 ml of YPAD medium. The culture was incubated at 30 °C and 250 rpm until an OD_{600} of 0.6. The cells were harvested by centrifugation in tabletop centrifuge for 5 mins and washed in 20 ml of solution A. After another centrifugation step, the cell pellet was resuspended in 2 ml of solution A and slowly cooled to minus 70 °C in 100 ul aliquots. The cells can be stored at minus 70 °C for several months.

Solution A (stored at 4°C)
10 mM Bicine
1 M Sorbitol
3% Ethylenglycol (w/v)
pH 8.35 with 1 M KOH

2.2.21.5 Transformation of Yeast Cells

The yeast cells were transformed with plasmid DNA using Dohmen et al., (1991) protocol. For yeast transformation, a mixture consisting of 5 µl of salmon sperm DNA carrier (denatured at 90 °C for 5 mins and cooled on ice) and 3 µl of DNA plasmids was added to the frozen competent cells. The mixture was incubated for 3 mins at 37 °C and the thawed cells were vortexed briefly before adding 1 ml of solution B. The suspension was mixed thoroughly and incubated for 1 hr at 30 °C. Cells were harvested by centrifugation for 20 sec in tabletop centrifuge and washed carefully with 800 µl solution C. Cells were centrifuged again for 20 sec in tabletop centrifuge and resuspended in 100µl of solution C. Thereafter, plated on yeast media (SD with appropriate drop-in-solution) and grown for 3-4 days at 30 °C.

Solution B
200 mM Bicine
40% Polyethylenglycol (w/v)
pH 8.35 with 1 M KOH, autoclave

Solution C
10 mM Bicine
150 mM NaCl
pH 8.35 with1 M KOH, autoclave

Salmon sperm DNA (carrier DNA), 10 mg/ml in TE

The solution was treated with ultrasound (breaking DNA into fragments) until its viscosity allowed a simple pipetting. The carrier DNA was incubated for 10 mins at 90 °C and then quenched for 10 mins on ice before use. The carrier DNA was stored at minus 20 °C.

2.2.21.6 Protein Extraction from Yeast Cells

Extraction of protein from yeast cells was carried out from a single colony. The colony was grown in a 5 ml liquid minimal medium and shaken o/n at 30 °C and 250 rpm. Then the culture was centrifuged for 5 mins at 5000 rpm and 4 °C in a 1.5 ml reaction vial. The cell pellet was washed with 1 ml of sterile pre-cooled water, and centrifuged again as before (cells were kept on ice). The cells were frozen for at least for 1 min in liquid nitrogen and were either processed immediately or stored at minus 70 °C. For analysis on SDS-gel, the cell pellet was re-suspended with 100 µl 1 × SDS loading buffer + 20 x 2M DTT and cooked for 5 mins at 95 °C in heating block. Samples were again centrifuged for 5 mins at 5000 rpm to precipitate the cell debri. The supernatant containing the proteins was transferred to a new reaction tube and analyzed via SDS-PAGE (Sec. 2.2.18.2). 20 µl of the supernatant was applied onto SDS gel. The remainder was stored at minus 20 °C.

2.2.21.7 Subcellular Localization Studies

To determine the sub-cellular localization in yeast green fluorescence protein (GFP) protein is used as reporter gene. GFP absorbs light with an excitation maximum at λ of 395 nm, and fluoresces with an emission maximum at λ of 510 nm (Morise et al., 1974; Ward et al., 1980). The cDNA of the protein to be analyzed for sub-cellular localization was cloned to the 5´ end of GFP reporter gene (resulting in translational fusion with GFP) in pEGFP C-FUS vector (Sec. 2.1.3.1.8). The plasmid constructs were subsequently transformed in yeast strain Cen.PK. The yeast strain Cen.PK has *ura3*-phenotype which is complemented by the *URA3*-gene from the pEGFP C-FUS vector. The transformed yeast cells were cultivated for 3-4 days on medium without uracil. The colonies were taken and grown in liquid SD-medium without uracil and methionine. Cultured cells were used for fluorescence microscopy (Sec. 2.2.21.8).

2.2.21.8 Fluorescence Microscopy

Transformed Cen.PK2 yeast cells were mounted in water and viewed with a Nikon Eclipse TS100 microscope (Nikon GmbH, Düsseldorf, GER). yEGFP was visualized with a filter block limiting fluorescence excitation in the range 450–490 nm and allowing low-pass emission detection beyond 515 nm. Fluorescence and bright field images were captured at x 200 magnification with an Olympus C7070 camera (Olympus Imaging Europa GmbH, Hamburg, GER). The images were merged and processed in software Adobe Photoshop.

2.2.21.9 The Yeast two Hybrid System for the Characterization of Protein-Protein Interactions

The Y2HS is a sensitive *in vivo* assay used for the detection of specific protein-protein interactions (Fields and Song, 1989). The Y2H analysis depends on modular structure of transcription activator proteins. Most of these proteins contain two domains the DNA-BD that mediates binding of the transcription factor to gene promoters by sequence specific DNA recognition and activation domain (AD) that recruits the transcriptional apparatus to the gene for mRNA production (Coates and Hall, 2003). The system used in this work is the MATCHMAKER two-hybrid system from Clontech (Heidelberg, GER). It is based on the yeast GAL4 transcription factor, the functional domains GAL4-BD and GAL4-AD are encoded by the plasmids pGBT9 and pGAD424, respectively. A cDNA can be fused to the sequence encoding DNA-BD of the GAL4 transcription factor contained in pGBT9, while the second cDNA can be fused to the sequence encoding GAL4-AD contained in pGAD424 vectors. Both plasmids were transformed into yeast strain HF7c (Sec. 2.1.2.2). In case of an interaction between the two inserted proteins, DNA-AD and DNA-BD come in close physical proximity and result in reconstitution of GAL4 transactivator. In yeast strain (HF7c) this reconstitution of the GAL4 transactivator ultimately results in GAL4 dependent reporter gene activation. As a reporter gene *HIS3* is used. When there is an interaction between the two GAL4 fusion proteins this results in the expression of the *HIS3* gene. Therefore, HF7c yeast cells expressing reporter gene will be able to make histidine and hence to grow in media lacking histidine. The genome of yeast strain contains another GAL4 regulated *lacZ* reporter gene which allows the quantitative measurement of the interaction based on the beta-galactosidase activity (Sec. 2.2.21.10).

2.2.21.10 The Yeast three Hybrid System for the Characterization of Protein-Protein Interactions

Several modifications of Y2HS have been established recommending the system for several different approaches (Brachmann and Boeke, 1997; Serebriiskii et al., 2001). For instance, the Y3HS makes it possible to investigate ternary protein complex formation by allowing the expression of three proteins together. In Y3HS, in addition to the two proteins fused to the activation and binding domain of the GAL4 transcription factor (Protein A and B) a third protein (Protein C) is additionally expressed from additional cassette in the binding domain vector (Tirode et al., 1997). Expression of the third protein is controlled by a $MET25_{pro}$ which can be regulated by methionine. In the presence of methionine in the medium, the $MET25_{pro}$ is repressed, however, in the absence of methionine, the gene under control of the $MET25_{pro}$ is induced. Simultaneous binding of protein A and B to the third protein C result in bringing the two functional domains of the GAL4 transcription factor in close proximity to each other as in Y2HS. The GAL4 transactivator is reconstituted and thereby results in the expression of reporter genes in yeast (HF7c). The GAL4-BD vector used in the Y3HS is designated is pBD-/ - (Sec. 2.1.3.1.7).

2.2.21.11 Quantitative Test of Protein-Protein Interactions in Yeast

The *lacZ* reporter gene is used for a quantitative measurement of the interactions in the above-mentioned yeast systems. The ß-galactosidase activity is proportional to the binding affinity of the two or in the case of the Y3HS system three interaction partners. The compound o-nitrophenyl-beta-D-galactopyranoside (ONPG) serves as a substrate for the enzyme β-galactosidase. The β-galactosidase hydrolysis the colorless substrate ONPG into the coloured o-Nitrophenole substance.

For this purpose, a single colony was inoculated in 5 ml in minimal medium with the appropriate drop-in solution. The yeast culture was grown o/n at 30 ˚C and 250rpm to an OD_{600} of 1.0 -1.5. For each interaction test 3 independent co-transformed yeast colonies were grown in liquid culture. From each culture 2 x 700 µl were used for assay as a replicate approach. The cells were centrifuged and the sediment was resuspended in 100 µl of Z-buffer in a 1.5 ml vial. Cells were flash-frozen for 1 min in liquid nitrogen and then thawed at 37 °C in a water bath (freezing and thawing is repeated thrice to disrupt the cells). Subsequently 700 µl of Z-buffer solution containing mercaptoethanol (2.7ml mercaptoethanol/l Z-buffer, stored

at 4 °C) and 160 µl ONPG (4mg/ml ONPG in Z-buffer, freshly prepared) substrate solution were added into the reaction tube. The mixtures were incubated at 30 °C and 250 rpm. A mixture without cells was performed in parallel as a blank sample. The expression of the *lacZ* reporter gene leads to the conversion of the substrate to a yellow product. Incubation could be from several mins to 4 hrs depending on enzyme activity. The color reaction is stopped with 400 µl 1 M Na_2CO_3. Sediment cell debris by centrifugation and transfer the supernatant to 1 ml plastic cuvette and measure OD_{420} in spectrophotometer.

The enzyme activity of beta-galactosidase is calculated as follows (Miller, 1972):

ß-Galactosidase units = $1000 \times OD_{420} / (t \times V \times OD_{600})$
t = incubation time
V = 0.7 ml (volume of cell suspension)
OD_{600} = cell density of o/n culture

The values obtained from three independent colonies are calculated as average of the interaction strength as well as serves as the standard deviation of the interaction.

Z-Puffer

60 mM $Na_2HPO_4 \times 7\ H_2O$
40 mM $NaH_2PO_4 \times H_2O$
10 mM KCl
1 mM $MgSO_4$
pH 7.0, autoclave
2.7ml mercaptoethanol/l Z-buffer, stored at 4 °C

ONPG Solution

4 mg/ml o-Nitrophenyl-ß-D-Galactopyranosid in Z-Puffer (freshly prepared).

3 Results

3.1 Optimization of transient Agrobacterium-mediated gene expression system in *N. benthamiana* to monitor *PR-1* gene expression

NIMIN proteins from plants including Arabidopsis, tobacco and rice have been found to interact with NPR1 (Weigel et al., 2001; Zwicker et al., 2007; Maier et al., 2011; Chern et al., 2005, 2012). The interaction of NIMIN proteins with NPR1 and their presence in whole plant kingdom (Chern et al., 2005; Zwicker et al., 2007), suggests NIMINs role as an important class of plant protein in SAR pathway. Present study is aimed to determine significance of different NIMIN proteins in *PR-1* gene induction. For this purpose, *N. benthamiana* TGES is preferred, to determine the function of different NIMIN proteins. Transient expression systems are being used globally to determine gene function and protein production. Transient expression is a simple, effective, economic and fast technique to monitor the gene function as compared to stable Agrobacterium-mediated transformation. SAR marker genes, i.e., *PR-1* and *PR-1a*, from Arabidopsis and tobacco, respectively, contain SA responsive *as-1*-like *cis*-acting elements in their promoter region (see Introduction). A *-1533PR-1a*$_{pro}$ sequence from tobacco is enough to yield SA-dependent full induction of *PR-1a* gene. Therefore, *-1533PR-1a*$_{pro}$ was fused to the *GUS* gene and resultant *-1533PR-1a*$_{pro}$*:GUS* reporter construct was stably integrated into the genome of *N. benthamiana* via Agrobacterium-mediated transformation. One transgenic line was selected and self fertilized. F2 generation plants were used for transient expression studies showing induction of reporter construct and an endogenous *PR-1* gene. Water treated plants showed no *GUS* activity, but 1mM SA treated plants showed quite high expression of the reporter gene construct (U.M Pfitzner, personal communication, data not shown). Therefore, *-1533PR-1a*$_{pro}$*:GUS* line showing high induction of reporter construct and endogenous *PR-1* was used for transient expression studies.

Initially, in order to check the potential of the TGES, trail experiment was performed. In trail experiment, an Agrobacterium strain carrying *35S*$_{pro}$*:GFP*, which encodes an irrelevant protein in SAR response, was infiltrated to check if infiltration of bacteria per se can induce the *PR-1a* reporter gene. Additionally, infiltration of *35S*$_{pro}$*:GFP* harboring Agrobacterium strain provides an advantage of monitoring visual protein expression level in infiltrated plant

leaves, when observed under UV-light. In order to enhance gene expression, *Carnation mottle virus* coat protein (*CarMVCP)* silencing suppressor was chosen. Therefore, Agrobacteria containing $35S_{pro}$*:GFP* were co-infiltrated with $35S_{pro}$*:CarMVCP* Agrobacteria, where *GFP* and *CarMVCP* were expressed constitutively under the control of strong *Cauliflower mosaic virus* (CaMV) *35S RNA* promoter. For infiltration, Agrobacterium cell suspensions were adjusted at OD_{600} to 1 (see Sec. 2.2.20.3). Non-infiltrated plants showed no induction of *PR-1a* reporter gene in water treated leaf disks, but showed significantly high induction of *PR-1a* reporter gene in 1mM SA treated leaf disks. In the same way, agroinfiltrated plants showed high induction and no significant *PR-1a* induction in 1mM SA and water treated leaf disks, respectively (Fig. 4A). Hence, it proved that the TGES could be used in exploring the functional significance of diverse *NIMIN* genes. Of note, the visual expression level of GFP protein when observed under UV-light was considerably higher in plants co-infiltrated with silencing suppressor than with only *GFP* agroinfiltrated plants (data not shown). This showed that the silencing system worked efficiently in newly developed *in planta* assay. Control experiment worked well, however, some subsequent agroinfiltration experiments showed quite high background in some water floated leaf disks. Figure (4B) shows that co-infiltration of Agrobacterium strains harboring $35S_{pro}$*:GFP* and $35S_{pro}$*:P19* (a silencing suppressor from *Tomato bushy stunt virus*) cause $PR\text{-}1a_{pro}$ activation in some water treated leaf disks.

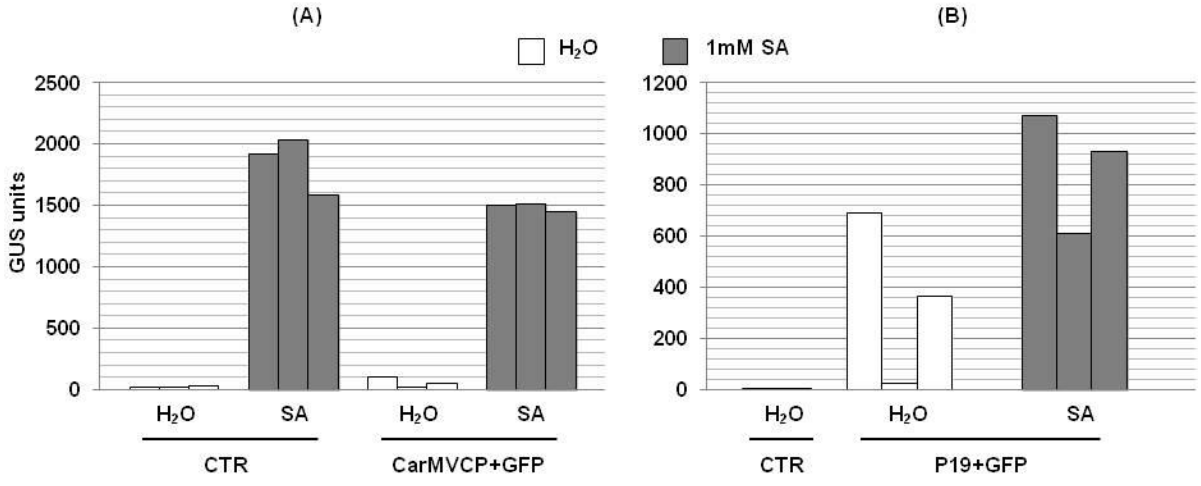


Fig. 4: GUS activities in agro-infiltrated *N. benthamiana* -1533PR-1a$_{pro}$:GUS reporter line plants. co-infiltration of Agrobacterium strains carrying **A)** $35S_{pro}$*:GFP* and $35S_{pro}$*:CarMVCP* or **B)** $35S_{pro}$*:GFP* and $35S_{pro}$*:P19*, to reporter line plants. Two or three non-infiltrated reporter line plants were used as a control (CTR). GUS assays were performed to monitor $PR\text{-}1a_{pro}$ activity in non and agro-infiltrated plant leaf disks treated with H_2O (white bars) or 1mM SA (grey bars). Each bar represents an individual plant. Horizontal line helps grouping the plant.

Therefore, in order to have a true and reliable TGES to determine significance of different NIMIN proteins in *PR-1* gene induction, optimization of the transient expression system was felt mandatory.

3.1.1 Infiltration of Agrobacterium growth or induction medium ingredients do not induce *PR-1a* gene in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants

The optimization of TGES was a challenge. There could be a single or multiple factors affecting the reliability of the system, thereby activating the *PR-1a* reporter gene in water treated agro-infiltrated control plant leaf disks. All potential factors that could induce the *PR-1a* reporter gene were taken into consideration and experiments were planned in order to set-up a reliable TGES in reporter line plants. The first possible potential cause of high background in water treated leaf disks could be the Agrobacterium growth or induction medium ingredients. Therefore, induction medium (10mM $MgCl_2$) with and without 150µM acetosyringone was infiltrated in right *N. benthamaina* leaf half together with co-infiltration of *35S$_{pro}$:GFP* and *35S$_{pro}$:P19* Agrobacteria in left leaf half. The infiltration of induction medium with and without 150µM acetosyringone was not able to induce the *-1533PR-1a$_{pro}$:GUS* reporter gene. Whereas, co-infiltration of *35S$_{pro}$:GFP* and *35S$_{pro}$:P19* carrying Agrobacterium strains brought high to medium induction of reporter gene (Fig. 5A). The results showed that induction medium, i.e., 10mM $MgCl_2$ or 150µM acetosyringone infiltration per se was not the cause for *PR-1a$_{pro}$* activation in agro-infiltrated water treated reporter line plants.

Therefore, antibiotics (2µl/ml of kanamycin and rifampicin) an ingredient of Agrobacterium growth medium was infiltrated in *N. benthamiana -1533PR1-a:GUS* leaf halves as described in Figure (5A). Co-infiltration of Agrobacterium strains harboring *35S$_{pro}$:GFP* and *35S$_{pro}$:P19* induced reporter gene, however, antibiotics were not responsible for unspecific induction of *PR-1a* reporter gene (Fig. 5B). Noteworthy, even washing of Agrobacterium cell suspension twice with 10mM $MgCl_2$ was not able to eradicate the elevated background problem (data not shown). Hence, data show that Agrobacterium growth or induction medium ingredients were not responsible for unspecific activation of *-1533PR-1a$_{pro}$*.

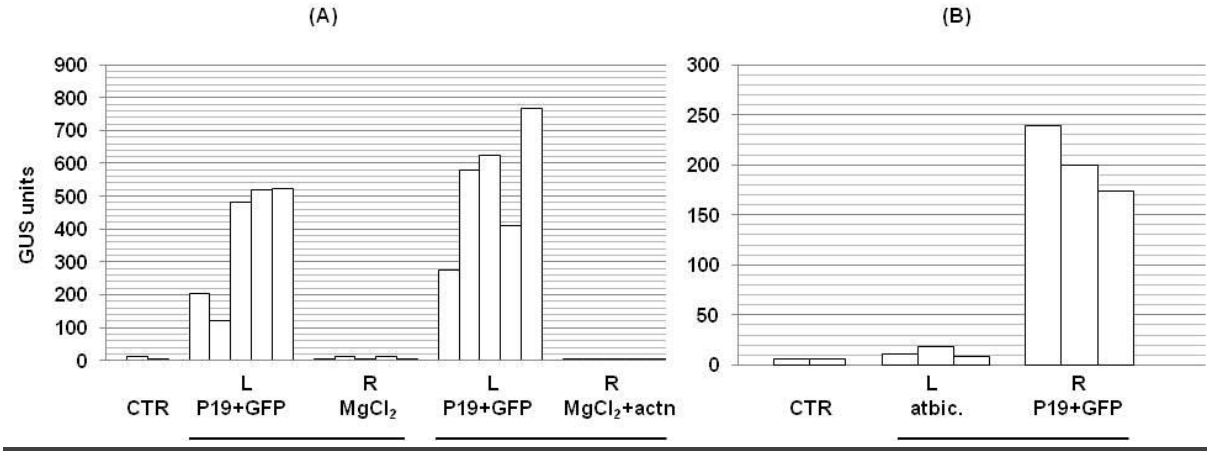


Fig. 5: GUS activities in growth or induction medium infiltrated *N. benthamiana -1533PR-1a:GUS* reporter line plants. A) co-infiltration of *$35S_{pro}$:GFP* and *$35S_{pro}$:P19* carrying Agrobacterium strains to abaxial left (L) leaf half of five reporter line plants and right (R) leaf half with induction medium containing 10mM $MgCl_2$ ($MgCl_2$) with or without 150µM acetosyringone (actn.) **B)** co-infiltration of *$35S_{pro}$:GFP* and *$35S_{pro}$:P19* Agrobacteria to R-leaf half of three *N. benthamiana* reporter line plants and L-leaf half with growth medium containing antibiotics (atbic.).

3.1.2 Agrobacterium strains and silencing suppressor agroinfiltrations are not able to induce *PR-1a* gene in *N. benthamiana -1533PR-$1a_{pro}$:GUS* plants

In previous agro-infiltration experiments binary plasmids *pBin19/$35S_{pro}$:GFP* and *pBin19/$35S_{pro}$:CarMVCP* or *pBin19/$35S_{pro}$:P19* mobilized to Agrobacterium strains LBA4404 and GV3101, respectively, were co-infiltrated in reporter line plants. The presence of two different Agrobacterium strains in same co-infiltration experiment was here suspected as a cause for the induction of reporter gene in *N. benthamiana -1533PR-$1a_{pro}$:GUS* reporter line plants. Therefore, binary plasmid *pBin19/$35S_{pro}$:GFP* mobilized to Agrobacterium strains LBA4404 or GV3101 was co-infiltrated with *pBin19/$35S_{pro}$:P19* mobilized to Agrobacterium strain GV3101. However, the Agrobacterium co-infiltration with same or two different Agrobacterium strains did not turn out to be the cause for *PR-1a* reporter gene induction in *N. benthamiana* (Fig. 6A). It shows that two different Agrobacterium strains co-infiltration were not responsible for induction of *PR-1a* reporter gene in water treated leaf disks, as the reporter gene was induced even when binary constructs in same Agrobacterium strains were co-infiltrated. Nevertheless, it could also be possible that individual infiltration of Agrobacterium strain harboring *$35S_{pro}$:GFP*, *$35S_{pro}$:CarMVCP* or *$35S_{pro}$:P19* can potentially induce *PR-1a* reporter gene. Secondly, it could not be excluded that silencing suppressor's aggressive nature could be the cause of unspecific activation of *PR-1a* promoter.

Therefore, another investigative experiment was performed in which individual infiltration of Agrobacterium strain containing *35S$_{pro}$:GFP*, *35S$_{pro}$:CarMVCP* or *35S$_{pro}$:P19* were carried out in plant leaf half to check their sole effect on *PR-1a* reporter gene induction. *35S$_{pro}$:GFP* and *35S$_{pro}$:P19* were available in Agrobacterium strain LBA4404 and GV3101, respectively. However, silencing suppressor *35S$_{pro}$:CarMVCP* construct was available in both understudy Agrobacterium strains. *35S$_{pro}$:GFP*, *35S$_{pro}$:CarMVCP* or *35S$_{pro}$:P19* availability in different Agrobacterium strains can provide further knowledge about the unspecific induction of *PR-1a* reporter gene. Nevertheless, the single agroinfiltration of *35S$_{pro}$:GFP*, *35S$_{pro}$:CarMVCP* or *35S$_{pro}$:P19* bacteria still activated the *PR-1a$_{pro}$:GUS* reporter gene. Moreover, two silencing suppressors tested here produced higher induction of *PR-1a* reporter gene as compared to GFP. It was also clear that P19 was the least aggressive between two different silencing suppressor tested here and that LBA4404 strain was found out to be a bit more aggressive strain than GV3101, although the difference was not significant (Fig. 6B).

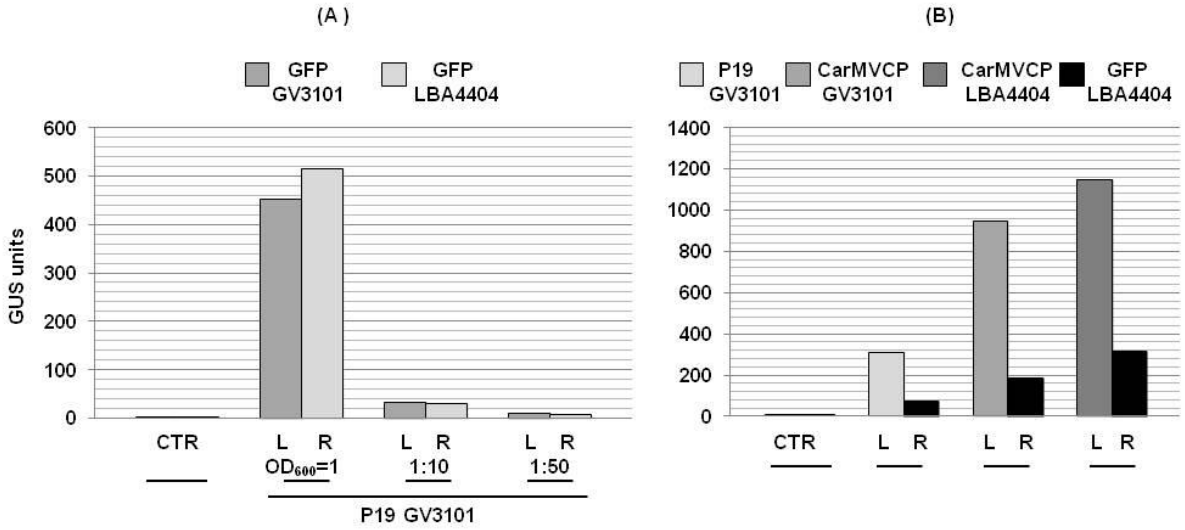


Fig. 6: GUS activities in *N. benthamiana -1533PR-1a$_{pro}$:GUS* reporter line plants infiltrated with different silencing suppressors and Agrobacterium strains. 1:50 and 1:10; dilution factors. **A)** *35S$_{pro}$:P19* and *35S$_{pro}$:GFP* carried in Agrobacterium strain GV3101 were infiltrated in L-leaf half of reporter line plants. *35S$_{pro}$:P19* and *35S$_{pro}$:GFP* carried in Agrobacterium strains GV3101 and LBA4404, respectively were infiltrated in R-leaf half of the reporter line plants. **B)** R-leaf half of *N. benthamiana* reporter line plants were infiltrated with Agrobacterium strain LBA4404 containing *35S$_{pro}$:GFP*. L-leaf halves were infiltrated with *35S$_{pro}$:P19* or *35S$_{pro}$*:CarMVCP carried in Agrobacterium strain GV3101 only or GV3101 as well as LBA4404, respectively.

Therefore, because of being least aggressive silencing suppressor Agrobacterium strain *35S$_{pro}$:P19* was used in future Agrobacterium infiltrations. Although Agrobacterium strain LBA4404 was found more aggressive than GV3101 strain, but this idea could be excluded as first, the difference between two strains was not found significant and secondly, individual infiltration with GV3101 strain still induced the *PR-1a* reporter gene induction.

3.1.3 High Agrobacterium density induces *PR-1a* gene in water treated *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants

The cause of unspecific induction of *PR-1a* reporter gene was neither Agrobacterium growth or induction medium ingredients nor the different Agrobacterium strains co-infiltration stress. Moreover, the induction of reporter gene was observed only in agroinfiltrated plants making it clear that reporter gene was induced because of Agrobacterium infiltration. To confirm the hypothesis if induction of reporter gene was because of the load of Agrobacterium cell suspension in single or co-infiltration experiments, Agrobacterium stains harboring *35S$_{pro}$:GFP* and *35S$_{pro}$:P19* cell suspension adjusted at OD_{600} to one was co-infiltrated together with corresponding dilution of 1:100 or 1:1000 in three individual *N.benthamiana -1533PR-1a$_{pro}$:GUS* plants. Figure (7A) shows that Agrobacterium density was the reason for the induction of reporter gene as the dilution of Agrobacterium cell suspension was not able to activate the *PR-1a$_{pro}$*. However, the visual expression level of *GFP* protein in 1:100 or 1:1000 dilutions was very low, when the leaves were observed under UV-light (data not shown). In Figure (6A) where Agrobacterium strains harboring *35S$_{pro}$:GFP* and *35S$_{pro}$:P19* were co-infiltrated together with their corresponding dilution, i.e., 1:50 and 1:10, also showed that the *PR-1a* reporter gene was not induced in diluted Agrobacterium cell suspension. Visual GFP expression levels under UV-light were low for 1:50, but were recorded medium for 1:10 (data not shown). Therefore, suggesting that Agrobacterium high density was responsible for the activation of *PR-1a$_{pro}$* in water treated leaf disks. Secondly, it showed that there should be a point in between OD_{600} one to 0.1 that could give maximum expression of understudy proteins and minimum unspecific induction of *PR-1a* reporter gene in control water treated agro-infiltrations. Therefore, Agrobacterium strains harboring *35S$_{pro}$:GFP* and *35S$_{pro}$:P19* were co-infiltrated at different OD ranging from OD_{600} 0.1 and OD_{600} 0.75 in reporter line plants. The infiltrated leaves disks were floated side by side on 1mM SA or water, to observe the standard OD point for maximum induction of *PR-1a* reporter gene in 1mM SA floated leaf disks with minimum induction of *PR-1a* reporter gene in water treated leaf disks. OD_{600} 0.1 to OD_{600} 0.5 were able to avoid the unspecific induction of reporter gene whereas at OD_{600} 0.75 the *PR-1a* reporter gene was induced in water treated infiltrated plants (Fig. 7B). The expression level of GFP protein under UV-light suggested that OD_{600} 0.5 and OD_{600} 0.75 were the most appropriate densities of Agrobacterium cell suspension in TGES (data not shown). Hence, OD_{600} set to 0.5 produced maximum expression of GFP protein with no unspecific induction of *PR-1a* reporter gene in water treated infiltrated plants.

Therefore, in all future agro-infiltration experiments OD_{600} to 0.5, was taken as a standard for gene characterization studies via TGES.

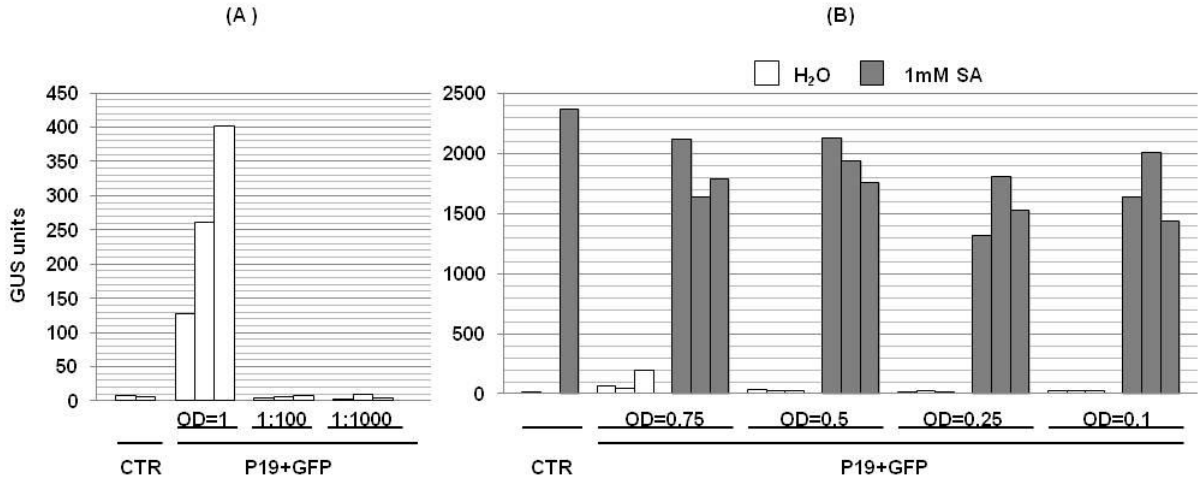


Fig. 7: GUS activities in *N. benthamiana -1533PR-1a$_{pro}$:GUS* reporter line plants infiltrated at different ODs and dilutions of Agrobacterium suspensions. A) Reporter line plants infiltrated with Agrobacterium strains harboring *35S$_{pro}$:P19* and *35S$_{pro}$:GFP* adjusted at OD_{600} one and corresponding dilution of 1:00 and 1:1000. **B)** Reporter line plants infiltrated with *35S$_{pro}$:P19* and *35S$_{pro}$:GFP* Agrobacteria. Agrobacterium cell suspension were adjusted at OD_{600} to 0.75, 0.5, 0.25 and 0.1.

3.1.4 Relative protein expression level at different plant leaf position in *N. benthamiana -1533PR-1a$_{pro}$:GUS* reporter line plants

The transient expression system is very sophisticated, as by using this technique not only the induction of reporter gene could be assayed, but also the phenotypic characteristics of expressed proteins could be monitored in agroinfiltrated *N. benthamiana* plant leaves. The above-mentioned studies showed that the transient expression system is extremely sensitive and much care is needed while handling the *N. benthamiana -1533PR-1a$_{pro}$:GUS* reporter line plants. Moreover, as the agro-infiltrations were done in plant leaves, it was equally important to examine the best *N. benthamiana* leaf position to achieve maximum expression of the constructs. GFP protein over-expression has an advantage as when protein is expressed in *N. benthamiana* plant leaves, the relative protein expression level could be directly monitored under UV-light. Therefore, the upper most and lower most *N. benthamiana -1533PR-1a$_{pro}$:GUS* reporter line plant leaves were infiltrated with Agrobacterium strains harboring *35S$_{pro}$:GFP* and *35S$_{pro}$:P19*. The upper and lower leaf photographs of agro-infiltrated plants were taken under bright and UV-light at four dpi. The infiltrated plants showed fluorescence when infiltrated plant leaves were observed under UV-light. The upper younger leaves showed high GFP protein expression as compared to lower older leaves (Fig.

8). Thus, for all subsequent experiments the upper younger leaves were preferred for agro-infiltrations as they showed the high expression of the constructs.

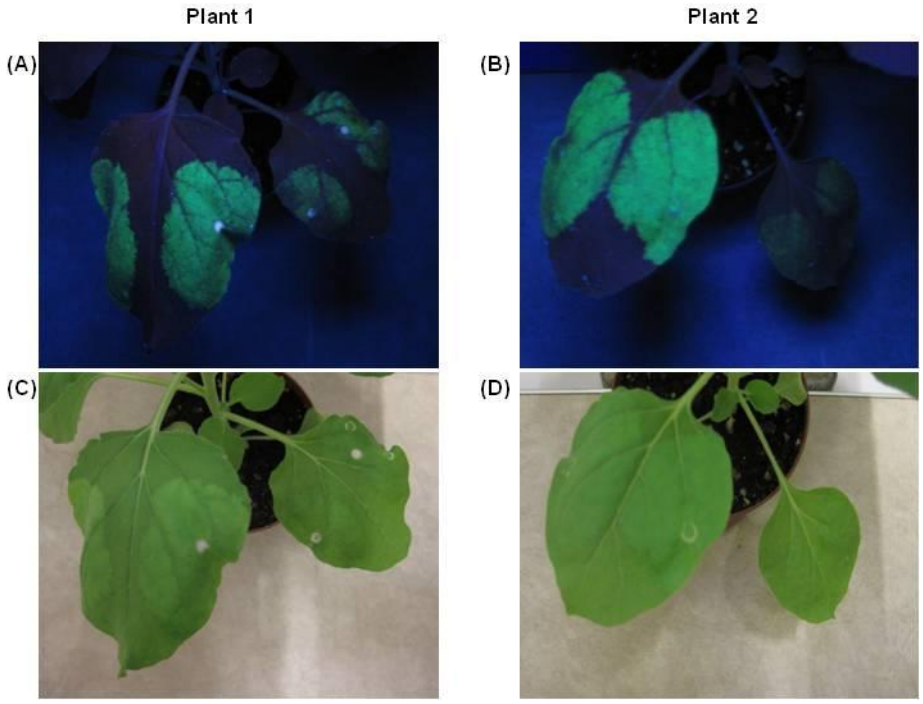


Fig. 8: Relative GFP expression level at different *N. benthamiana* plant leaf positions. Two leaf positions, i.e., the upper younger leaves and lower older leaves were co-infiltrated with Agrobacterium strains carrying *$35S_{pro}$:P19* and *$35S_{pro}$:GFP* adjusted at OD_{600} 0.5. Plant pictures were taken under bright (C and D) and UV-light (A and B) after four dpi.

3.2. Effects of Arabidopsis NIMIN proteins on the *PR-1* gene expression via transient expression system

As mentioned previously (Sec. 3.1), the main purpose of the current research is to characterize the NIMIN proteins for their role in the course of SAR via the TGES. After optimization, the TGES is now ready for functional studies of NIMIN proteins. Arabidopsis contains four *NIMIN* genes namely *NIMIN1*, *NIMIN1b*, *NIMIN2* and *NIMIN3*. General descriptions about the NIMIN proteins have been shown in (Fig. 2). *NIMIN1* over-expression in transgenic Arabidopsis plants has been reported to suppress *PR-1* gene expression and SAR, suggesting *NIMIN1* role as a negative regulator of SAR (Weigel et al., 2005). However, the functional role of other NIMIN proteins is unknown. So, current study is focused to dissect the role of NIMIN proteins in the *PR-1* gene expression using the TGES.

3.2.1 Effects of Arabidopsis NIMIN1 and NIMIN1b

Initially, Agrobacterium strains carrying two different *NIMIN1* constructs, i.e., *$35S_{pro}$:NIMIN1* and *$35S_{pro}$:6xHis:NIMIN1* were infiltrated in *N. benthamiana -1533PR-$1a_{pro}$:GUS* plants to challenge the reliability of the TGES. As shown in (Fig. 9A), *PR-1a* gene expression was strongly induced in non-infiltrated and *$35S_{pro}$:GFP* agro-infiltrated control plants after SA application. On the contrary, both *NIMIN1* containing Agrobacterium strains suppressed the SA mediated *PR-$1a_{pro}$* activation significantly. Hence, the TGES confirms suppressive effect of NIMIN1 protein in *PR-1* gene induction as found previously in transgenic Arabidopsis plants (Weigel et al., 2005). This nevertheless proved that the TGES is trustworthy technique and could be used to characterize the other members of NIMIN protein family. Therefore, *$35S_{pro}$:NIMIN1b* harboring Agrobacterium strain was brought forward to be infiltrated in *N. benthamiana* reporter line plants for validation of its effect on SA-mediated *PR-1a* reporter gene induction via the TGES. NIMIN1b was preferred, as it is highly similar to the NIMIN1 protein (Fig. 2). It was not surprising to learn that NIMIN1b, like NIMIN1, also suppresses the SA-mediated *PR-$1a_{pro}$* induction (Fig. 9B).

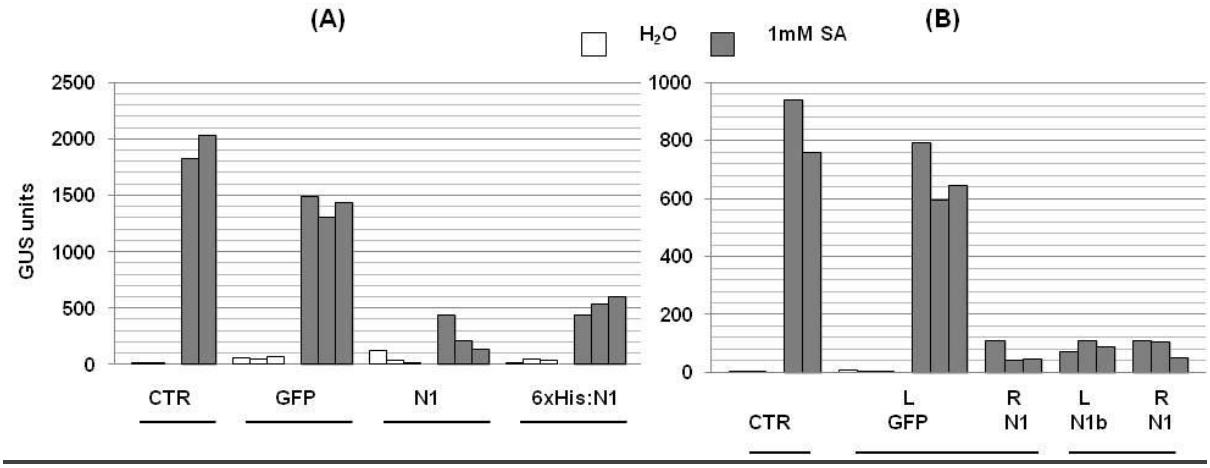


Fig. 9: Transient expression of *NIMIN1* and *NIMIN1b* in *N. benthamiana* -1533PR-1a$_{pro}$:GUS plants. Results presented here were confirmed at least three times. L and R; left and right leaf half infiltrations, respectively, N; *35S$_{pro}$:NIMIN* constructs; 6xHis, hexa histidine tag. *N. benthamiana* reporter line plants infiltrated with **A)** *35S$_{pro}$:GFP*, *35S$_{pro}$:NIMIN1* or *35S$_{pro}$:6xHis:NIMIN1* Agrobacteria. **B)** *35S$_{pro}$:GFP*, *35S$_{pro}$:NIMIN1* or *35S$_{pro}$:NIMIN1b* harboring Agrobacterium strains.

3.2.2 Effects of Arabidopsis NIMIN3

Convincing results encouraged to explore for another *NIMIN* gene family member *NIMIN3* for its effect on the SA-mediated *PR-1a* reporter gene induction via TGES. NIMIN3 shares similar structural domains with other Arabidosis NIMIN proteins, but it is clearly different from other NIMIN protein in terms of its interaction with NPR1. NIMIN1 and NIMIN2 bind to the C-terminus of NPR1, whereas NIMIN3 binds to the N-terminus of NPR1 (Weigel et al., 2001). Additionally, NIMIN3 protein sequence does not contain a clear NLS sequence unlike rest of NIMIN proteins. All Arabidopsis NIMIN proteins excluding NIMIN3 have clear NLS sequence composed of R/K-K-R-R/K-R amino acids (Fig. 10B). In order to check if NIMIN3 although lacking clear NLS sequence still targets cell nucleus, sub-cellular localization of NIMIN3 was carried out together with NIMIN1. NIMIN1 was used as a positive control as it contains a clear NLS sequence and has been shown as nuclear localizing protein in transformed tobacco (Weigel et al., 2001; Fig. 10B). To analyze the sub-cellular targeting, NIMIN1:yEGFP:GUS and NIMIN3:yEGFP:GUS fusion proteins were expressed in yeast. NIMIN proteins are very small and can easily pass through the nuclear pore. Therefore, *GUS* gene was fused to *NIMINs* in order to avoid their free localization to cell nucleus. Figure (10) shows that NIMIN3 like NIMIN1 is also targeted to nucleus, although it does not contain a clear NLS sequence. The nuclear localization of NIMIN3 and its interaction with NPR1 in yeast, confirm NIMIN3 involvement in SAR mechanism (This study; Weigel et al., 2001; Maier et al., 2011). This encouraged to check for NIMIN3 effect

on SA-mediated *PR-1a* gene induction in established *N. benthamiana* transient gene expression system. Therefore, Agrobacterium strain carrying *35S$_{pro}$:NIMIN3* was agro-infiltrated in reporter line plants. The *NIMIN3* agro-infiltrated plants also suppress the SA-mediated *PR-1* gene induction, but to a lesser extent than *NIMIN1* agroinfiltrated plants (Fig. 11). This finding files NIMIN3 protein as yet another negative regulator of SAR together with NIMIN1 and NIMIN1b.

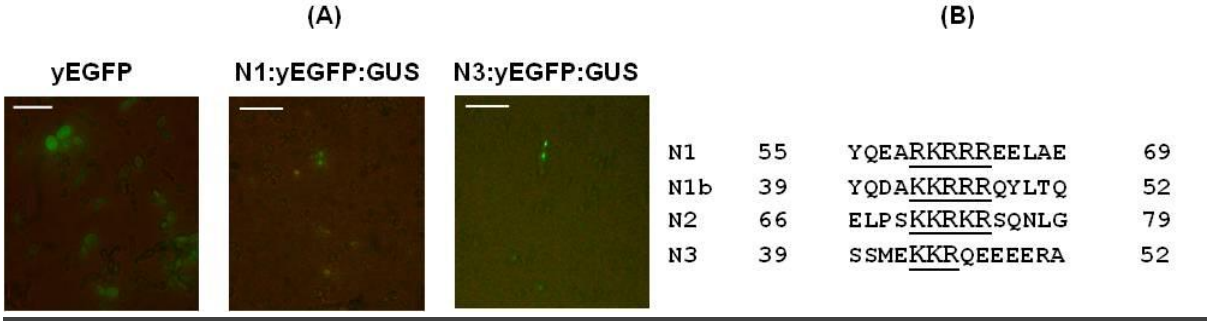


Fig. 10: Nuclear localization of NIMIN1 and NIMIN3 proteins in yeast. A) Sub-cellular localization of yEGFP, NIMIN1:yEGFP:GUS and NIMIN3:yEGFP:GUS fusion proteins in yeast. Yeast cells were examined under fluorescent microscopy. Scale bars represent 25µm. **B)** Alignment of Arabidopsis NIMIN proteins at the region of NLS sequence. The amino acids known to be involved in nuclear localization of protein are underlined and marked in bold letters.

3.2.3 Effects of Arabidopsis NIMIN2

All Arabidopsis NIMIN proteins tested so far suppress the SA-mediated *PR-1a* reporter gene induction in transient assays. NIMIN2 is the only member left, which yet needs to be characterized for its effects on SA-mediated *PR-1a* promoter activation. Therefore, *35S$_{pro}$:NIMIN2* Agrobacteria were infiltrated in reporter line plants. The results obtained were pretty surprising that NIMIN2, unlike other NIMIN proteins, does not suppress the SA-mediated *PR-1a* reporter gene induction in transient assay system (Fig. 11B). The water treated *NIMIN2* agroinfiltrated plants further clarifies that NIMIN2 does not induce the reporter gene on its own. Accumulation of NIMIN2 protein in agro-infiltrated plant tissues was confirmed via immunodetection (U.M. Pfitzner, personal communication), thereby eliminating the fact that the non-suppressive effect on *PR-1a$_{pro}$:GUS* reporter gene was due to mere absence of NIMIN2 protein. With these results, NIMIN2 qualifies as a non-suppressive protein in SA-mediated *PR-1* gene expression unlike less suppressive NIMIN3 and strong suppressors NIMIN1 and NIMIN1b (Fig. 11C).

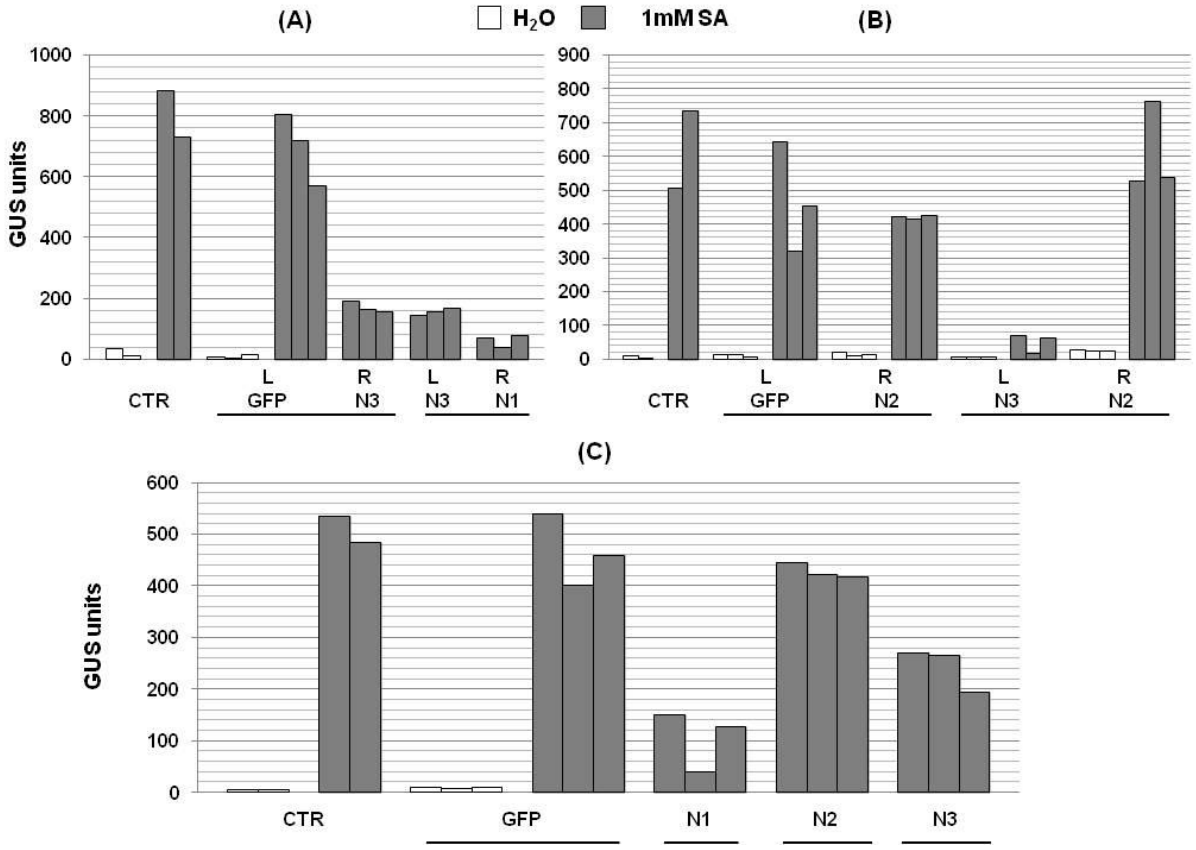


Fig. 11: Transient expression of *NIMIN1, NIMIN2* and *NIMIN3* in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants. A) Reporter line plants infiltrated with Agrobacterium strain harboring *35S$_{pro}$:GFP, 35S$_{pro}$:NIMIN3* or *35S$_{pro}$:NIMIN1*. **B)** Reporter line plants infiltrated with Agrobacterium strain carrying *35S$_{pro}$:GFP, 35S$_{pro}$:NIMIN2* or *35S$_{pro}$:NIMIN3*. **C)** Direct comparison of *NIMIN1*, *NIMIN2* and *NIMIN3* agroinfiltrated reporter line.

3.2.4 Effects of NIMIN1 and NIMIN1b mutants

Arabidopsis NIMIN1 and NIMIN1b contain similar structural motifs and are the strongest repressors of SA-mediated *-1533PR-1a$_{pro}$:GUS* reporter gene induction among the Arabidopsis NIMIN proteins (Fig. 2, 9 and 11A,C). Therefore, mutations in structural motifs identified in NIMIN1 and NIMIN1b may help to understand the mechanism behind the suppressive role of these proteins in SAR scenario.

3.2.4.1 Construction of NIMIN1 and NIMIN1b mutants

It has been shown previously that NIMIN1 F49/50S mutant does not interact with NPR1 in plant extracts and transgenic plants overexpressing mutant protein, are not impaired in *PR-1* gene induction after SA application unlike wild type NIMIN1 (Weigel et al., 2005). NIMIN1 F49/50S carries a mutation in NPR1 interacting (FFK) domain where two consecutive large

non-polar hydrophobic phenylalanine (Phe) residues (at position 49/50), are replaced by slightly polar serine (Ser) residues (Weigel et al., 2005; Fig. 14A). NIMIN1 F49/50S was cloned as *Bam*HI fragment into binary plasmid *pBin19/35S$_{pro}$:NOS* as described by Weigel et al., (2005). NIMIN1 E94A D95V mutagenesis was created via overlap extension PCR technology using specific primers (Tab. 1; Sec. 2.2.14). NIMIN1 E94A D95V carries a mutation in EDF domain where two consecutive large negatively charged glutamic acid (Glu) and aspartic acid (Asp) residues (at position 94/95), are replaced by non-polar alanine (Ala) and valine (Val) residues, respectively (Fig. 15A). The mutant was cloned as *Bam*HI fragment in yeast vector pGBT9 after verification of nucleotide sequence. For cloning *Bam*HI fragments a vector *pBin19/35S$_{pro}$:NOS* was created by transferring *Eco*RI cut *35S$_{pro}$:NOS* cassette from *puc18/35S$_{pro}$:NOS* donor vector to *pBin19/35S$_{pro}$:mGFP4* (Fig. 12). NIMIN1 E94A D95V mutant was cloned as *Bam*HI fragment into newly constructed binary vector *pBin19/35S$_{pro}$:NOS* (U.M. Pfitzner, personal communication; Fig. 12).

Tab. 1: Oligonucleotides for amplification of *NIMIN1* E94A D95V and *NIMIN1* L138A L140A

Mutant	Template	N-terminus		C-terminus	
		Forward primer	Back primer	Forward primer	Back primer
NIMIN1 E94A D95V	pGBT9/NIMIN1	N1 fwd	N1-6	N1-5	N1-4
NIMIN1 L138A L140A	pGBT9/NIMIN1	N1 fwd	-	-	N1-7

NIMIN1 L138A L140A mutation was introduced via PCR technology using specific primers (Tab. 1; Sec. 2.2.14). NIMIN1 L138A L140A carries a mutation in EAR domain (conserved in all Arabidopsis NIMIN proteins) where two large non-polar hydrophobic leucine (Leu) residues (at position 138/140), are exchanged by small Ala residues (Fig. 16A). The mutant nucleotide sequence was verified and cloned as *Bam*HI fragment in yeast vector pGBT9. Eventually, the NIMIN1 L138A L140A sequence was cloned into newly constructed binary vector *pBin19/35S$_{pro}$:NOS* (U.M. Pfitzner, personal communication; Fig. 12). Amino acid sequence shows that C-terminus of NIMIN1 and NIMIN1b proteins is very similar holding EAR domain with a *Bgl*II restriction site. Therefore, NIMIN1 and NIMIN1b were cut by using *Bgl*II restriction enzyme site which deletes five amino acids including three Leu residues of the C-terminal EAR domain (Fig. 16B; 17A). The mutants were named NIMIN1 1/137 and NIMIN1b 1/135 which were constructed and thereafter cloned as *Bam*HI and *Bgl*II fragments into binary vector *pBin19/35S$_{pro}$:NOS* (U.M. Pfitzner, personal communication; Fig. 12). All NIMIN mutant constructs were mobilized to Agrobacterium strain LBA4404 via TPM (Sec. 2.2.19)

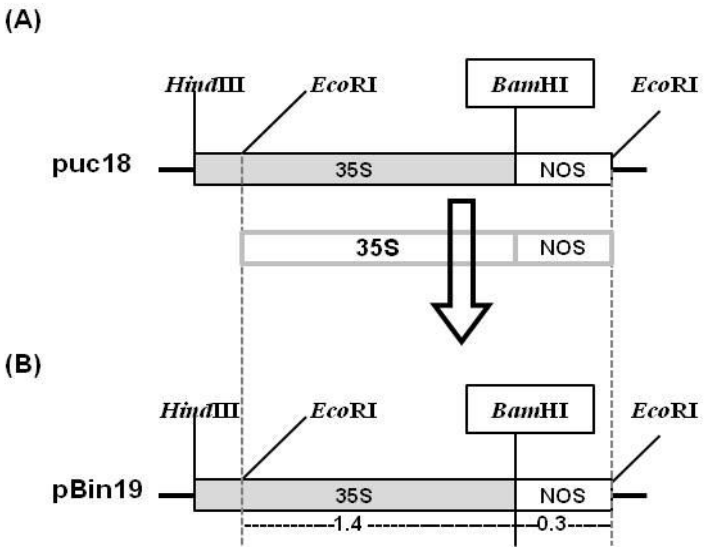


Fig. 12: Schematic representation of newly constructed plant expression vector *pBin19/35S$_{pro}$:NOS.* The diagramme is drawn approximately to scale. Schematic illustration of **A)** *puc18/35S$_{pro}$:NOS* donor vector and **B)** *pBin19/35S$_{pro}$:NOS* newly prepared plant expression vector for cloning *Bam*HI fragments.

3.2.4.2 Analysis of NIMIN1 mutants in yeast

Presence of EDF motif from NIMIN proteins in plant species other than Arabidopsis, e.g., rice NRR and its homologue (Chern et al., 2005; 2012) and tobacco NIMIN-like1 (This study), advocates for EDF motif functional purpose in plants. Therefore, mutation in EDF motif will provide information about its role in *PR-1* gene regulation. But before further analysis of NIMIN1 E94A D95V via transient assay system, it was important to know if mutant still binds to core SAR regulatory protein NPR1. Therefore, in order to explore NIMIN1 E94A D95V interaction with AtNPR1, Y2H assays were performed (Sec. 2.2.21.9). To determine this, DNA fragments containing full length *NIMIN1* and *NIMIN1 E94A D95V* genes were cloned in frame with the GAL4BD domain into the pGBT9 vector. The resulting constructs were co-transformed with the pGAD424/AtNPR1 plasmid (encoding GAL4AD-AtNPR1) into the yeast cells HF7c (strain), carrying the *HIS3/lacZ* reporter genes under the control of *UAS^{GAL4}* element. Quantitative *lacZ*-reporter gene assay (Sec.2.2.21.11) shows that the NIMIN1 E94A D95V mutant still interacts with AtNPR1 in yeast assay but not as strongly as wild type NIMIN1. Moreover, it shows that the protein-protein interaction, like wild type NIMIN1, is also sensitive to SA (Fig. 13). Hence, it proves that NIMIN1 E94A D95V mutant protein is still a SA-sensitive NPR1 interactor. Likeweise, NIMIN1 1/137 and NIMIN1 L138A L140A interact with NPR1 to similar level as wild type NIMIN1 (Späth, 2012).

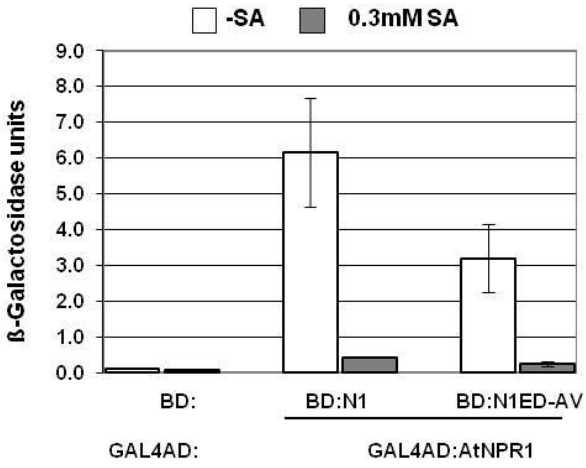


Fig. 13: Interaction of NIMIN1 and NIMIN1 E94A D95V with AtNPR1 in yeast. Y2H assay for interaction of GAL4BD:NIMIN1 and GAL4BD :NIMIN1 ED-AV (E94A D95V) fusion protein with GAL4AD:AtNPR1 was performed in the presence (gray bar) and absence (white bar) of 0.3mM SA. Quantitative yeast two hybrid assays were performed under standard conditions. Y-axis shows *lacZ*-reporter gene activity in term of β-Galactosidase units. β-Galactosidase activites shown here are mean of three independent colonies tested, with three replicates of each colony. The presented results were reproduced at least three time.

3.2.4.3 Analysis of NIMIN1 mutants in *N. benthamiana*

NIMIN1 F49/50S mutant as mentioned in previous Sec. (3.2.4.1) does not interact with AtNPR1 in yeast and *in planta* experiments. Therefore, already known characteristics of NIMIN1 F49/50S in yeast and transgenic plants and availability of the Agrobacterium strain carrying *$35S_{pro}$:NIMIN1 F49/50S* encouraged to explore for the mutant function in established TGES. Therefore, *$35S_{pro}$:NIMIN1 F49/50S* Agrobacteria were infiltrated in *N. benthamiana -1533PR-$1a_{pro}$:GUS* plants. However, it comes as no surprise that NIMIN1 F49/50S does not suppress the *PR-1a* gene induction in reporter line plant after SA application (Fig. 14). The similar effect of NIMIN1 F49/50S on *PR-1a* reporter gene in TGES like in transgenic plants once more proclaims that the transient gene expression system is reliable and fast technique for gene characterization.

Yeast results support the view that NIMIN1 E94A D95V mutant still interacts with AtNPR1, but not as strongly as wild type NIMIN1 protein (Fig. 13). Mainly focused on NIMIN1 E94A D95V mutant for its effect on *PR-1* gene induction, Agrobacterium strain carrying *$35S_{pro}$:NIMIN1 E94A D95V* was infiltrated in *N. benthamiana* reporter line plants. NIMIN1 protein suppresses the SA-mediated activation of *PR-1a* promoter, however, much to surprise NIMIN1 E94A D95V mutant protein that still interacted with AtNPR1 in yeast (Sec. 3.2.4.2) does not suppress the SA-mediated *PR-1a* gene induction in *N.benthamiana -1533PR-$1a_{pro}$:GUS* reporter line plants (Fig. 15B).

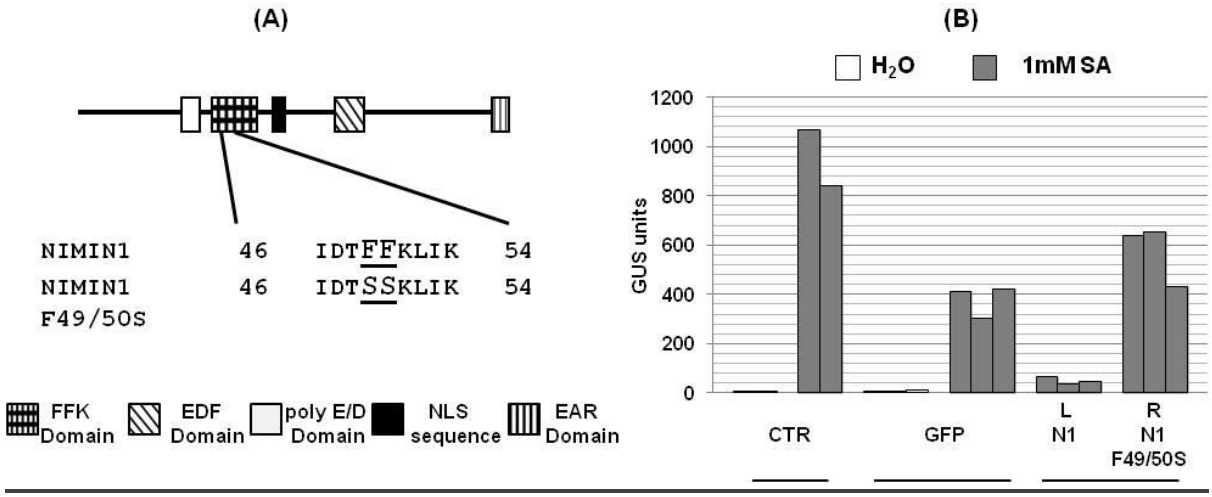


Fig. 14: Transient expression of *NIMIN1 F49/50S* in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants. A) Diagrammatic representation of different structural domains found in NIMIN1 indicating site of point mutation and amino acid changes. The underlined amino acids are the switched amino acid as a result of NIMIN1 F49/50S mutagenesis. **B)** *N. benthamiana* reporter line plants infiltrated with Agrobacterium strains carrying *35S$_{pro}$:GFP, 35S$_{pro}$:NIMIN1* or *35S$_{pro}$:NIMIN1 F49/50S.*

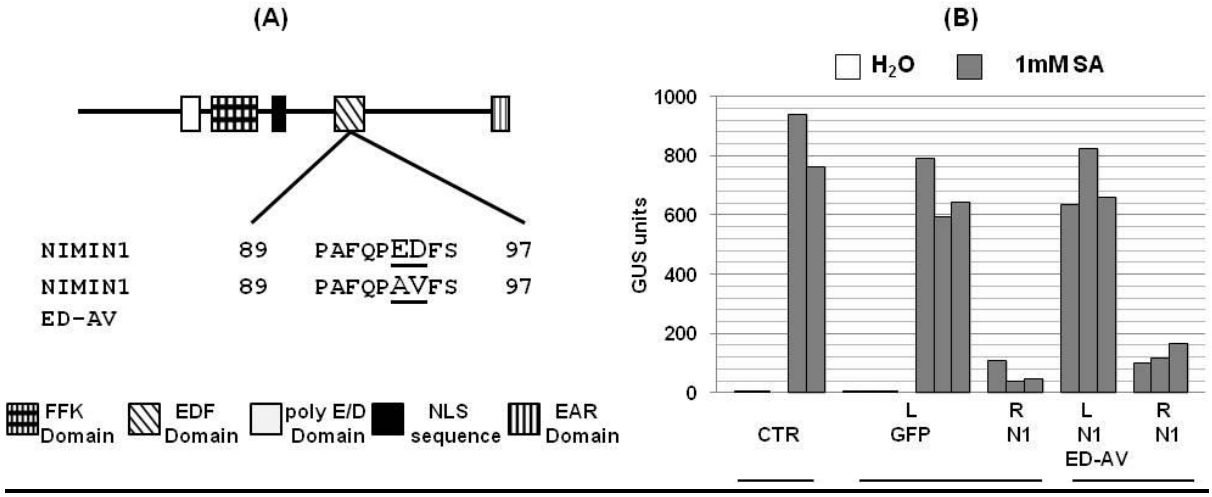


Fig. 15: Transient expression of *NIMIN1* E94A D95V mutant in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants. A) Diagrammatic representation of different structural domains found in NIMIN1 indicating site of point mutation and amino acid changes. **B)** *N. benthamiana* reporter line plants infiltrated with Agrobacterium strains carrying *35S$_{pro}$:GFP, 35S$_{pro}$:NIMIN1* or *35S$_{pro}$:NIMIN1 E94A D95V (ED-AV).* Part of this experiment has already been shown in Figure (9B).

It has been reported that TOPLESS (TPL) interacts with diverse transcription factors having repression domains (containing variable sequences) suggesting that repression domains are necessary for recruitment of TPL (Causier et al., 2012). Interaction of a co-repressor TPL, with transcription complexes involved in auxin and JA signaling, meristem maintenance and in defense responses has been shown in several studies (Kieffer et al.,2006; Szemenyei et al., 2008; Gallavotti et al., 2010; Pauwels et al., 2010; Zhu et al., 2010). In addition to that, TPL potential involvement in SAR-pathway has been shown through its interaction with NIMIN proteins (Arabidopsis Interactome Mapping Consortium, 2011). Taking this into

consideration, NIMIN1 L138A L140A and NIMIN11/137 mutants were constructed as mentioned in previous section (3.2.4.1). Both above mentioned NIMIN1 mutants carry a mutation in EAR repression domain (Fig. 2; 16A andB). EAR motif which is also present in NIMIN proteins at their C-terminal end is considered as hallmark of transcriptional repressors (Kazan et al., 2006). Therefore, reporter line plants were infiltrated with Agrobacterium strains carrying *35S$_{pro}$:NIMIN1 L138A L140A*, *35S$_{pro}$:NIMIN1* 1/137 together with *35S$_{pro}$:NIMIN1*. Transient assay shows that wild type NIMIN1 protein suppresses *PR-1a* gene induction as always, NIMIN1 1/137 deletion mutant suppresses to intermediate levels. However, the NIMIN1 L138A L140A mutant does not suppress the SA-mediated *PR-1a $_{pro}$:GUS* transgene induction in *N. benthamiana* reporter line plants (Fig. 16C).

It was astonishing to learn that, although missing three Leu residues of EAR domain, NIMIN1 1/137 still represses the SA-mediated *PR-1a* reporter gene induction, but to a lesser extent than wild type NIMIN1.

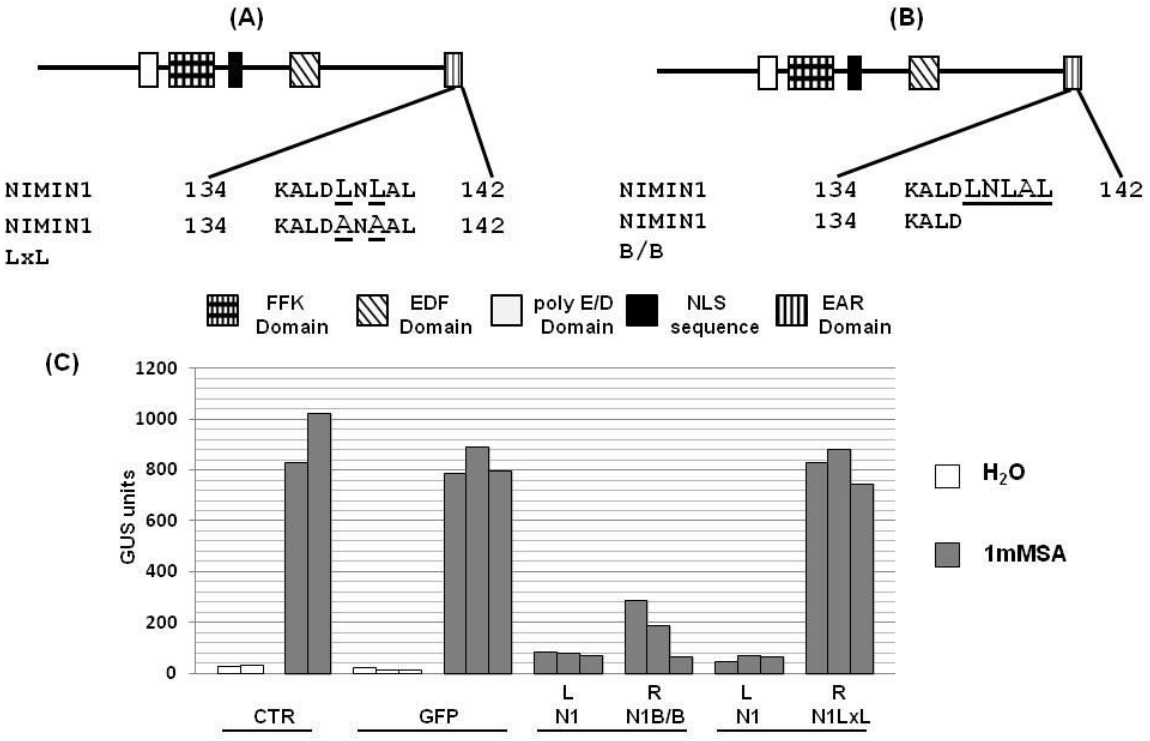


Fig. 16: Transient expression of *NIMIN1* 1/137 and *NIMIN1* L138A L140A mutants in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants. A,B) Diagrammatic representation of different structural domains found in NIMIN1 indicating the site of point mutation and amino acid changes. The underlined amino acids are **A)** the changed amino acid as a result of NIMIN1 L138A L140A (LxL) mutagenesis **B)** the missing amino acid as a result of NIMIN1 1/137 (B/B) deletion mutagenesis. **C)** Reporter line plants infiltrated with Agrobacterium strains carrying *35S$_{pro}$:GFP*, *35S$_{pro}$:NIMIN1*, *35S$_{pro}$:NIMIN1* 1/137 or *35S$_{pro}$:NIMIN1 L138A L140A.*

As mentioned previously (Sec. 3.2.4.1) that very C-terminus of NIMIN1 and NIMIN1b proteins are pretty similar, it was not surprising to find that infiltration of *35S$_{pro}$:NIMIN1b 1/135* Agrobacteria in reporter line plants suppressed the SA-induced *-1533PR-1a$_{pro}$:GUS* reporter gene (Fig. 17). Therefore, it shows that both NIMIN1 1/137 and NIMIN1b 1/135 mutants still repress the SA-mediated *PR-1a* gene induction, concluding that repression does not solely depend on EAR motif.

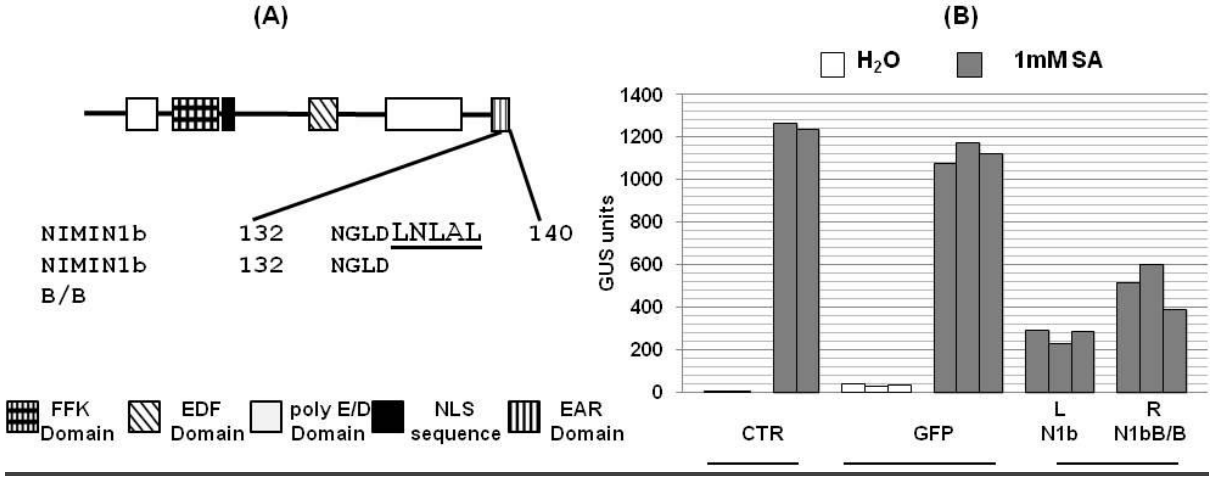


Fig. 17: Transient expression of *NIMIN1b 1/135* mutant in *N. benthamiana -1533PR-1a$_{pro}$:GUS* reporter line plants. A) Diagrammatic representation of different structural domains found in NIMIN1 indicating the mutation site. The underlined amino acids are the missing amino acid as a result of NIMIN1 1/135 (B/B) deletion mutagenesis. **B)** *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants infiltrated with Agrobacterium strains carrying *35S$_{pro}$:GFP, 35S$_{pro}$:NIMIN1b* or *35S$_{pro}$:NIMIN1b 1/135*.

3.2.5 Effects of NIMIN3 mutants

NIMIN3, like NIMIN1, suppresses the SA-mediated *PR-1a* reporter gene induction in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants. Secondly, NIMIN3 also contains similar domains like NIMIN1, i.e., EAR and EDF domains which have already been characterized for NIMIN1 (Sec.3.2.4). Therefore, mutations in NIMIN3 were planned to explore further about functional significance of these domains in NIMIN proteins.

3.2.5.1 Construction of NIMIN3 mutants

NIMIN3 E63A D64V mutagenesis, like NIMIN1 E94A D95V was created via overlap extension PCR technology using specific primers (Tab. 2). NIMIN3 E63A D64V carries a mutation in EDF domain where two consecutive large negatively charged Glu and Asp residues (at position 63/64), are replaced by non-polar Ala and Val residues respectively (Fig.

19A). After verification of nucleotide sequence, mutant was cloned as *Bam*HI fragment in yeast vector pGBT9. *NIMIN3 E63A D64V* mutant was cloned as *Bam*HI fragment into binary vector *pBin19/35S$_{pro}$:NOS* (Fig. 12).

NIMIN3 L108A L110A, like NIMIN1 L138A L140A, mutagenesis was introduced via PCR technology using specific primers (Tab. 2). NIMIN3 L108A L110A carries a mutation in EAR domain where two large non-polar hydrophobic Leu residues (at position 108/110), are exchanged by small Ala residues (Fig. 20A). The mutant was cloned as *Bam*HI fragment in yeast vector pGBT9 after verification of nucleotide sequence. The *NIMIN3 L108A L110A* mutant sequence was cloned as *Bam*HI fragment into newly designed binary vector *pBin19/35S$_{pro}$:NOS* (U.M. Pfitzner, personal communication; Fig. 12).

Tab. 2: Oligonucleotide combination for amplification of *NIMIN3* E63A D64V and *NIMIN3* L108A L110A

Mutant	Template	N-terminus		C-terminus	
		Forward primer	Back primer	Forward primer	Back primer
NIMIN3 E63A D64V	pGBT9/NIMIN3	N3 fwd	N3-3	N3-2	N3-1
NIMIN3 L108A L140A	pGBT9/NIMIN3	N3 fwd	-	-	N3-4

3.2.5.2 Analysis of NIMIN3 mutants in yeast

Mutagenesis NIMIN3 E63A D64V was created as mentioned (Sec. 3.2.5.1). In order to explore NIMIN3 E63A D64V interaction with AtNPR1, Y2H assays were performed (Sec. 2.2.21.9). Therefore, DNA fragments encoding NIMIN3 and NIMIN3 E63A D64V were cloned downstream the GAL4BD domain into the pGBT9 vector and were co-transformed with the pGAD424/AtNPR1 plasmid into the yeast cells HF7c (strain), carrying the *HIS3/lacZ* reporter gene under the control of *UAS^{GAL4}* element. Quantitative *lacZ*-reporter gene assay was performed for wild type NIMIN1 and NIMIN3 together with their corresponding EDF mutant. Figure (18A) shows that the NIMIN3 E63A D64V does not interact with AtNPR1 in yeast assay unlike wild type NIMIN3 and the protein-protein interaction is not influenced by SA. Here, NIMIN1 and its NIMIN1 E94A D95V mutants tested once more confirm the result presented in (Fig. 13). Hence, it shows that NIMIN3 E63A D64V mutant unlike its wild type protein is not able to interact with AtNPR1 in Y2H. Western blot analysis failed to show the expression of NIMIN3 E63A D64V mutant in yeast extracts. However, the wild type NIMIN3 was expressed at high level (Fig. 18B).

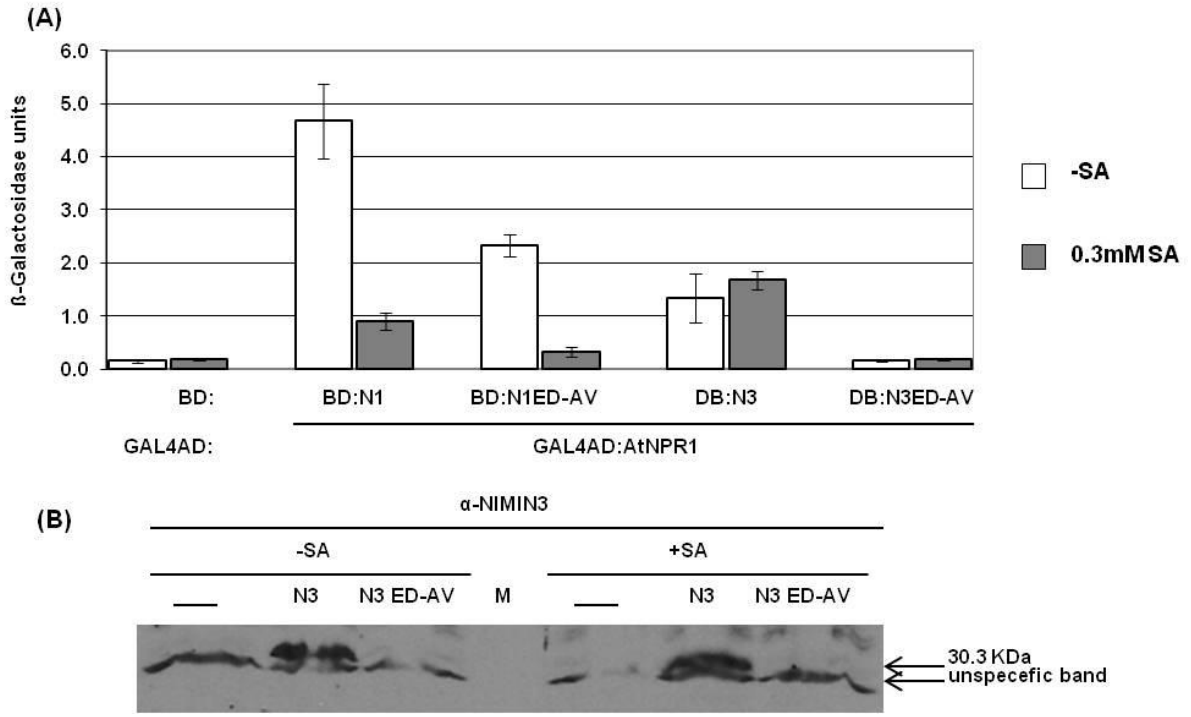


Fig. 18: Interaction of NIMIN3 and NIMIN3 E63A D64V with AtNPR1 in yeast. A) Y2H assay was performed for interaction of GAL4BD:NIMIN3 and GAL4BD:NIMIN3 E63A D64V (ED-AV) with GAL4AD:AtNPR1 as described in Figure (13). **B)** Immunodetection of GAL4BD:NIMIN3 and GAL4BD:NIMIN3 ED-AV in yeast extracts. Protein extracted from yeast cells carrying GAL4BD:NIMIN3 and GAL4BD:NIMIN3 ED-AV treated with and without 0.3mM SA were subjected to SDS-PAGE using 15% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane using western transfer buffer. The protein was detected by using α-NIMIN3 Antiserum. Molecular marker indicated the protein to be expected 30 KDa size.

3.2.5.3 Analysis of NIMIN3 mutants in *N. benthamiana*

Yeast results shown in (Fig. 18), prompted to explore NIMIN3 E63A D64V mutant via TGES for its effect on *PR-1a* gene induction. Therefore, Agrobacterium strain harboring *$35S_{pro}$:NIMIN3 E63A D64V* or *$35S_{pro}$:NIMIN3* were infiltrated in *N. benthamiana* reporter line plants. The results show that NIMIN3 protein suppresses SA-mediated activation of *PR-$1a_{pro}$*. However, the NIMIN3 E63A D64V mutant failed to suppress the SA-mediated *PR-1a* reporter gene induction in *N. benthamiana -1533PR-$1a_{pro}$:GUS* plants (Fig. 19B).

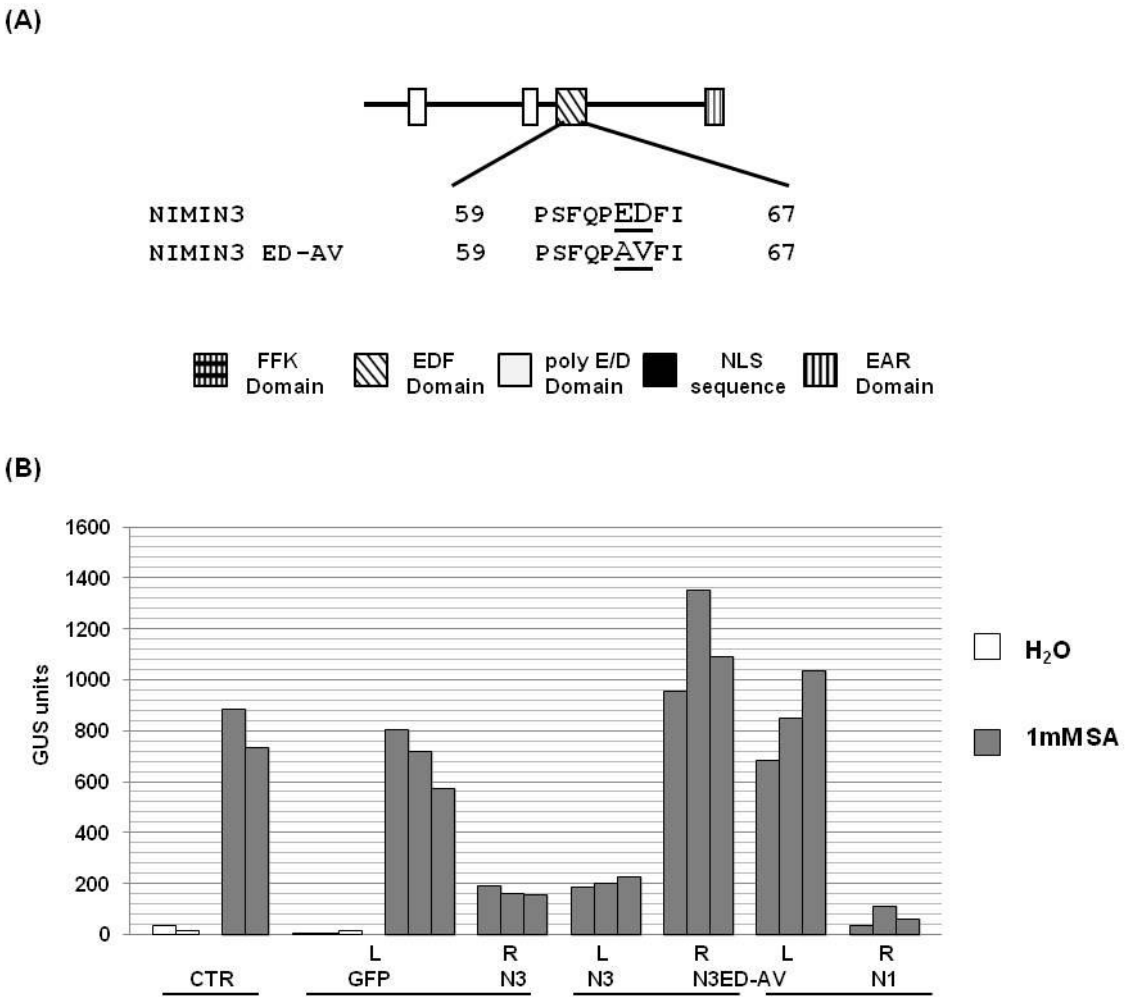


Fig. 19: Transient expression of *NIMIN3 E63A D64V* mutant in *N. benthamiana -1533PR-1a*$_{pro}$*:GUS* plants. A) Diagrammatic representation of different structural domains found in NIMIN3, indicating the point mutation and amino acid exchanges. The underlined amino acids are the changed amino acid as a result of NIMIN3 E63A D64V (ED-AV) mutagenesis. **B)** Reporter line plants infiltrated with Agrobacterium strain carrying *35S*$_{pro}$*:GFP*, *35S*$_{pro}$*:NIMIN1*, *35S*$_{pro}$*:NIMIN3* and *35S*$_{pro}$*:NIMIN3* E63A D64V . Part of this experiment has already been show in Figure (11A).

On the other hand, infiltration of *35S*$_{pro}$*:NIMIN3 L108A L110A*, or *35S*$_{pro}$*:NIMIN3* harboring Agrobacterium strain in reporter line plants clearly shows that NIMIN3 protein suppresses whereas, the NIMIN3 L108A L110A mutant does not suppress the SA-mediated *PR-1a* gene induction (Fig. 20).

The thorough studies of Arabidopsis NIMIN proteins for their functional significance on *PR-1a* gene induction via transient expression assay, advocate that Arabidopsis NIMIN proteins have different roles in course of SAR (see discussion).

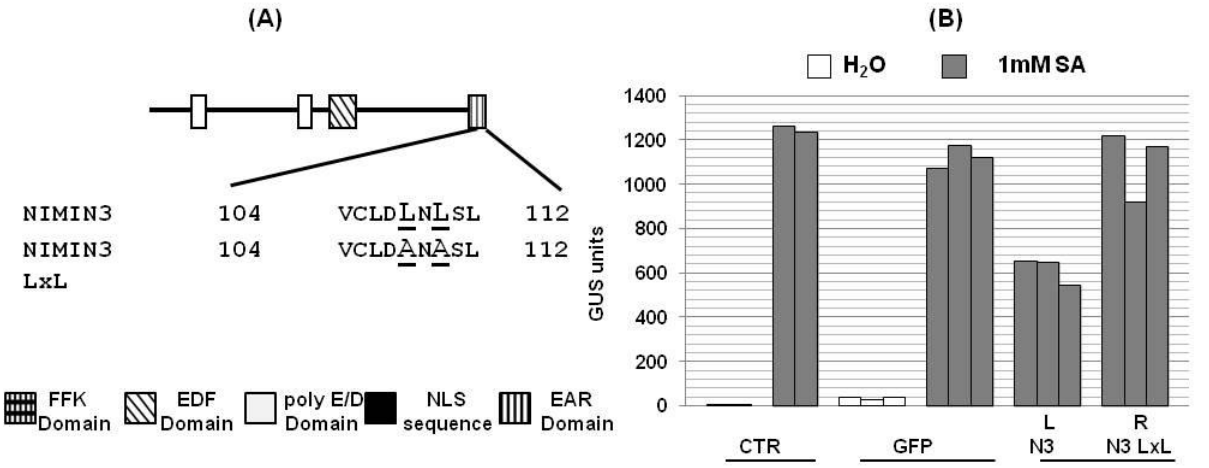


Fig. 20: Transient expression of *NIMIN3 L108A L110A* mutant in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants. A) Diagrammatic representation of different structural domains found in NIMIN3 indicating the point mutation and amino acid exchanges. The underlined amino acids are the altered amino acid as a result of NIMIN3 L108A L110A (LxL) mutagenesis. **B)** *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants infiltrated with Agrobacterium strains carrying *35S$_{pro}$:GFP, 35S$_{pro}$:NIMIN3* or *35S$_{pro}$:NIMIN3 LxL*. Part of this experiment has already been shown in Figure.(17B).

3.3. Effects of transient over-expression of Arabidopsis *NIMIN* genes on *N. benthamiana* leaf tissue phenotype

As mentioned in previous section, the TGES additionally helps in monitoring the phenotypic characteristics of expressed genes. Therefore, careful inspection of agroinfiltrated *N. benthamiana* leaves was carried out. Keen observation uncovered that transient over-expression of NIMIN1 protein yielded necrotic spots on the infiltrated *N. benthamiana* leaf tissues. This unanticipated effect was analyzed in more detail together with other NIMIN proteins.

3.3.1 Phenotypic effects of transient over-expression of Arabidopsis *NIMIN1*, *NIMIN1b* and *NIMIN3* genes

As reporter line plants over-expressing *NIMIN1* yielded necrotic spots leading to cell death, more careful observation about this novel phenotypic effect was studied in comparison with known cell death inducer, pro-apoptotic *Bax* gene (as a reporter gene). *Bax* is a eukaryotic gene of the human Bcl-2 protein family, which promotes cell death. It has been shown that expression of *Bax* gene in transgenic plants results in necrosis (Roth, 2003). Therefore, reporter line plants were infiltrated with Agrobacterium strains harboring *NIMIN1*$_{pro}$*:Bax*, *35S*$_{pro}$*:NIMIN1* and *35S*$_{pro}$*:GFP* as negative control. Here, *Bax* gene has been expressed under the control of *NIMIN1* full-length promoter because *35S*$_{pro}$*:Bax* containing Agrobacteria are not able to survive (U.M Pfitzner, personal communication). The phenotypic characteristics of agroinfiltrated reporter line plants were monitored in time course study and photos were recorded from 4 dpi to 11 dpi. Cell death was recorded in *Bax* as well as in plant leaf tissue transiently over-expressing *NIMIN1*, but no cell death was observed in *GFP* agroinfiltrated reporter line plants (Fig. 21). Detailed examination revealed that cell death symptoms were pretty prominent in *Bax* expressing plant leaves even as early as at 4 dpi and heavy cell death was observed at 8 dpi or later. *NIMIN1* transient over-expression produced similar phenotypic effects on *N. benthamiana* reporter line plants as *Bax*, however, the cell death symptoms appeared at later point in time, i.e., 6 dpi and reached peak at 10 dpi or later. On the contrary, tissues infiltrated with *35S*$_{pro}$*:GFP* Agrobacteria produced no cell death, even when followed up to 11dpi. Additionally, the GFP expression

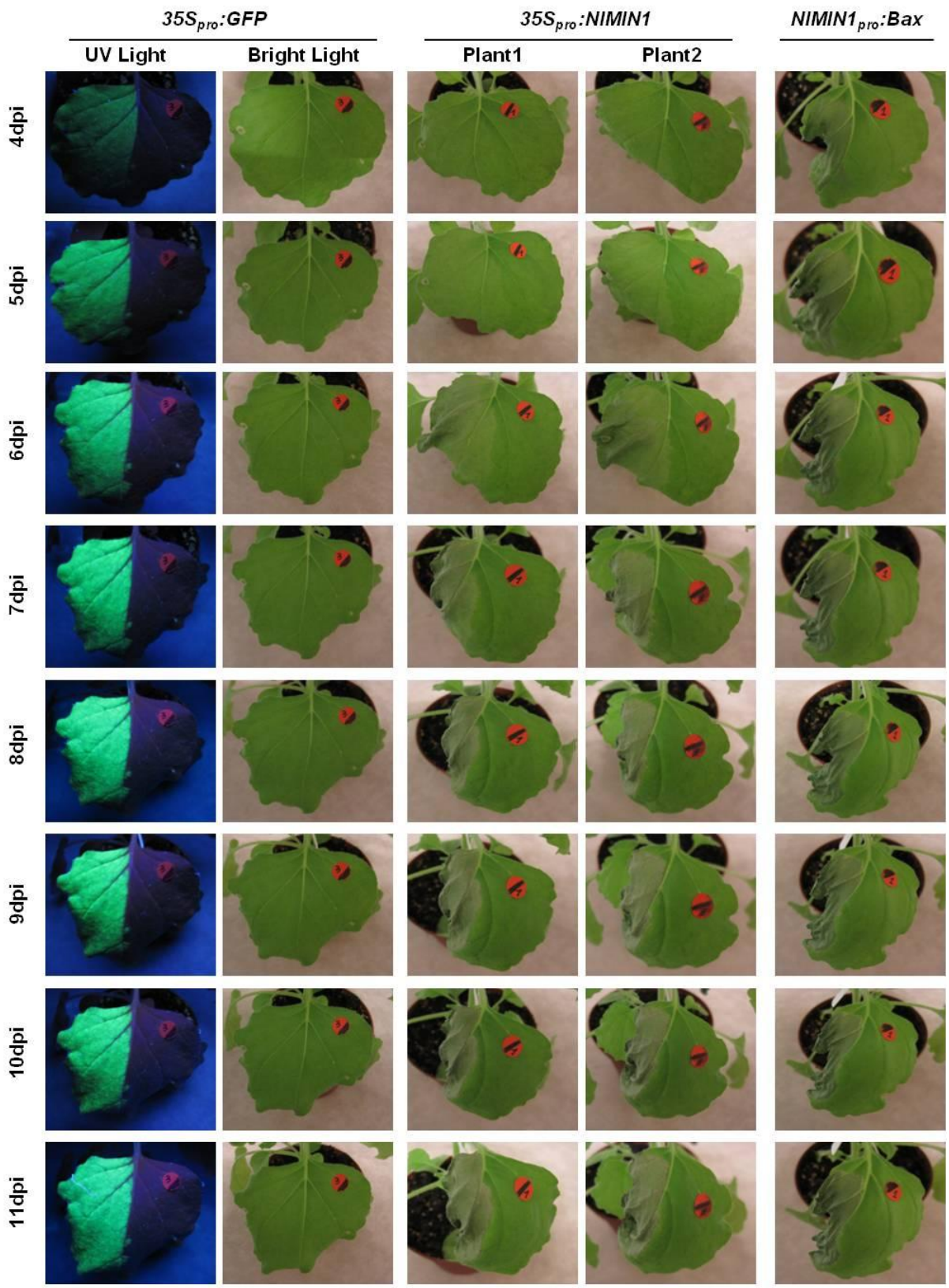


Fig. 21: Phenotypic analysis of *N. benthamiana* -1533PR-1a$_{pro}$:*GUS* plants over-expressing *GFP*, *NIMIN1* or *Bax* during time course studies. Left leaf halves of reporter line plants were infiltrated with *NIMIN1$_{pro}$:Bax*, *35S $_{pro}$:NIMIN1* or *35S $_{pro}$:GFP* Agrobacteria. Photos were taken under bright or UV-light at 4 dpi to 11 dpi.

level when infiltrated plant leaves were observed under UV-light announces that the maximum expression level was reached at 7 dpi, which exactly fits to the mentioned experimental schedule (Sec. 2.2.20.3).

The result presented here nevertheless shows that *NIMIN1* transient over-expression displays cell death symptoms like *Bax*, during time course experimental studies carried out in *N. benthamiana PR-1a$_{pro}$:GUS* transgene. Although results presented in Figure (21) are rather clear, but in order to be sure that the cell death phenomenon displayed in tissues infiltrated with *35S$_{pro}$:NIMIN1* Agrobacteria is not because of an artifact or technical mistake, two parallel strategies were adopted. The first was to revive mother culture freeze and raise the Agrobacterium strain harboring *35S$_{pro}$:NIMIN1* from a single freshly grown colony. This practice will eliminate the fact that the culture used was contaminated with cell death inducing factors. The other approach to use wild type plants instead of reporter line *N. benthamiana* plants will confirm that this novel cell death phenomenon was only because of *NIMIN1* over-expression, but not because of any accompanied feature of the transgenic line being used. Nevertheless, as shown in Figure (22), the cell death was pretty clear in wild type *N. benthamiana* plants infiltrated with freshly raised Agrobacterium strain carrying *35S$_{pro}$:NIMIN1* at 8 dpi. Routine expression was recorded when *GFP* agroinfiltrated plant tissues were exposed to UV-light.

In addition to that, Figure (19B), which is an independent experiment shows that infiltration of reporter line plants with freshly raised Agrobacterium strain harboring *35S$_{pro}$:NIMIN1* also show same repression effect on SA-mediated *PR-1a$_{pro}$* activation as observed in previous experiments. Hence, the presented result advocates that *NIMIN1* transient over-expression indeed generates cell death in *N. benthamiana* plants. Therefore, it was interesting to check for the phenotypic characteristics of other members of NIMIN protein family.

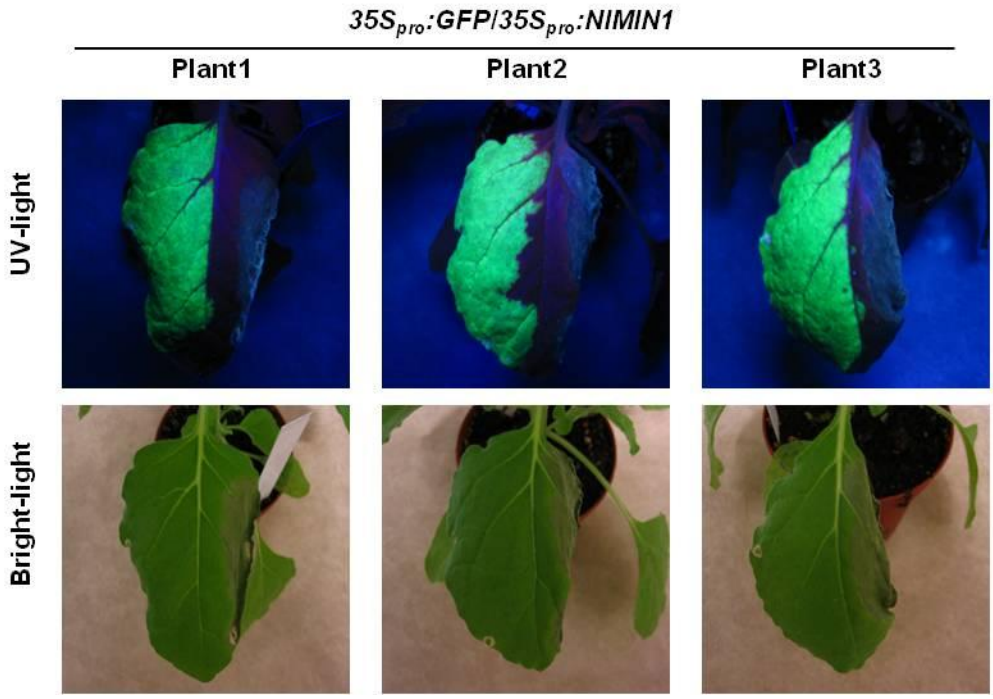


Fig. 22: Phenotypic analysis of wild type *N. benthamiana* plants over-expressing *GFP* or *NIMIN1*. Wild type *N. benthamiana* plant leaf halves were infiltrated with Agrobacterium strains harboring *35S$_{pro}$:GFP* on the left side and *35S$_{pro}$:NIMIN1* on the right side. Photographs were taken at 8 dpi.

The next lined up member for phenotypic characterization was NIMIN1b, as like NIMIN1 it represses the SA-mediated activation of *PR-1a$_{pro}$:GUS* reporter gene (Sec. 3.2.1) and secondly, the amino acid sequence reveals that both proteins are extremely similar (Fig. 23).

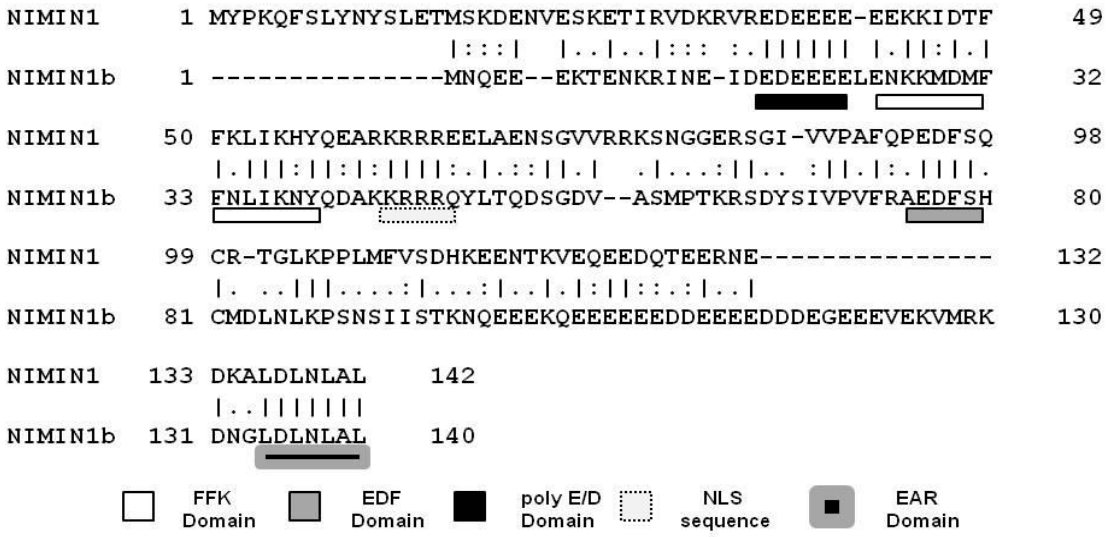


Fig. 23: Alignment of Arabidopsis NIMIN1 and NIMIN1b proteins. The sequence alignment was accomplished using EMBOSS Needle-alignment tool (http://www.ebi.ac.uk/Tools/psa/). Software determined proteins to be 38.1% identical and 52.5% similar. Horizontal dashes indicate gaps introduced to maximize alignment. Vertical dashes in between two aligned protein sequences represent the perfectly conserved amino acid residues.

However, *N. benthamiana* leaf halves over-expressing *NIMIN1* or *NIMIN1b* present quite astonishing results, as even though being so similar, NIMIN1b does not induce cell death (Fig. 24A). These unexpected phenotypic characteristics of *NIMIN1b* over-expression, felt mandatory to be carefully examined once more for a longer period of time in *N. benthamiana* plants. Therefore, time course studies were performed on *N. benthamiana* plant leaf halves infiltrated with $35S_{pro}$*:NIMIN1* or $35S_{pro}$*:NIMIN1b* Agrobacteria. However, much to surprise no cell death symptoms were recorded in plant leave tissues transiently over-expressing *NIMIN1b* even at 18 dpi (Fig. 24B). Thus, phenotypic studies clearly show that, despite many similarities, both NIMIN1 and NIMIN1b proteins show different phenotypic characteristics, when transiently over-expressed in *N. benthamiana* plants.

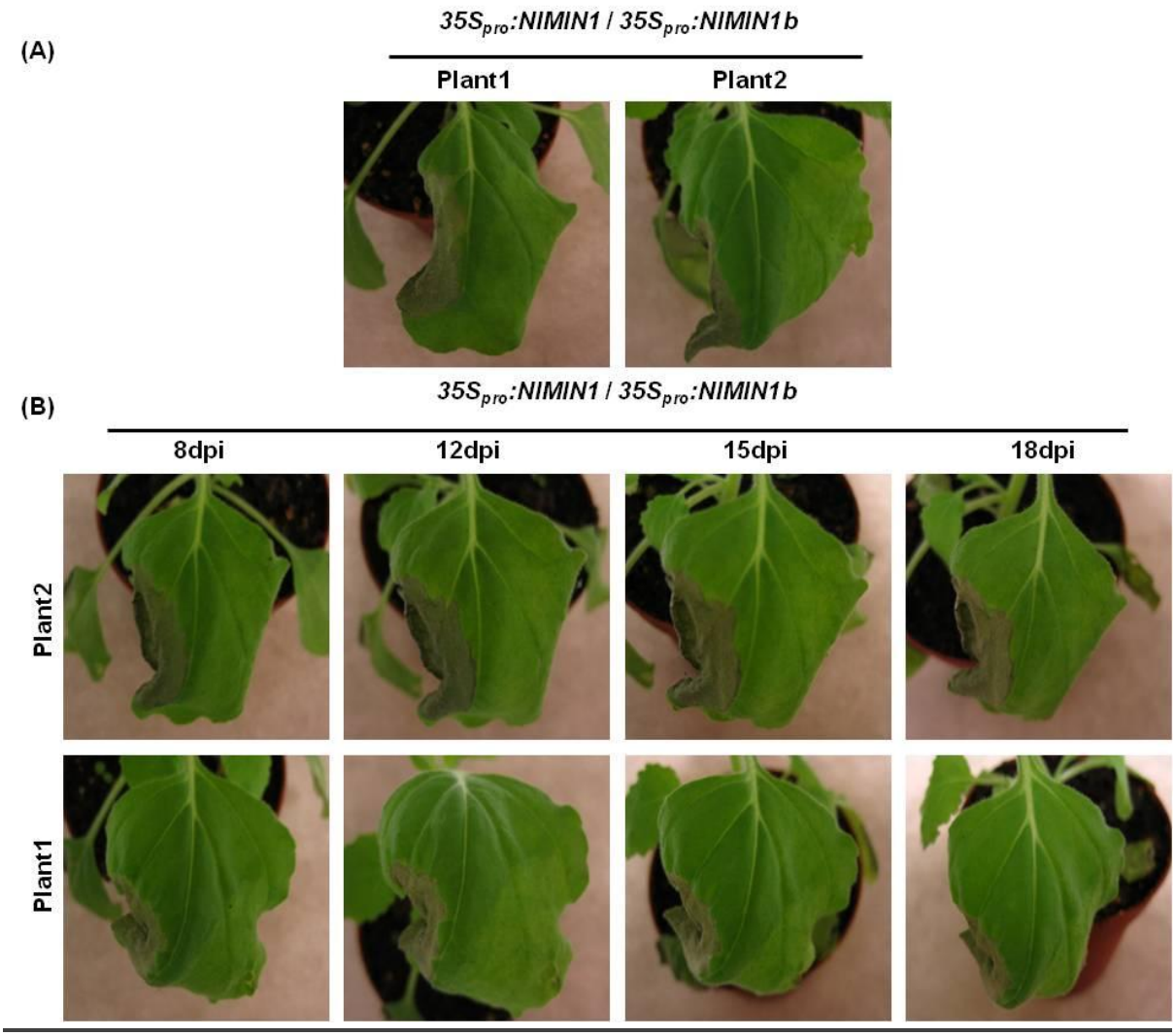


Fig. 24: Phenotypic effects of over-expression of *NIMIN1* and *NIMIN1b* in *N. benthamiana* - *1533PR-1a$_{pro}$:GUS* plants. Phenotypic effects on plant leaf halves infiltrated with Agrobacterium strains harboring $35S_{pro}$*:NIMIN1* (L-leaf halves) or $35S_{pro}$*:NIMIN1b* (R-leaf halves), recorded **A)** at 8 dpi and **B)** Time course studies carried out at 8, 12, 15 and 18 dpi**.**

As shown in Sec. 3.2.2, NIMIN3, like NIMIN1 and NIMIN1b proteins, also suppresses the SA-mediated *PR-1a* reporter gene induction. Therefore, Agrobacterium strains carrying *$35S_{pro}$:NIMIN3* or *$35S_{pro}$:NIMIN1* were infiltrated in *N. benthamiana* plant leaf halves to check, if *NIMIN3* like *NIMIN1* over-expression generates cell death. The result shows that *NIMIN3* over-expression does not produce cell death in *N. benthamiana* plant leaf halves when monitored until 8 dpi (Fig. 25A). More careful observation for cell death phenomenon in time course studies shows that *NIMIN3* agroinfiltrated leaf tissues indeed manifest cell death (Fig. 25B).

However, the cell death phenomenon induced by *NIMIN3* was clearly later than *NIMIN1* over-expressing plant leaf halves. Hence, this shows that, like *NIMIN1*, *NIMIN3* agroinfiltrated tissue display cell death albeit late, i.e., 12 dpi or later.

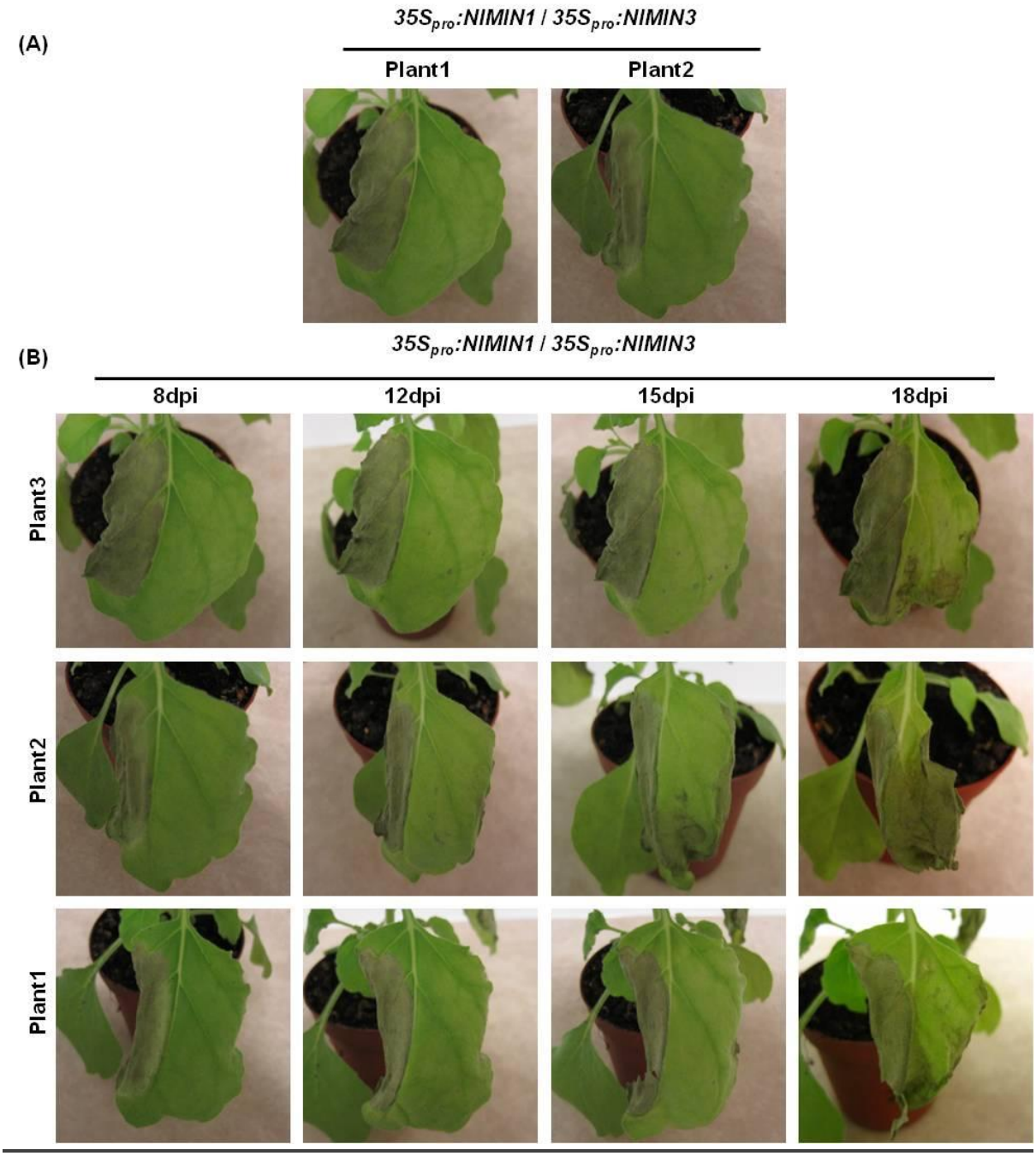


Fig. 25: Phenotypic effects of over-expression of NIMIN1 and NIMIN3 proteins in *N. benthamiana* -1533PR-1a$_{pro}$:GUS plants. Phenotypic effects on plant leaf halves infiltrated with Agrobacterium strains harboring *35S$_{pro}$:NIMIN1* (L-leaf halves) or *35S$_{pro}$:NIMIN3* (R-leaf halves) recorded **A)** at 8 dpi and **B)** Time course studies carried out at 8, 12, 15 and 18 dpi**.**

The role of H_2O_2 as a regulatory signal molecule of defense gene expression and cell death has been shown in several model systems (Levine et al., 1994; Willekens et al., 1994; Bestwick et al., 1997; Thordal-Christensen et al., 1997; Alvarez et al., 1998; Chamnongpol et al., 1998; Pellinen et al., 1999; Pellinen et al., 2002). To find the correlation between cell death and H_2O_2 accumulation, *NIMIN1* or *GFP* were transiently over-expressed in *N. benthamiana* leaf halves. H_2O_2 was visually detected in the leaves of plants by using 3,3-

diaminobenzidine (DAB) as substrate. H_2O_2 accumulation detected by DAB staining (Sec. 2.2.20.6), showed strong spatial correlation with cell death and was visible as dark-brown coloration only at and in near vicinity of the agro-infiltrated sites in plant leaf halves over-expressing *NIMIN1* (Fig. 26).

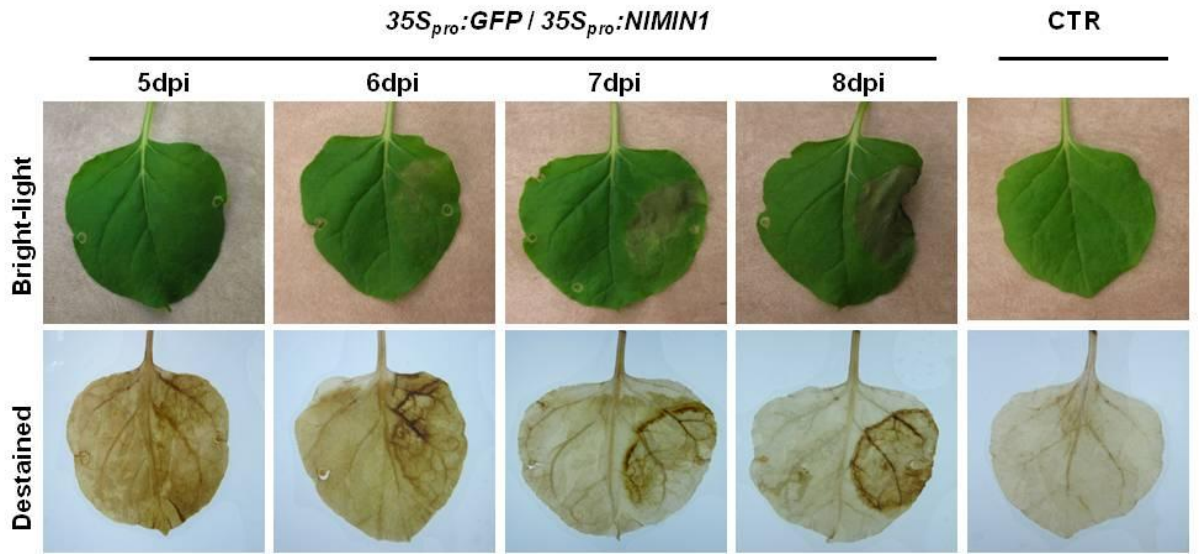


Fig. 26: DAB staining for H_2O_2 detection in *GFP* and *NIMIN1* over-expressing *N. benthamiana* plants. *N. benthamiana* leaf halves were infiltrated with *35S$_{pro}$:GFP* (L-leaf halves) or *35S$_{pro}$:NIMIN1* (R-leaf halves) harboring Agrobacterium strains. Photographs were recorded under bright-light or de-stained conditions at different time intervals. Non-infiltrated control leaf was photographed at 8 dpi.

Although minute phenotypic effects and H_2O_2 accumulation could be observed at 5dpi in *NIMIN1* agroinfiltrated *N. benthamiana* leaf halves, however, prominent results were only recorded at 6 dpi or later. On the other hand, no cell death or H_2O_2 accumulation was observed in non-infiltrated or *GFP* over-expressing plant leaf halves even at 8 dpi. This nevertheless proves that *N. benthamiana* leaf tissues over-expressing *NIMIN1* manifest cell death and this cell death is correlated with H_2O_2 accumulation. On the other hand, *N. benthamiana* plants leaf halves infiltrated with *35S$_{pro}$:GFP* Agrobacteria, does neither manifest cell death nor H_2O_2 accumulation, like non-infiltrated control plants.

As shown previously, infiltration of *NIMIN3* Agrobacteria like *NIMIN1* Agrobacteria cause cell death in *N. benthmiana* leaf tissues, but symptom appeared considerably later in case of NIMIN3. Therefore, comparison studies were done to find correlation between cell death and H_2O_2 accumulation in leaf halves of *N. benthamiana* plants over-expressing *NIMIN1* or *NIMIN3*. Keeping in mind that *NIMIN3* transient over-expression produces cell death albeit late, DAB staining for H_2O_2 determination was performed at two stages, i.e., 7 dpi and 12 dpi. Unlike *NIMIN1*, no clear cell death or H_2O_2 was detected in *NIMIN3* over-expressing

plant leaf halves when observed at 7 dpi (Fig. 27A). However, after 12 dpi, cell death was recorded in both *NIMIN1* and *NIMIN3* agroinfiltrated leaf halves (Fig. 27B). Hence, it shows that agroinfiltration of *N. benthamiana* leaf tissues with $35S_{pro}$*:NIMIN3* or $35S_{pro}$*:NIMIN1* harboring bacteria causes cell death accompanied by H_2O_2 accumulation, however, this phenomenon is faster in latter case.

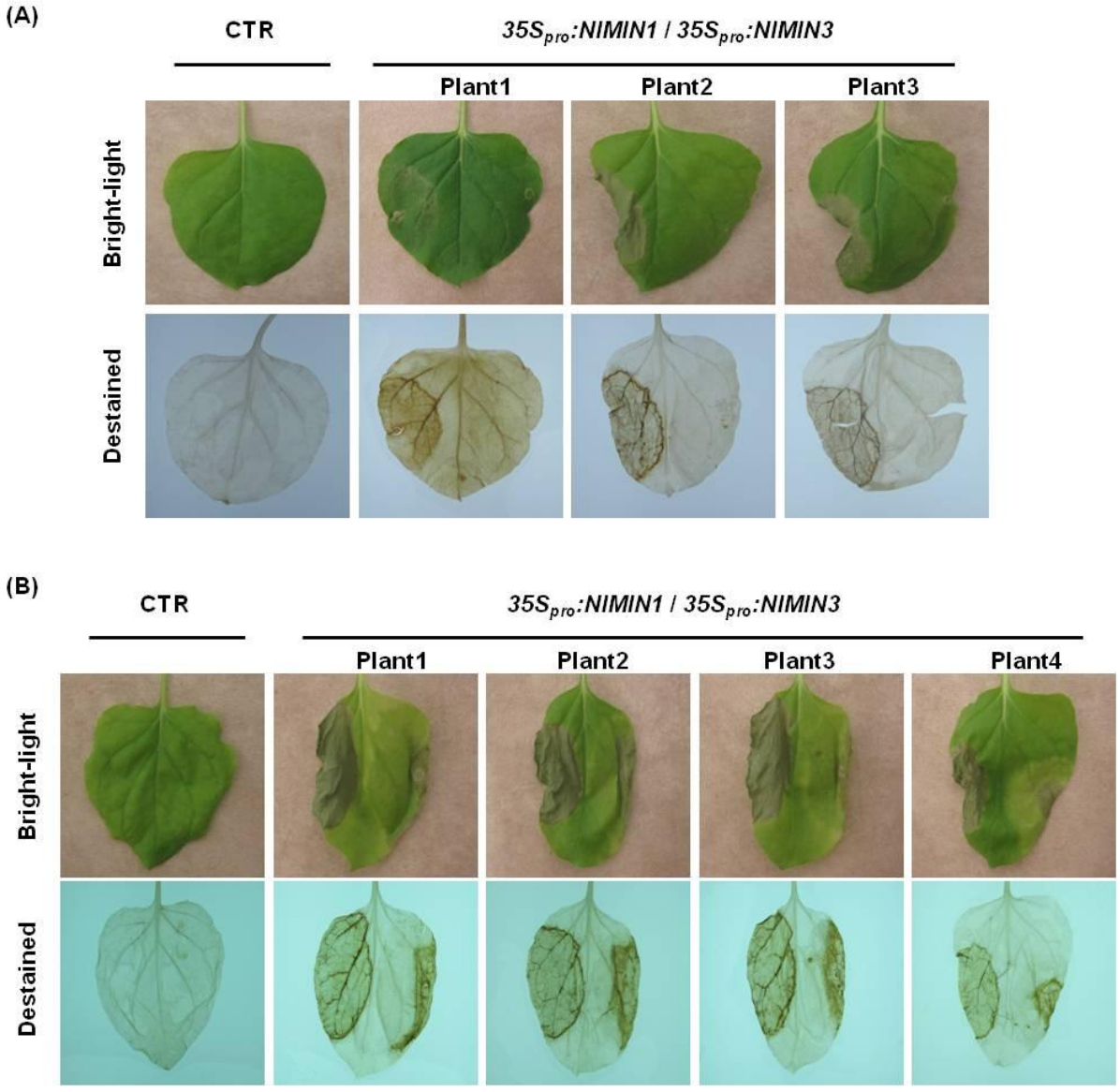


Fig. 27: DAB staining for H_2O_2 detection in *NIMIN1* and *NIMIN3* over-expressing *N. benthamiana* plants. *N. benthamiana* leaf halves were infiltrated with Agrobacterium strains harboring $35S_{pro}$*:NIMIN1* (L-leaf halves) or $35S_{pro}$*:NIMIN3* (R-leaf halves). Agroinfiltrated and control plant leaves were detached and DAB staining was performed. Photographs were recorded under bright-light or de-stained conditions **A)** at 7 dpi and **B)** at 12 dpi.

3.3.2 Phenotypic effects of over-expression of *NIMIN2*

The transient expression studies carried out in *N. benthamiana* reporter line plants, advocates NIMIN2 role as non-suppressive protein in SA-mediated *PR-1a* reporter gene induction (Sec. 3.2.3). The characteristic mentioned above declares NIMIN2 as a unique protein among the whole NIMIN gene family in Arabidopsis. Therefore, efforts were made to reveal the phenotypic characteristics of over-expression of NIMIN2 on *N. benthamiana* plants leaf tissue. Figure (28A) clearly shows that after 8 dpi in opposition to *NIMIN1*, there were no cell death symptoms in *NIMIN2* over-expressing plant leaf halves. Although results presented here were pretty convincing nevertheless, it was felt necessary to look for the *NIMIN2* over-expression effect for a longer period of time, as practiced for NIMIN1b and NIMIN3. Therefore, parallel agroinfiltration of *NIMIN1* or *NIMIN2* in *N. benthamiana* plants leaf halves was carried out. As usual, *NIMIN1* over-expression was accompanied by heavy cell death after 8 dpi, but *NIMIN2* over-expressing plant leaf halves showed no cell death when followed up to 18 dpi (Fig. 28B). This idea was further confirmed by H_2O_2 accumulation in *NIMIN1*, but not in *NIMIN2* over-expressing leaf halves (Fig. 28C). Hence, it clearly shows that *NIMIN2* neither manifests cell death nor H_2O_2 accumulation when transiently over-expressed in plants.

Previously it has been shown that NIMIN1 and NIMIN2 harbor structurally related NPR1 interaction motifs and both proteins bind to the C-terminus of NPR1 (Weigel et al., 2001). However, results presented in this study clearly suggest that NIMIN1 and NIMIN2 are different from each other in two aspects, i.e., manifestation of cell death and suppression of SA-mediated *PR-1a*$_{pro}$*:GUS* transgene. This knowledge brought motivation to study competition between the two NIMIN proteins in terms of their phenotype development as well as effects on SA-mediated *PR-1a* reporter gene induction. Therefore, cell suspensions containing equal proportion of *35S*$_{pro}$*:NIMIN1* and *35S*$_{pro}$*:NIMIN2* carrying Agrobacterium strains were co-infiltrated in *N. benthamiana* reporter line leaf halves. Additionally, to witness the individual effect of NIMIN1 protein, *NIMIN1* was transiently over-expressed in parallel leaf halves of *N. benthamiana* in the absence of *NIMIN2* and *vice versa*. Induction medium (10mM $MgCl_2$ and 100µM acetosyringone) was mixed in cell suspensions to facilitate equal amount infiltration of *35S*$_{pro}$*:NIMIN1* or *35S*$_{pro}$*:NIMIN2* Agrobacteria in both leaf halves of reporter line. Figure (29A) shows that individual over-expression of *NIMIN1* in *N. benthamiana* reporter line plants induces cell death at 8 dpi and proceeds with the passage

of time, i.e., 10 and 12 dpi. The co-expression of both *NIMINs* however, develops late cell death, but ultimately results in same amount of damage at 12 dpi. On the contrary, *$35S_{pro}$:NIMIN2* agroinfiltrated plant leaf tissues showed no cell death when monitored up to 12 dpi. Transient assays were performed on plant extracts made from the very same plants to check for effect of individual or co-expression of *NIMIN1* and *NIMIN2* on SA-mediated *PR-$1a_{pro}$* activation (Fig. 29B). The data clearly demonstrate that NIMIN1 is the stronger candidate between the two, as the co-expression experiments always suppressed the SA-mediated *PR-$1a_{pro}$:GUS* reporter gene induction in *N. benthamiana*. The results shown in Figure (29C) reconfirm the data presented in Figure (29B). Here, additional water floating makes it clearer that there is no induction of reporter gene in *NIMIN2* only or *NIMIN1* and *NIMIN2* co-agroinfiltration, thereby removing the fact that infiltration of *$35S_{pro}$:NIMIN2* harboring Agrobacteria can induces *PR-1a* reporter gene itself, i.e., without SA dose. The data suggest that although *NIMIN2* delays cell death when co-infiltrated with *NIMIN1*. Thus, TGES clearly demonstrates that NIMIN1 is dominant over NIMIN2 in terms of reporter gene induction as well as cell death phenotype.

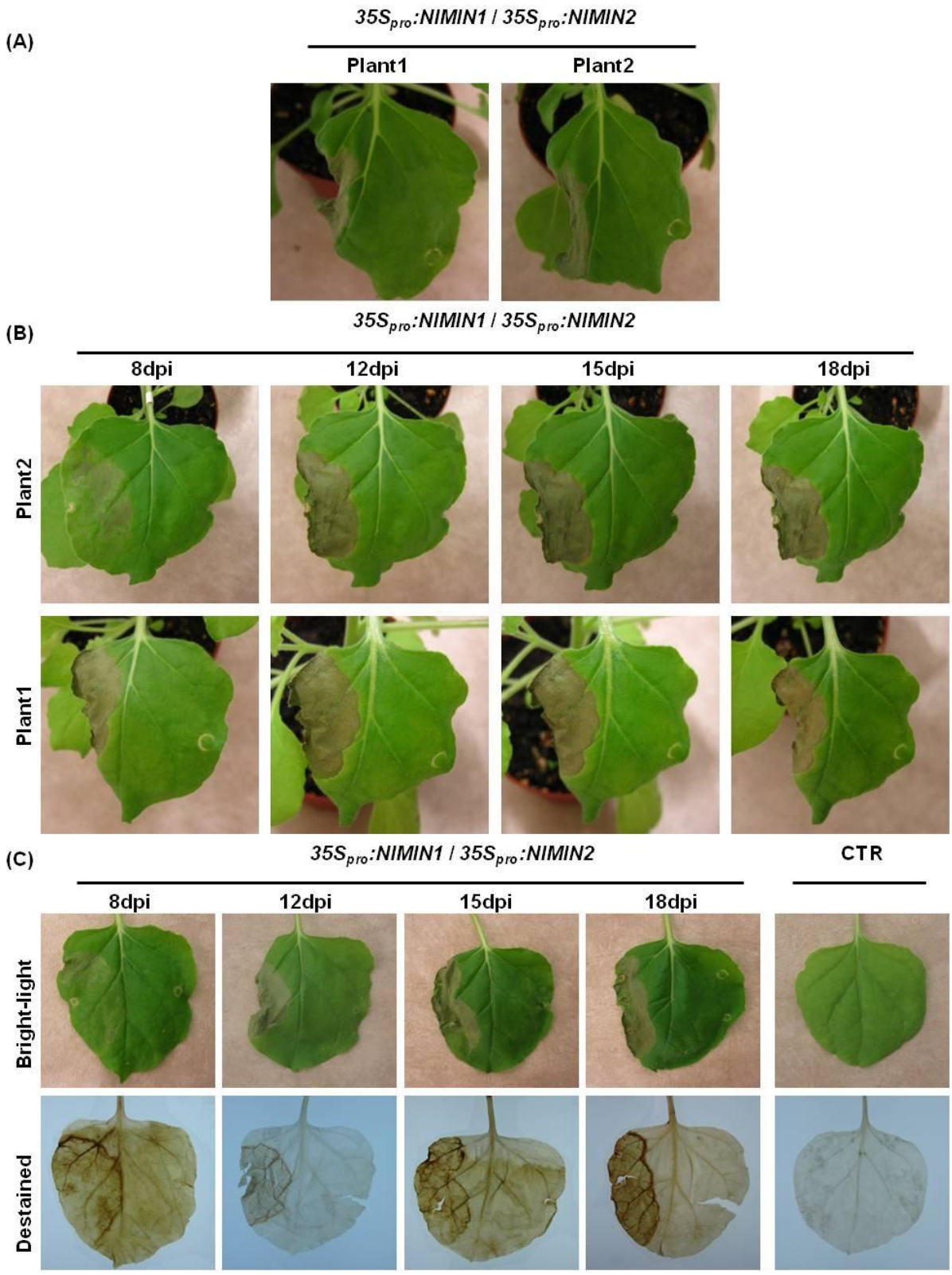


Fig. 28: Phenotypic effects of over-expression of *NIMIN1* and *NIMIN2* in *N. benthamiana* plants. Phenotypic effects on *N. benthamiana* plant leaf halves infiltrated with Agrobacterium strains harboring *35S$_{pro}$:NIMIN1* (L-leaf halves) or *35S$_{pro}$:NIMIN2* (R-leaf halves), recorded **A)** at 8 dpi. **B)** Time course studies carried out at 8, 12, 15 and 18 dpi. **C)** DAB staining for H_2O_2 detection in detached agroinfiltrated plant leaf halves. Photographs were recorded under normal or de-stained conditions at 8, 12, 15 and 18 dpi. Non-infiltrated control leaf photograph was taken at 18 dpi.

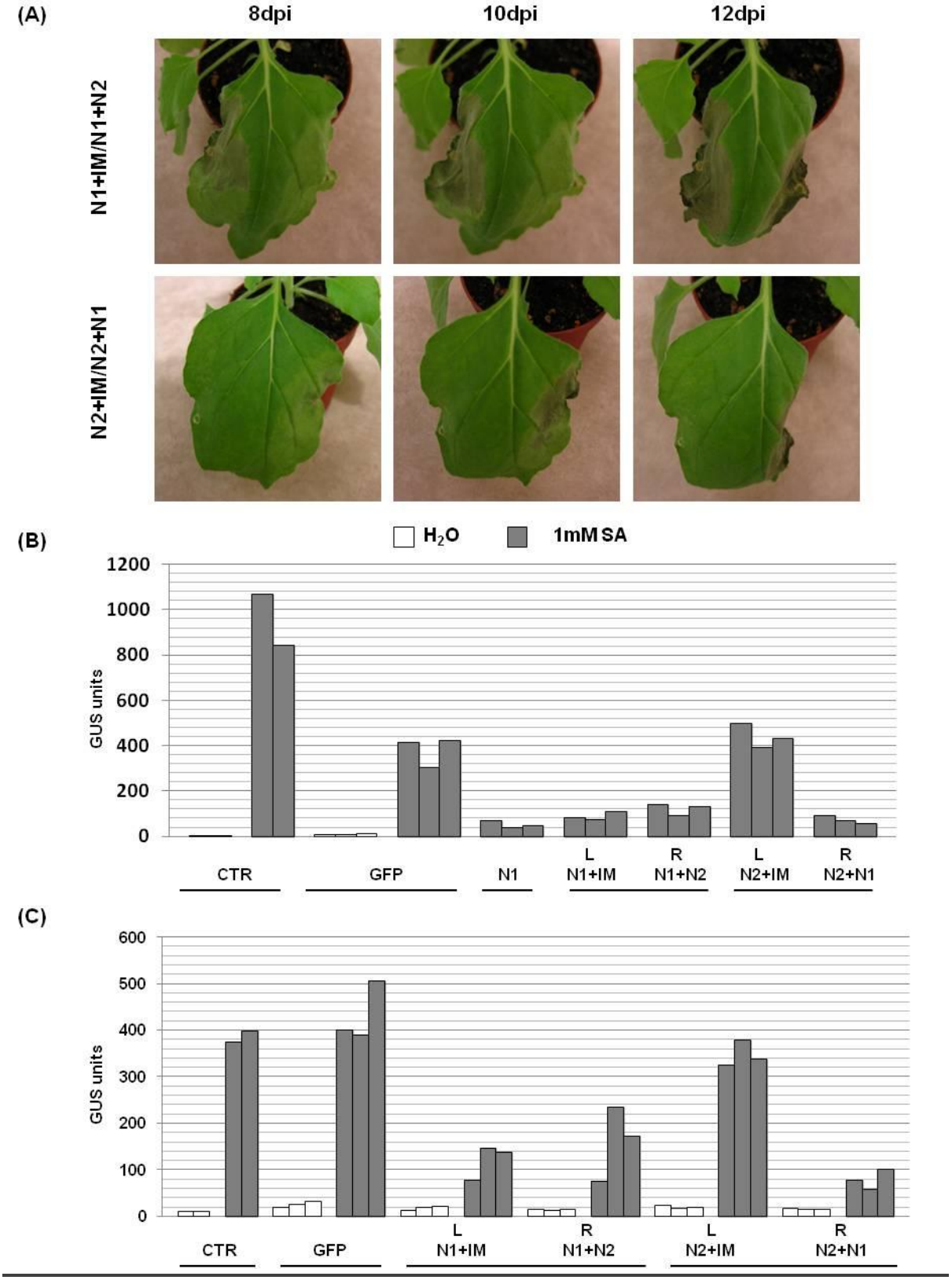


Fig. 29: Co-expression of *NIMIN1* and *NIMIN2* in *N. benthamiana* -1533PR-1a$_{pro}$:*GUS* plants. Reporter line plant leaf halves over-expressing *NIMIN1* plus induction medium (IM) or *NIMIN1* plus *NIMIN2* mixture and *vice versa*, **A)** Time course studies in *N. benthamiana* plant leaf tissues for gradual symptom development was recorded at 8, 10 and 12 dpi. **B)** GUS activities from leaf tissue shown in A. Part of this experiment has already been shown in Figure (14B). **C)** GUS activities of an independent infiltration experiment.

3.3.3 Effects of transient over-expression of *NIMIN1* and *NIMIN3* mutants

It is pretty clear that *NIMIN1* transient over-expression manifests cell death in infiltrated plant leaf tissues. Utilizing availability of different *NIMIN1* mutants in over-expression studies in *N. benthamiana* plants will help in understanding the cell death phenomenon in detail. Therefore, *NIMIN1* mutants which have already been characterized for their influence on *PR-1a* gene induction (Sec. 3.2.4.3) were transiently over-expressed in *N. benthamiana* plant leaf tissues. Mutant protein NIMIN1 F49/50S is known not to be able to bind NPR1 and it has been shown in this study via *N. benthamiana* TGES that NIMIN1 F49/50S does not suppress the SA-mediated *PR-1a*$_{pro}$*:GUS* reporter gene induction (Fig. 14). However, Figure (30) shows that *NIMIN1* transient over-expression in *N. benthamiana* leaf halves induced quite prominent cell death at 8 dpi, whereas no cell death was recorded in *NIMIN1 F49/50S* agroinfiltrated leaf halves when followed until 18 dpi.

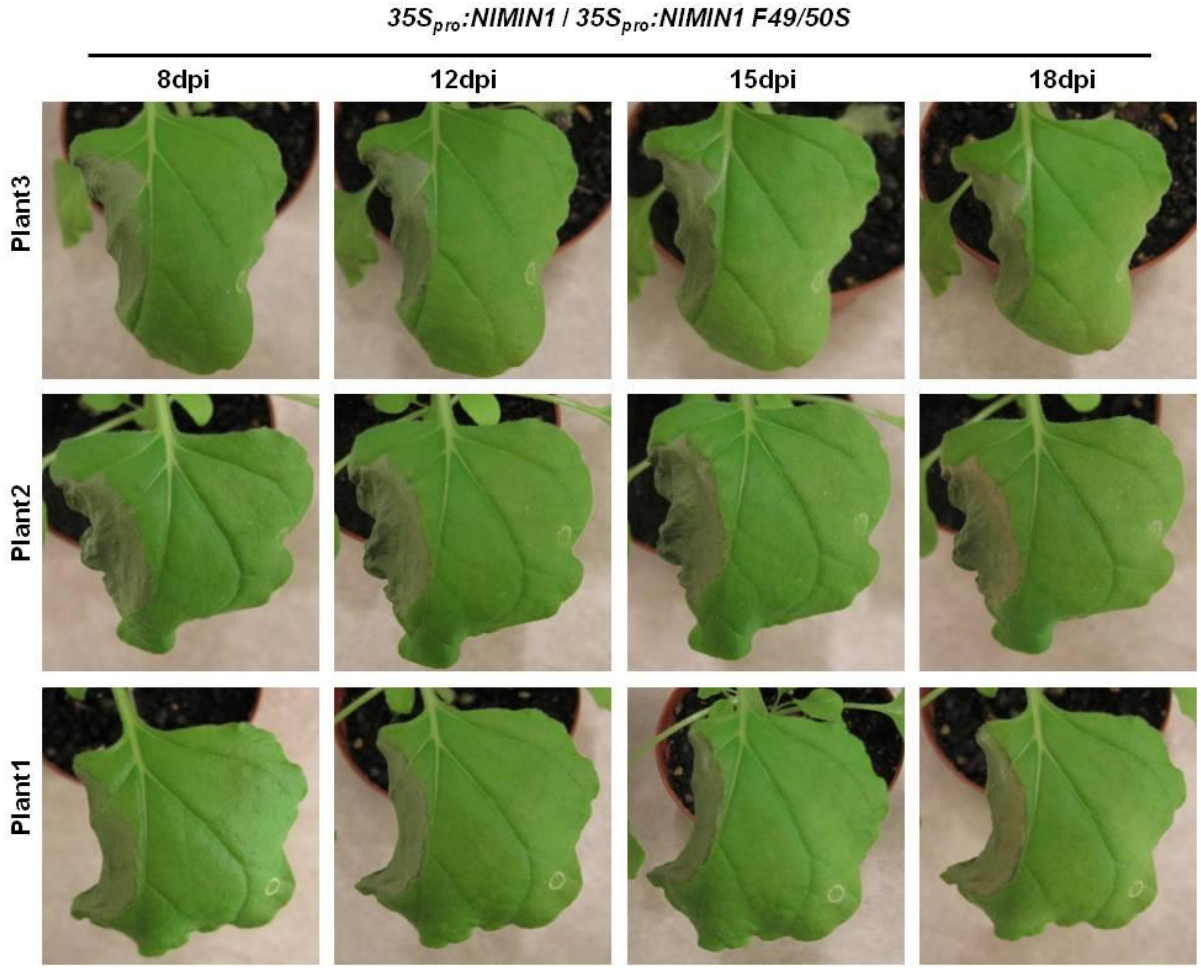


Fig. 30: Phenotypic effects of over-expression of *NIMIN1* and *NIMIN1 F49/50S* in *N. benthamiana* plants. *N. benthamiana* leaf halves were infiltrated with Agrobacterium strains harboring *$35S_{pro}$:NIMIN1* (L-leaf halves) or *$35S_{pro}$:NIMIN1 F49/50S* (R-leaf halves). Gradual symptom development was recorded at 8, 12, 15 and 18 dpi**.**

Transient over-expression of *NIMIN1 E94A D95V* mutant in *N. benthamiana* plant leaf halves does not suppress the SA-mediated *PR-1a_{pro}* activation and Y2H data show that the mutant protein still interacts with NPR1(Figs. 13 and 18A). Therefore, it was of interest to know if *$35S_{pro}$:NIMIN1 E94A D95V* harboring Agrobacterium strain infiltration can still induce cell death in infiltrated leaf halves. However, the data show that *NIMIN1 E94A D95V* agroinfiltration in *N. benthamiana* plant leaf halves produces cell death albeit weaker and later, i.e., at 12 dpi compared to NIMIN1 which develops stronger and faster cell death, i.e., at 8 dpi (Fig. 31).

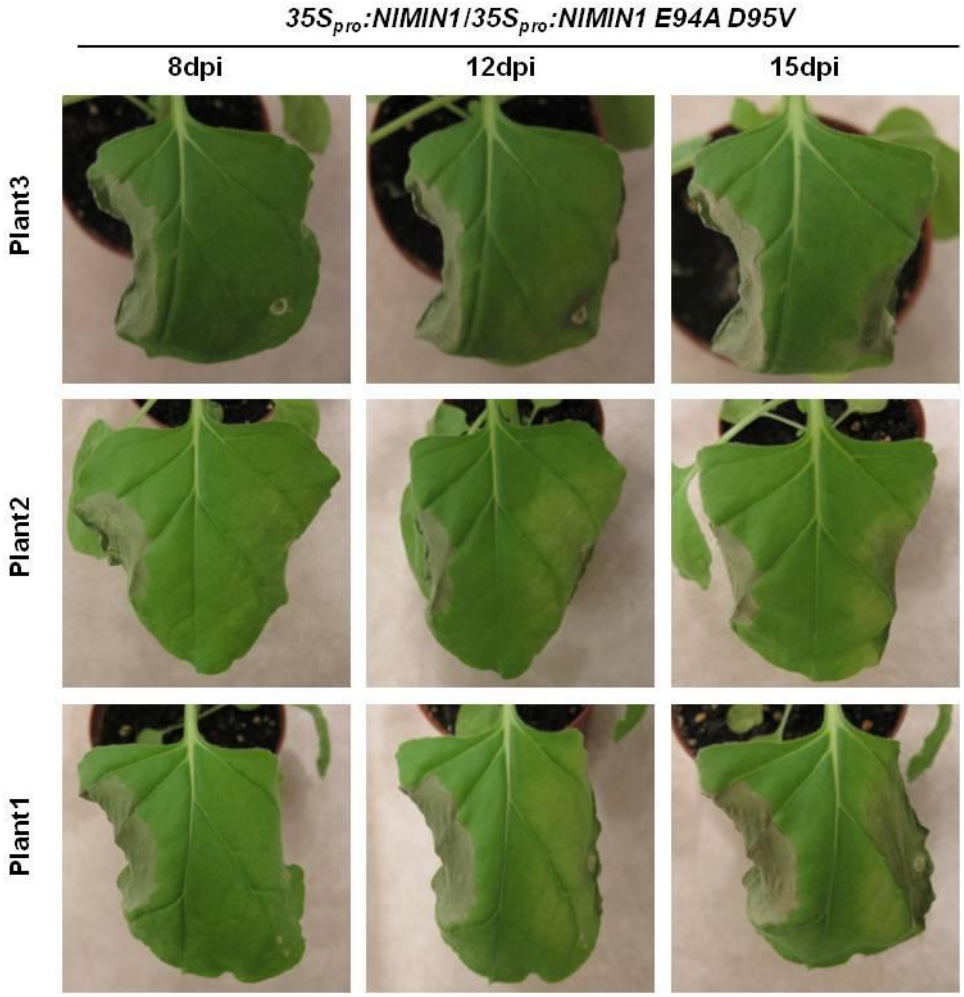


Fig. 31: Phenotypic effects of over-expression of *NIMIN1* and *NIMIN1 E94A D95V* in *N. benthamiana* plants. *N. benthamiana* plant leaf halves were infiltrated with Agrobacterium strains harboring *$35S_{pro}$:NIMIN1* (L-leaf halves) or *$35S_{pro}$:NIMIN1 E94A D95V* (R-leaf halves). Gradual symptom development was recorded at 8, 12 and 15 dpi**.**

The situation with NIMIN1 L138A L140A protein was not much different from NIMIN1E94A D95V protein, the transient over-expression of *NIMIN1 L138A L140A* in *N. benthamiana* plant leaf halves generates cell death, but late and weaker, i.e., at 12 dpi, in comparison with *NIMIN1* (Fig. 32).

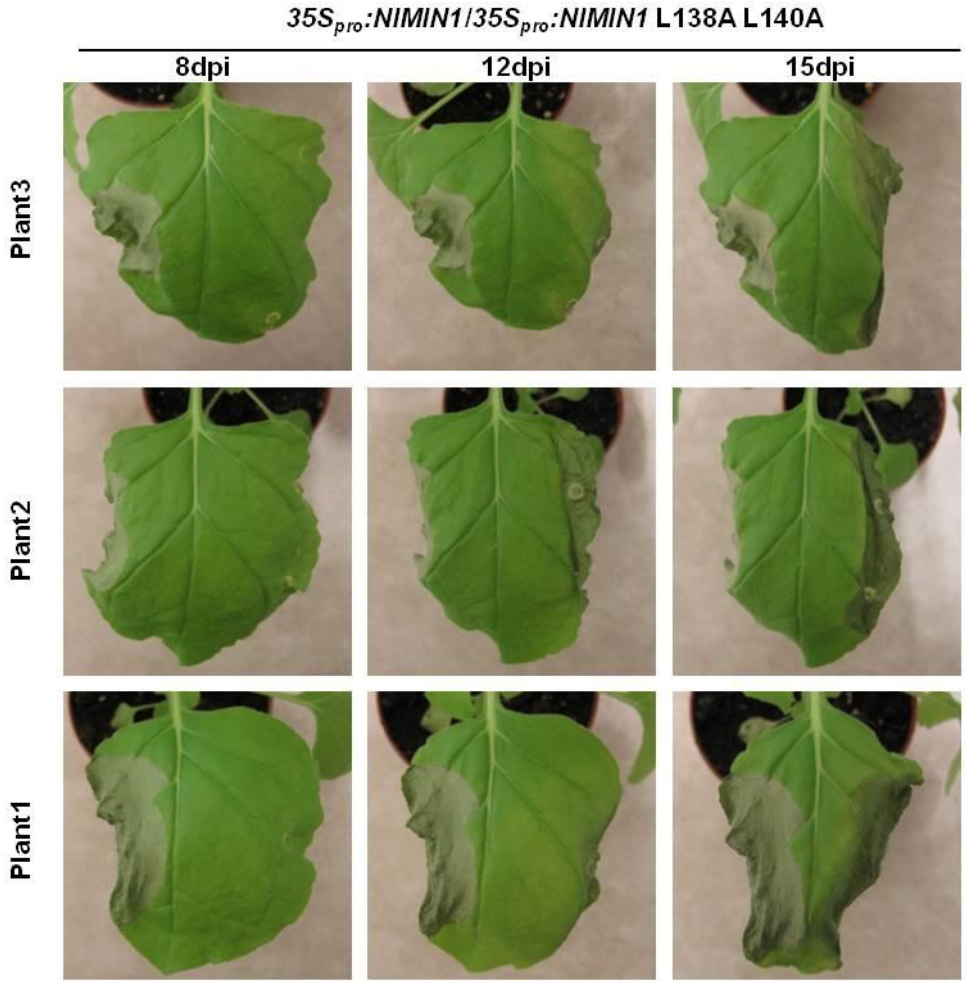


Fig. 32: Phenotypic effects of over-expression of *NIMIN1* and *NIMIN1 L138A L140A* in *N. benthamiana* plants *N. benthamiana* leaf halves were infiltrated with Agrobacterium strains harboring *35S$_{pro}$:NIMIN1* (L-leaf halves) or *35S$_{pro}$:NIMIN1* L138A L140A (R-leaf halves). Gradual symptom development was recorded at 8, 12 and 15 dpi**.**

NIMIN1 1/137 over-expression interestingly showed clear cell death in *N. benthamiana* plant leaf halves at 8 dpi which became clearer at 12, 15 (Fig. 33A) and 18 dpi (Fig. 33B). Although the symptom emergence in case of *NIMIN1 1/137* over-expression was delayed as compared to *NIMIN1* over-expression, but *NIMIN1 1/137* indeed showed strongest cell death among the entire *NIMIN1* mutants tested in this study.

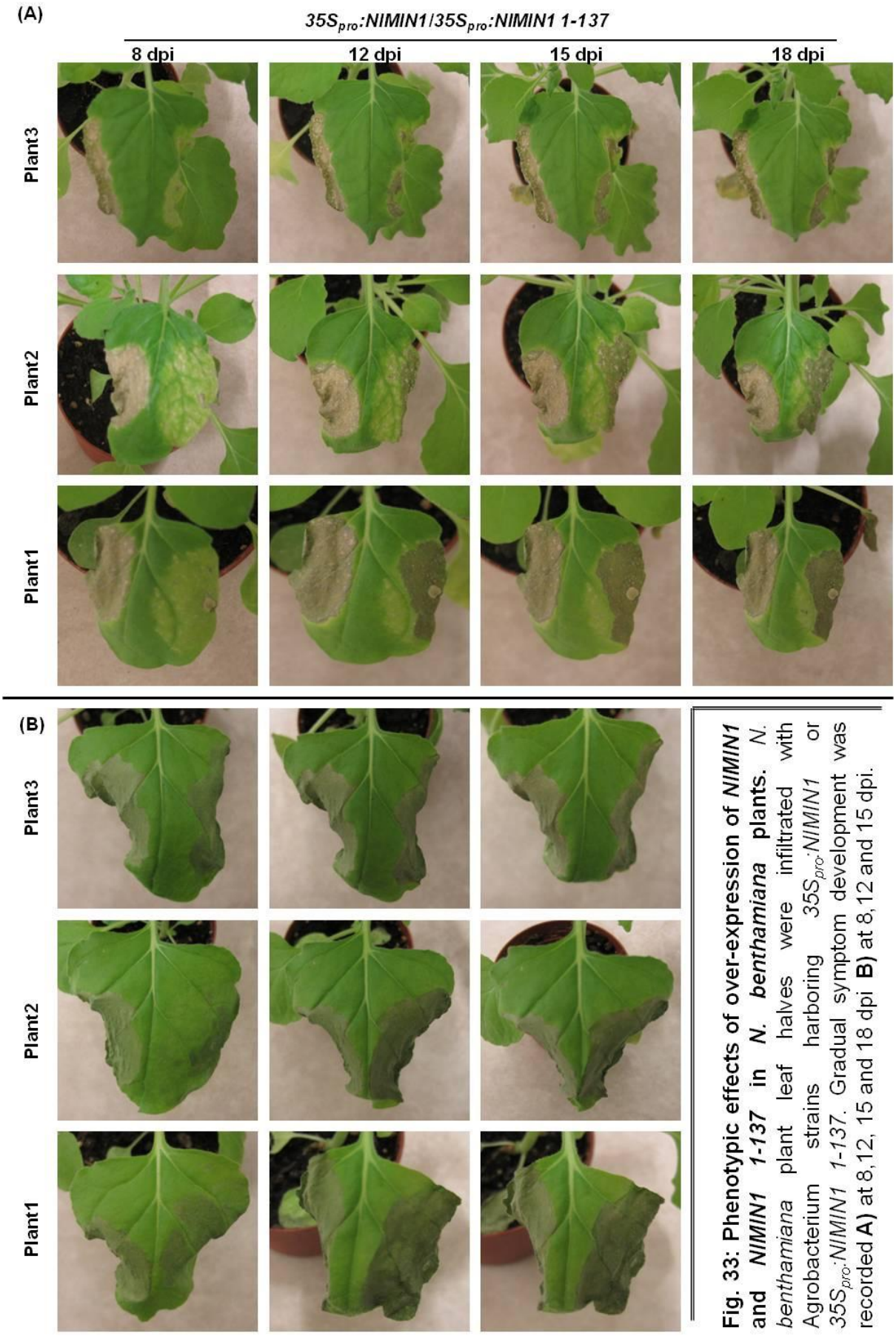


Fig. 33: Phenotypic effects of over-expression of *NIMIN1* and *NIMIN1 1-137* in *N. benthamiana* plants. *N. benthamiana* plant leaf halves were infiltrated with Agrobacterium strains harboring 35S$_{pro}$:*NIMIN1* or 35S$_{pro}$:*NIMIN1 1-137*. Gradual symptom development was recorded **A)** at 8,12, 15 and 18 dpi **B)** at 8,12 and 15 dpi.

Infiltration of Agrobacterium strain harboring *$35S_{pro}$:NIMIN3* like *$35S_{pro}$:NIMIN1* also develops cell death in *N.benthamiana* plants. Along with NIMIN1 mutant, two NIMIN3 mutants namely NIMIN3 E63A D64V and NIMIN3 L108A L110A were available that can help in revealing the mechanism behind the emergence of cell death. Unfortunately, the phenotypic characteristics were not recorded because the transient over-expression of *NIMIN3* mutants did not show clear and satisfactory data (see discussion).

3.4. Characterization of novel *Nicotiana tabacum* (Nt) *NIMIN* genes

Existence of *NIMIN* genes in higher plants has been approved by two independent research groups (Weigel et al., 2001; Chern et al., 2005; Zwicker et al., 2007). Like other higher plants, tobacco also contains *NIMIN* genes. In tobacco, *NIMIN2a*, also called *G8-1*, was the first cloned *NIMIN* gene. It was characterized as a gene rapidly induced by SA, however, its functional significance has not been determined (Horvath et al., 1998). Some other tobacco *NIMIN* genes, e.g., *NIMIN2b* and *NIMIN2c*, have also been cloned (Zwicker et al., 2007). In addition to that, even more *NIMIN*-like sequences from tobacco are available in Genbank database. This information gave hint that tobacco NIMIN proteins might function in somewhat similar fashion as NIMIN proteins in Arabidopsis.

3.4.1 Cloning of two novel tobacco *NIMIN* genes

Using the amino acid sequence of Nt NIMINs as the probe sequence, a BLAST search against the expressed sequence tags (ESTs) of the Genbank database was performed. This enabled the identification of NMIN-like sequences, harboring highly conserved NPR1 binding site. From Genbank data, two EST sequences (i.e., BP531936 and FS401103) were chosen for further analysis (Fig. 34). Primers (Tab. 3) were designed based on the available sequences and genes were amplified from genomic DNA of tobacco cultivar Samsun NN using standard PCR reaction. The sizes of the PCR products correspond to the predicted sizes (Fig. 35). As a control, the *NIMIN2a* gene was amplified in parallel. PCR fragments were cloned into T-vector and then sequenced. The sequences of two amplified genes were found identical to EST sequences described in Genbank (Fig. 34). From this point onwards, ESTs BP531936 and FS401103 will be called *NIMIN-like1* and *NIMIN-like2*, respectively. The two novel NIMIN-like proteins contain all characteristic structural domains, as found in other known NIMIN proteins, thereby fitting them easily in growing tobacco NIMIN protein family (Fig. 36A and B). Amino acid multiple alignments show that, among all tobacco NIMIN proteins, NIMIN-like1 is unique one, as it contains EDF domain which is not present in any other Nt NIMIN protein (Fig. 34; Fig. 36B and C).

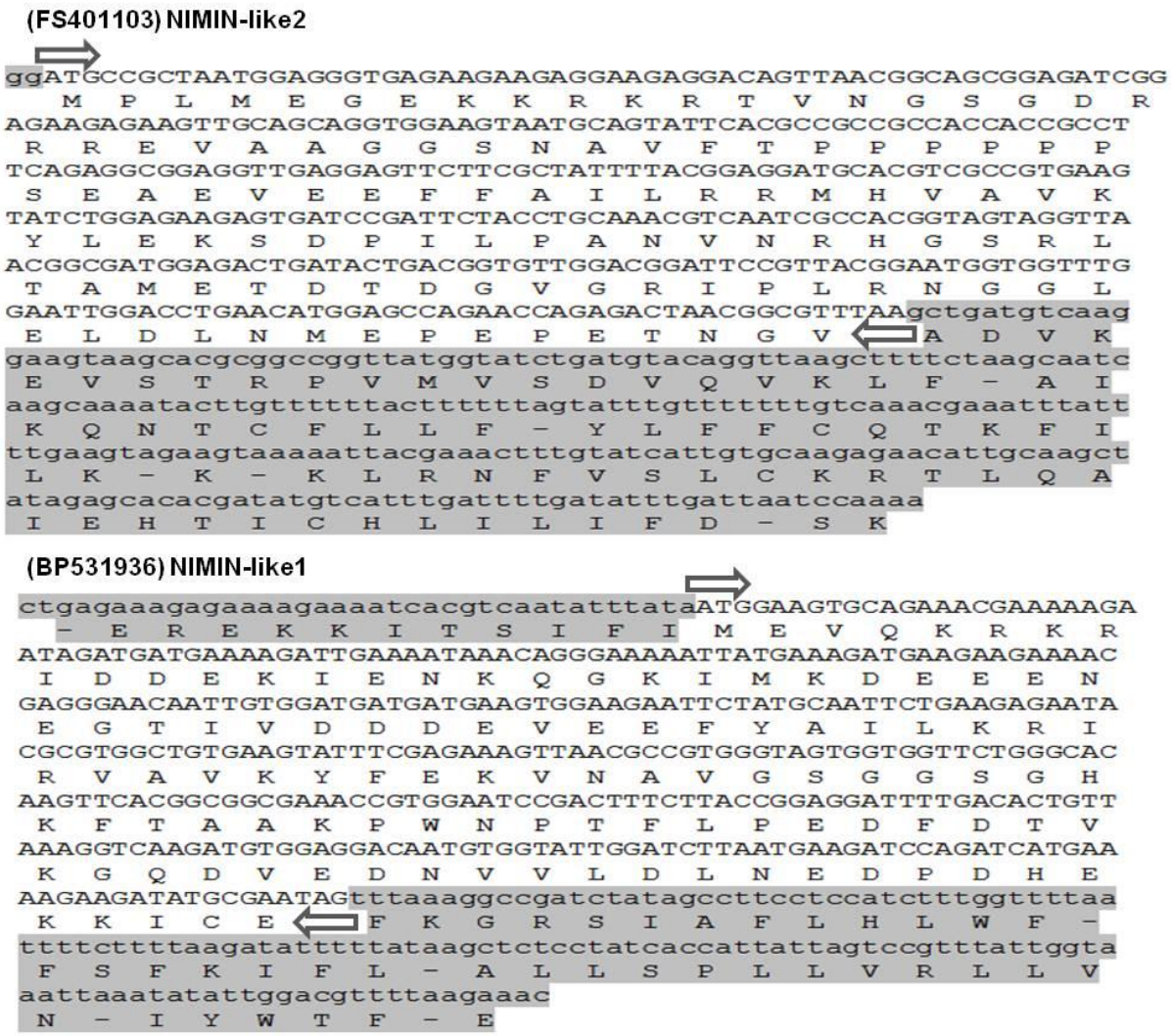


Fig. 34: Nucleotide and amino acid sequences of two novel tobacco *NIMIN* genes. Full-length cDNA and deduced amino acid sequences of NIMIN-like1 and NIMIN-like2. Arrow indicates the site of primer sitting. Area outside start and stop codon are represented in gray color. The protein sequence was obtained after cDNA translation using the translate tool in the ExPASy server (http://web.expasy.org /translate/).

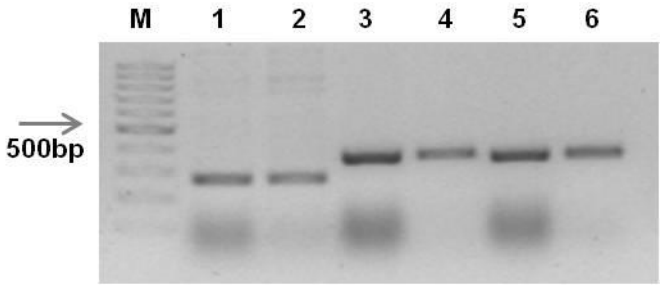
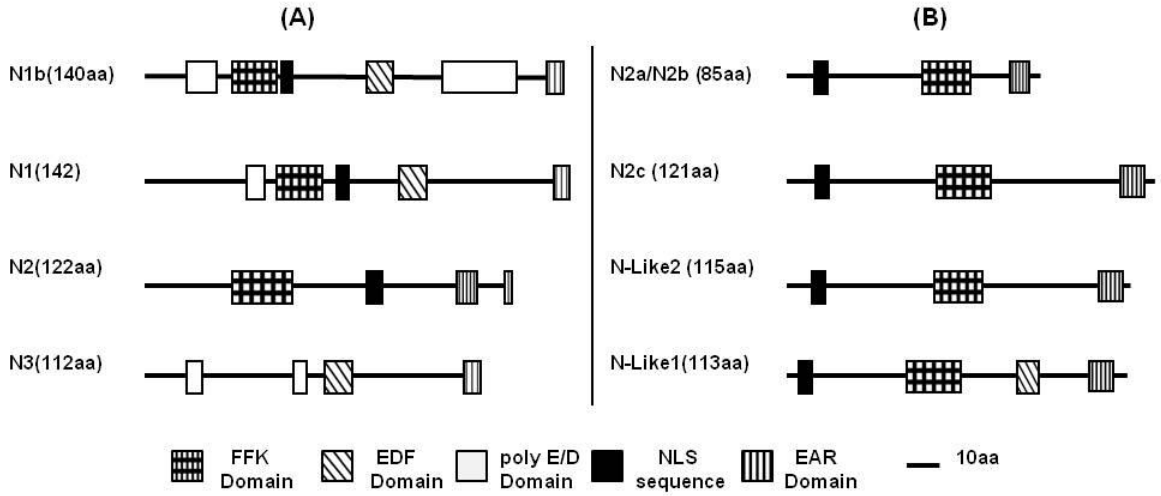


Fig. 35: Amplification of two novel *NIMIN* genes from tobacco. PCR amplification of *NIMIN-like2* (lane 5 and 6, 363bp), *NIMIN-like1* (lane 3 and 4, 357bp) and *NIMIN2a* (lane 1 and 2, 273bp) by using *N. tabacum* cv. Samsun NN genomic DNA). M stands for 100bp marker.



(C)

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NIMIN2a          ----MDGEKKRKRTENGKANGGDRNRHERKSAAN--EHTAVSPPPSEAEVDEFFAILRRM
NIMIN2b          ----MDGEKKRKRTNNGEANGGDWNKHERESAAN--EDIAVAPPPSEAEVDEFFAILRRM
NIMIN2c          MLLTMDGEKKRKRTENDEVNGGDRSKHEIENAVKEKEETVVLPPPSEAEVDEFFAILRRM
NIMIN-Like2      -MPLMEGEKKRKRT----VNGSGDRRREVAAGGSNAVFTPPPPPPSEAEVEEFFAILRRM
NIMIN-Like1      ----MEVQKRKRIDDEKIENKQGKIMKDEEENEG--------TIVDDDEVEEFYAILKRI
                     *: :*::: *  .   ::             .  .: **:**:***:*:

NIMIN2a          HVAVRYLQESGQKR-----------------------------VVPKGDLDLNTLPGNG
NIMIN2b          HVAVKYLQKSGQKR-----------------------------VVPKGDLDLNTLPENG
NIMIN2c          HVAVKYLQKNSQILPIVNDHGSKLTASPAGVNSIVVAGQRRTQGIVRKGGLDLNALPEGG
NIMIN-Like2      HVAVKYLEKSDPILPANVNRHG----SRLTAMETDTDGVGRIPLRNGGLELDLNMEPEPE
NIMIN-Like1      RVAVKYFEKVNAVGSGGSGHKFTAAKPWNPTFLPEDFDTVKGQDVEDNVVLDLNEDPDHE
                 :***:*::: .                                        ****  *

NIMIN2a          D----
NIMIN2b          D----
NIMIN2c          D----
NIMIN-Like2      TNGV-
NIMIN-Like1      KKICE
```

Fig. 36: Different Arabidopsis and tobacco NIMIN proteins. Schematic representation of NIMIN proteins showing their characteristic domains A) Arabidopsis NIMIN proteins **B)** Tobacco NIMIN proteins. N stands for NIMIN. **C)** Multiple alignment of NIMIN proteins from tobacco was performed using CLUSTALW program (http://www.genome.jp/tools/clustalw/). EDF motif (underlined here) was found only in NIMIN-like1.

After verification of sequences, novel tobacco genes were cloned as *Bam*HI fragments into yeast vector pGBT9 (Fig. 37). To further reveal functional significance of Nt NIMINs in plants, *NIMIN-like1* and *NIMIN2c* genes were PCR amplified as *Bam*HI/*Sac*I fragments using specific primers and template (Tab. 3). The PCR yielded the expected fragments of 360bp and 384bp for *NIMIN-like1* and *NIMIN2c*, respectively. PCR fragments were cloned into a T-vector and sequence was verified.

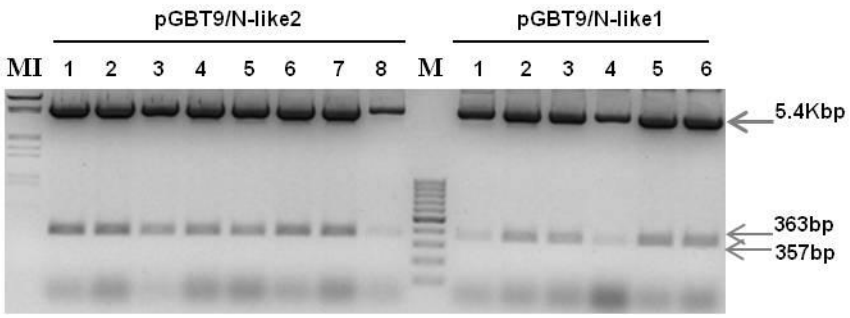

Fig. 37: Cloning of *NIMIN-like1* and *NIMIN-like2* genes into yeast vector pGBT9. Restriction analysis of pGBT9/NIMIN-like1 (357bp) and pGBT9/NIMIN-like2 (363bp) with *Bam*HI restriction enzyme. MI stands for lambda DNA marker.

Tab. 3: Oligonucleotide combination for amplification of *NtNIMIN* genes

Gene	Template	Primers	
		Forward primer	Back primer
Nt NIMIN-Like1 (*Bam*H1 fragment)	Nt Genomic DNA	BP-1	BP-2
Nt NIMIN-Like2 (*Bam*H1 fragment)	Nt Genomic DNA	FS-1	FS-2
Nt NIMIN2a (*Bam*H1 fragment)	Nt Genomic DNA	G8-1/1	G8-1/2
Nt NIMIN-like1 (BamHI/SacI fragment)	pGBT9/NIMIN-Like1	BP-1	BP-3
Nt NIMIN2c (BamHI/SacI fragment)	pGBT9/NIMIN2c	AD10/2	N2c-5
Nt NIMIN2c (cloned fragment)	pBin19/35S_{pro}:NtNIMIN2c:Nos	35S	N2c-5
Nt NIMIN-like1 (cloned fragment)	pBin19/35S_{pro}:NtNIMIN-like1:Nos	35S	BP-3

Figure (38A) confirms the successful cloning of *NIMIN-like1* and *NIMIN2c Bam*HI and *Sac*I fragments via restriction digest analysis. Thereafter, *NIMIN-like1* and *NIMIN2c* were cloned into *Bam*HI and *Sac*I cleaved *pBin19/35S_{pro}:GUS* vector from which GUS had been removed. The cloning was confirmed via *Eco*RI restriction digest analysis as well as via PCR by using specific primers (Fig. 38B and C; Tab. 3). *Eco*RI restriction enzyme cuts twice in vector, in addition to that *NIMIN-like1* and *NIMIN2c* also contain internal *Eco*RI restriction sites albeit at different positions. In *NIMIN-like1* it cuts at N-terminus, however, in case of *Nt NIMIN2c* it cuts at C-terminus, thereby yielding different fragment sizes of approx. 485/1475bp and 315/1585bp, respectively (Fig. 38B). The smaller fragments are not visible on the gel.

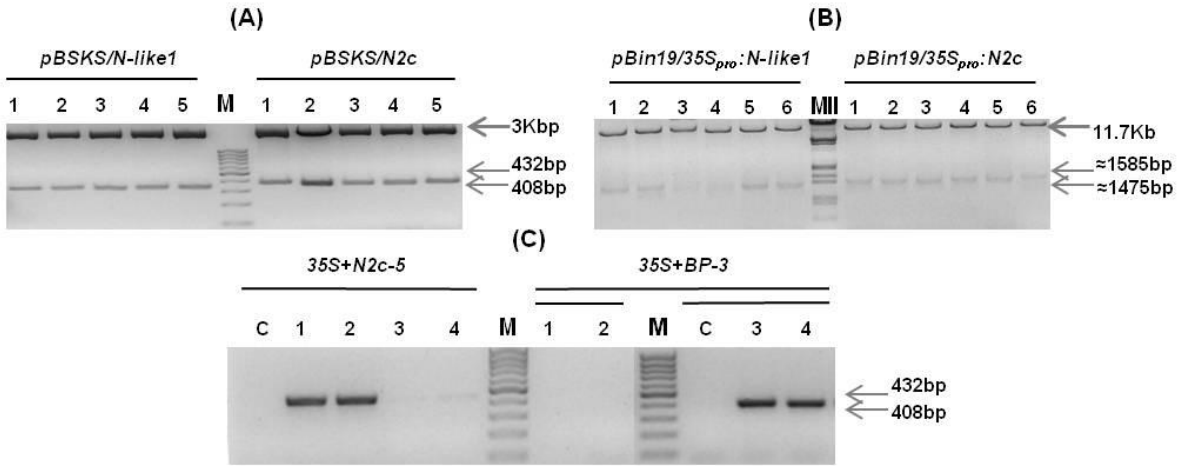

Fig. 38: Cloning of *NIMIN-like1* and *NIMIN2c* genes. A) Plasmidprep DNA of pBSKS cloned *NIMIN* genes, digested with *Bam*HI and *Sac*I restriction enzymes. Cloning of *NIMIN-like1* and *NIMIN2c* genes into plant expression vector pBin19 confirmed via **B)** *Eco*RI restriction enzyme analysis and **C)** PCR technology using specific primers. Sample lane numbered 1 and 2 corresponds to *pBin19/35S$_{pro}$:NIMIN2c:Nos,* 3 and 4 correspond to *pBin19/35S$_{pro}$:NIMIN-like1:Nos* and C corresponds to water. MII stands for lambda marker.

Thereafter, constructs *pBin19/35S$_{pro}$:NIMIN-like1:NOS* and *pBin19/35S$_{pro}$:NIMIN2c:NOS* were mobilized to Agrobacterium strain LBA4404 via tri-parental mating (Sec. 2.2.19). Successful mobilization was once more confirmed via restriction digest and specific primers (Fig. 39A and B). Restriction analysis was not able to show insert band as the fragment sizes are very small and DNA recovery from Agrobacteria is very low, nevertheless it shows clear and accurate fragment size of vector (Fig. 39A). Therefore, successful mobilization was once more confirmed via specific primer in PCR reaction (Fig. 39B).

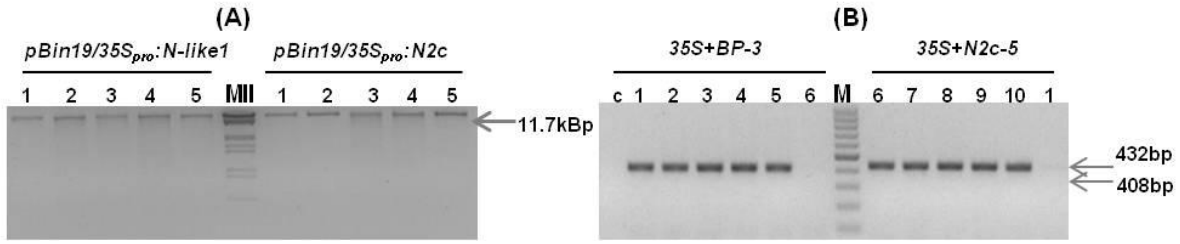

Fig. 39: Mobilization of *pBin19/35S$_{pro}$:NIMIN-like1:NOS* and *pBin19/35S$_{pro}$:NIMIN2c:NOS* constructs from *E.coli* into Agrobacteria. A) *Bam*HI and *Sac*I restriction analysis of Agrobacteria mobilized *pBin19/35S$_{pro}$:NIMIN-like1:NOS* and *pBin19/35S$_{pro}$:NIMIN2c:NOS* constructs, showing only vector band. **B)** Insert presence was confirmed via PCR technology using specific primers. Sample lane numbered 1 to 5 corresponds to *pBin19/35S$_{pro}$:NIMIN-like1:NOS,* 6 to 10 correspond to *pBin19/35S$_{pro}$:NIMIN2c:NOS* and C correspond to water control.

3.4.2 Binding of NIMIN-like1 and NIMIN-like2 to NgNPR1 in Y2H and Y3H

As mentioned earlier, NIMIN-like1 and NIMIN-like2 carry a NPR1 interacting domain, by which NIMIN proteins interact with NPR1. Here, Y2H assays were performed to confirm the interaction of two novel tobacco NIMIN proteins with NtNPR1. Therefore, *NIMIN-like1* and *NIMIN-like2* were cloned in frame to the GAL4BD into pGBT9 vector. The resulting constructs were co-transformed with pGAD424/NgNPR1 plasmid (encoding GAL4AD-NgNPR1) into the yeast cells HF7c (strain), carrying the *HIS*/*lacZ* reporter genes under the control of UAS^{GAL4} element. Quantitative *lacZ*-reporter gene assays (Sec. 2.2.21.11) confirm that NIMIN-like1 and NIMIN-like2 themselves do not exert transcriptional activity (Fig. 40A). Y2H assays show that both NIMIN-like1 and NIMIN-like2 interact with NgNPR1 to reasonable strength as monitored for NIMIN2a which is used here as positive control.

So far, all Nt NIMIN protein interactions with NtNPR1 have been found sensitive to SA application. To monitor the effect of SA on two novel proteins, interaction assays with NtNPR1 in yeast with different SA doses ranging from 0.5µM-300µM were performed. Like observed for NIMIN2a-NgNPR1, interaction between NIMIN-like1-NgNPR1 is sensitive to SA, however, the interaction was less sensitive at lower SA doses in latter case (Figure 40B). Hence, it proves that both tobacco proteins having NPR1 interacting domain indeed interact with NgNPR1 in Y2H system. Moreover, NIMIN-like1 protein interaction with NgNPR1 is found sensitive to SA. Unfortunately, the α-NIMIN2a:MBP antiserum used in immunodetection studies for determining NIMIN-like1 accumulation was not able to detect the NIMIN-like1 in yeast extracts, although, the antiserum was able to detect NIMIN2a accumulation to high level (Fig. 40C).

All Nt NIMIN proteins cloned so far interact at same site of NPR1 (Zwicker et al., 2007). Therefore, in order to confirm if NIMIN-like1 interacts at the same binding site on NPR1, Y2H assays were performed for interaction of NIMIN-like1 with NgNPR1 F505/506S. NgNPR1 F505/506S mutant harbors mutation at NIMIN1, NIMIN1b or NIMIN2 binding site where two phenylalanine residues at position 505 and 506 were changed to serine residues. Maier et al, (2011) have demonstrated that the F505/506S mutation specifically destroys the NIMIN2 binding ability to NgNPR1 without interfering with the protein's interaction with TGA factors, its transcription activation potential and its responsiveness to SA.

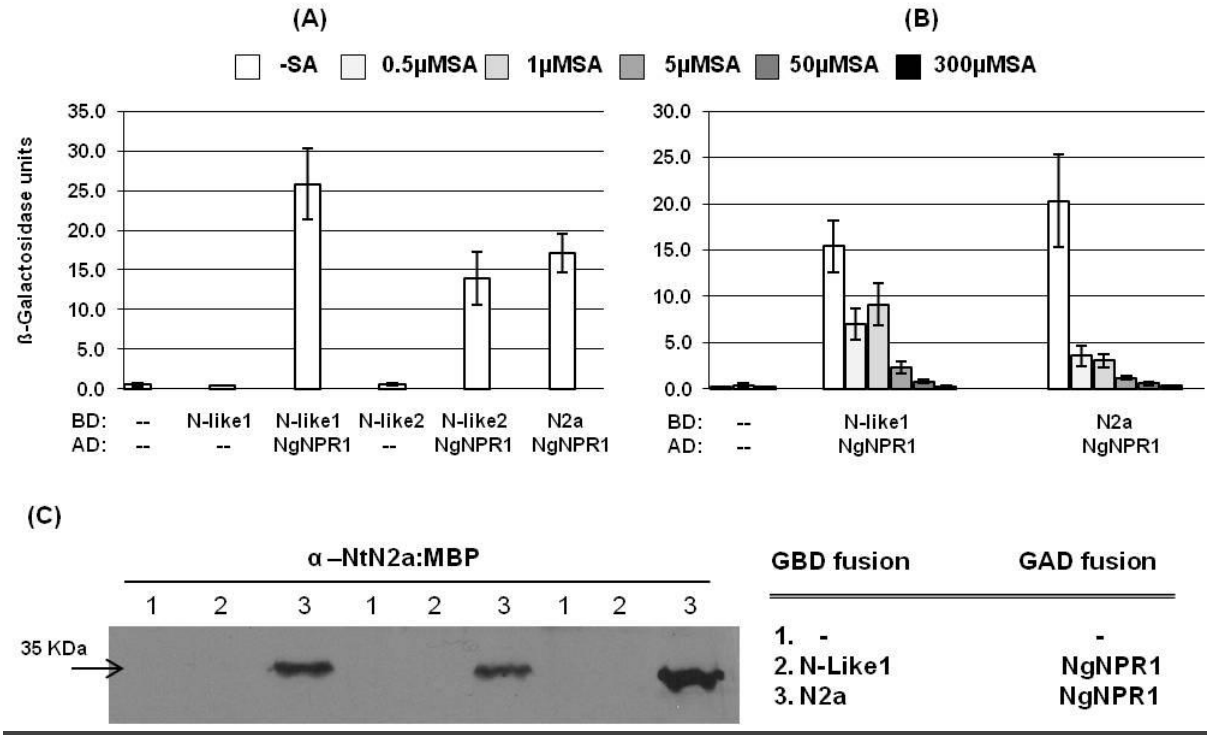

Fig. 40: Interaction of NIMIN-like1 and NIMIN-like2 with NgNPR1 in yeast. A) Y2H assay for interaction of GAL4BD:NIMIN-like1, GAL4BD:NIMIN-like2 or GAL4BD:NIMIN2a fusion proteins with GAL4AD:NgNPR1. Quantitative Y2H assays were performed under standard conditions. **B)** Influence of different SA concentrations on NIMIN-like1 interaction with NgNPR1 in yeast. For control SA doses of 0, 50 and 300µM are used. **C)** Immunodetection of NIMIN2a and NIMIN-like1 in yeast extracts via western blot analysis. Lanes numbered 1 corresponds to empty vectors, 2 corresponds to NIMIN-like1 and 3 corresponds to NIMIN2a.

pGBT9/NIMIN-like1 or pGBT9/NIMIN2a were co-transformed with pGAD424/NgNPR1 F505/506S plasmid (encoding GAL4AD-NgNPR1 F505/506S) into the yeast cells HF7c (strain), carrying the *HIS*/*lacZ* reporter genes under the control of UAS^{GAL4} element. Y2H result presented in Figure (41A) clearly shows that NIMIN-like1 does not interact with NgNPR1 F505/506S mutant, however, the proteins interact strongly with wild type NPR1. Thereby, the data clearly show that NtNIMIN-like1 also binds to the same site as reported for other Nt NIMIN proteins.

In addition to *NPR1*, Arabidopsis possesses *NPR1*-related genes, but, not much is known about the functional relevance of these *NPR1-like* genes. However, it has been demonstrated that *NPR1-like* genes from Arabidopsis are not positive regulators of SA-induced SAR gene expression (Liu et al., 2005; Zhang et al., 2006). Like At, Nt also possesses one *NPR1* related gene, i.e., *NtNIM1-like1*, also called *NtNPR3* (Maier et al., 2011). NtNPR3 shows 43% (61%) overall identity (similarity) to NtNPR1. In yeast, NtNPR3 shows binding properties to TGA factors and NIMIN proteins as observed for NtNPR1 (Zwicker et al., 2007; Maier et al., 2011).

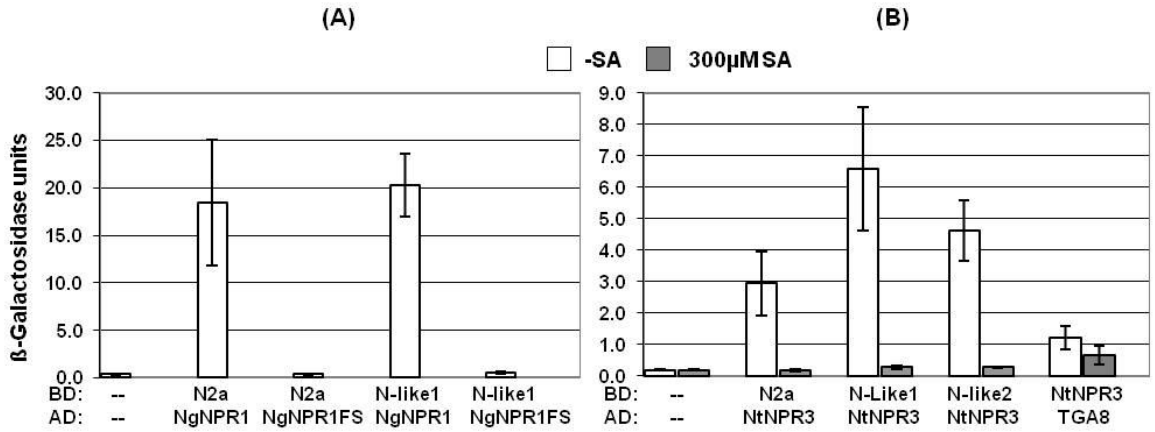

Fig. 41: Interaction of Nt NIMIN proteins with NgNPR1 F505/506S and NtNPR3 in yeast. A) Y2H assay for interaction of GAL4BD:NIMIN-like1 and GAL4BD:NIMIN-like2 fusion proteins with GAL4AD:NgNPR1. **B)** Y2H assay for interaction of NIMIN-like1, NIMIN-like2 and TGA8 protein with NtNPR3.

Therefore, to know if NtNPR3 also interacts with Nt NIMIN-like1 and Nt NIMIN-like2 proteins, Y2H assays were performed. pGBT9/NIMIN-like1, pGBT9/NIMIN-like2, pGBT9/NIMIN2a or pGBT9/NtNPR3 constructs were co-transformed with pGAD424/NtNPR3 or pGAD424/TGA8 plasmid into the yeast cells HF7c (strain). Y2H assays show that NtNPR3 does interact with NIMIN-like1 as well as NIMIN-like2 and their interaction is sensitive to SA. In contrast, interaction of NtNPR3-TGA8 was not found significantly sensitive to SA application (Fig. 41B).

The two novel Nt NIMIN proteins bind to the C-terminus of NPR1, where TGA8 factor also binds (Stos, 2007). TGA8 factor is the only Nt TGA factor that binds to NtNPR1. To find out if the two classes of proteins affect each other for binding to NtNPR1, the Y3H system was utilized. Unlike the Y2H system, Y3H assay additionally makes it possible to investigate ternary protein complex formation (Sec. 2. 2.21.10). As NgNPR1 386/588 deletion mutant was available which carries C-terminus of protein (comprising amino acid 386-588), it was interesting to know how Nt NIMINs and TGA8 interact with truncated NPR1 protein in yeast. *NIMIN2a* or *NIMIN-like1* expression was put under the control of the $MET25_{pro}$ in three-hybrid vector pBD (Sec. 2.1.3.1.7) yielding pBD:-/NIMIN2a or pBD:-/NIMIN-like1, respectively, and the NgNPR1 386-588 sequence was fused to the GAL4BD sequence in the three-hybrid vector to give pBD:NgNPR1 386-588/NIMIN2a and pBD:NgNPR1 386-588/NIMIN-like1. The result presented in Figure (42A) clearly shows that like NIMIN2a, expression of NIMIN-like1 rather inhibited the interaction of NgNPR1 with the TGA factors.

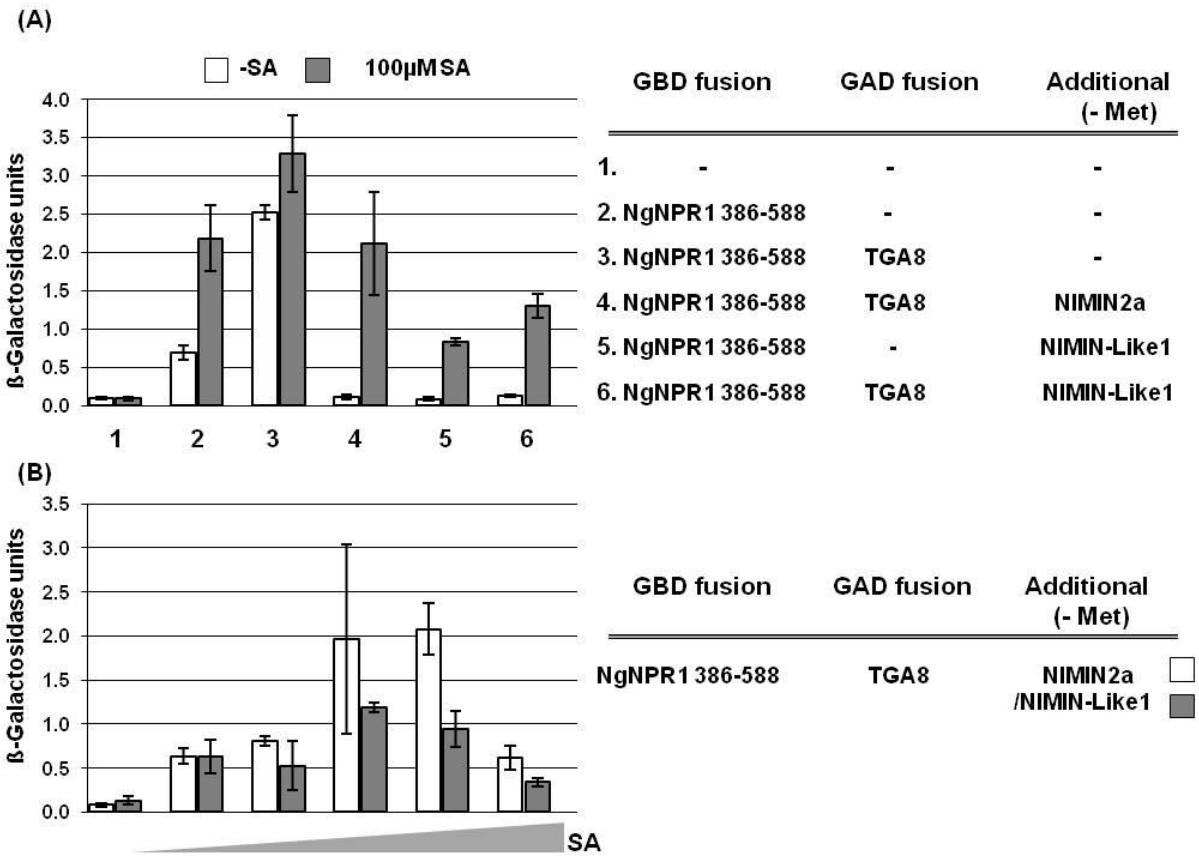

Fig. 42: NIMIN-like1 interferes with NgNPR1 386-588-TGA8 complex in yeast. (A) Y3H interaction of GAL4AD:NgNPR1 386-588 and GAL4BD:TGA8 fusion proteins in the presence of Nt NIMINs. *NIMIN-like1* and *NIMIN2a* were expressed from the $Met25_{pro}$ which is de-repressed in the absence of methionine. **B)** Influence of different SA concentrations on NIMIN-like1 or NtTGA8 interaction with NgNPR1 in yeast. Different SA doses, i.e., 0, 1, 5, 50, 100 and 300µM were applied to the yeast growth medium.

However, upon SA application NPR1 is relieved from Nt NIMINs resulting in establishing NgNPR1-TGA8 interaction. Moreover, it fixes the transcription activity of NgNPR1 which was lost due to Nt NIMINs binding. However, interaction between NgNPR1 and TGA8 was not found sensitive to SA application. Hence, it proves that, like NIMIN2a, NIMIN-like1 interferes with NgNPR1 transcriptional activity as well as NgNPR1 interaction with TGA8 in yeast.

As mentioned above, NIMIN-like1 interferes with NgNPR1 386/588-TGA8 protein-protein interaction in yeast. But as soon as NgNPR1 386/588 is relieved from NIMIN-like1, this results in establishing the interaction of NgNPR1 386/588 and TGA8. This could mean that both proteins have the same or an overlapping binding domain, or it could also mean that interaction of one protein makes major conformational changes in NgNPR1 protein which masks the binding site for other protein. To address this, different doses of SA, i.e., 1-300µM were applied to yeast culture medium to monitor the pattern of relief of NIMIN-like1 or

establishment of NgNPR1-TGA8 complex utilizing Y3H system. As usual, NIMIN2a was used as a positive control. The data show that SA doses of 50 and 100µM are most efficient doses, enough to relieve NIMIN2a or NIMIN-like from NgNPR1 leading NgNPR1 386/588 interaction with TGA8 (Fig. 42B). Concentration higher than 100µM might influence yeast cell negatively.

3.4.3 Effects of transient over-expression of tobacco *NIMIN* genes in *N. benthamiana*

After successful characterization of At NIMIN proteins, it was of interest to characterize tobacco NIMIN proteins for their effect on *PR-1a*$_{pro}$ activation via the TGES. Like Arabidopsis, many Nt *NIMIN* genes have been cloned, e.g., *NIMIN2a* (Horvath et al., 1998) *NIMIN2b* and *NIMIN2c* (Zwicker at al., 2007), *NIMIN-like1* and *NIMIN-like2* (this study). In order to determine the functional significance of different Nt NIMIN proteins in terms of *PR-1a* gene induction, *N. benthamiana* TGES was utilized as practiced previously for At NIMIN proteins (Sec. 3.2). Therefore, Agrobacterium strains carrying different tobacco NIMIN constructs, i.e., *35S*$_{pro}$*:NIMIN2a, 35S*$_{pro}$*:NIMIN2c* or *35S*$_{pro}$*:NIMIN-like1* were infiltrated in leaf halves of *N. benthamiana -1533PR-1a*$_{pro}$*:GUS* plants. As shown in Figure (43A and B), *PR-1a*$_{pro}$ was strongly activated in non-infiltrated and *35S*$_{pro}$*:GFP* agro-infiltrated control plants after SA application.

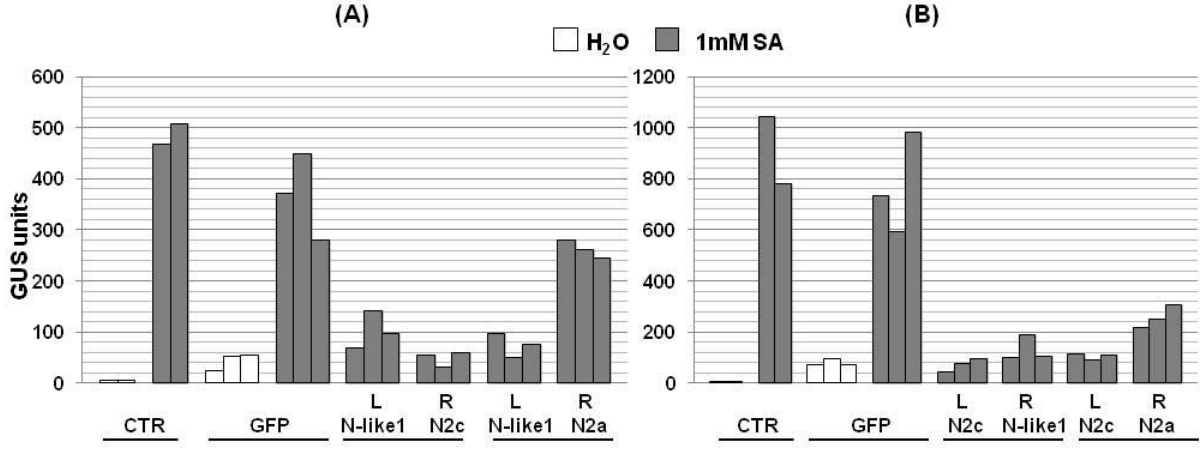

Fig. 43: Transient expression of Nt NIMIN proteins in *N. benthamiana -1533PR-1a*$_{pro}$*:GUS* plants. A and **B)** *N. benthamiana -1533PR-1a*$_{pro}$*:GUS* plants infiltrated with Agrobacterium strains carrying *35S*$_{pro}$*:GFP, 35S*$_{pro}$*:NIMIN2c, 35S*$_{pro}$*:NIMIN-like1* or *35S*$_{pro}$*:NIMIN2a*. A and B show independent experiments.

On the contrary, all tobacco NIMIN proteins suppressed the SA mediated *PR-1a*$_{pro}$ activation to different intensity. Keen observation of GUS assay results revealed NIMIN2c and NIMIN-like1 as strong suppressors of SA mediated *PR-1a*$_{pro}$ activation in mentioned order, however, NIMIN2a appeared as the least suppressor protein.

Transient over-expression of some At NIMIN proteins manifests cell death in *N. benthamiana* leaf tissue. Therefore, effects of transient over-expression of tobacco NIMIN proteins were also examined on plant phenotype. *N. benthamiana* leaf halves over-expressing different Nt NIMIN proteins present quite variable results. Among all tobacco NIMIN proteins tested here, NIMIN2c appeared as the only NIMIN protein in tobacco which induces cell death in plant leaf tissue (Fig. 44). However, NIMIN-like1 and NIMIN2a have not shown any signs of cell death when monitored till 18 dpi.

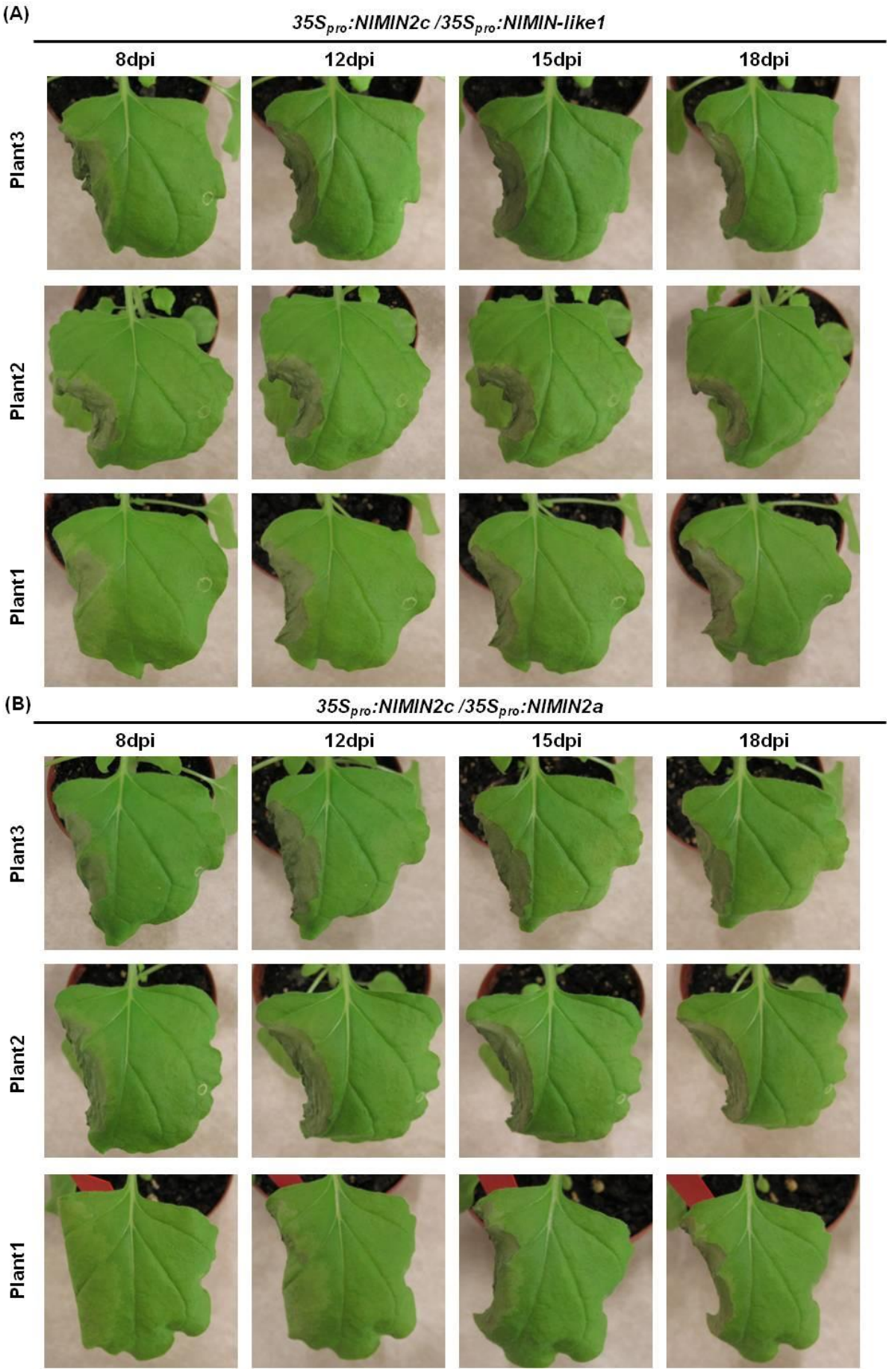
(A)
35Spro:NIMIN2c /35Spro:NIMIN-like1
8dpi
12dpi
15dpi
18dpi
Plant3
Plant2
Plant1
(B)
35Spro:NIMIN2c /35Spro:NIMIN2a
8dpi
12dpi
15dpi
18dpi
Plant3
Plant2
Plant1

Fig. 44: Phenotypic effects of over-expression of Nt NIMIN proteins in *N. benthamiana -1533PR-1a$_{pro}$:GUS* plants. *N. benthamiana* leaf halves were infiltrated with Agrobacterium strains as mentioned in Figure (43B). The very same plants were used for phenotypic studies. For time course study gradual symptom development was recorded at 8, 12, 15 and 18 dpi**.**

Hence, it proves that NIMIN2c is the strongest suppressor of *PR-1a* activation plus the only NIMIN protein in tobacco that manifests cell death when transiently over-expressed in *N. benthamiana* leaves. This finding brings forth an interesting correlation between NIMIN2c and NIMIN1. As NIMIN2c in tobacco, NIMIN1 in Arabidopsis is the strongest suppressor of SA-mediated *PR-1a$_{pro}$* activation and also shows clear cell death. Taking all this into consideration, it was interesting to confirm this correlation via direct and side by side comparison of the effects produced by both proteins. Therefore, Agrobacterium strains carrying *35S$_{pro}$:NIMIN2c* or *35S$_{pro}$:NIMIN1* were infiltrated in leaf halves of *-1533PR-1a$_{pro}$:GUS N. benthamiana* plants. As usual, infiltration of Agrobacterium strain harboring *35S$_{pro}$:GFP* construct served as a control. Infiltration of *35S$_{Pro}$:NIMIN1* or *35S$_{Pro}$:NIMIN2c* bacteria suppressed SA-mediated *PR-1a$_{pro}$* activation to nearly background levels, comparable to GUS levels observed in *GFP* expressing leaf disks floated on water (Fig. 45A). Repression with NIMIN2c was, however, slightly weaker than with NIMIN1. The very same over-expressing plants used for GUS activity in Figure (45A) were also used for recording phenotypic characters of both proteins (Fig. 45B). It is quite clear from the graph that cell death emerges earlier in NIMIN1 over-expressing plant leaf halves than with NIMIN2c. However, the damage assessed in both NIMIN protein over-expression experiments was similar at later stages, i.e., 12 dpi.

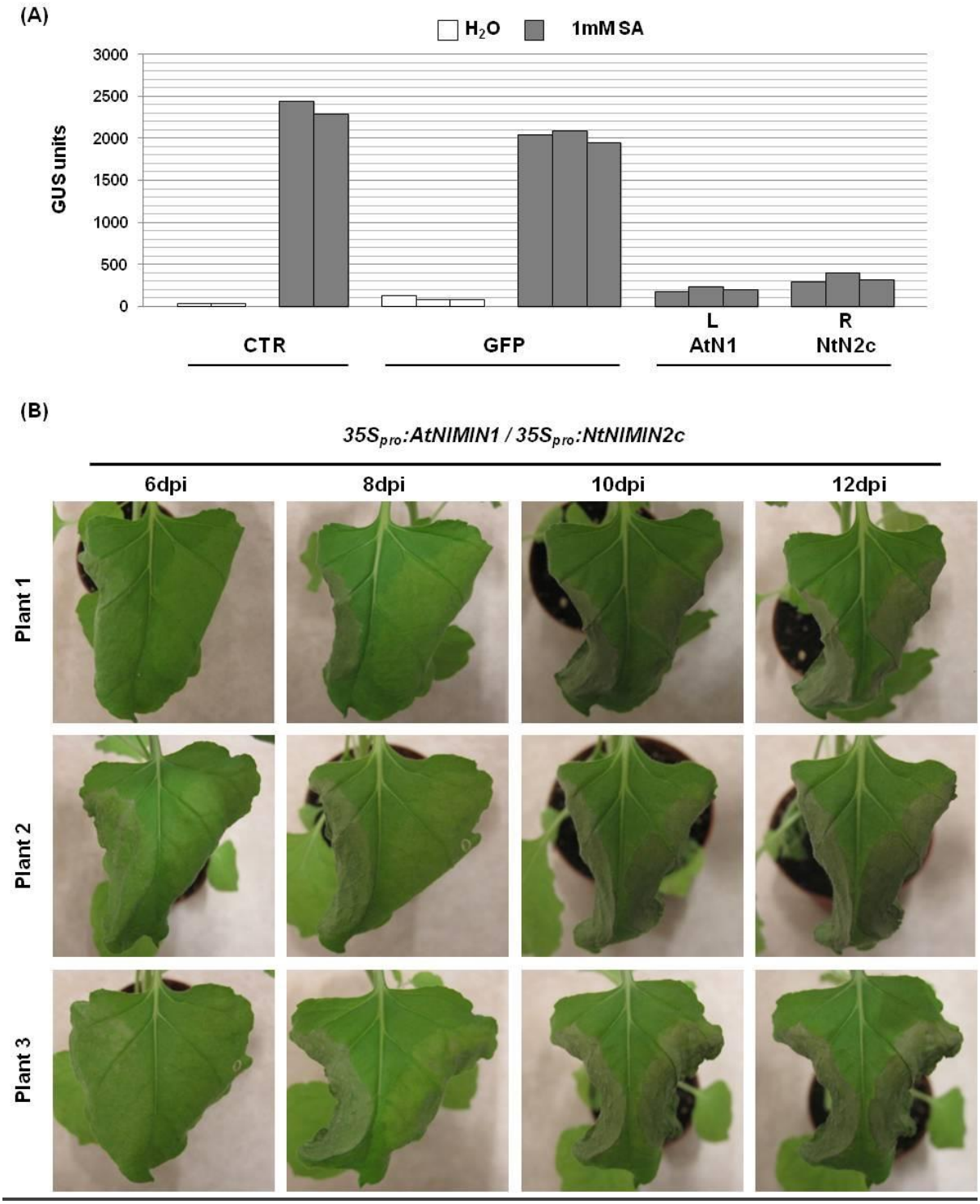

Fig. 45: Effects of over-expression of AtNIMIN1 and NtNIMIN2c proteins in *N. benthamiana* - *1533PR-1a$_{pro}$:GUS* plants. A) Plant leaf halves were infiltrated with Agrobacterium strain carrying *35S$_{pro}$:GFP, 35S$_{pro}$:NIMIN2c or 35S$_{pro}$:NIMIN1*. The very same plants were used for recording phenotypic characteristics. **B)** Time course studies carried out in plant leaves over-expressing NIMIN2c or NIMIN1 proteins. Gradual symptom development was recorded at 6, 8, 10 and 12 dpi.

4 Discussion

4.1 *N. benthamiana* transient transformation system: A reliable tool for *NIMIN* gene characterization

Functional characterization of gene products is not an easy task for scientists to understand the biological function of large gene sets even in the postgenomic era (Levy et al., 2005). Transient transformation techniques are being used for rapid *in vivo* analyses of gene function such as protein-protein interaction, protein subcellular localization and promoter activity. TGES offers a number of advantages over stable expression. For instance, in TGES gene expression can be assayed shortly after DNA delivery (Kapila et al., 1997), system is readily applicable as it does not require expensive materials and equipment (Wroblewski et al., 2005). Moreover, TGES allows many constructs to be analyzed in parallel, thereby, considerably enhancing the pace of research (Li et al., 2009). TGES efficiency in several model species, such as Arabidopsis, tobacco, tomato, potato, grapevine and rose has already been reported (Tsuda et al., 2012; Yasmin and Debener 2010; Zottini et al., 2008; Kościańska et al., 2005; Bhaskar et al., 2009; Kim et al., 2009).

N. benthamiana is considered by scientific community as a model organism for performing plant research. *N. benthamiana* has very delicate leaves which are an excellent target for agroinfiltration (Reiner et al., 2000). Therefore, in this study *N. benthamiana* based TGES was used to determine the role of NIMINs in SAR pathway. In this TGES, *N. benthamiana* -*1533PR-1a*$_{pro}$*:GUS* reporter gene line was utilized in which *GUS* reporter gene was placed under the control of -*1533PR-1a*$_{pro}$ sequence containing elements mediating the transcriptional regulation of the *PR-1a* gene (Grüner and Pfitzner, 1994; for detail Sec. 3.1).

In novel *N. benthamiana* established TGES, non-infiltrated plants were used as zero/reference control and a parallel infiltration of *35S*$_{pro}$*:GFP* containing Agrobacterium strain in reporter line plant leaves was used as an infiltration control. GFP is an excellent choice used here as infiltration control. Firstly, GFP is not known to be involved in SAR pathway and therefore, works in TGES as a neutral protein. Secondly, GFP fluorescence provides an extra advantage by keeping check of the gene expression level, when infiltrated tissues are observed under UV-light. Moreover, with the help of GFP fluorescence, it was possible to determine that upper (younger) leaves are more competent for gene expression

studies and that co-infiltration with P19 suppresses the post transcriptional gene silencing, thereby resulting in high-level GFP fluorescence (Fig. 8 and data not shown).

Initial experiment performed to check the reliability of the TGES worked fine (Fig. 4A). Leaf discs harvested from non infiltrated or *GFP* agroinfiltrated tissues showed strong induction of *-1533PR-1a$_{pro}$:GUS* reporter gene after 1mM SA floating and no induction in case of water floatings. However, the subsequent experiment showed unspecific promoter activation in water floated control infiltrations (i.e., infiltration of *35S$_{pro}$:GFP* harboring Agrobacterium strains). This clearly indicated need for the TGES optimization. The result presented in section (3.1) show that among different suspected causes tested in this study, the load of Agrobacterial cell suspension was the cause for unspecific activation of *PR-1a$_{pro}$* (Fig.7). Therefore, following agroinfiltrations were carried out at OD_{600} set to 0.5 instead of 1.

Noteworthy, in rare occasions during sunny hot days infiltration of bacterial suspension adjusted at OD_{600} to 0.5 per se can even induce the reporter gene to slightly above background level. Therefore, in broader sense, load of Agrobacterium, wounding reaction and hot sunny weather can cumulatively impose stress which ultimately leads to the unspecific induction of *-1533PR-1a$_{pro}$:GUS* reporter gene. In this line, the agroinfiltrations were carried out with much care avoiding any additional physical damage to the reporter line plants. The agroinfiltration experiments were planned and carried out depending on weather conditions. However, no change in the density of bacterial cell suspension (i.e., OD_{600} set to 0.5) for infiltration was carried out, as this optical density is enough for maximum gene expression with minimum unspecific background induction.

4.2 At NIMIN proteins effect the SA-mediated induction of reporter gene differentially

In Arabidopsis, there are four *NIMIN* genes, encoding small molecular weight proteins. The encoded proteins share several conserved regions in common, which hint for NIMIN proteins involvement and function in very same pathway. Y2H studies have revealed that AtNIMIN proteins interact at different sites on AtNPR1. NIMIN3 interacts at the N-terminus, however, NIMIN1, NIMIN1b and NIMIN2 interact at the C-terminus of AtNPR1 (Weigel, 2001). The effect of NIMIN1 protein on NPR1 activity has been shown in overexpression studies carried out in transgenic Arabidopsis plants, i.e., NIMIN1 suppresses the *PR-1* gene induction and

causes enhanced susceptibility of plant to bacterial pathogen *Pseudomonas syringae* pv *maculicola* (Weigel et al., 2005). Apart from this report, the biological significances of other AtNIMIN proteins were not known. Influence of *NIMIN1* overexpression on *PR-1* gene induction in transgenic plants encouraged to check for the effect of *NIMIN1* transient overexpression on SA-mediated *-1533PR-1a$_{pro}$:GUS* reporter gene induction.

In the TGES, transient overexpression of *NIMIN1* strongly suppressed the SA-mediated *-1533PR-1a$_{pro}$:GUS* reporter gene induction (Fig. 9). Hence, TGES generated similar results as obtained previously for *NIMIN1* overexpression in transgenic plants, thereby, pronouncing the reliability of novel TGES established in *N. benthamiana* plants. After gaining quite clear results from NIMIN1 the focus was then shifted to other NIMIN proteins. Except NIMIN1, none of the other AtNIMINs have ever been studied for their effect on *PR-1* gene induction neither in stable nor in transient transformation system.

NIMIN1b was the next NIMIN candidate dissected via TGES for its effect on SA-induced reporter gene expression. NIMIN1b was identified in Genbank database by running computer software program BlastP. The protein shows 44% identity and 64% similarity to NIMIN1 protein (Weigel, 2001). NIMIN1b shares several signature sequences in common with NIMIN1 at protein level, i.e., an EAR motif, NLS motif, EDF motif and DFK motif (Figs. 2 and 23). As NIMIN1b is pretty similar to NIMIN1 at the amino acid level, it was not of a surprise to find that *NIMIN1b* transient overexpression suppresses the *PR-1a$_{pro}$* activation in TGES (Fig. 9B).

NIMIN3 is the smallest protein (i.e., 112aa) among four Arabidopsis NIMIN proteins, and Y2H data make it even more interesting as NIMIN3 is the only protein that interacts at the N-terminus of AtNPR1. However, the transient overexpression of *NIMIN3* like *NIMIN1* and *NIMIN1b*, also suppresses the SA-mediated reporter gene induction, although to less extent (Fig. 11A). Surprisingly, NIMIN2 was not found as suppressor of SA-mediated *PR-1a$_{pro}$* activation when transiently expressed in *N. benthamiana* leaf tissues (Fig. 11B). Although in Y2H assays NIMIN2 binds to almost the same affinity and site in AtNPR1 like NIMIN1 (Weigel et al., 2001), NIMIN2 imposes contrasting effects on SA-mediated *PR-1a$_{pro}$* activation in TGES compared with NIMIN1. Therefore, no correlation is found between NIMINs binding affinity to NPR1 and suppression of SA-mediated *-1533PR-1a$_{pro}$:GUS* reporter gene. In summary, the data show that Arabidopsis NIMIN proteins impose differential effects on SA-mediated *PR-1a$_{pro}$* activation via TGES. Arabidopsis NIMIN

family comprises members which are strong repressors, e.g., NIMIN1 and NIMIN1b, an intermediate suppressor NIMIN3 and a weak repressor NIMIN2.

This led to the question to look for a common signature in NIMIN proteins to understand the suppression phenomena observed via TGES. Arabidopsis NIMIN1, NIMIN1b and NIMIN3 share two similar regions in their C-terminus, i.e., an EAR motif and an EDF motif.

EDF domain was first identified in Arabidopsis NIMINs as a sequence composed of eight nearly identical amino acids (PA/SFQPEDF) found in NIMIN1 and NIMIN3 (Weigel et al., 2001). Not only NIMIN1 and NIMIN3, but NIMIN1b which was discovered later, also contains EDF domain. However, the functional significance of this domain in Arabidopsis NIMIN proteins has not been worked out. Of note, NIMIN2 does not contain this very domain and does not suppress the SA-mediated reporter gene induction. The presence of EDF domain in NIMIN1, NIMIN1b and NIMIN3 and SA-mediated suppression of reporter gene exerted by these proteins was pretty interrogative. Therefore, NIMIN1 and NIMIN3 mutants were created harboring mutations in the EDF domain, i.e., NIMIN1 E94A D95V and NIMIN3 E63A D64V. The mutation in EDF domain was targeted to explore the functional significance of this domain in NIMIN1 and NIMIN3 if any. NIMIN1 ED-AV and NIMIN3 E63A D64V were tested via Y2H assays, if mutants can interact with NPR1. Y2H assays performed showed that NIMIN1 E94A D95V still interacts with AtNPR1, although, not as strongly as wt NIMIN1 (Figs. 13 and 18A). On the other hand, NIMIN3 E63A D64V was not able to interact with AtNPR1 at all. The argument that NIMIN1 and NIMIN3 harboring mutation in EDF motif are affected in their interaction with NPR1 in yeast suggests that EDF domain might have a functional relevance in protein interaction with NPR1. It is known from Y2H assays that NIMIN1 interacts strongly with AtNPR1 via the NPR1 interacting DFK domain (Weigel et al., 2001). However, mutation in EDF domain results in considerable loss of interaction to AtNPR1 possibly because NIMIN1 E94A D95V might end up in an improper folding of protein which could mask the NPR1 interacting domain leading to considerable loss of interaction.

On the other hand, NIMIN3 E63A D64V loses its complete interaction with NPR1 suggesting that NIMIN3 might interact with NPR1 via EDF domain as it does not contain DFK domain. This is further supported by the argument that interaction of NIMIN3 with NPR1 is weaker than NIMIN1 interaction with NPR1. Moreover, previously scanned NIMIN3 region responsible for interaction with NPR1 also includes the EDF motif (Weigel et al., 2001).

In TGES, GUS assays showed that transient overexpression of NIMIN1 E94A D95V and NIMIN3 E63A D64V do not suppress the reporter gene induction, further supporting the significance of EDF domain. However, unfortunately the NIMIN3 E63A D64V protein did not accumulate to detectable levels neither in yeast nor in plants, while wt NIMIN3 protein was detected to high levels in immunodetection (Fig. 18B). On the other hand, NIMIN1 was found out to be an unstable protein via immunodetection (U.M. Pfitzner, personal communication). Therefore, clear conclusion could not be drawn to determine the functional significance of EDF domain from these experiments.

Interestingly, it is known that NIMIN3 does neither interact with NtNPR1 nor NtNPR3 in yeast (Zwicker et al., 2007; Maier et al., 2011). Therefore, *N. benthamiana* established TGES results are pretty unforeseen as NIMIN3 transient overexpression suppresses the SA-mediated *PR-1a*$_{pro}$ activation. Thus, present data cannot provide a concrete basis for understanding the mechanism, i.e., how NIMIN3 can suppress the SA-mediated *PR-1a*$_{pro}$ activation in *Nicotiana* species. However, presence of EDF domain in several cDNAs from *N. tabacum* and *N. benthamiana* in the databanks proclaims the functional relevance of EDF domain in tobacco. Furthermore, NIMIN-like proteins containing EDF motif are found in multiple plant species and EDF domain functional significance has been shown with detail in rice NRR and its paralogs (Chern et al., 2012). This supports the view that EDF domain in NIMIN-like proteins has functional importance for interaction with NPR1 thereby, regulating induction of *PR-1*. However, the significance of the EDF domain for *PR-1* gene induction may vary among different plant species. Hence, the mechanism by which EDF domain in NIMIN proteins could suppress the *PR-1* gene induction remains elusive.

Among other transcription repression motifs i.e., TLLLR motif (Matsui et al., 2008), R/KLFGV motif (Ikeda et al., 2009), LxLxPP motif (Paponov et al., 2009), EAR motif was the first repression motif reported in plants (Ohta et al., 2001). The EAR motif, defined by the consensus sequence patterns of LxLxL, DLNxxP, or an overlapping LxLxL and DLNxxP, is the most predominant form of transcriptional repression motif so far identified in plants (Kagale et al., 2010). The repression function of EAR motif was shown in experiments where its fusion with transcription activators leads to the repression of target genes (Hiratsu et al., 2003). Identification of the repression potential of the EAR motif in several other transcriptional regulators involved in diverse biological functions, e.g., SUPERMAN (Hiratsu et al., 2002), AUX/IAA proteins (auxin/indole-3-acetic acid; Tiwari et al., 2004), HSI2 and related proteins (high-level expression of sugar inducible gene 2; Tsukagoshi et al., 2007;

2005), AGL15 (agamous-like 15; Hill et al., 2008) announce role of this motif in mediating transcriptional repression.

Arabidopsis NIMINs also contain EAR motif, Arabidopsis NIMIN1, NIMIN1b and NIMIN3 contain monopartite EAR motif sequence composed of amino acids LxLxLxL. However, NIMIN2 contains a bipartite EAR motif (LxLxL and LxL, separated by a spacer of 10 amino acids). Therefore, significance of this motif in NIMIN proteins for *PR-1* suppression is addressed in this study by generating different EAR motif mutants. The data obtained via TGES indicate that mutants missing three Leu residues of the EAR domain (i.e., NIMIN1 1/137 and NIMIN1b 1/135; Sec. 3.2.4.1) still repress the SA-mediated reporter gene, but not as strongly as wild type proteins (Figs. 16C and 17B), thus indicating that the EAR motif is not the only repression domain existing in NIMIN proteins. NIMIN1 1/137 and NIMIN1b 1/135 suppression effect on SA-mediated *PR-1a*$_{pro}$ activation also indicates that the mutant proteins are expressed in plants.

On the other hand, NIMIN1 L138A L140A and NIMIN3 L108A L110A harboring amino acid exchanges in EAR motif do not suppress the SA-induced *PR-1a* gene activation. This might be because of major conformational changes in protein due to the amino acid exchanges which lead to very low or no expression at all for the mutant proteins. This fact is indeed true for NIMIN3 L108A L110A mutant which results in very low protein accumulation in plant extracts via western blot (U.M. Pfitzner, personal communication).

Thus, in summary it could be deduced that EAR domain is not the only repression domain existing in NIMIN proteins. The possible play of EDF domain in suppressing SA-induced *PR-1*$_{pro}$ activation proclaims that the domain is functional in NIMINs, however, further confirmations are needed.

4.3 Tobacco NIMIN proteins effect the SA-mediated induction of reporter gene differentially

The Arabidopsis NIMIN protein family comprises four members. In tobacco, three *NIMIN* genes are already known, i.e., *NIMIN2a* (Horvath et al., 1998), *NIMIN2b* and *NIMIN2c* which are induced by SA and are closely related to At NIMIN2 (Zwicker et al., 2007).

During this study, two novel *NtNIMIN* genes were analyzed namely, *NIMIN-like1* and *NIMIN-like2*. NIMIN-like2 is not studied in detail as the amino acid sequence does not harbor a stop codon before the start methionine of the protein. However, both genes were cloned and proteins found to interact with NtNPR1 and NtNPR3 in Y2H and the interaction was found sensitive to SA. Nevertheless, further studies were carried out for NIMIN-like1. The interaction of NIMIN-like1 with NPR1 was found sensitive to increasing concentration SA (Fig. 40B). All known NtNIMINs, interact at the C-terminus of NPR1 via a domain called NIMIN1/NIMIN2 binding domain (Maier et al., 2011). NIMIN-like1 also interacts with NPR1 at the same site, discovered via its inability to interact with NPR1 F505/506S mutant that carries mutations in the NIMIN1/NIMIN2 binding domain (Fig. 41A). Interestingly, NtTGA8 also interacts at the C-terminus half discovered by using C-terminus deletion NgNPR 386-588 and the interaction is enhanced in the presence of SA. Y3H shows that NIMIN-like, as known for NIMIN2a compete with TGA8 for interaction with NgNPR1 at C-terminus. However, after SA application NtNIMIN-NPR1 complex is relieved from NIMIN-like1 or NIMIN2a, thus enabling the TGA8 interaction with NgNPR1 (Fig. 42).

Interestingly, NIMIN-like1 also contains the EDF motif found in some Arabidopsis NIMIN proteins. Interestingly, tobacco NIMINs have another type of EAR domain, i.e., DLNxxP. Currently, it is not clear about the functional significance of multiple SA-sensitive NIMIN2 type proteins in tobacco. Thus, the SA-induced NIMIN2 type family is more complex in tobacco than in Arabidopsis.

Therefore, functional significance of tobacco NIMIN proteins for their effect on SA-mediated *PR-1a* gene induction was determined using *N. benthamiana* established TGES. The Nt NIMIN proteins tested here show different degrees of suppression, e.g., NIMIN2c suppresses the SA-mediated reporter gene induction to high degree, NIMIN-like1 suppresses, but comparatively to lesser extent. NIMIN2a is the least suppressive in terms of SA-mediated *PR-1a*$_{pro}$*:GUS* gene induction. It was however, interesting to note that NIMIN-like1, which contains the EDF domain was less suppressive than NIMIN2c which does not contain the EDF domain. Therefore, suggesting that EDF domain might not work as a major repressive domain in tobacco NIMIN proteins (unlike Arabidopsis NIMINs). From these experiments it is not clear, which is the repressive domain in tobacco NIMINs and what is the functional significance of EDF domain in NIMIN-like1. Further, experiments are needed to explore which region in the tobacco NIMINs suppresses NPR1 activity, e.g., EAR motif mutation in NIMIN2c and EDF domain mutation in NIMIN-like1. In accordance with At NIMIN2, Nt

NIMIN2a is only a weak suppressor, consistent with transgenic results by Zwicker et al., (2007).

4.4 Some NIMIN proteins manifest cell death upon transient overexpression in *N. benthamiana* leaf tissue

The most surprising discovery of this work was the phenotypic manifestation of some NIMIN proteins when transiently overexpressed in *N. benthamiana* leaf tissue. Keen observation revealed that *NIMIN1* as well as *NIMIN3* transient overexpression yield cell death like symptoms. The symptoms appeared after 4-6 dpi in case of NIMIN1. However, in case of NIMIN3 symptoms emerged comparatively late, i.e., 12 dpi. On the other side, overexpression of NIMIN1b or NIMIN2 does not yield any symptoms. As the symptoms showed much resemblance to typical cell death, a known cell death inducer, the proapoptotic *Bax* gene, was used as reference, in order to confirm if the novel phenotypic effects observed during NIMIN1 overexpression are similar compared to cell death elucidated by *Bax*. The parallel comparison showed that indeed the symptoms development on leaves infiltrated with $35S_{pro}$*:NIMIN1* containing Agrobacterium strain are same as developed on leaves infiltrated with a $NIMIN1_{pro}$*:Bax* harboring Agrobacteria (Fig. 21). However, the symptoms appeared a bit late in *NIMIN1* overexpressing leaf tissues. *Bax* gene is expressed under the control of $NIMIN1_{pro}$, because the bacteria did not survive when *Bax* gene was expressed under the control of the constitutively active $35S_{pro}$. The *NIMIN1* promoter has been shown to be active in *N. benthamiana* infiltrated with Agrobacterium strain harboring $NIMIN1_{pro}$*:GUS* and activity of $NIMIN1_{pro}$ was found comparable to *35S* promoter (Hirth, 2011).

Of note, the fact that transgenic line being used could be the reason for manifestation of cell death is excluded since, first of all the cell death is specific only for NIMIN1 and NIMIN3, but not for the other AtNIMINs. Secondly, wild type *N. benthamiana* leaf tissue overexpressing NIMIN1 also showed cell death to the same extent (Fig. 22). Furthermore, cell death caused by transient overexpression of *NIMIN1* or *NIMIN3* is accompanied by the accumulation of H_2O_2, which is a regulatory signal molecule in cell death (Figs. 26 and 27). Thus, in summary it could be said that *NIMIN1* or *NIMIN3* overexpression induces cell death in plant leaf tissue. The symptoms are associated with accumulation of H_2O_2 and resemble to the cell death symptoms manifested in *Bax* overexpressing leaf tissues. Of note, SA-mediated $PR\text{-}1a_{pro}$ suppression is not parallel to the cell death manifestation, e.g., NIMIN1 and

NIMIN1b suppress the SA-mediated reporter gene induction, however, NIMIN1b in contrary to NIMIN1 does not manifest cell death. In addition to that, no correlation was found between the NIMINs binding to NPR1 and cell death as NIMIN2 and NIMIN1b bind to almost the same strength as NIMIN1, but impose differential effects on plant phenotype.

Arabidopsis NIMINs harbor various structural domains in common. Therefore, availability of different mutants was utilized to explore if there is a common motif in NIMIN1 and NIMIN3 mediating cell death. The data support the view that NIMIN1 EDF motif mutant, i.e., NIMIN1 E94A D95V still manifests cell death, but symptom appearance was delayed to 12 dpi (Fig. 31). Similar results were obtained from EAR motif amino acid exchange mutant *NIMIN1 L138A L140A* which also yields cell death upon overexpression in *N. benthamiana* leaf halves at 12dpi or later (Fig. 32). Overexpression of *NIMIN1 F49/50S* carrying mutations at NPR1 interacting site, however, did not induce cell death. The cell death data was not recorded from NIMIN3 E63A D64V and NIMIN3 L108A L110A overexpressing leaf tissues. Firstly, because overexpression of *NIMIN3* in *N. benthamiana* yields pretty late cell death (i.e., 12 dpi) as compared to NIMIN1. Secondly, after 18 dpi sometime *GFP* overexpressing leaf tissues also manifest very mild symptoms of cell death.

Of note, among all mutants tested, an EAR domain deletion mutant NIMIN1 1/137 was found to be the most aggressive in terms of cell death phenotype. Thus, results presented here support the view that EDF and EAR motifs are probably not involved in cell death induction. This interpretation is further consolidated by the fact that NIMIN1b which encompasses both domains does not induce cell death. As several mutants tested in this study do not suppress the SA-mediated reporter gene, but still yield cell death, so no correlation was found between suppression of *PR-1* and cell death manifestation.

Among tobacco *NIMIN* genes, *NIMIN2a* and *NIMIN-like1* transient overexpression do not induce cell death. However, *NIMIN2c* transient overexpression generates cell death in *N. benthamiana* plant leaf halves. The cell death symptoms appear after 7-8 dpi in *NIMIN2c* overexpressing leaf tissues, which are comparatively later than *NIMIN1* overexpressing leaf tissues (Fig. 45B). Interestingly, NIMIN-like1 contains an EDF domain. However, it does not manifest cell death when transiently overexpressed in plant leaf tissues. On the contrary, NIMIN2c harbors no EDF domain, but manifests cell death. Therefore, it could be concluded that as in Arabidopsis, in tobacco NIMIN proteins the EDF motif is not the cause of cell death.

Now taking all this into consideration, it is not clear what is the cause of cell death in *N. benthamiana* leaf tissue transiently overexpressing some members of *NIMIN* genes. However, it has been published recently that NPR1 suppresses cell death in SAR tissue (Fu et al., 2012). In this line, NIMIN1, NIMIN3 and NtNIMIN2c could function as suppressors of this specific activity of NPR1. However, in order to understand this assumption, the mechanism needs to be explored in future experiments.

4.5 A Working model for the consecutive action of Arabidopsis NIMIN proteins in course of SAR

Since their discovery, NIMIN proteins have gained much attention. Interactions with NPR1 portray them as an important group of proteins, with possible play in SAR pathway. Indeed, data obtained previously and presented in this study propose a working model for consecutive action of AtNIMINs on AtNPR1 at distinct stages of SAR (Fig. 46). The model presented is deduced from the studies conducted by Weigel et al., (2001 and 2005), Hermann et al., (2013) and this study. It is known that *NIMIN3* is expressed constitutively at a low-level and may repress the *PR-1* gene activation in unchallenged plants. *NIMIN2* is a very early SA-induced and *NPR1*-independent gene. Based on data, it is likely that *NIMIN2* is expressed in plant leaf tissues with low SA level (e.g., early after pathogen attack) to relieve NIMIN3 repression by binding to NPR1. This interaction may activate the early SA and *NPR1*-dependent genes, e.g., *NIMIN1*. NIMIN2 interaction at the C-terminus of NPR1 does not appear to be sufficient for the strong induction of late SAR gene *PR-1*. *NIMIN2* expression is transient and NIMIN2 interaction with NPR1 is replaced by NIMIN1. NIMIN1 suppresses the activation of *NPR1*-dependent genes, i.e., *PR-1* gene. NIMIN1 action on NPR1 seems to be even more transient than NIMIN2. NIMIN1 is an instable protein, which results in less overall accumulation of NIMIN1 protein. Eventually, the *PR-1* gene would be activated through direct action of SA on NIMIN-NPR1 complex, which leads to the removal of NIMIN1 suppressor from NPR1 (Maier et al., 2011; Fu et al., 2012; Wu et al., 2012). Thus, the sequential actions of NIMINs on NPR1 mount differential effects, which lead to coordinate induction of defense genes to warfare against the invading pathogens. NIMINs-NPR1 complexes respond to and monitor the ambient concentration of SA in diseased plant, which leads plant to decode an increasing SA level into two decision steps, early and late SAR gene expression. The model presented in Figure (46) also proposes the events

happening in pathogen infected plant, leading to cell death in local pathogen infected leaf, which ultimately leads to the SAR induction in distal plant tissues. It is known that local pathogen infected leaf tissues undergo apoptosis by sacrificing some neighboring cells, which prevent the further spread of pathogens. This leads to increased tissue levels of the defense hormone SA. At molecular level, sequential NIMIN-NPR1 complex formation and influence of the signal molecule SA in SAR pathway has been reported in Figure (46). Moreover, the model also proposes the parallel phenotypic events happening in pathogen infected plants, i.e., cell death progression in local pathogen infected leaf leading to SAR in distal plant tissues.

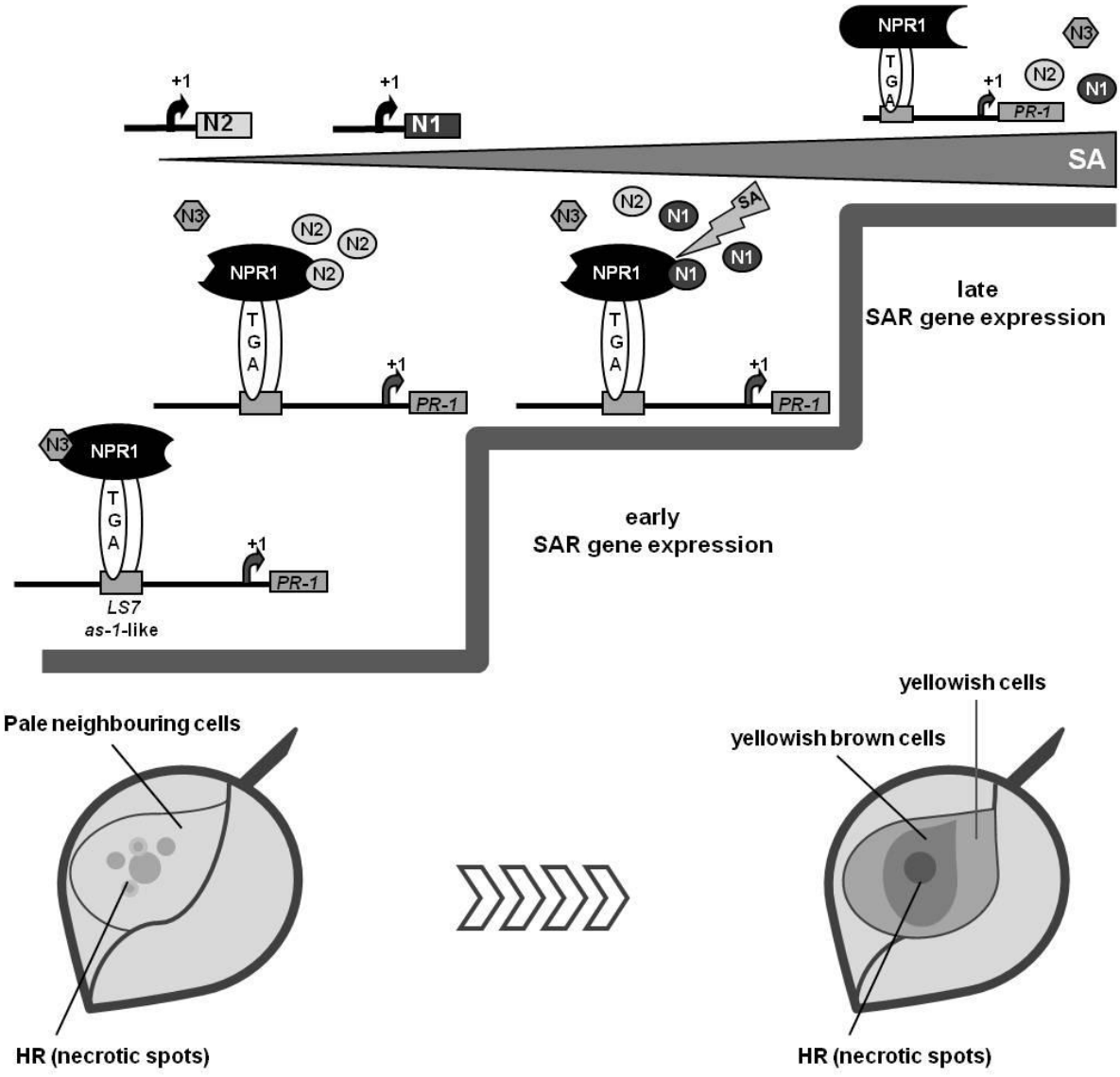

Fig. 46: Working model for the NIMIN-mediated control of NPR1 activity and progress of cell death. The model explains the consecutive interaction of NIMIN proteins with NPR1 and effects of ambient SA levels on NIMIN-NPR1 complexes in infected plant in activating *PR -1* gene. The model also presents the gradual cell death progression in infected ETI leaf.

The research conducted here suggests that NIMIN proteins are regulators of NPR1 activity during SAR. This study has contributed in deducing a revised model of SAR pathway, by established novel transient gene expression system in *N. benthamiana* reporter line. The data also suggest that NIMIN may have other targets. Indeed, the Arabidopsis Interactome Mapping Consortium (2011; Fig. 47) showed that TOPLESS (TPL) interacts with NIMIN2 and NIMIN3. Interaction between NIMINs and TPL has been shown in yeast (Späth, 2012). This supports the view that NIMINs are part of a complex hormone signaling network. Thus, role of NIMINs might be much more complex than initially anticipated. However, still a lot needs to be done to find out the contribution of NIMINs in cell death, which still remains a mystery. Gene chimera study between NIMIN1 and NIMIN1b domain swapping experiments might help in understanding the cell death phenomenon. Tobacco NIMINs contain a different EAR domain, therefore, it will be interesting to check for the role of Nt NIMINs EAR domain in *PR-1a* gene regulation. The role of EDF domain in NtNIMIN-like1 also remains a future challenge.

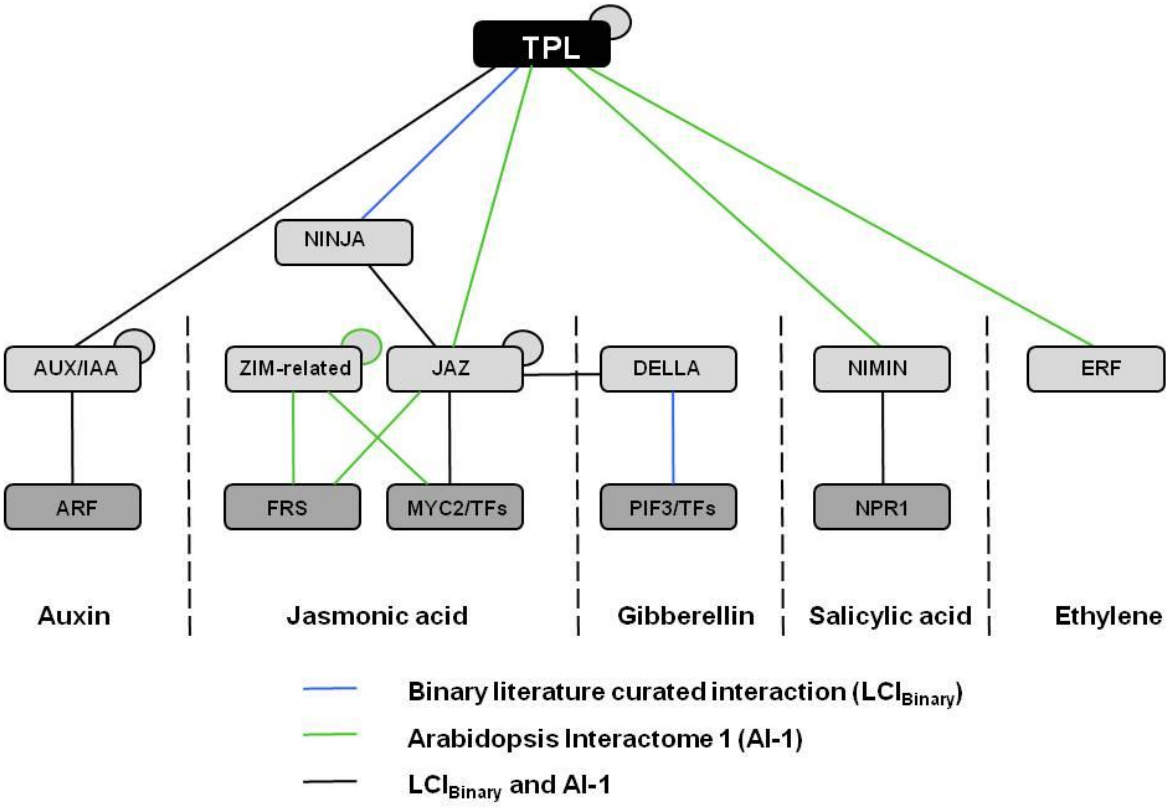

Fig. 47: Protein-protein interactions in AI-1 suggest a modular assembly of transcriptional hormone-response regulators and support a global regulatory role for TPL. (Arabidopsis Interactome Mapping Consortium, 2011)

5 Summaries

5.1 Summary

Systemic acquired resistance (SAR) is a whole plant resistance mechanism, launched after initial exposure to a necrotizing pathogen. At molecular level, SAR is characterized by elevated level of plant hormone salicylic acid (SA) and induction of pathogenesis-related (PR) proteins. During SAR, SA signal is transduced through regulatory protein NPR1 (Non-Expressor of PR Genes1; also known as NIM1 or SAI1) leading to the induction of the SAR marker *PR-1*. Present data strongly suggest that the SA signal is directly perceived by NPR1. NPR1 interacts with two classes of proteins. DNA binding TGA factors link the SA sensor NPR1 to the *as-1* like *cis-acting* elements present in the promoter region of *PR-1* gene. In addition, NPR1 interacts with NIM1-interacting (NIMIN) proteins. In Arabidopsis, there are four *NIMIN* genes, i.e., *NIMIN1*, *NIMIN1b*, *NIMIN2* and *NIMIN3*. Initially, it was hypothesized that, although structurally related to each other, NIMIN proteins might play diverse functions during SAR response. Indeed, it has been shown that the *NIMIN* genes are expressed differentially and that the encoded proteins interact differentially with NPR1. Based on these observations, NIMIN proteins have gained much attention. The functional significance of NIMIN proteins in SAR pathway has been addressed in overexpression studies. Overexpression of *NIMIN1* yielded strong suppression of *PR* gene induction and enhanced susceptibility to a bacterial pathogen in transgenic Arabidopsis. Apart from NIMIN1, the functional significance of other Arabidopsis NIMIN family members has not yet been addressed. Therefore, present research is conducted to explore the biological significance of other NIMIN family members from Arabidopsis as well as tobacco. To this end, transient gene expression in a *N. benthamiana* reporter line containing a *-1533PR-1a$_{pro}$:GUS* construct was employed. The research achievements of this work are listed below.

1. The *N. benthamiana* infiltration procedure was optimized for reliable determination of *-1533PR-1a$_{pro}$:GUS* reporter gene activation in presence of different Agrobacterium strains.
2. After optimization, transient gene expression system (TGES) was successfully used to uncover the functional significance of NIMIN proteins on SA-mediated *PR-1a* gene induction. NIMIN1 and NIMIN1b are categorized as strong, NIMIN3 as an intermediate and NIMIN2 as a non-suppressor of SA-mediated *PR-1a* reporter gene activation.

3. Interestingly, suppressing NIMIN1, NIMIN1b and NIMIN3 all contain an EDF (glutamic acid, aspartatic acid, phenylalanine) motif. Therefore, EDF mutants were generated in NIMIN1 and NIMIN3, i.e., NIMIN1 E94A D95V and NIMIN3 E63A D64V, respectively. Yeast two-hybrid (Y2H) data show that NIMIN1 E94A D95V still interacts with NPR1, while NIMIN3 E63A D64V interaction with NPR1 could not be validated due to undetectable accumulation of the mutant fusion protein in yeast. In the TGES, NIMIN1 E94A D95V and NIMIN3 E63A D64V were not able to suppress the SA-mediated *PR-1a* promoter activation. The data support the fact that the EDF motif may have a function in NIMIN proteins interaction with NPR1, thereby, regulating *PR-1* gene induction.
4. EAR domain is generally considered as a repression domain and also exists in NIMIN proteins. The deletion mutants, i.e., NIMIN1 1/137 and NIMIN1b 1/135 still suppress the SA-induced *-1533PR-1a*$_{pro}$*:GUS* gene activation. On the other hand, NIMIN1 L138A L140A and NIMIN3 L108A L110A do not suppress the SA-mediated reporter gene induction. But that is because of low overall accumulation of mutant proteins in *N. benthamiana* leaf tissues. Thus, the data support the view that EAR domain is not the only repressional domain active in NIMIN proteins.
5. Like Arabidopsis, tobacco also contains *NIMIN* genes. During this study, a novel *NIMIN* gene from tobacco, *NIMIN-like1*, was cloned and characterized. Using Y2H analyses, it was shown that NIMIN-like1 binds to NgNPR1 and that interaction is sensitive to SA. Thus, NIMIN-like1 falls into the tobacco NIMIN family. Thereafter, functional significance of diverse tobacco NIMIN proteins for their effects on SA-mediated *PR-1a* gene induction via TGES was carried out. NIMIN2c and NIMIN-like1 are categorized as strong suppressors, whereas NIMIN2a is a weak suppressor of SA-mediated *PR-1a* reporter gene induction.
6. *NIMIN1* and *NIMIN3* overexpression manifests cell death in *N. benthamiana*, and cell death is accompanied by the accumulation of H_2O_2. No correlation was found between NIMIN proteins binding intensity to NPR1 and cell death. Similarly, no correlation was found between *PR-1a* reporter gene suppression and cell death. The data support the view that the EDF and EAR motifs are not involved in cell death phenomenon.

Based on previous and data gathered in this study, a model for the hypothetical play of sequential interaction of NIMIN proteins with NPR1 in course of SAR is presented.

5.2 Zusammenfassung

Systemisch aktivierte Resistenz (SAR) ist ein Resistenzmechanismus der Pflanze, der nach einem initialen Kontakt mit nekrotisierenden Pathogenen in Gang gesetzt wird. Auf molekularer Ebene zeichnet sich SAR durch erhöhte Konzentrationen des Pflanzenhormons Salicylsäure (SA) und der Induktion von pathogenesis-related (PR) Proteinen aus. Während der SAR wird das SA-Signal durch das Regulatorprotein NPR1 (Non-Expressor of PR Genes 1; auch bekannt als NIM1 oder SAI1) übermittelt, was zur Induktion des SAR-Markers *PR-1* führt. Das SA-Signal wird vermutlich direkt von NPR1 erkannt. NPR1 interagiert mit zwei Klassen von Proteinen. DNA-bindende-TGA-Faktoren verbinden den SA-Sensor NPR1 mit *as-1* ähnlichen *cis*-aktivierenden Elementen in der Promotorregion des *PR-1* Gens. Zusätzlich interagiert NPR1 mit NIM1-interagierenden (NIMIN) Proteinen. In Arabidopsis gibt es vier *NIMIN*-Gene: *NIMIN1*, *NIMIN1b*, *NIMIN2* und *NIMIN3*. Anfangs wurde die Hypothese aufgestellt, dass die verschiedenen NIMIN-Proteine trotz ihrer ähnlichen Struktur verschiedene Funktionen bei der SAR-Antwort erfüllen. Tatsächlich wurde gezeigt, dass die *NIMIN*-Gene verschieden exprimiert werden und die kodierten Proteine auf unterschiedliche Weise mit NPR1 interagieren. Die Rolle der NIMIN-Proteine beim SAR-Mechanismus wurde in Überexpressionsstudien untersucht. Überexpression von *NIMIN1* führte zur starken Suppression der *PR*-Gen-Induktion und erhöhte die Anfälligkeit transgener Arabidopsis-Planzen gegenüber bakteriellen Pathogenen. Abgesehen von NIMIN1 wurde die Funktion der anderen NIMIN-Proteine aus Arabidopsis noch nicht untersucht. Daher wurden in der vorliegenden Arbeit die Aktivitäten der anderen Mitglieder der Arabidopsis NIMIN-Familie wie auch von Tabak NIMIN-Proteinen untersucht. Dazu wurde transiente Genexpression in einer *N. benthamiana* Reporter-Linie mit einer *-1533PR-1a$_{pro}$:GUS* Konstruktion verwendet. Die Forschungsergebnisse dieser Arbeit sind im Folgenden aufgelistet.

1. Die Infiltration von Agrobacterium in *N. benthamiana* wurde optimiert, um die Aktivierung des Reportergens *-1533PR-1a$_{pro}$:GUS* verlässlich messen zu können.
2. Nach der Optimierung wurde das transiente Genexpressionssystem (TGES) erfolgreich eingesetzt, um die funktionelle Bedeutung der NIMIN-Proteine für die SA-vermittelte *PR-1a* Geninduktion zu bestimmen. NIMIN1 und NIMIN1b sind starke, NIMIN3 ein mittlerer und NIMIN2 ein Nicht-Suppressor des SA-regulierten *PR-1a* Reportergens.

3. Interessanterweise enthalten alle reprimierenden Proteine, NIMIN1, NIMIN1b, und NIMIN3, ein EDF-Motiv (Glutaminsäure, Asparaginsäure, Phenylalanin). In NIMIN1 und NIMIN3 wurden EDF-Mutanten generiert, nämlich NIMIN1 E94A D95V bzw. NIMIN3 E63A D64V. Hefe-zweihybridsystem (Y2H)-Daten zeigen, dass NIMIN1 E94A D95V immer noch mit NPR1 interagiert, während die Interaktion von NIMIN3 E63A D64V mit NPR1 nicht validiert werden konnte, da das mutante Protein in Hefezellen nicht akkumulierte. Im TGES konnten NIMIN1 E94A D95V und NIMIN3 E63A D64V die Aktivierung des *PR-1a* Promotors durch SA nicht unterdrücken. Die Daten lassen den Schluss zu, dass das EDF-Motiv eine Funktion bei der Interaktion der NIMIN-Proteine mit NPR1 einnehmen, und so die *PR-1* Geninduktion regulieren könnte.
4. Die EAR-Domäne wird generell als eine Repressions-Domäne angesehen und existiert auch in NIMIN-Proteinen. Die Deletions-Mutanten, NIMIN1 1/137 und NIMIN1b 1/135, unterdrücken dennoch die SA-induzierte Aktivierung des *-1533PR-1a*$_{pro}$*:GUS*-Gens. Auf der anderen Seite unterdrücken NIMIN1 L138A L140A und NIMIN3 L108A L110A nicht die SA-vermittelte Induktion des Reportergens. Aber das ist auf die niedrige Akkumulation von mutanten Proteinen in Blattgewebe von *N. benthamiana* zurückzuführen. Die Daten lassen also den Schluss zu, dass die EAR-Domäne nicht die einzige Repressions-Domäne ist, die in NIMIN-Proteinen aktiv ist.
5. Wie Arabidopsis enthält auch Tabak *NIMIN*-Gene. Es wurde ein neues *NIMIN*-Gen aus Tabak, *NIMIN-like1,* kloniert und charakterisiert. Durch Y2H-Analyse wurde gezeigt, dass NIMIN-like1 an NgNPR1 bindet und diese Interaktion sensitiv für SA ist. Folglich gehört NIMIN-like1 zur Tabak-NIMIN-Familie. Daraufhin wurde die funktionelle Bedeutung diverser Tabak-NIMIN-Proteine auf die SA-vermittelte *PR-1a*-Geninduktion mithilfe von TGES ausgetestet. NIMIN2c und NIMIN-like1 werden als starke Suppressoren kategorisiert, während NIMIN2a ein schwacher Suppressor der SA-vermittelten *PR-1a* Reportergen-Induktion ist.
6. Die Überexpression von *NIMIN1* und *NIMIN3* führt in *N. benthamiana* zu Zelltod; der von H_2O_2-Akkumulation begleitet ist. Es wurde keine Korrelation zwischen der Bindungsintensität der NIMIN-Proteine an NPR1 und Zelltodinduktion gefunden. Gleichermaßen wurde keine Korrelation zwischen der *PR-1a* Reportergen-Suppression und Zelltod gefunden. Die Daten lassen den Schluss zu, dass die EDF- und EAR-Motive nicht an der Auslösung des Zelltod-Phänomens beteiligt sind.

Basierend auf anderen und den in dieser Studie generierten Daten wird ein Modell für die aufeinanderfolgende Interaktion von NIMIN-Proteinen mit NPR1 und ihr hypothetisches Zusammenspiel im Laufe der SAR präsentiert.

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Author's Declaration

Anlage 2 zur Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

Eidesstattliche Versicherung gemäß § 7 Absatz 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

1. Bei der eingereichten Dissertation zum Thema

   Effects of overexpression of *NIMIN* genes on Salicylic acid-mediated *PR-1* gene activation and phenotype in *Nicotiana benthamiana* (Domin)

   handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich: Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Stuttgart, June 17, 2013

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Ort und Datum Unterschrift

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Publications

Hermann, M., Maier, F., Masroor, A., Hirth, S., Pfitzner, A.J.P., and Pfitzner, U.M. (2013). The Arabidopsis NIMIN proteins affect NPR1 differentially. Frontiers in Plant Science *4*, 88.

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Poster Presentations

Masroor, A., Maier, F., and Pfitzner, U.M. (2011). The NIMIN proteins have differential effects on the expression of *PR-1* genes. Poster presented at PR-Proteins and induced resistance against pathogens and insects (conference), Neuchâtel, Switzerland 4-8 September 2011.

Masroor, A., Maier, F., and Pfitzner, U.M. (2011). The NIMIN proteins have differential effects on the expression of *PR-1* genes. Poster presented at 5th Regio Plant Science Meeting Stuttgart-Ulm-Tübingen, Ulm, November 5th, 2011.

Masroor, A., Maier, F., and Pfitzner, U.M. (2012). The Arabidopsis NIMIN proteins affect *PR-1* gene induction differentially. Poster presented at 6th Regio Plant Science Meeting Stuttgart-Tübingen-Ulm, ZMBP Tübingen, October 12th, 2012.