Functional Analysis of the Salicylic Acid-Responsive *PR-1* Promoter in *Arabidopsis thaliana*

Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultäten

der Georg-August-Universität zu Göttingen

vorgelegt von

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Göttingen 2009

D7

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Tag der mündlichen Prüfung: 9. Juli 2009

Contents

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
aa	amino acids
A	Ampere
A	adenosine
ABA	abscisic acid
amp	ampicilin
APS	ammoniumpersulfate
as-1	*activating sequence 1*
AT	marks a protein from *Arabidopsis thaliana*
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumine
bZIP	basic leucine zipper
C	cytosine
CaMV	cauliflower mosaic virus
CAT	catalase
cDNA	copy DNA
ChIP	chromatin immunoprecipitation
C-terminal	carboxy-terminal
Da	Dalton
ddNTPs	didesoxy nucleotides
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotides

DTT	dithiotreitol
EB	elusion buffer
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ET	ethylene
et al.	*et alii* (and others)
EtOH	ethanol
F	Farad
g	gravitation
g	gram
G	guanine
GA	gibberellic acid
β-Gal	β-galactosidase
GFP	green fluorescence protein
GST (U)	glutathione-S-transferase (τ-class)
GUS	glucuronidase
h	hours
HIS	marker gene for histidine prototrophy
H_2O_2	hydrogen peroxide
hpi	hours past infection
HR	hypersensitive response
HSP	herring sperm DNA
IAA	auxin
INA	isonicotinic acid
JA	jasmonic acid
k	kilo (10^3)
kan	Kanamycine
L	litre

lacZ	gene coding for β-galactosidase
LB	left border
LUC	Luciferase
μ	micro (10^{-6})
m	mili (10^{-3})
M	molarity [mol/L]
Me-SA	methyl salicylate
min	minutes
mRNA	messenger RNA
MS	Murahige and Skoog
n	nano (10^{-9})
NPR1	NON-EXPRESSOR of *PR*-GENES 1
Ω	Ohm
OD	optical density
OE-PCR	overlap extension polymerase chain reaction
o/n	over night
p	pico (10^{-12})
PAA	polyacrylamide
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
PCD	programmed cell death
PDA	potato dextrose agar
PDB	potato dextrose broth
pH	negative $\log_{10}$ of proton concentration
PMSF	phenyl-methyl-sulfonyl-fluoride
PR	*Pathogenesis related*
PR-1	*pathogenesis-related protein 1*
RNA	ribonucleic acid
RNase	ribonuclease

ROS	reactive oxygen species
rpm	rotations per minute
RT	room temperature
s	second
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecylsulfate
SNI1	SUPPRESSOR OF NPR1, INDUCIBLE 1
SOD	superoxide dismutase
spec	Spectinomycine
T	thymine
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetraethylenediamine
TGA	*as-1* (TGACG motive) binding bZIP transcription factors
TIBA	2,3,5-triiodobenzoic acid
Tris	tris-hydroxymethylamino methane
u	unit (quantity for enzyme activity)
U	uracil
UV	ultra violet
V	Volt
v/v	volume per volume
W	Watt
wt	wildtype plants
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1   Summary

The Arabidopsis *PR-1* gene belongs to a subset of genes upregulated during "systemic acquired resistance" (SAR), a plant defense response against a broad spectrum of pathogens mediated by the signaling molecule salicylic acid (SA). Genetic analysis has revealed that the promoter is repressed by SNI1 and that this repression has to be overcome by the SA-sensitive positive regulator NPR1. NPR1 activates transcription after association with TGA factors which bind to the *as-1*-like element of the *PR-1* promoter. This study reveals that NPR1 and SNI1 regulate the *PR-1* promoter through the *as-1*-like element and through WRKY boxes. The SNI1/NPR1 antagonism at the *PR-1* promoter is explained by the existence of two alternate activation pathways. In the wildtype promoter, activation is mediated by the TGA-NPR1 complex at the *as-1*-like element. The *as-1*-like element represses at the same time the alternate activation pathway, presumably through indirect interactions with SNI1. In the absence of the *as-1* element or SNI1, the alternate pathway can be activated through WRKY transcription factors, which are expressed in an NPR1-dependent manner. NPR1 cannot activate the promoter if the distance between the two TGA binding sites of the *as-1*-like element is 4- instead of 9bps, but such an *as-1*-like element can still repress the alternate pathway. The *as-1* element of the *CaMV 35S* promoter, which contains 4bps between the TGA binding sites, confers strong constitutive activation when replacing the *as-1*-like element of the *PR-1* promoter, as a binding site for a strong activator is encoded in the sequence of this *cis*-regulatory element. Thus, the *as-1* element of the *CaMV 35S* promoter cannot mediate repression of the alternate pathway in the *PR-1* promoter context. However, the repression capacity is re-established in the absence of the TGA subclass II members TGA2, TGA5 and TGA6.

2 Introduction

2.1 Plant immune responses

Plant-microbe interactions display a well-studied example for co-evolutionary development, as the strong selective pressure favors constant modulations of attack- and counterattack mechanisms (Chisholm et al. 2006). The most common form of plant defense response against a wide range of microorganisms is referred to as "non-host resistance" (Lipka et al. 2008). This kind of defense mechanism protects the plant by preventing the penetration or the establishment of the pathogen in early phases of attack and thereby prevents infection. Plants encountered with non-host pathogens show no visible symptoms at all (type I non-host resistance) or a rapid induction of cell death (type II non-host resistance) (Mysore and Ryu 2004). Important mediators involved in non-host resistance against fungi belong to the PEN (PENETRATION) class of proteins, which act at the cell periphery and limit fungal growth by execution of apoplastic immune responses (Meyer et al. 2009). The induction of essential processes concerning the establishment of the non-host resistance response can be elicited by molecules presented by a variety of microbes. These molecules are therefore designated as pathogen- or microbe-associated molecular pattern (PAMPs/MAMPs) and the induced defense response is thus frequently termed PAMP-triggered immunity (PTI) (Murray et al. 2007). Typical MAMPs are the 22-amino-acid epitope of flagellin, flg22 (Felix et al. 1999), or the most abundant bacterial protein, the elongation factor Tu (EF-Tu) (Kunze et al. 2004). The sensing of these substances is followed by the activation of a complex defense signaling network at the local site of infection and a subsequent activation of systemic acquired resistance (SAR), which renders the plant more resistant in distal tissues (Mishina and Zeier 2007). This barrier of inducible defense contributes to "basal resistance" of the plant, since the recognized molecular charateristics are shared by large groups of microbes and are therefore relatively unspecific with regard to the attacking pathogen. However, pathogens developed mechanisms to prevail over basal resistance by blocking essential steps in the PAMP-stimulated signaling cascade via the evolution of virulence factors referred to as effector proteins (Stahl and Bishop

2000). A more efficient and specific second layer of defense can be triggered upon recognition of these bacterial effector proteins by specialized resistance proteins (*R*-proteins) of the plant, a process designated as *R* gene-mediated resistance or effector triggered immunity (ETI) respectively. Sensing of bacterial effector (*avr*) proteins can elicit a hypersensitive response (HR), a rapid and effective defense reaction against biotrophic pathogens which is initiated by accumulation of reactive oxygen species (ROS) and subsequently causes local cell death of infected tissue. The establishment of a HR goes along with the activation of SAR (Staskawicz et al. 1995; Ryals et al. 1996; Grant and Lamb 2006). The downstream events leading to resistance against attacking pathogens are dependent on the interplay between several phytohormone pathways whose positive and negative feedback regulation is critical for the efficiency of the defense response.

2.2   Plant hormones and their role in host defense

The communication between plant cells and tissues is mediated via phytohormone signaling in analogy to the hormone system known from animals. Important hormonal regulators in the context of defense responses are jasmonate (JA), salicylic acid (SA) and ethylene (ET) (Glazebrook 2001; Pieterse and van Loon LC 1999; Thomma et al. 2001). Resistance is achieved by processes like cell wall strengthening, lignifications, *pathogenesis-related (PR)*-gene expression, phytoalexin synthesis and, in case of R-gene-mediated resistance, induction of localized cell death (Jalali et al. 2006). Although the three mentioned phytohormones all contribute to plant defense, they are functioning in an antagonistic way and their synthesis is dependent on the kind of pathogen that is attacking the plant. For instance, the SA signaling pathway plays a major role in the defense against biotrophic pathogens like *Pseudomonas syringae*, *Pernospora parasitica* and different viruses, while JA/ET signaling is activated after infection with necrotrophic pathogens or by herbivorous insects (Dong 2001; Feys and Parker 2000; Kachroo et al. 2000; Kessler and Baldwin 2002; Takahashi et al. 2002; Thomma et al. 1998). Biotrophic pathogens require living host cells to establish their propagation, whereas necrotrophic attackers obtain their nutrition from dead plant tissue.

JA is an oxygenated fatty acid-derivative (oxylipin), which is produced via oxidative metabolism of polyunsaturated fatty acids by enzymes of the octadecanoid pathway. The synthesis of this phytohormone is induced after attack of pathogens with necrotrophic lifestyles or by wounding. Infestation of plants with the necrotrophic fungus *Botrytis cinerea* causes the formation of necrotic lesions due to toxic enzymes and metabolites secreted by the attacker and by elicitation of an oxidative burst during penetration (van Kan 2006). The dead tissue serves the invaded microorganism as a source for nutrients. The receptor protein of the JA conjugate JA-isoleucine (JA-Ile) is COI1 (CORONATINE INSENSITIVE 1), an essential regulator of JA signaling (Katsir et al. 2008). *coi1-1* mutants exhibit increased susceptibility to necrotrophic fungi and herbivores and induction of JA-responsive marker genes like *LOX* and *VSP* or the plant defensin *PDF1.2* is completely abolished (Reymond et al. 2004; Stintzi et al. 2001). The COI1 protein is an E3-ligase that forms the multi protein complex SCF^{COI1} (skip-cullin-F-box) to target proteins of the JAZ (Jasmonate ZIM-domain) family for ubiquitination and subsequent degradation in the 26S proteasome (Chini et al. 2007; Thines et al. 2007). ET signaling also contributes to resistance against necrotrophic pathogens beside its well characterized functions in fruit ripening and senescence (Thomma et al. 1999; Yang and Hoffman 2003). Central regulators of this pathway are the ET receptors ETR1 (ENHANCED TRIPLE RESPONSE 1), the suppressor CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), the membrane -located EIN2 (ETHYLENE INSENSITIVE 2) protein and the transcription factor EIN3 (ETHYLENE INSENSITIVE 3) (Chen et al. 2005; Alonso et al. 1999). CTR1 suppresses ET signaling in the absence of the hormone and is inactivated upon binding of ET to the CTR1 receptor, which subsequently leads to a MAP-kinase (mitogen-activated protein)-mediated phosphorylation cascade and a stabilization of EIN3 (Gao et al. 2003). JA- and ET signaling show a synergistic cross-talk on the expression of important defense related genes like *osmotins* and *PDF1.2* (Thomma et al. 1998).

The SA pathway is generally activated by pathogens exhibiting a biotrophic lifestyle and is crucial for basal and *R*-gene mediated resistance (Tsuda et al. 2008). Biotrophic microorganisms receive their nutrients from living plant cells and recognition by the plant leads in case of ETI to the establishment of a hypersensitive response (HR) and systemic acquired resistance (SAR). During HR, reactive oxygen species (ROS) are

produced following programmed cell death (PCD) and disintegration of the infested tissue (Lam et al. 2001).

Due to the different kinds of defense responses required against biotrophic, hemibiotrophic and necrotrophic pathogens as well as herbivorous insects, a tightly regulated fine tuning of the hormonal pathways is essential for the fitness of the plant. Activation of the SA signaling cascade causes a negative cross-talk on JA signaling, as revealed by a lack of *PDF1.2* induction after combined exogenous application of SA and JA (Kunkel and Brooks 2002). This negative regulation depends on NPR1 (NONEXPRESSOR OF PR-GENES 1), a key regulator in SA- dependent signal transduction (Spoel et al. 2003), but the NPR1 dependency is lost when ET signaling modulates the SA/JA cross-talk (Leon-Reyes et al. 2009). A positive effect of ET on SA-dependent defense signaling and strengthened accumulation of the marker gene *PR-1* has been described previously (De Vos et al. 2005; Lawton et al. 1994). The biological relevance of the negative influence of SA on JA-mediated resistance was shown by experiments employing biotrophic *Pseudomonas syringae* and necrotrophic *Alternaria brassicicola* in cross-talk experiments. The results revealed that pathogens with different lifestyles force the plant to mediate tradeoffs between distinct defense signaling pathways (Spoel et al. 2007). Due to the complex interplay between hormonal signaling pathways, pathogens evolved strategies to manipulate the immune response of the plant to increase pathogenicity. Some *Pseudomonas syringae* strains are able to produce the phytotoxin coronatine (COR) (Bender et al. 1999), a molecule that structurally resembles JA-derivates like JA-Ile (Staswick 2008). COR induces a similar subset of genes as MeJA (Uppalapati et al. 2005) and experiments with COR-deficient *P. syringae* strains and plants impaired in the JA pathway demonstrate that *P. syringae* utilizes COR to mimick JA-signaling and to suppress SA-dependent defense (Nomura et al. 2005).

Beside SA, JA and ET, there are other hormones and genes involved in plant defense. For instance the *PAD3* (PHYTOALEXIN DEFICIENT 3) gene plays an indispensible role in camalexin biosynthesis, a phytoalexin important for resistance against necrotrophic pathogens. The camalexin production is independent of SA, JA and ET and is induced by oligogalacturonides (OG) accumulating due to cell wall degradation processes caused by the pathogen (Ferrari et al. 2007). Furthermore the phytohormone

abscisic acid (ABA) has in impact on plant defense, partly by affecting JA biosynthesis (Adie et al. 2007), as well as Auxin (IAA), which is antagonized by SA-signaling (Wang et al. 2007).

2.3   SA signaling and systemic acquired resistance (SAR)

SAR initiation provokes an increased protection against a wide range of microorganisms and is associated with an activation of signal transduction pathways and the induction of *PR*-genes (Uknes et al. 1992; Van Loon 1997; Durrant and Dong 2004). The onset of SAR causes an endogenous increase in salicylic acid (SA) levels in local and systemic tissues (Malamy et al. 1990), resulting in a primed defense state of the plant (Durrant and Dong 2004). Several *PR*-genes exhibit chitinase or glucanase activities and their synergistic action yields a strong antipathogenic potential. However, the role and molecular function of *PR-1* remains elusive (Van Loon 1997).

SA is a critical molecule for the establishment of SAR, proven by experiments conducted with tobacco and *A. thaliana* plants carrying a transgenic *NahG* gene. *NahG* encodes a bacterial hydroxylase which converts SA to catechol and thereby depletes endogenous SA levels. The *NahG* plants are no longer able to accumulate SA and *PR-1* transcripts after pathogen infection, resulting in an impaired SAR (Gaffney et al. 1993). The observation that SA accumulates in phloem exudates led to the hypothesis that SA is the mobile signal produced at the site of infection and transported in systemic tissues to render the whole plant more resistant to secondary pathogen attacks (Métraux et al. 1990). Tobacco grafting experiments with *NahG* rootstocks and wildtypic scions revealed that SA is not important for the generation of the mobile signal, but reciprocal grafting experiment illustrated the importance of SA for perceiving the mobile signal and assuring systemic resistance (Vernooij et al. 1994). A recent study has shown that a volatile ester of SA, methyl SA (MeSA), plays a critical role as mobile SAR signal, at least in tobacco plants. MeSA is synthesized at the site of infection by SA carboxyl methyltransferase (SAMT) and cleaved in systemic tissue by SA binding protein 2 (SABP2) (Park et al. 2007). The methyl esterase activity of SABP2 is essential for the perception of the signal and initiation of SAR.

Other studies suggest a lipid-based molecule to be the decisive mobile signal in SAR. The *dir1* (<u>D</u>EFECTIVE <u>I</u>N <u>I</u>NDUCED <u>RESISTANCE</u> <u>1</u>) mutant carries a mutation in a gene similar to lipid transfer proteins (LTPs) and displays normal local resistance to pathogens, while the generation of SAR and induction of *PR*-genes in systemic tissues fails (Maldonado et al. 2002).

After pathogen attack, SA is synthesized from chorismate, derived from the shikimate pathway, by isochorismate synthase (ICS1) (Wildermuth et al. 2001). The components of the shikimate pathway are transcriptionally upregulated after infection to provide sufficient amounts of chorismate for SA biosynthesis (Truman et al. 2006). The ICS1 protein shows a high chorismate binding affinity and is localized in the stroma of chloroplasts (Strawn et al. 2007). Mutation of the ICS1 protein in *sid2* (<u>S</u>A <u>I</u>NDUCTION-<u>D</u>EFICIENT <u>2</u>) causes a reduction of SA accumulation after infection to only 5-10% of the wildtypic level and a decrease in basal and systemic resistance (Wildermuth et al. 2001). Regulatory steps upstream of *ICS1* are dependent on the attacking pathogen and the defense pathway elicited by it. In case of resistance mediated via the *R*-gene RPS4 (<u>R</u>ESISTANCE <u>TO</u> <u>P</u>SEUDOMONAS <u>S</u>YRINGAE <u>4</u>), key regulatory proteins upstream of ICS1 are the two lipase-like proteins EDS1 (<u>EN</u>HANCED <u>D</u>ISEASE <u>S</u>YMPTOMS <u>1</u>) and PAD4 (<u>PHYTOA</u>LEXIN <u>D</u>EFICIENT <u>4</u>), which function in a positive feedback loop to increase SA biosynthesis and their own expression (Feys et al. 2001). Furthermore, the initiation of SAR and the production of SA are accompanied by the accumulation of ROS. Earlier experiments showed, that SA can directly inhibit the H_2O_2 scavanging enzymes catalase and ascorbat peroxidase (APX) (Chen et al. 1993; Durner and Klessig 1995), while later studies suggest ROS signaling upstream of SA biosynthesis (Bi et al. 1995; Neuenschwander et al. 1995). Application of high H_2O_2 concentrations to plants leads to a dose-dependent induction of SA synthesis and *PR-1* expression (Leon et al. 1995). On the other hand, H_2O_2 is not detectable in systemic tissue (Ryals et al. 1995), making the attributed second messenger function of H_2O_2 questionable. Taken together, it seems that the combinatorial action of SA and ROS, produced during microburst formations, increases the defense response in systemic tissues synergistically (Du and Klessig 1997; Shirasu et al. 1997). Additionally, SA changes the intracellular redox potential, observable by alterations in absolute glutathione levels and differences in the ratio of oxidized (GSSG)

to reduced (GSH) glutathione. In the first hours after pathogen attack or SA treatment, an initial oxidative burst occurs followed by a recovery and rebounce to a reduced environment in local as well as in systemic tissues (Mou et al. 2003). Interestingly, these changes only take place when a compatible interaction is given, illustrating the importance of the variations in redox potential for SAR (Schafer and Buettner 2001). The distinct redox states go along with the activation of different sets of target genes. During the oxidizing phase, early SA responsive genes are induced that play a role in detoxifying oxidative stress, like glutathione-S-transferases or glucosyltransferases (Blanco et al. 2009; Uquillas et al. 2004), while under later reducing conditions *PR*-gene expression takes place (Dong 2004).

SA is an electrophilic compound and high concentrations can cause detrimental effects due to xenobiotic stress. Therefore plants are able to form the bioinactive SA conjugate SA 2-o-ß-D-glucoside (SAG), which can be stored in the vacuole and serves as a hydrolysable source for SA. The enzyme responsible for this conversion is the UDP-glucosyltransferase (UGT) (Dean et al. 2005). The establishment of SAR and induction of *PR*-genes can be elicited by exogenous application of SA or synthetic compounds like 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Durner and Klessig 1995; Friedrich et al. 1996). The advantage of these SA-analogs is their less toxic effect on the plant.

2.4   NPR1-, WRKY- and SNI1 dependent SA signaling

Mutant screens designed to identify central regulators involved in SAR downstream of SA revealed that the ankyrin repeat protein NONEXPRESSOR OF PR-GENES 1 (NPR1) is essential for SAR-mediated plant defense and *PR*-gene expression (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996). NPR1 contains a BTB/POZ (Broadcomplex, Tramtrack, brick-a-brac/Poxvirus, Zinkfinger) protein-protein interaction domain, an ankyrin repeat domain, a putative nuclear localization signal and phosphorylation sites (Cao et al. 1997; Ryals et al. 1997), but a DNA binding domain is missing. Signaling by NPR1 is controlled via SA-stimulated modification of the cellular redox status, leading to a reduction of oligomeric- to monomeric NPR1. Oligomeric NPR1 is sequestered in the cytosol, whereas the monomeric NPR1 is shuttled to the nucleus (Kinkema et al., 2000; Mou et al., 2003). When the protein enters the nucleus, it is able to interact with members of the TGA basic leucine zipper (*bZIP*) family of transcription factors and increases their DNA binding affinity to induce target gene expression (Johnson et al. 2003). A recent study has illustrated that S-nitrosylation and thioredoxins play crucial roles in the regulation of conformational changes affecting the nuclear import and export of the NPR1 protein (Tada et al., 2008).

Oppositional observations have been made by ChIP (chromatin immunoprecipitation) experiments using a NPR1 antibody to detect protein recruitment to the *PR-1* promoter under inducing- and non- inducing conditions. The ChIP experiments revealed constitutive recruitment of NPR1 to the *PR-1* promoter independent of the presence of subclass II TGA transcription factors (Rochon et al. 2006). Additionally, this study showed that the protein possesses co-activator capacities due to TGA2-conditioned enhanceosome formation.

A genomic approach utilizing plants expressing an NPR1-GR (glucocorticoid receptor) fusion protein was performed to identify important regulatory components involved in direct NPR1-dependent signaling (Wang et al. 2006). The transgenic plants were treated simultaneously with cycloheximide (CHX) to suppress protein translation and dexamethasone to shuttle the fusion protein to the nucleus. Genes activated by NPR1-GR were direct targets of NPR1 as protein biosynthesis and subsequent activation of secondary NPR1 dependent genes was blocked by CHX. The results obtained by

microarray analysis illustrate that members of the WRKY protein family are transcriptionally upregulated by NPR1and that these target genes are also implicated in SAR. The WRKY family of transcription factors consists of 74 members in *Arabidopsis* which all contain at least one conserved WRKYGQK amino-acid DNA binding motif within their protein sequences (Eulgem and Somssich 2007). Several WRKY proteins are induced by SA and show an impact on plant defense (Dong et al. 2003; Eulgem and Somssich 2007). The putative DNA recruitment sites for WRKY proteins are W-boxes, which consist of the core-binding motif (T)(T)TGAC(C/T) (Eulgem 2005; Rushton et al. 1995). Interestingly, the induction of NPR1 itself is dependent on WRKY transcription factors, since *npr1-1* plants transformed with a transgenic NPR1 construct driven by an endogenous promoter lacking certain W-boxes abolish NPR1 induction and complementation of the *npr1-1* phenotype (Yu et al. 2001). These results illustrate, that WRKY proteins are important regulators for upstream- and downstream events in the NPR1-dependent signaling cascade. A transcriptional profiling study uncovered an overrepresentation of W-boxes within the promoters of genes sharing a similar induction pattern as *PR-1,* emphasizing once more the role of WRKY transcription factors in SAR (Maleck et al. 2000).

SA-mediated initiation of defense gene expression can broadly be classified in three categories, dependent on the time-point of induction and NPR1 requirement. Some early defense genes like *GST6* (GLUTATHIONE-S-TRANSFERASE 6) are not oblique to NPR1 and even show an increased expression in *npr1* mutant plants after SA treatment (Uquillas et al. 2004). These genes are often associated with TGA factor-mediated regulation and show furthermore sensitivity to 2,4D or xenobiotic chemicals (Chen and Singh 1999; Fode et al. 2008). Early NPR1-dependent genes are frequently related to the protein secretory pathway and contain a conserved *TL1 cis*-motif within their promoters. The *TL1* motif is critical for NPR1-dependent regulation, though the corresponding transcription factor is still unknown (Wang et al. 2005). In the later stages of SA signaling, NPR1-dependent *PR*-proteins are induced and released in the apoplastic space due to previous activation of the secretory pathway. These late NPR1-dependent transcriptional processes rely on TGA transcription factors, at least in case of *PR-1*, a marker gene for late NPR1 dependent gene expression (Zhang et al. 2003).

A negative regulator involved in NPR1-dependent gene regulation is SNI1 (SUPPRESSOR OF NPR1;INDUCIBLE 1), a repressor discovered in a mutant screen for rescued *PR*-gene inducibility in *npr1* mutant background. *sni1-1* shows increased basal- and INA-induced *PR-1* expression levels in comparison to wildtype plants and a restored SAR (Li et al., 1999). Interestingly, the *sni1-1* mutation causes a specific derepression of a subset of NPR1-dependent SA-responsive genes, illustrating a tight interconnection between these two proteins (Wang et al. 2006). Like NPR1, SNI1 does not contain a DNA-binding domain and it is speculated, that the structural similarity to armadillo repeat proteins enables SNI1 to form a scaffold to interact with other proteins to suppress gene expression. Changes in *PR*-gene transcription after INA- or pathogen stimulus are accompanied with chromatin modifications. It has been shown that SNI1 seems to play an essential role in this process by *sni1* plants revealing higher rates of activating histone modifications under uninduced conditions (Mosher et al. 2006). A mutant screen for suppressors of *sni1* discovered RAD51D, a factor involved in DNA recombination, to be an essential regulator of NPR1-independent *PR*-gene expression. The analysis of recombination frequencies in *sni1* and *rad51d* showed that both proteins fulfill dual functions by co-regulating gene expression and homologous recombination (Durrant et al. 2007). Direct evidence for SNI1 recruitment to promoter sequences via DNA-binding proteins is missing so far.

Beside the key function in SAR, NPR1 plays an important role in another induced resistance response, termed ISR (Induced systemic resistance) (Pieterse et al. 1998). ISR is induced by non-pathogenic rhizobacteria and renders the plant more resistant to a wide range of pathogens. Suprisingly, ISR is not dependent on SA-, but on JA- and ET-signaling.

2.5   TGA transcription factors and their role in SAR

Taking into account that NPR1 does not contain any known DNA interaction domains, a number of yeast two hybrid screens have been performed to identify interacting transcription factors. Several proteins found in these screens belong to the group of TGA transcription factors, a subgroup of the large family of *bZIP* transcription factors (Jakoby et al. 2002). The name of the family is derived from the ability to bind TGACG motifs in regulatory promoter sections (Katagiri et al. 1989).   From the 10 group members in *Arabidopsis*, TGA2, TGA3, TGA5, TGA6 and TGA7 are able to interact with NPR1 (Després et al. 2000; Kim and Delaney 2002; Zhang et al. 2003; Zhou et al. 2000), whereas TGA1 and TGA4 only show weak interaction in yeast unless two unique cysteins are reduced (Després et al. 2003). Beside TGA transcription factors, three small proteins termed NIMIN1, NIMIN2 and NIMIN3 (NIM1-INTERACTING) were also found to interact with NPR1 (Weigel et al. 2001). Overexpression of NIMIN1 leads to an attenuated expression of *PR-1* and a compromised SAR, while *nimin1* mutants display a hyperactivation of *PR-1* though disease resistance stays unaltered. These results suggest that NIMIN proteins act as negative modulators of NPR1-dependent processes (Weigel et al. 2005).
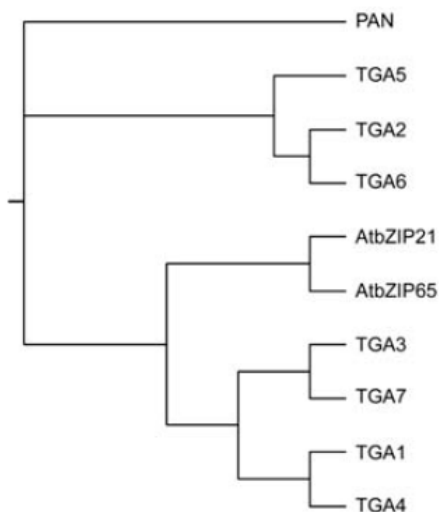
Figure2.1: Phylogenetic tree of the TGA transcription factor family in *Arabidopsis thaliana* (Hepworth et al. 2005).

The TGA factors 1 and 4 form the subgroup I, TGA2, 5 and 6 subgroup II, while TGA3 and TGA7 are designated as subgroup III.

TGA factors fulfill divergent functions in the plants, as some are involved in flower development (Chuang et al. 1999) while others play a role in defense gene expression and SAR (Kesarwani et al. 2007), crosstalk (Ndamukong et al. 2007) or detoxification issues (Fode et al. 2008). PAN (PERIANTHIA) for instance is important for a proper arrangement of first whorle floral organs. Interestingly, the protein interacts with two NPR1 homologs called BOP1 and BOP2 (BLADE-ON-PETIOLE) and plants carrying mutations in both BOP genes show the same pentamerous flower phenotyp like *pan* (Hepworth et al. 2005). Another study provided evidence, that TGA4 influences flowering via interaction with CO (CONSTANS), a keyregulator of floral transition (Song et al. 2008). A further feature of TGA4 and the second subgroup I member TGA1 is the sensitivity to changes in the cellular redox state. Although both proteins show no interaction with NPR1 in yeast, the interaction takes place *in planta* following SA induction, linking the two TGA factor to defense gene expression and resistance. A unique trait of subgroup I members are two conserved cysteine residues (Cys260 and Cys266) which form a disulfide bound under uninduced conditions. When the redox potential changes to a more reducing environment, the disulfide bound is set off and the TGA factors are able to commit interaction with NPR1 (Després et al. 2003). Analysis of the *tga1* and *tga4* single- and double mutants revealed an impact on basal resistance, with TGA1 playing a more dominant role in this issue (Kesarwani et al. 2007). The influence of TGA1 and TGA4 on SAR has not been subject of investigation yet.

The subclass II TGA transcription factors are redundant regulators of *PR*-gene expression and SAR. *tga2 tga5 tga6* triple mutants display a similar phenotype like *npr1-1* concerning a compromised SAR and an increased sensitivity to higher doses of SA (Zhang et al. 2003). Furthermore, the expression of *PR*-genes is increased under non-inducing conditions, enlightening a dual function for TGA factors as transcriptional activators and repressors (Kesarwani et al. 2007). The subclass II is also involved in NPR1-independent detoxification processes induced by electrophilic phytoprostanes, formed due to accumulation of lipophilic xenobiotic compounds or ROS (Fode et al. 2008; Mueller et al. 2008). Another regulator implicated in the NPR1-independent induction of detoxification related stress responses is the GRAS protein SCL14 (SCARECROW-LIKE 14), which interacts with subclass II TGA factors (Fode et al. 2008). ChIP experiments performed in this study revealed, that the SCL14 protein is

recruited to target gene promoters via subclass II TGA factors and the *scl14* and *tga2 tga5 tga6* triple mutants show a similar negative impact on SCL14 target gene expression. Furthermore the two mutants suffer more than wildtype control plants when grown on plates supplemented with toxic INA or TIBA (2,4,6-triiodobenzoic acid) concentrations, while a SCL14 overexpressor shows enhanced resistance to the treatment. Further proteins known to interact with TGA subclass II members belong to the glutaredoxin (GRX) family. GRXs catalyze thiol disulfide reductions and are therefore implicated in regulatory processes regarding the redox state of the cell (Lemaire 2004). GRX480 is a SA-inducible glutaredoxin which antagonizes JA-dependent upregulation of the defensin gene *PDF1.2* in a TGA-dependent manner, linking TGA factor and GRX function to SA/JA crosstalk (Ndamukong et al. 2007).

The subclass III TGA factor TGA3 is positive regulators of *PR*-gene expression and basal resistance, shown by experiments conducted with the *tga3-1* single mutant. Additionally, the increased *PR-1* background expression observed in the *tga2 tga5 tga6* triple mutant is vanished in the *tga2 tga3 tga5 tga6* quadruple mutant, once more displaying the complex interconnection of the TGA family members (Kesarwani et al. 2007). The function of the final two TGA factors *AtbZIP21* and *AtbZIP65* still remains elusive, mutant analyses with regard to plant defense have not been performed yet. Due to the redundancy of different subgroup members and even different subgroups, an accurate analysis of additional multiple knock out mutants is needed to further decipher the elaborate network of TGA regulation.

2.6 The regulatory element *activating sequence 1 (as-1)*

TGA factors specifically bind to *as-1*-like sequences located in various defense gene promoters and activate transcription after perception of certain signals like SA and/or auxin (Liu and Lam 1994; Qin et al. 1994). bZIP proteins like TGA factors bind to DNA as hetero- or homodimers, formed by hydrophobic interactions of their leucine zipper domain (Landschulz et al. 1988). The *as-1* element was originally described in the *Cauliflower Mosaic Virus (CaMV) 35S* promoter as being a *cis*-regulatory element relevant for promoter activity (Benfey and Chua 1990). *as-1*-like elements can differ in

the composition of their two palindromic TGAC/GTCA core-binding motifs and the distance between the two palindromic centers. SA- and auxin-responsive elements feature a 12bp spacing between the TGA binding motifs, whereas late SA-responsive genes like *PR-1* show alterations in spacer length of the *as-1*-like element (Krawczyk et al. 2002; Strompen et al. 1998). Such differing linker regions are often correlated with NPR1-dependency of the genes, though functional data concerning this issue are missing to date (Blanco et al. 2005). Modifications in spacer length lead to changes in TGA binding affinities and thus reduce the recruitment of these factors to the corresponding *cis*-element (Krawczyk et al. 2002). The function of the TGA factor binding motifs of the *as-1*-like element of the *PR-1* promoter differ, as revealed by analysis of promoter derivatives with substitutions in each of the two TGACG motifs. Substitution of the upper motif *LS5* leads to slightly enhanced basal and induced expression levels, substitution of the more downstream located *LS7* leads to loss of SA sensitivity (Lebel et al. 1998). Further differences between promoters containing *as-1*-like elements are the timing of induction after SA treatment and the need of protein biosynthesis. In contrast to *PR-1*, NPR1-independent "immediately early"-genes like GST6 or early NPR1-dependent genes like WRKY transcription factors do not need *de novo* protein synthesis for induction (Chen and Singh 1999; Wang et al. 2006). Another interesting aspect of TGA factor dependency with regard to the structure of the *as-1* element can be observed in the *tga2 tga5 tga6* triple mutant. While mutation of the three subgroup II members leads to an increased background expression of uninduced *PR-1*, transcription of genes comprising conserved *as-1* elements in their promoters is abolished (Fode et al. 2008; Zhang et al. 2003). This result demonstrates that the structure of the TGA-binding *cis*-element is critical for the activating or repressing properties of the recruited TGA transcription factors.

Figure 2.2: *as-1* element sequences of SCL14 target genes and the *as-1*-like element sequence of the *PR-1* promoter (Fode et al. 2008; modified).

The NPR1-independent target genes of SCL14 contain *as-1* elements with a conserved spacer length of 12bp, similar to the *CaMV 35S as-1* element (*as-1:GUS*), while the NPR1-dependent *PR-1* gene features an *as-1*-like element with increased distances between the palindromic repeats.

2.7   Aim of the study

The signal transduction mechanism leading to the expression of *PR-1* is of major interest for the understanding of NPR1-mediated regulation and has been subject of several studies in the last couple of years (Després et al. 2000; Kesarwani et al. 2007; Johnson et al. 2003; Rochon et al. 2006). Although much progress concerning NPR1, TGA and SNI1 biochemistry has been made, the function of *cis*-regulatory sequences in NPR1-dependent- and independent regulation has still to be elucidated in more detail. It has been published previously, that distinct *cis*-elements within the *PR-1* promoter are essential for *PR-1* induction (Lebel et al., 1998). Substitution of a palindromic TGA factor binding motif (*LS7*) by a linker sequence causes a complete loss of INA-sensitivity, while other *cis*-element substitutions increase reporter gene expression after induction. Based on these observations, several models have been proposed which describe the regulation of *PR-1* via recruitment of NPR1, SNI1 and TGA factors to distinct *cis*-elements located in the *PR-1* promoter (Durrant and Dong 2004; Eulgem 2005; Johnson et al. 2003; Kesarwani et al. 2007). However, a detailed analysis of *cis*-regulatory elements within the *PR-1* promoter considering different mutant backgrounds for a more accurate functional characterization of these sequences is missing so far. Therefore, different *PR-1* promoter:reporter constructs carrying discrete mutations, deletions or substitutions in regulatory sequences were generated, and transformed in Col-0, *npr1-1*, *sni1-1* and *sni1 npr1* to obtain stable transgenic lines. Additionally, electrophoretic mobility shift assays (EMSAs) were performed to analyze protein recruitment to these critical promoter sections. The resulting data enhance the knowledge of *PR*-gene regulation mediated via a concerted regulatory network of NPR1, SNI1, TGA factors and additional NPR1-independent proteins.

3 Materials and methods

3.1 Materials

3.1.1 Devices

Device	Model	Source
Autoclave	3870 ELV	Tuttnauer
Automatic pipettes		Gilson
Blotting device		University of Göttingen
Chambers for gel electrophoreses		University of Göttingen
Cooling centrifuge	Sorvall RC 5B Plus	DuPont
Cooling micro centrifuge with overhung rotor		Hettich
Counting chamber	Fuchs-Rosenthal	Brand
Electroporator	Gene Pulser$^{®}$ II	BioRad
Fluorometer	CytoFluorII Plate Reader	PerSeptive
Gel documentation device		MWG Biotech
Heat block		Boekel Scientific
Heated stirrer	RCT basic	IKA Labortechnik
Heated shaker	Thermomixer 5436	Eppendorf
Locker for incubations		WTC binder; Memmert
Luminometer	FLUOstar Optima	BMG Labtech
Micro centrifuge	Biofuge pico	Heraeus Christ
Micro centrifuge, cooled	5403	Eppendorf
PCR cycler	MiniCycler™ PTC-150	MJ Research
pH-Meter	HI 9321	Hanna Instruments
Photometer	Unikon 720 LC	Kontron
Photometer for microtiter	MRX Dynex Plate Reader	Dynex

Device	Model	Source
plates		
Realtime PCR cycler	iCycler	BioRad
RNA-/DNA-Calculator	GeneQuant II	Pharmacia
Rotation platform	IntelliMixer	
Scanner for array slides	G2505B	Agilent Technologies
Sequencer	ABI PRISM 3100	Perkin-Elmer
Sterile bench	Microflow Laminar	Nunc
Sterile bench	Microflow Biohazard	Nunc
UV transilluminator	FLX-20 M	Vilber Lourmat
Water deionization device	Option 4, Maxima	ELGA
Vacuum pump	Cyclo 1	Roth
Vortex	L46	Labinco BV, Niederlande

3.1.2 Materials

Product	Source
Electroporation cuvettes	BioRad
Filter paper Miracloth	Calbiochem
Flow paper 3MM	Whatman
LIA- plates, white, 96 well flat- bott	Greiner bio-one
Microtiter plates	Roth
Para-film M	American National Can™
Plastics one-way material	Biozym; Eppendorf; Greiner; Roth; Sarstedt
PVDF membrane Immobilon™-P	Millipore
X-ray film Cronex 5	Agfa, Belgium

3.1.3 Chemicals

Chemical	Source
30 % (w/v) Acrylamide: N,N´-Methylenebisacrylamide (37,5:1)	Roth

Chemical	Source
Agarose SeaKem LE	Biozym
Ampicillin	AGS
APS (Ammonium persulfate)	Biometra
Bradford-Reagent	Roth
Bromophenol blue	Roth
BSA	Serva
Diethylpyrocarbonat (DEPC)	Roth
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
dNTPs	MBI; Roth
Ethylene diaminetetraacetate (EDTA)	AppliChem
Ethidiumbromide	Roth
Gentamycine	Duchefa
PIPES	Roth
Hydrogen peroxide	Roth
Herring sperm DNA (HSP)	Sigma
Isonicotinic acid (INA)	Sigma
Isopropylthiogalactosid (IPTG)	Bio Tech Trade
Kanamycine	Sigma
β-Mercaptoethanol	Roth
MES	Roth
Murashige and Skoog medium	Duchefa
Orange G	Sigma
Phenol	Sigma
Phenylmethane sulfonylchloride (PMSF)	Fluka
Ponceau S	Sigma
Rifampicine	Duchefa
X-ray developer LX24	Kodak
X-ray fixer AL4	Kodak
Salicylic acid (SA)	Merck
Select Agar	Life Technologies

Chemical	Source
Select Yeast Extract	GIBCO BRL
TEMED	Roth
Triton X-100	Roth
Trypton	Oxoid
Tween20	Roth
X-Gal	Bio Tech Trade
X-Gluc	Roth

3.1.4   Kits

Kit	Source
BCA Protein Assay Kit	Thermo Scientific
BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v.3.1 and v.1.1	Perkin-Elmer Corporation
HiDi-Mix	ABI PRISM™
iProof™ high fidelity PCR kit	Bio-Rad
Nucleo Spin® Extract II	Macherey-Nagel
Nucleo Spin® Plasmid	Macherey-Nagel
Qiagen Plasmid Preparation Kits (Midi, Maxi)	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Micro Spin™ G25	Pharmacia

3.1.5   Enzymes

Enzyme	Source
Biotaq DNA polymerase	Bioline
Cellulase "Onozuka R-10"	Serva
Immolase DNA polymerase	Bioline
iProof high fidelity DNA polymerase	BioRad
Klenow DNA polymerase exo⁻	MBI Fermentas
Lyticase	Sigma
Macerozyme R-10	Serva
Reverse transcriptase H-	MBI Fermentas
Restriction enzymes	MBI Fermentas, New England Biolabs
RNase A (DNase-free)	Qiagen
RNase inhibitor	MBI Fermentas
T4 DNA-ligase	MBI Fermentas
T4 DNA-polymerase	MBI Fermentas

3.1.6   Standards

Standard	Source
GeneRuler DNA Ladder Mix	MBI Fermentas

3.1.7   Antibodies

Antibody	Specificity	Properties	Reference
αTGA2/5 (serum) (SA 4364)	TGA2 and TGA5 C-terminal region including the zipper domain	polyclonal from rabbit (final bleeding) (1:1000)	(Fode et al. 2008)

3.1.8   Nucleic acids

3.1.8.1   Primers and Oligos

Primer	Sequence5´→3´	Tm °C
3`D Oligo ChIP-R + w1+w2 F	TGAAACACTAAGAAACAAATAATTCTTGACTTTTT TTCTTTTATTTGAAAATTGACTGTAGTTGCA	82°C
3`D Oligo ChIP-R + w1+w2 R	ACTACAGTCAATTTTCAAATAAAAGAAAAAAGTC AAGAATTATTTGTTTCTTAGTGTTTCATGCA	82°C
3`D Oligo ChIP-R + w1+w2+w3F	TGAAACACTAAGAAACAAATAATTCTTGACTTTTT TTCTTTTATTTGAAAATTGACTGTAGATATAAACTT TTATTTTTTCTGACTGTATTGCA	86°C
3`D Oligo ChIP-R + w1+w2+w3R	ATACAGTCAGAAAAAATAAAAGTTTATATCTACAG TCAATTTTCAAATAAAAGAAAAAAGTCAAGAATT ATTTGTTTCTTAGTGTTTCATGCA	86°C
3`D Oligo Chip-R + w1F	TGAAACACTAAGAAACAAATAATTCTTGACTTTTT TGCA	72°C
3`D Oligo Chip-R + w1R	AAAAAGTCAAGAATTATTTGTTTCTTAGTGTTTCA TGCA	72°C
3`D Oligo ChIP-R F2	TGAAACACTAAGAAACAAATAATTTTGCA	63°C
3`D Oligo ChIP-R R	AAATTATTTGTTTCTTAGTGTTTCATGCA	63°C
35S-35Slinker-PR1-F	GACTGTTTCTTGACGTAAGGGATTACGTCATAGA TGTGGCGGCATATATTCTTCAGG	86,5°C
35S-35Slinker-PR1-R	GCCACATCTATGACGTAATCCCTTACGTCAAGAA ACAGTCAATAGATCACCCATTGAG	86,5°C
35S-AS1-AGGA-F	GACTGTTTCTTGACGTAAAGGATGACGCACTAGA TGTGGCGGCATATATTCTTCAGG	88°C
35S-AS1-AGGA-R	GCCACATCTAGTGCGTCATCCTTTACGTCAAGAA ACAGTCAATAGATCACCCATTGAG	88°C
35S-AS1-F	GACTGTTTCTTGACGTAAGGGATGACGCACTAGA TGTGGCGGCATATATTCTTCAGG	89°C
35S-AS1-R	GCCACATCTAGTGCGTCATCCCTTACGTCAAGAA ACAGTCAATAGATCACCCATTGAG	89°C
35S-AS1-GAAA-F	GACTGTTTCTTGACGTAAGAAATGACGCACTAGA TGTGGCGGCATATATTCTTCAGG	87°C
35S-AS1-GAAA-R	GCCACATCTAGTGCGTCATTTCTTACGTCAAGAA ACAGTCAATAGATCACCCATTGAG	87°C

Primer	Sequence 5´→3´	Tm °C
35S-AS1-GAGA-F	GACTGTTTCTTGACGTAAGAGATGACGCACTAGATGTGGCGGCATATATTCTTCAGG	88°C
35S-AS1-GAGA-R	GCCACATCTAGTGCGTCATCTCTTACGTCAAGAAACAGTCAATAGATCACCCATTGAG	88°C
35S-AS1-PR1linker-F	GACTGTTTCTTGACGTAACTATTTTACTGACGCACTAGATGTGGCGGCATATATTCTTCAGG	86,5°C
35S-AS1-PR1linker-R	GCCACATCTAGTGCGTCAGTAAAATAGTTACGTCAAGAAACAGTCAATAGATCACCCATTGAG	86,5°C
35Slinker-F	GACTGTTTCTCTACGTCAGGGATTACGTCATAGATGTGGCGGCATATATTCTTCAGG	87°C
35Slinker-R	GCCACATCTATGACGTAATCCCTGACGTAGAGAAACAGTCAATAGATCACCCATTGAG	87°C
35Slinker-variationB-F	GACTGTTTCTCTACGTCAGAAATTACGTCATAGATGTGGCGGCATATATTCTTCAGG	85°C
35Slinker-variationB-R	GCCACATCTATGACGTAATTTCTGACGTAGAGAAACAGTCAATAGATCACCCATTGAG	85°C
35Slinker-variation-F	GACTGTTTCTCTACGTCAAAAATTACGTCATAGATGTGGCGGCATATATTCTTCAGG	85°C
35Slinker-variation-R	GCCACATCTATGACGTAATTTTTGACGTAGAGAAACAGTCAATAGATCACCCATTGAG	85°C
35S-PR1linker-PR1-F	GACTGTTTCTTGACGTAACTATTTTACTTACGTCATAGATGTGGCGGCATATATTCTTCAGG	84°C
35S-PR1linker-PR1-R	GCCACATCTATGACGTAAGTAAAATAGTTACGTCAAGAAACAGTCAATAGATCACCCATTGAG	84,5°C
CAT235F-Eco91I	AATGGTCACCGTGAATACCACGACGATTTCC	76°C
CAT235R-Mph1103	CCAATGCATATCAGCACCTTGTCGCCTTGC	78°C
CAT483F	GATAGTCGACCTCAATGTACCTATAACCAGACCG	71,5°C
CAT483R	AATGGTGACCGCAACACGCAGACGCTACTACCTTCTTGCGTATAATATTTGCCCATG	90°C
ChIP-R +w1 Competition-F	CATGAAACACTAAGAAACAAATAATTCTTGACTTTTTTTC	70,5°C
ChIP-R +w1Competition-R	GAAAAAATTCTTGACTTTATTTGTTTCTTAGTGTTTCATG	70,5°C
ChIP-R +w1mutated Competition-F	CATGAAACACTAAGAAACAAATAAAAGACTATCTTTTTTC	69°C

Primer	Sequence5´→3´	Tm °C
ChIP-R +w1mutated Competition-R	GAAAAAAGATAGTCTTTTATTTGTTTCTTAGTGTT TCATG	69°C
ChIP-R+wrky1+2 shift-F	AGCTTTTGCGTCTTCAAACACTAAGAAACAAATAA TTCTTGACTTTTTTTCTTTTATTTGAAAATTGACTG TAGAGAAGACGCAAAAG	86°C
ChIP-R+wrky1+2 shift-R	AATTCTTTTGCGTCTTCTCTACAGTCAATTTTCAA ATAAAAGAAAAAAAGTCAAGAATTATTTGTTTCTT AGTGTTTGAAGACGCAAA	86°C
DOF-F	CTTCGTTCTAGAGGAGCCATAGGCAAGAGTGATA GAGATAC	76°C
DOF-R	CACTCTTGCCTATGGCTCCTCTAGAACGAAGAAT ATATGCCGCCAC	85°C
Egfp QTect -F	ACCACTACCAGCAGAACACCC	59°C
Egfp QTect -R	GAACTCCAGCAGGACCATGTG	60°C
Egfp-R	CCTCGCCCTTGCTCACCATCC	68,5°C
GFP-R	CCGTTTACGTCGCCGTCCAGC	69°C
GUS169R	GGCGAACTGATCGTTAAAACTGC	63°C
GUS431R	GGATAGTCTGCCAGTTCAGTTCG	61°C
GUS-F	CGCTGGACTGGCATGAACTTCG	67,5°C
HA-Tag forward	ATGGCATACCCATACGACGTTCC	65°C
HA-Tag reverse	CATAGTCCGGGACGTCATAGGG	63°C
LS4-LS8 shift-F	AGCTTTTGCGTCTTCTCTATTGACTGTTTCTCTAC GTCACTATTTTACTTACGTCATAGATGTGGCGGC ATGAAGACGCAAAAG	89°C
LS4-LS8 shift-R	AATTCTTTTGCGTCTTCATGCCGCCACATCTATG ACGTAAGTAAAATAGTGACGTAGAGAAACAGTCA ATAGAGAAGACGCAAA	89°C
LS5-F	AGAATAATCTAGATATATTTTACTTACGTCATAGA TGTGGCGGCATATATTC	76°C
LS5-R	CGTAAGTAAAATATATCTAGATTATTCTAGAATAT AGATCACCCATTGAG	71,5°C
LS5onlyF	GGTGATCTATTGACTGTTTCAATCTAGATATATTT TACTTACGTCATAGATGTGGC	79°C
LS5onlyR	ATATATCTAGATTGAAACAGTCAATAGATCACCCA T	66,7°C

Primer	Sequence5´→3´	Tm °C
LS5-LS10 shift-F	AGCTTTTGCGTCTTCTCTACGTCACTATTTTACTTACGTCATAGATGTGGCGGCATATATTCTTCAGGACTTTTCAGGAAGACGCAAAAG	89°C
LS5-LS10 shift-R	AATTCTTTTGCGTCTTCCTGAAAAGTCCTGAAGAATATATGCCGCCACATCTATGACGTAAGTAAAATAGTGACGTAGAGAAGACGCAAA	89°C
LS5-LS7F	GGTGATCTATTGACTGTTTCAATCTAGATATATTTTACTTTTTCTAGATGGTGGC	78,5°C
LS6-9bp-F	GACTGTTTCTCTACGTCACTCTAGATTTTACGTCATAGATGTGGCGGCATATATTCTTCAGG	85°C
LS6-9bp-R	GCCACATCTATGACGTAAAATCTAGAGTGACGTAGAGAAACAGTCAATAGATCACCCATTGAG	86°C
LS7-F	CTTTTTCTAGATGTGTGGCGGCATATATTCTTCAGGACTTTTCAGCCATAGGCAAGAG	87°C
LS7-LS5-wrky-R	CCTGAAGAATATATGCCGCCACACATCTAGAAAAAGTAAAATATATCTAGATTATTCTAG	79°C
LS7onlyR	AATATATGCCGCCACACATCTAGAAAAAGTAAAATAGTGACGTAGAGAAACAGTCAATAG	82°C
LS7-R	CCTGAAGAATATATGCCGCCACACATCTAGAAAAAGTAAAATAGTGACGTAGAATTCTAG	82°C
LS8-F	AGAAATCTAGAGCTATATTCTTCAGGACTTTTCAGCCATAGG	74°C
LS8-R	GTCCTGAAGAATATAGCTCTAGATTTCTATGACGTAAGTAAAATAGTGACGTAGAG	77°C
PR1 ChIP-R+w1+2+3FΔ	GAGATACTCATATGCATAAATATAATCTTAATTGCCAAACTGTCCG	76°C
PR1 ChIP-R+w1+2+3RΔ	GGCAATTAAGATTATATTTATGCATATGAGTATCTCTATCACTCTTGC	74,5°C
PR1 ChIP-R-FΔ	GAGATACTCATATGCATCTTGACTTTTTTTCTTTTATTTGAAAATTGACTGTAG	78°C
PR1 ChIP-R-R Δ	GAAAAAAAGTCAAGATGCATATGAGTATCTCTATCACTCTTGC	74,5°C
PR1 w1 w2RΔ	GAAACAAATAATTATATAAACTTTTATTTTTTCTGACTGTAAATATAATCTTAATTGCC	74°C
PR1 w1 w2RΔ	GAAAAAATAAAAGTTTATATAATTATTTGTTTCTTAGTGTTTCATGC	71°C

Primer	Sequence5´→3´	Tm °C
PR1 w1+w2+w3FΔ	GAAACAAATAATTAAATATAATCTTAATTGCCAAA CTGTCC	70°C
PR1 w1+w2+w3RΔ	GGCAATTAAGATTATATTTAATTATTTGTTTCTTAG TGTTTCATGC	71°C
PR1 w1FΔ	GAAACAAATAATTCTTTTATTTGAAAATTGACTGT AGATATAAAC	70°C
PR1 w1RΔ	ACAGTCAATTTTCAAATAAAAGAATTATTTGTTTC TTAGTGTTTCATGC	76°C
PR1 w2FΔ	CTTGACTTTTATATAAACTTTTATTTTTTCTGACTG TAAATATAATCTTAATTGCC	74°C
PR1 w2RΔ	CAGTCAGAAAAAATAAAAGTTTATATAAAGTCAA GAATTATTTGTTTCTTAGTGTTTCATGC	79°C
PR1-1293F-Sall	GATAGTCGACGTAATAATATCCTATGGTGTCATTT TATAAGTTAGC	73°C
PR1-3`D F	GCAAGAGTGATAGAGATACTCATATGCATAAAAA AAAAGAAAAAAATAGTTTTCAAATCTC	79,5°C
PR1-3`D R	TTTATGCATATGAGTATCTCTATCACTCTTGCCTA TGG	71°C
PR1-35Slinker variationB-F	GACTGTTTCTCTACGTCACAAATTACGTCATAGAT GTGGCGGCATATATTCTTCAGG	85,5°C
PR1-35Slinker variationB-R	GCCACATCTATGACGTAATTTGTGACGTAGAGAA ACAGTCAATAGATCACCCATTGAG	85,5°C
PR1-35Slinker variation-F	GACTGTTTCTCTACGTCAAAAATTACGTCATAGAT GTGGCGGCATATATTCTTCAGG	85°C
PR1-35Slinker variation-R	GCCACATCTATGACGTAATTTTTGACGTAGAGAA ACAGTCAATAGATCACCCATTGAG	85°C
PR1-35Slinker-35S-F	GACTGTTTCTCTACGTCAGGGATGACGCACTAGA TGTGGCGGCATATATTCTTCAGG	89°C
PR1-35Slinker-35S-R	GCCACATCTAGTGCGTCATCCCTGACGTAGAGA AACAGTCAATAGATCACCCATTGAG	89°C
PR1-387F	TAAAGCCAGTGCATATCAGTAGTC	56,5°C
PR1-483R	AATGGTGACCGCAACACGCAGACGCTACTACCT TTTCAGTATACCTAATTTTGTACCG	87°C
PR1-5`UTR-pBGWFS7	GTAGTCTAGACATTTTTCTAAGTTGATAATGGTTA TTGTTG	69°C
PR1-617F	CTTTTTGGATAAATCTCAATGGGTG	61,5°C

Primer	Sequence 5´→3´	Tm °C
PR1-725F	TATATGCATGCAGAGACCAACGAGGACTACAGG TTGACTTTTTTTCTTTTATTTGA	84°C
PR1-835R	AATCGTATCGGACAGTTTGGC	59°C
PR1-PR1linker-35S-F	GACTGTTTCTCTACGTCACTATTTTACTGACGCA CTAGATGTGGCGGCATATATTCTTCAGG	87°C
PR1-PR1linker-35S-R	GCCACATCTAGTGCGTCAGTAAAATAGTGACGTA GAGAAACAGTCAATAGATCACCCATTGAG	87°C
w1+w2+w3 / w1+w2+w3 mutated Competition-F	AAAGACTATCTTTTTTCTTTTATTTGAAAAAAGACT ATCGATATAAACTTTTATTTTTAAGACTATCAA	77,5°C
w1+w2+w3 / w1+w2+w3 mutated Competition-R	TTGATAGTCTTAAAAATAAAAGTTTATATCGATAG TCTTTTTTTCAAATAAAAGAAAAAGATAGTCTTT	77,5°C
w1+w2+w3 / w1+w2mutated Competition-F	AAAGACTATCTTTTTTCTTTTATTTGAAAAAAGACT ATCGATATAAACTTTTATTTTTTCTGACTGTAA	79°C
w1+w2+w3 / w1+w2mutated Competition-R	TTACAGTCAGAAAAAATAAAAGTTTATATCGATAG TCTTTTTTTCAAATAAAAGAAAAAGATAGTCTTT	79°C
w1+w2+w3 / w1mutated Competition-F	AAAGACTATCTTTTTTCTTTTATTTGAAAATTGACT GTAGATATAAACTTTTATTTTTTCTGACTGTAA	78,5°C
w1+w2+w3 /w1 mutated Competition-R	TTACAGTCAGAAAAAATAAAAGTTTATATCTACAG TCAATTTTCAAATAAAAGAAAAAGATAGTCTTT	78,5°C
wrky_shift-F	AGCTTTTGCGTCTTCCTTGACTTTTTTTCTTTTATT TGAAAATTGACTGTAGATATAAACTTTTATTTTTTC TGACTGTAAAGAAGACGCAAAAG	86°C
wrky_shift-R	AATTCTTTTGCGTCTTCTTTACAGTCAGAAAAAAT AAAAGTTTATATCTACAGTCAATTTTCAAATAAAA GAAAAAAGTCAAGGAAGACGCAAA	86°C
WRKY18seq-F	CCGAAGAAGGAGGTCTCAGTTTTGG	67°C
WRKY18seq-R	CAACGCTAGTCTATGACAGCCATTAACC	66°C
WRKY46-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAT GATGATGGAAGAGAAACTTGTGATCAACG	91°C
WRKY46-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTA CGACCACAACCAATCCTGTCCG	92°C
WRKY46seq-F	GAGGAAAGTATCGGAGAAGAACACAGAG	65°C
WRKY46seq-R	CGTTCTGAAAATGTTCTTCTTGTTCTCC	65°C
WRKY53-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAT GGAAGGAAGAGATATGTTAAGTTGGGAGC	89,5°C

Primer	Sequence5´→3´	Tm °C
WRKY53-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAA TAATAAATCGACTCGTGTAAAAACGCG	88°C
WRKY53seq2-F	CGGCTGTTGCTGAGACTAACG	61°C
WRKY53seq2-R	ACTCGCCGTTGATAGTTCCG	60°C
WRKY53seq-F	CCTCACCGAGCGTACAACTTATTCC	65,5°C
WRKY53seq-R	CCATCATCAAGCCCATCGGTTCG	70°C
WRKY-Box 1X F	CCGGGGATCTATTGACTGTTTCG	65°C
WRKY-Box 1X R	TCGACGAAACAGTCAATAGATCC	59°C
WRKY-Box 3X F	CCGGGGATCTATTGACTGTTTCTCTACGTCGATC TATTGACTGTTTCTCTACGTCGATCTATTGACTGT TTCG	90°C
WRKY-Box 3X R	TCGACGAAACAGTCAATAGATCGACGTAGAGAAA CAGTCAATAGATCGACGTAGAGAAACAGTCAATA GATCC	90°C

3.1.8.2   Plasmids

3.1.8.2.1   Plasmids for promoter analysis purposes

pUC18-Entry2	pBGWFS7	pBGWL7	Description
3`D	Yes	Yes	Deletion of 502bp between -68bp and -570bp; Insertion of a sequence derived from the *CAT* gene between -1294bp and -817bp
3`D + ChIP-R	No	Yes	481bp deletion between -65bp and -546bp. *CAT* sequence between -1294bp and -817bp
3`D + w1	No	Yes	The first w-box is attached to the *3`D + ChIP-R* construct

pUC18-Entry2	pBGWFS7	pBGWL7	Description
3`D + w1 + w2	No	Yes	The two w-boxes *w1* and *w2* are attached to the *3`D + ChIP-R* construct
3`D + w1 + w2 + w3	No	Yes	All three w- boxes are attached to *3`D + ChIP-R*
3`D as-1 linker	No	Yes	*3`D Mph* deletion construct containing a substituted as-1 linker (GGGA) from *CaMV35S*
3`D Mph	Yes	Yes	Deletion of 504bp between -65bp and -569bp; Insertion of a sequence derived from the *CAT* gene between -1294bp and -817bp; reconstituted Mph1103I restriction site
3`D Mph as-1	Yes	Yes	*3`D Mph* construct with substituted *as-1* element from *CaMV35S*
35S-35S-PR-1	No	Yes	The downstream TGA binding motif in *as-1* is substituted against the TGA binding motif from the original *PR-1* promoter
as-1	Yes	Yes	Substitution of the *as-1*-like element against *CaMV35S as-1*
as-1 - AGGA	No	Yes	The sequence of the *as-1* linker region is changed to AGGA
as-1 - GAAA	No	Yes	The sequence of the *as-1* linker region is changed to AGGA
as-1 - GAGA	No	Yes	The sequence of the *as-1* linker region is changed to GAGA
as-1 linker	No	Yes	The linker sequence between the TGA binding motifs is substituted against the *CaMV35S* linker sequence
as-1 LS10	No	Yes	Mutated *LS10* element and substituted *as-1* element from *CaMV35S*
as-1-LS10	No	Yes	Substituted *as-1* element and mutated *LS10* box
CAT235	Yes	Yes	235bp substitution in the position -810bp to -575bp; coding sequence was obtained from the chloramphenicol acetyltransferase gene (*CAT*)
CAT477	No	No	477bp substitution in the position -1294bp to -817bp; the sequence was obtained from the chloramphenicol acetyltransferase gene *(CAT)*; The construct was used for *3`D* cloning
LS10	Yes	Yes	Mutation of the putative DOF transcription factor binding box *LS10*

pUC18-Entry2	pBGWFS7	pBGWL7	Description
LS4	Yes	Yes	w-box mutation *LS4*
LS4-as-1	Yes	Yes	Mutated w-box *LS4* in combination with substituted *as-1* element
LS4-as-1	Yes	Yes	Mutated *LS4* element and substituted *as-1* element from *CaMV35S*
LS4-LS5	Yes	Yes	w-box *LS4* and TGA motif *LS5* mutation
LS4-LS5-LS7	Yes	Yes	w-box LS4 and both TGA binding motifs of the as-1-like element are substituted
LS4-LS7	Yes	Yes	w-box *LS4* and TGA motif *LS7* mutation
LS5	Yes	Yes	TGA binding motif mutation *LS5*
LS5-LS7	Yes	Yes	Mutation of the TGA binding motifs of the as-1-like element
LS7	Yes	Yes	TGA binding motif mutation *LS7*
LS7-LS10	No	Yes	Sequence substitutions in *LS7* and *LS10*
PR-1	Yes	Yes	1294bp full length PR-1 promoter
PR-1 as-1 VAR	Yes	Yes	The sequence of the *as-1*-like element linker region is changed to AAAA
PR-1 linker in as-1	No	Yes	The TGA binding motifs of the *as-1*-like element are substituted against the binding motifs of *CaMV35S as-1*
W1	No	Yes	Deletion of the first w-box downstream of the as-1-like element
W1-2	No	Yes	Deletion of the first two w-boxes downstream of the *as-1*-like element
W1-3	No	Yes	Deletion of the whole w-box cluster downstream of the *as-1*-like element
W2	No	Yes	Deletion of the second w-box downstream of the *as-1*-like element

All plasmids were transformed in different plant genotypes via *agrobacterium tumefaciens* mediated gene transfer. A detailed list of transgenic plants generated with these plasmids is attached in the "supplemental data"-section of this work.

3.1.8.2.2   Plasmids for cloning- and transient assay purposes

Plasmid	Description	Reference
pHBT	Vector for transient expression in plant cells, pHBTL-sGFP derivative, deletion of sGFP gene by *Nco*I / *Not*I restriction, Klenow fill in and religation, *amp^r*	(Heinekamp et al. 2002)
pSK-T	Vector for cloning and sequencing, *lac*Zα, *amp^r*	Kriete, unpublished

3.1.9   Organisms

3.1.9.1   Bacteria

Species	Properties	Reference
Agrobacterium tumefaciens GV3101	PMP90RK *rif^r*, *gm^r*	(Koncz and Schell, 1986)
Escherichia coli DB3.1	F⁻, *gyr*A 462, *end*A1, D(*sr1-rec*A), *mcr*B, *mrr*, *hsd*S20 (r_B^- m_B^-), *sup*E44, *ara*-14, *gal*K2, *lac*Y1, *pro*A2, *rps*L20(Sm^r), *xyl*-S, λ-leu, *mtl*-1	(Bernard et al. 1993)
Escherichia coli DH5α	F⁻, *gyr*A 96 (Nalr), *rec*A1, *end*A1, *thi*-1, *hsd*R17 (rk-mk+), *gln*V44, *deo*R, D(*lac*ZYA-*arg*F) U169 [p80dD(*lac*Z)M15]	(Hanahan 1983)

3.1.9.2   Plant genotypes

Genotype	Description	Reference
Columbia, Col-0	Wildtype	NASC Stock Nr. N1092, NASC 2002
npr1-1 mutant	Knock out line lacking functional NPR1	(Cao et al. 1994)
sni1-1 mutant	Knock out line lacking functional SNI1; Bgl2:*GUS*	(Li et al. 1999)
sni1-1/npr1-1 mutant	*sni1-1* and *npr1-1* double mutant; Bgl2:*GUS* reporter	(Li et al. 1999)
tga2 tga3 tga5 tga6 quadruple mutant	Knock out line lacking all three class II TGA transcription factors and TGA3, kindly provided by X. Dong	(Kesarwani et al. 2007)
tga6 mutant	Single knock out of TGA6, kindly provided by Y. Zhang	(Zhang et al. 2003)
tga2 tga5 double mutant	TGA2 and TGA5 double mutant, kindly provided by Y. Zhang	(Zhang et al. 2003)
tga2 tga5 tga6 triple mutant (*tga2,5,6* mutant)	Knock out line lacking all three class II TGA transcription factors, impaired in SAR, kindly provided by Y. Zhang	(Zhang et al. 2003)

3.1.10 Growing media

Medium	Content
dYT medium for bacteria	20 g/L Tryptone; 10 g/L Yeast extract; 10 g/L NaCl
LB medium for bacteria	10 g/L Tryptone; 5 g/L Yeast extract; 10 g/L NaCl
MS medium for plants	4.4 g/L MS medium; 1g/L MES; pH 5.7 with KOH; 6,8g/L select agar

3.1.11 Standard buffers

Buffer	Content
PBS (10 x)	1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3
PBS-T (1 x)	1 x PBS with 0.05 % (v/v) Tween-20
TAE (20 x)	0.8 M Tris, 2.3 % (v/v) acetic acid, 20 mM EDTA
TE	10 mM Tris, 1 mM EDTA, pH 7.5
Buffer O+	50 mM Tris-HCl (pH 7,5 at 37°C); 10 mM $MgCl_2$; 100 mM NaCl; 0,1 mg/ml BSA
Buffer B+	10 mM Tris-HCl (pH 7,5 at 37°C); 10 mM $MgCl_2$; 0,1 mg/ml BSA
Buffer G+	10 mM Tris-HCl (pH 7,5 at 37°C); 10 mM $MgCl_2$; 50 mM NaCl; 0,1 mg/ml BSA
Buffer R+	10 mM Tris-HCl (pH 8,5 at 37°C); 10 mM $MgCl_2$; 100 mM KCl; 0,1 mg/ml BSA
Buffer Y+	33 mM Tris-Acetat (pH 7,9 at 37°C); 10 mM magnesium acetat ; 66 mM potassium acetat; 0,1 mg/ml BSA

3.2 Methods

3.2.1 Standard molecular methods

3.2.1.1 Isolation of plasmid DNA from *E. coli*

3.2.1.1.1 Alkaline lysis

Small amounts of plasmid DNA for analytical purposes were isolated from *E. coli* using a modification of the alkaline lysis method. *E. coli* overnight culture (stationary phase) of 1.5 mL was collected by centrifugation at 13000 rpm for 1 minute. The supernatant was removed and the cells were resuspended in 100 µL of buffer I for plasmid DNA isolation (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ µL RNase A). The cell suspension was lysed for 5 minutes on ice using 200 µL of buffer II (0.2 M NaOH; 1 % (w/v) SDS). The suspension was neutralized with 150 µL of buffer III (29.4 g potassium acetate; 5 mL formic acid and water till 100 mL). The solution was mixed well by inverting 6-8 times and the suspension was centrifuged for 10 minutes at 13000 rpm at room temperature. The aqueous solution (~400 µL) was transferred into a new microcentrifuge tube containing 1 mL of 96 % (v/v) ethanol. The DNA was left to precipitate for 20 min at -20°C. Plasmid DNA was collected by centrifugation for 10 minutes at 13000 rpm and 4°C. The pellet was washed with 70 % (v/v) ethanol and air-dried for 10 minutes at 37°C. The DNA was dissolved in 20 µL of EB buffer (10 mM Tris-HCl, pH 8.5).

3.2.1.1.2 Isolation of high- quality plasmid DNA

For sequencing and Gateway® cloning purposes, high-purity plasmid DNA was isolated using QIAprep (Qiagen) or Nucleospin Mini kit (Machery&Nagel) following the manufacturer's instructions. Optional steps were always followed according to the manufacturer's recommendation. A 5 mL overnight culture was used to isolate plasmid and the isolated DNA was eluted with 50 µL (high copy) or 30 µL (low copy) EB buffer or water (ultra pure).

Larger amounts of plasmid DNA from E. coli with high purity were isolated using Qiagen or Macherey-Nagel Midi and Maxi kit depending upon the required end concentration. Manufacturer's protocol including the optional recommendations was followed and final elution volume depended on the plasmid copy number, size of the DNA pellet to be eluted and final concentration required.

3.2.1.2   Measurement of DNA- and RNA concentrations

The concentration of nucleic acids was estimated by measuring their absorption in a spectrophotometer at a wavelength of 260 nm (maximum nucleic acid absorption value; due to the π-electron systems of the heterocycles of the nucleotides). In a cuvette having 10 mm path-length where OD_{260} reading is 1 corresponds to 50 and 40 µg/ mL double-stranded DNA and RNA,respectively. Absorption at 280 nm (for the presence of aromatic rings from amino acids and phenol compounds) was used to give information about the purity of the DNA or RNA sample, where an optimal ratio OD_{260}/OD_{280} is in the range of 1.9-2.0 for RNA and 1.8 for DNA. DNA concentrations lower than 100 ng/ µL were measured on an agarose gel using the Gene Ruler Ladder Mix.

3.2.1.3   Separation of DNA on agarose gels

The electrophoretic separation of DNA for analytical and preparative purpose was done in a horizontal agarose gel (10 cm x 7 cm x 0.3 cm, 16 lanes) with 1x TAE as running buffer. DNA fragments ranging between 500 bp and 14 kb were run in an agarose gel concentration of 1 % where DNA fragments with lower size were run in a 2 % agarose gel. DNA samples were mixed with 1/10 volume of 10x DNA loading buffer, loaded in separate lanes and run at 120 V for 40-45 min. Ethidiumbromide solution (0.1 % w/v) was used to stain the DNA fragments. The detection of DNA was done under UV light (260 nm). When a preparative gel was run and particular band fragments were needed to cut out, detection was done using larger wavelength UV light (320 nm). Before exposure to the UV light, the gel was rinsed briefly in H_2O to reduce background staining. In a gel-documentation station, gels were visualized on a UV-transilluminator and documented. The sizes and amount of the DNA fragments were determined using DNA standards.
The elution of DNA fragments from agarose gel was done using the QIAquick or Nucleospin Extract II Gel Extraction kit following the manufacturer's instructions. The eluted fragments were verified by electrophoresis as described above.

3.2.1.4   Separation of DNA on polyacrylamid gels (PAA)

The use of PAA-gels enables an accurate separation of small DNA fragments (1-300kb). In this work, vertical 5% PAA-gels (17cm x 15,5cm x 0,15cm, 11 wells) were utilized for preparation of radioactively labeled EMSA fragments (**3.2.1.9.8**) and gel retardation experiments (**3.2.1.9.9**). Each gel (50ml) consisted of: 5% (w/v) acrylamid:N,N´-methylenbisacrylamid (19:1), 10 % (v/v) glycerin and 1 x TBE (5 x TBE: 0,5M tris; 0,5M boric acid; 10mM EDTA), supplemented with 0,04 % (v/v) TEMED and 0,06 % (w/v) APS. Polymerization of the gel took place under 4°C over 5 hours. The electrophoresis was performed in 1x TBE buffer at 60-150V and 4°C. As markers, bromphenolblue and xylencyanol FF were used, running on the heights of 35bp- and 130bp DNA fragments, respectively.

3.2.1.5   Restriction digestion of DNA

Type II endonucleases were used to digest a double stranded DNA molecule for analytical and cloning purposes. The enzymes cut the DNA either as 5´ or 3´ "sticky" overhangs or as blunt ends. The digestion reactions were incubated in a buffer system optimized for the used enzyme and in the case of double digestion a universal buffer system was used. The activity of the restriction enzymes was estimated in "units" (U), where 1 U was defined as that amount of enzyme cutting completely 1 µg of λ DNA in 60 minutes at optimal conditions. The minimal amount of enzyme necessary for each restriction was determined according to the following formula:

U = (bp[λ] x No. of restriction sites in target DNA) / (No. of restriction sites in [λ] x bp of target DNA) with λ = 48500 bp

The incubation temperature was 37°C unless otherwise mentioned for particular restriction enzyme. Due to the adverse effect of high glycerol concentration, the total volume of restriction enzymes should not extend more than 10% in the restriction mix.

3.2.1.6   Ligation of DNA fragments

The conventional cloning of a DNA fragment into a selected plasmid was performed using the T4-DNA ligase enzyme, which is able to catalyze the formation of a phosphodiesther chemical bond between free 5´-phosphate and 3´-OH groups of double stranded DNA fragments and vectors. The donor DNA fragment (10x accesses over the vector) was incubated with the vector DNA, 2 µL of ligation buffer and 1 µL of T4-DNA ligase for 2 hours at room temperature. The ligation of DNA fragments with blunt ends was performed in the presence of 5 % (w/v) PEG 4000 with the ligation mix described above. Ligase activity was destroyed by heating at 65°C for 10 min before using the ligated DNA for transformation.

3.2.1.7   Gateway® cloning

The cloning of binary vectors for transient and stable plant transformation purposes has been performed with the gateway® cloning system from Invitrogen. The gateway® technology is based on the site specific recombination of bacteriophage lambda and thereby provides a fast method to move DNA sequences between multiple vector systems without the use of restriction enzymes (Landy 1989; Hartley et al. 2000). All cloning steps have been performed as mentioned in the Invitrogen manual, Version E, 22. September 2003.

3.2.1.8   Sequencing of DNA

The DNA sequencing was done using the BigDye Terminator RR Mix Cycle Sequencing kit. The principle of DNA sequencing is based on the chain-termination method (Sanger et al., 1977). In the chain-termination method, dideoxynucleotides (terminators) are incorporated into a newly synthesized complementary chain that will lead to stop its elongation in a PCR reaction. Each of dideoxynucleotides is labeled with a specific fluorescent dye and the terminated chains can be specifically detected using an ABI Prism 3100 Capillary Sequencer (Applied Biosystems). The PCR sequencing reaction was performed using 500-1000 ng plasmid DNA, 5 pmol primer, 2 µL RR mix (ready reaction) and H_2O up to 10 µL. The samples were subjected to 25 cycles of: 10 seconds at 95°C, 5 seconds at 50°C, 4 minutes at 60°C in a thermocycler. The DNA product was precipitated using 9.5 µL water and 30.5 µL of absolute ethanol and left for 1 hour. The DNA was collected by centrifugation for 20 minutes at 13000 rpm. The pellet was washed using 125 µL 70% ethanol and then centrifuged for 10 minutes at 13000 rpm. The pellet was dried at 95°C for one minute and resuspended in 10 µL of HiDi-reagent. The samples were placed on ice. The reactions were loaded on an ABI-Prism 3100 capillary electrophoresis sequencing station for analysis.

3.2.1.9   Cloning of vectors

3.2.1.9.1   Hybridization of complementary DNA fragments

1nmole of complementary DNA oligonucleotides were added in a total volume of 20µl (screw cap reaction tube) and heated for 10 min in a 100°C water bath. To assure a smooth hybridization, the samples were cooled down to room temperature in the water bath over night.

3.2.1.9.2   Cloning of DNA fragments for EMSA studies

The fragments for EMSAs were obtained by hybridization of complementary oligonucleotides. All oligonucleotides contained open restriction sites (*Hind*III at the 5` end and *Eco*RI at the 3` end) for ligation in the pUC18 or pSK Vectors and *Bpi*I restriction sites for generation of polyT overhangs and subsequent radioactive labeling.

3.2.1.9.2.1   as-1

77bp fragment inserted in pUC18-*as-1* (Thurow et al. 2005)

*Hind*III -
gcatgcctgcaggtcgactctaga**TGACGTAA**ggga**TGACGCAC**tctagaggatccccgggtaccgagctc
– *Eco*RI

The TGA recruitment sites within the *as-1* element are labeled by bold capital letters.

3.2.1.9.2.2   LS4-LS8

78bp fragment inserted in pSK-*LS4-LS8*

*Hind*III – ttgc**GTCTTC**tctattgactgtttct**ctacgtcac**tattttac**ttacgtca**tagatgtggcggcat**GAA GAC**gcaaaa - *Eco*RI

The bold capital letters show the *Bpi*I restriction sites while the bold letters in the center of the fragment show the TGA binding sites of the *as-1-* like element.

3.2.1.9.2.3   LS5-LS10

84bp fragment inserted in pSK-*LS5-LS10*

*Hind*III – ttgc**GTCTTC**t**ctacgtcac**tattttac**ttacgtca**tagatgtggcggcatatattcttcaggacttttcag **GAAGAC**gcaaaa - *Eco*RI

The capital letters show the *Bpi*I restriction sites while the bold letters in the center of the fragment show the TGA binding sites of the *as-1-* like element.

3.2.1.9.2.4   w1+w2+w3

89bp fragment containing the whole w-box cluster downstream of the *as-1*-like element inserted in pSK-*W1+W2+W3*..

*Hind*III – ttgc**GTCTTC**ctt**tgact**ttttttcttttatttgaaaat**tgact**gtagatataaacttttatttttc**tgact**gtaaa **GAAGAC**gcaaaa - *Eco*RI

The bold capital letters show the *Bpi*I restriction sites while the bold labeled letters in the center of the fragment show the putative w-boxes.

3.2.1.9.3   Cloning of full length *PR-1* promoter constructs

All full length *PR-1* promoter derivates shown in this work were constructed as translational fusions reaching from +34bp to -1294bp relative to the transcriptional start site. The original fragment was amplified from Col-0 gDNA using the iProof ™ high-fidelity PCR kit from Bio-Rad. For amplification, the primers *PR-1*-1293-F-*Sal*I and *PR-1*-5`UTR-pBGWFS7 were used considering the standard protocol and PCR cycler program from the manual. The substitution and deletion constructs were obtained via overlap extension PCR (OE-PCR). Therefore, the two mentioned primers were utilized seperately in two iProof™ PCRs in combination with primers carrying the mutation of interest (see primer list in 2.1.8.1). The two different fragments yieded by the first PCR were purified after separation on a 1% agarose gel and diluted 1:200. The two diluted fragments were used as templates in a second PCR, again employing the primers *PR-1*-1293-F-SalI and PR-1-5`UTR-pBGWFS7. The resulting PCR fragment contained the desired DNA modification and was digested subsequently with restriction endonucleases to ligate the fragment in pUC18-Entry2-*PR-1*. If the mutation was located within the linker scanning region described by Lebel et al., *Mph1103*I and *Eco91*I were chosen for cloning, while w-box deletion fragments located downstream of this sector were digested with *Mph1103*I and *Bpi*I prior to ligation. The ligated vectors have been transformed in *E. coli* to accumulate plasmid DNA. All plasmids

were sequenced, at least in the region of the substituted DNA fragment, to exclude the possibility of randomly created sequence mutations due to PCR mistakes.

3.2.1.9.4   Cloning of *PR-1* promoter deletion constructs

The *PR-1* promoter construct lacking 495bp between -74bp and -569bp has been cloned in several steps. The initial promoter sequence for this derivate was not the full length promoter described in **3.2.1.9.3**, but a promoter amplified from Col-0 gDNA with the primer combination *PR-1*-1293-F-SalI and *PR-1-1*-R. This means, that the 5`UTR of *PR-1* is missing in this sequence. Furthermore, a fake sequence has been inserted by substitution of region -1294bp to -569bp against a stretch of coding sequence derived from the *CAT* gene using the restriction sites *Sal*I and *Eco91*I. For PCR mediated amplification of the *CAT* gene, the primer combination *CAT483F / CAT483R* has been used on the template plasmid pTAX-*CAT*. The deletion between -569 and -68 was created by ligation of the vector fragment pUC18-Entry2-CAT-PR-1 cut with *Eco91*I (-816) and *Hpa*I (-68), and the promoter fragments from *Eco91*I (-816) to *Nde*I$_{(fill\ in)}$(-573). As the fill in reaction of the *Nde*I overhanging ends had destroyed the *Mph1103*I restriction site an overlap extension PCR was performed to obtain a *PR-1* deletion construct that is more versatile concerning cloning purposes due to restoration of this restriction site. The primers used for this modification were *PR-1 3`D*-F and *PR-1 3`D*-R on the pUC18-Entry2-*3`D* plasmid as template.

3.2.1.9.5   Cloning of PR-1$_{816-509}$-w-box constructs

The pUC18-Entry2-PR-1$_{816-573}$ plasmid has been used to insert oligomers of different sizes in the Mph1103I restriction site. These oligomeres contain no w-box (3`D oligo Chip-R), one w-box (3`D oligo ChIP-R + w1), two w-boxes (3`D oligo ChIP-R + w1 +w2) or all three w-boxes downstream of the *as-1*-like element (3`D ologo ChIP-R +w1 +w2 +w3) of the *PR-1* promoter.

3.2.1.9.6   Klenow fragment-mediated filling of 5`end overhangs

The klenow fragment of *E. coli* DNA polymerase I shows 5`$\rightarrow$ 3` polymerase- and 3`$\rightarrow$ 5` exonuclease activity. The enzyme is frequently used to fill up DNA fragments with "sticky" 5`- overhangs or to digest fragments with 3`- overhangs to obtain blunt ended DNA fragments. The klenow fragment is active in all restriction buffers mentioned in the standard buffer table (2.1.11).
Procedure for 3µg of digested DNA in a volume of 30 µl:
0,33mM dATPs, dCTPs, dGTPs and dTTps 2U klenow fragment; 1x restriction buffer. The reaction was incubated for 1h at RT and subsequently heated to 75°C for 10 minutes to inactivate the enzyme. A following gel elution yielded the cleaned up DNA fragment.

3.2.1.9.7   T4 polymerase-mediated filling of 5`end overhangs

T4 is a bacteriophage of *E. coli*. The activities of T4 DNA polymerase are very similar to Klenow fragment of DNA polymerase I - it functions as a $5` \rightarrow 3`$ DNA polymerase and a $3` \rightarrow 5`$ exonuclease, but does not have $5` \rightarrow 3`$ exonuclease activity. The advantage of the T4 polymerase is the 200 times higher efficiency of the $3` \rightarrow 5`$ exonuclease activity in comparison to Klenow fragment, making it a preferred choice for blunting DNAs with 3`overhangs. 1µg of cleaned up DNA fragments were supplemented with 5x reaction buffer for T4 polymerase, 0,1mM of each dNTP, 1U T4 DNA polymerase and water to a final volume of 20µl. The mixture was incubated for 5 minutes at RT, while a subsequent 10 minutes heating step at 75°C inactivated the enzyme. After gel elution, the modified fragments were used for further cloning purposes.

3.2.1.9.8   Radioactive labeling of EMSA fragments

The radioactive labeling of EMSA fragments with α-^{32}P-dATPs has been achieved by filling up 5`overhangs of restricted vectors utilizing klenow fragment as described under **3.2.1.9.6**. The depletion of non-integrated α-^{32}P-dATPs from the mixture has subsequently been performed via gelfiltration using Micro Spin^{TM} G25 columns from Pharmacia and the appropriate protocol. Afterwards, the labeled fragment was separated from the labeled vector by PAA-gel electrophoresis (**3.2.1.4**), cut out and solved in 800µl EB buffer. Detection of the radioactive fragments was realized by autoradiography.

3.2.1.9.9   Electrophoretic mobility shift assays (EMSAs)

The binding reaction occurred in an overall volume of 30µl in 1x binding buffer (5x binding buffer: 125mM HEPES-KOH, pH7,6; 50mM $MgCl_2$; 1mM $CaCl_2$; 5mM DTT; 2mM PMSF; 50 % (v/v) glycerin). 3µg of PolydI/dC has been added to the protein samples (10µg) to avoid unspecific DNA binding and the extracts were pre-incubated on ice for 10 minutes. In case of supershift assays, 1µl of αTGA2/5 serum or the corresponding pre-immune serum were supplemented. After 4µl of the labeled fragment has been added, there was a further 10 minutes incubation step at RT. The samples were subsequently mixed with 10µl loading buffer (50% (v/v) glycerin; 42% (v/v) 5x binding buffer) and transferred in the wells of a 5% PAA-gel (**3.2.1.4**). Before loading, the gel had to run for at least 1h at 150V to assure a better quality of the gel. The loaded gel has been running over night at 65V and 4°C. On the next day, the gel was positioned on 3MM blotting paper and wrapped in plastic foil. Drying of the gel has been performed on an 80°C vacuum gel drying device over a period of 2h. For exposition, the gel was placed in a cassette together with an IP-screen. After 4h, the signal intensities could be detected using a bioimager (BAS-1000 from Fuji). The images were analyzed with the programs PCBAS®2.09 and TINA®2.0 from Raytest.

3.2.1.10   Gene transfer to *E. coli*

E. coli cells have no competent nature, *i.e.* they are not able to accept foreign 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. Two transformation methods were used to transform competent bacteria cells: The heat shock and the electroporation. The heat shock method was used only to transform *E. coli* chemical competent cells (Hanahan, 1983). In brief, 200 μL competent *E. coli* cells were thawed on ice for 20 min, 50 ng of plasmid DNA was added to the cells and mixed gently. The mixture was incubated on ice for 30 minutes. The cells were heat shocked for 90 s at 42°C. 700 μL of LB medium were added to the tube and the suspension was mixed on a roller for 45-60 min at 37°C depending on selectable antibiotic resistance marker. Different volumes of the culture were plated on plates containing LB medium supplemented with antibiotics. The plates were incubated overnight at 37°C.

The transformation using electroporation was done for *E. coli* cells according to a slight modification of (Dower et al., 1988). The electroporation was done using a Gene Pulser II. Bacterial competent cells were thawed on ice slowly before adding 2 μL of plasmid DNA. The mixture was transferred into an ice-cooled electroporation cuvette (2 mm electrode distance). The cuvette was subjected to electroporation at 25 μF, 2.5 kV, 200 Ω. The cells were suspended immediately with 1 mL LB medium and incubated for 45-60 min at 37°C. Different volumes of the culture were plated on LB media supplemented with antibiotics and incubated overnight (12-16 hours) at 37°C.

3.2.1.11   Gene transfer to *A. tumefaciens*

Competent cells of *Agrobacterium tumefaciens* GV3101 were transformed by electroporation method. Cells were thawed on ice, mixed with respective plasmid DNA and transferred to an electroporation cuvette. Electric pulse (2.5 kV, 25 μF, 400 Ω) was applied for ~5 s. Subsequently, cells were incubated with 1 mL LB medium for 2 h at 30°C and spread on selective YEB-plates. Incubation of plates was performed for 2-3 days at 30°C. Transformed cells from plates were grown in 25 mL selective YEB liquid medium o/n at 30°C. From 5 mL of this pre-culture plasmid-DNA was extracted (QIAprep kit) to control the transformed cells. The rest of the pre-culture was transferred into 400 mL selective YEB liquid medium and incubated o/n at 30°C. Cells were harvested by centrifugation (2500 rpm, 30 min) and resolved in 5 % sucrose solution to an OD_{600} of 0.8. Silvet-L77 (0.05 %) was added to this solution prior to *A. thaliana* transformation.

3.2.1.12 *Agrobacterium*- mediated gene transfer to *A. thaliana*

Transformation of *A. thaliana* with *Agrobacterium* was performed after (Clough 2005). Therefore, flowering plants were dipped into an *Agrobacterium* solution (OD=0,8). Plants were subsequently cultured to seed development. Selection was performed with respective selection markers on the integrated transgenic DNA.

3.2.1.13 Plant growth conditions

3.2.1.13.1 Growth of transgenic reporter gene lines

Approximately 200 F2 seeds of selected reporter gene lines were surface sterilized and sown on sterile 0x MS-plates comprising 0,5g/L MES. The control plates contained 15µl/L DMSO, while the plates used for induction treatment were supplemented with 30µM INA. The plants were grown for 18 days at 22°C under long day conditions (14h light/ 10h dark) and 60% humidity.

3.2.1.13.2 Plant growth conditions for transient expression studies

Plants for protoplast transformation were grown on steamed soil under long day- (16h light/ 8h dark) and weak light conditions over a period of three weeks at 22°C and 60% humidity. For each pot three plants were sown out.

3.2.1.13.3 Plant growth conditions for EMSA studies

Surface sterilized seeds were sown on sterile 0x MS- plates containing 0,5g/L MES and grown for 14 days under long day conditions (14h light/ 10h dark) at 22°C. On day 13, some of the plates were sprayed with 1mM SA. After 24h of induction treatments, 100mg plant material was harvested for preparation of protein extracts. Alternatively, 0x MS- plates supplemented with 30µM INA were used to trigger chemically induced SAR.

3.2.1.14   RNA extraction

The extraction method based on TRIZOL extraction can be used to extract RNA, DNA and proteins from plants (Chomczynski, 1993). This method uses a Phenol/ Chloroform (dichloromethane) extraction to solve RNA in the aqueous phase while other parts like chlorophyll is solved in the hydrophobic chloroform phase. The two thiocyanates in the extraction buffer inhibit RNAses. After grinding of the plant material under liquid nitrogen 1 mL extraction buffer was added to ~150 mg plant material. After shaking for 15 min at RT, chloroform (200 µL) was added to each sample. After an additional shaking step (15 min, RT) and centrifugation (12000 rpm, 35 min, 4°C) the supernatant (700 µL) was transferred to new microcentrifuge tubes. Precipitation buffer (HSPB) and 2-propanol (each 250 µL) were added and the samples were incubated for 10 min at RT and centrifuged (12000 rpm, 20 min, 4°C). After removing the supernatant, samples were dried at RT. The dried pellets were resolved in $50 - 100$ µL water (ultra pure). Concentration was measured as described in 2.2.1.2.

3.2.1.15   Quantitative Realtime RT-PCR (qRT-PCR)

RNA extraction of plant leaf material was performed as described above. DNaseI restriction was done before cDNA synthesis. 1 µg of RNA, 1 µL of 10x reaction buffer with $MgCl_2$ (Fermentas, St. Leon-Roth, Germany) and desoxyribonuclease I (DNaseI), RNase-free was added with water to a final reaction volume of 10 µL. The mixture was incubated at 37°C for 30 minutes. To denatured the DNaseI 1 µL 25 mM EDTA was added and incubated at 65°C for 10 minutes. cDNA synthesis was performed with 1 µg total RNA (DNA-free), 20 pmol of oligo-dT primer and 200 pmol of random nonamer oligonucleotides. Water was added to a final reaction volume of 12.5 µL. The mixture was heated to 70°C for 10 min, 20 nmol dNTPs, 4 µL 5x reaction buffer (Fermentas, St. Leon-Roth, Germany) and 30 u ribonuclease inhibitor (Eppendorf, Hamburg, Germany) were added and the mixture was heated to 37°C for 10 min. 100 u of RevertAid^{TM} H Minus M-MuLV reverse transcriptase (Fermentas, St. Leon-Roth, Germany) was added (final volume 20 µL) and the mixture was incubated at 42°C for 70 min, then heated to 70°C for 10 min. The iCycler System (Bio Rad, Hercules, CA, USA) was used for the amplification and quantification of cDNA using QuantiTect Primer Assays (Qiagen, //www1.qiagen.com/Products/Pcr/QuantiTect/PrimerAssays.aspx) for the respective genes and for ubiquitin 5 (UBQ5) as reference gene. The amplification mix consisted of 1x NH$_4$-reaction buffer (Bioline, Luckenwalde, Germany); 2 mM $MgCl_2$; 100 µM of dNTPs; 0.4 µM of primers, 0.25 u BIOTaq DNA polymerase (Bioline Luckenwalde, Germany); 10 nM Fluoresceine (BioRad, Hercules, CA, USA); 100,000 times diluted SYBR Green I solution (Cambrex, Rockland, ME, USA); 1 µL of a 1:10 dilution of cDNA as template and water (ultra pure) added to a total volume of 25 µL. PCR consisted of a 6 min initial denaturation step at 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 55°C and 40 s at 72°C.

3.2.1.16   Preparation of protein extracts for LUC assays

Protein extractions for luciferase activity assays have been prepared by application of 700 µl 1x cell culture lysis reagent (Promega) to approximately 300 – 500 mg of grinded and frozen plant material. The samples were shaken in an Eppendorf mixer until the plant powder was completely solved in the buffer. Afterwards the samples were placed on ice immediately. After centrifugation (10 min, 12.000 rpm at 4°C) 10 µl of protein extract could be added in each well of a pre- cooled (ice bath) Greiner lumitrac 200 plate. The plate was subsequently covered under a hood (20 min; ice bath) to avoid background illumination during the LUC activity measurement. The LUC assays were started 5-10 min after insertion of the plate in the FLUOstar® Ultima luminescence plate reader. It is important to note that the samples should be used immidiately after preparation, because they cannot be stored over night without a severe decrease in LUC activity.

3.2.1.17   Preparation of protein extracts for MUG assays

Extraction of proteins for MUG assay purposes was conducted by addition of 700µl GUS extraction buffer pH7.5 (supplemented with 0,05% ß-mercaptoethanol) to grinded plant material (300 - 500mg). After solvation of the powder, the samples were centrifuged (10 min, 12.000 rpm at 4°C) and the supernatant was transfered to a new 1.5 ml reaction tube. The samples could be stored at -80°C.

3.2.1.18   Determination of protein concentrations

Protein concentrations were estimated by two different methods. A colorimetric assay was used to determine the concentration from proteins extracted without detergent usage (for MUG assay purposes) according to (Bradford and Williams, 1976). The assay was conducted by pipetting equal amounts of protein extract into a microtiter plate containing 200 µL of 5-fold diluted Bradford reagent. The OD595 was measured with a MRX plate reader (Dynex). Protein concentrations were calculated with the help of a standard curve derived from different BSA protein amounts (1, 3 and 6 µg) on the same plate. Proteins isolated from LUC reporter gene plants were measured with the BCA protein assay kit (Thermo scientific) according to the instruction manual.

3.2.1.19   Measurement of RLU using the FLUOstar® Ultima platereader

For the detection of relative light units emitted by protein extracts of transgenic reporter gene lines, the FLUOstar® platereader was used. All measurements have been performed under the same conditions, using a detection interval of 10s and a relative gain value of 3600. The volume of 50µl luciferase substrate buffer (Promega) was applied to each well via one of the pumps integrated in the platereader. After detection of the emitted light, the values were calculated considering the protein concentrations of the extracts to obtain the RLU.

3.2.1.20   Measurement of relative GUS activities

For the determination of GUS activities from protein extracts, 1 μl of a 1:100 dilution was employed in a MUG assay using a flat bottomed multititerplate. After addition of 99μl of MUG extraction buffer pH7,5 + ß-mercaptoethanol, 100μl of MUG-solution were added to each well and the plate was placed in a 37°C incubator to start the enzymatic reaction. 10 minutes later, a t_0 value was taken by pipetting 100μl of the sample to 100μl of GUS-stop buffer (200mM Na_2CO_3). The remaining reaction was stopped 60 minutes later to gain a t_{60} value. The t_0 and t_{60} values were used to calculate the specific GUS activities of the samples.

3.2.1.21   Transient expression analysis by protoplast transformation

Protoplast isolation and transformation was performed according to the method described by (Sheen 2001). For *PR-1* promoter activity measurements, 20 μg of promoter:GUS plasmids (pBGWFS7) were transformed in *Arabidopsis* protoplasts. For standardization 3 μg of 35S:NAN plasmid was used (Kirby and Kavanagh 2002), alternatively, the protein concentrations were measured by colorimetric Bradford assays. GUS and NAN enzyme assays were performed according to (Kirby and Kavanagh 2002).

3.2.1.22   Buffers used for protoplast transformations

Buffer	Content
Enzyme solution	1-1,5% cellulase R10 0,2-0,4% macerozyme R10 0,4M mannitol 20mM KCl; 20mM MES, pH 5,7 Heat the enzyme solution at 55°C for 10 min (to inactivate proteases and enhance enzyme solubility), The enzyme solution is light brown but clear (passed through a 0,45 μm filter)
PEG solution (40%, v/v)	4g PEG4000; 3ml H_2O; 2,5ml 0,8 M mannitol 1ml 1M $Ca(NO_3)_2$ or $CaCl_2$
Washing and incubation solution (WI)	0,5M mannitol ; 4mM MES, pH 5,7 ; 20mM KCl
W5 solution	154mM NaCl ; 125mM $CaCl_2$; 5mM KCl 2mM MES (pH 5,7)
MMg solution	0,4M mannitol ; 15mM $MgCl_2$; 4mM MES (pH 5,7)

4   Results

4.1   The *PR-1* promoter contains at least two different integration sites for SNI1-mediated suppression

The transcriptional activator NPR1 and the repressor SNI1 are two key regulatory proteins for *PR-1* induction. While it is assumed that NPR1 positively influences expression via interaction with members of the TGA factor family (Rochon et al. 2006), not much is known about the recruitment of SNI1 to promoter sequences to mediate repression. Since SNI1 does not contain a DNA binding domain a direct recruitment to regulatory sequences is unlikely (Mosher et al. 2006). However, at least one *cis*-element within the *PR-1* promoter which contributes to SNI1-mediated repression has to be postulated. Repression can either be realized by recruitment of SNI1 to the promoter through a sequence specific DNA binding domain, or, alternatively, by recruitment of a transcriptional activator, whose expression is negatively regulated by SNI1. The critical sequence information for SNI1-dependent regulation is encoded in a 1294 bp *PR-1* promoter fragment as revealed by its expression pattern after INA induction in distinct mutants, which corresponds to endogenous *PR-1* regulation (figure **4.1A**). (1) The reporter gene shows an increased basal expression in *sni1-1* accompanied by a hyperinduction when INA is applied. (2) The 1294 promoter shows no sensitivity to INA in *npr1-1* and (3) this lack of induction can be partially rescued in the *sni1 npr1* double mutant. (4) The *sni1 npr1* mutant displays an increased background expression level similar to *sni1-1* but the hyperinduction fails likely due to the absence of the transcriptional co-activator NPR1. In conclusion, these experiments show that the SNI1/NPR1 independent pathway of *PR-1* induction is operational on this promoter fragment.
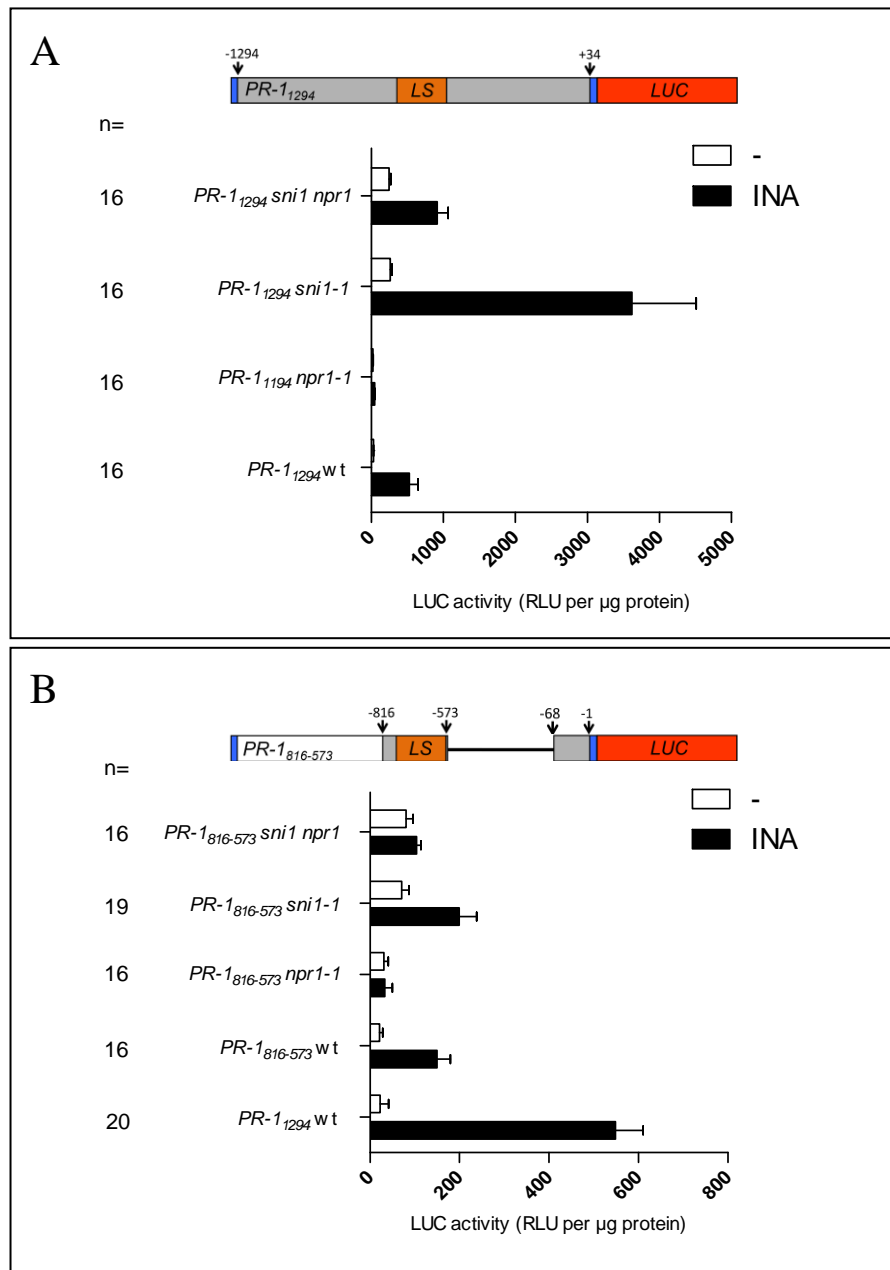
Figure 4.1: Average LUC activities of the *PR-1_{1294}* (A) and the *PR-1_{816-573}* (B) promoter fragment in wt and mutant plants.

Luciferase activities of 18-day-old plants grown on MS-plates supplemented without (white columns) or with (black columns) 30 µM INA were measured. The numbers of single transgenic lines investigated for each genotype is displayed on the left (n=). Error bars represent the standard deviation of at least two independent experiments. The promoter derivative illustrations show *PR-1* promoter sequences fused to a *LUC* reporter gene. The blue boxes display the gateway® cloning sites, grey shaded regions illustrate *PR-1 cis*-regulatory region and the white box shows sequence substitution against coding sequence derived from the *CAT* gene. The orange *LS*-labeled region in the center of the two constructs displays the location of the *LS*-region. The gap in *PR-1_{816-573}* illustrates sequence deletion and the arrows show relative base pair positions.

Previous promoter deletion and linker scan analysis has shown that the region between -706 to -579 (called *LS*-region for linker scan from here on) encodes different *cis*-

elements that positively or negatively influence *PR-1* promoter activity (Lebel et al. 1998). Therefore, the *LS*-region has been hypothesized to be the target for NPR1 and SNI1 activity (Kesarwani et al. 2007). One important *cis*-element within this region is the *as-1*-like element which consists of two TGACG motifs designated as *LS5* and *LS7*. Substitution of *LS5* by a linker sequence leads to slightly activated promoter activities both in the uninduced- and induced state, whereas linker scan mutagenesis of *LS7* completely abolishes promoter activity, indicating that the two TGA motifs have distinct function. Because of the positive impact of *LS7* on *PR-1* induction it was hypothesized that it recruits NPR1 via interaction with TGA transcription factors.

 In order to gain direct evidence for the critical function of this promoter sequence with regard to NPR1- and SNI1-dependent regulation the region between -816 and -573 was fused to the basal *PR-1* promoter (-68bp to -1bp). The *PR-1$_{816-573}$* construct was transformed in the different plant genotypes (wt, *npr1-1*, *sni1-1* and *npr1 sni1*) to validate the promoter activity in comparison to the *PR-1$_{1294}$* promoter (figure **4.1 B**). In the wildtype background, the modified promoter construct was still inducible by INA indicating that central regulatory elements within this region are not only necessary but also sufficient for induction. The induced expression levels of *PR-1$_{816-573}$* only reach approximately one third of the intensity measured in *PR-1$_{1294}$* control plants suggesting additional positive *cis*-elements outside the *LS*-region. Interestingly, the *PR-1$_{816-573}$* construct shows slightly increased basal expression levels when transformed in *sni1-1* while the hyperinduction displayed by the *PR-1$_{1294}$* construct is missing. These results suggest that SNI1 affects *PR-1* expression in two ways. On the one hand, SNI1 suppresses basal levels of the *PR-1* promoter via a regulatory sequence present in the *PR-1$_{816-573}$* derivate. On the other hand, in contrast to the INA-induced transcription of *PR-1$_{1294}$*, INA induced transcription of *PR-1$_{816-573}$* is not influenced by SNI1. This illustrates that *PR-1$_{816-573}$* misses *cis*-elements that can serve as target sites for positive regulators that are up-regulated in the *sni1-1* mutant. Though the influence of SNI1 on this modified promoter is altered, the induction of the construct is still strictly dependent on NPR1, illustrated by a complete lack of INA sensitivity in *npr1-1*. This confirms the notion that NPR1 may acts on the promoter through one or both of the TGACG motifs within the *LS*-region. The induction of *PR-1$_{816-573}$* in the *sni1 npr1*double mutant is abolished, likely due to the absence of sequence information that confers induction when no functional NPR1 protein is present. In conclusion, the analysis of the *PR-1$_{1294}$*- and the deleted *PR-1$_{816-573}$* promoter in the different mutant backgrounds revealed that other positive acting regulatory sequences exist outside the *LS*-region. Moreover these

cis-regulatory sequences contribute to hyperinduction in *sni1-1* illustrating that the corresponding transcriptional activators are exclusively active in *sni1-1* mutant background.

4.2   W-boxes downstream of the *LS*-region contribute to *PR-1* induction and SNI1-dependent regulation

Further characterization of regulatory sequences outside of the *LS*-region was performed by deletion analysis of a W-box cluster located between -544 and -485. Several WRKY proteins are identified to be primary target genes of NPR1 (Wang et al. 2006) and play a role in PAMP-triggered- and SA-mediated defense responses (Dong et al. 2003; Lippok et al. 2007). Consequently it is reasonable that the W-boxes within the *PR-1* promoter contribute to *PR-1* regulation although it has already been reported that this region of the promoter is not sufficient for induction (Lebel et al. 1998). Nevertheless, transcriptional regulation is often associated with synergistic actions of transcription factors and the function of these boxes was examined in the *PR-1$_{1294}$* promoter context. Deletion of W-box sequences leads to a considerable reduction of reporter gene activity (figure **4.2 A**). The removal of the WRKY box designated as *W1* leads to an 8-fold decrease on inducible expression compared to the *PR-1$_{1294}$* control. Deletion of both or even all three W-boxes does not yield an enhanced reduction in reporter activity, indicating that *W2* and *W3* cannot substitute for *W1*. The impact of *W2* seems less severe as displayed by a moderate 3-fold reduction of induced expression levels detectable in the *ΔW2* lines. This can be taken as evidence that *W1* may functions only if *W2* is present. In summary, the most upstream located W-box (*W1*) seems to act as a recruitment site for a transcriptional activator to sustain the expression of *PR-1* after induction.

Since the expression of several WRKY proteins is dependent on NPR1 and SNI1 (Mosher et al. 2006; Wang et al. 2006), it can be assumed that the lacking hyperinduction of the *PR-1$_{816-573}$* reporter construct in *sni1-1* might be due to the absence of the W-boxes. The promoter derivative *PR-1$_{816-573}$* was therefore modified by attaching the W-boxes *W1* and *W2*. Figure **4.2 B** shows that *W1* and *W2* do not alter the general expression levels under inducing- and non-inducing conditions in comparison to *PR-1$_{816-573}$* in wildtype plants illustrating that further enhancer sequences are needed to reach the expression capacity of the *PR-1$_{1294}$* promoter. Interestingly, the addition of *W1*

and *W2* to *PR-1_{816-573}* shows the hyperinducibility seen with *PR-1_{1294}* in *sni1-1*. This result implies that the W-boxes *W1* and *W2* integrate SNI1-dependent signals by recruitment of different amounts or combinations of SNI1-regulated WRKY transcription factors. These WRKY factors are thus candidates which mediate the increased transcriptional activation in *sni1-1* and are necessary for the induction of the *PR-1* promoter in *sni1 npr1*.

4.3 The *as-1*-like element is dispensable for INA induced activation of *PR-1*

The *LS*-region between -704 and -577 contains an *as-1*-like element, which is assumed to be the putative integration site for NPR1-dependent *PR-1* regulation. The TGACG motifs of this element are also referred to as *LS5* and *LS7*, since they were identified by linker scan mutagenesis. Surprisingly, substitution of both motifs generates a promoter derivative, that reveals a similar induction like the *PR-1_{1294}* promoter though basal- and induced expression levels are increased (Figure **4.3**). This result illustrates that the *as-1*-like element is dispensable for *PR-1* induction and that an *as-1*-independent mechanism can complement the loss of TGA factor-controlled regulation. This *as-1*-independent regulation depends on the W-box containing element *LS4* as shown by substantially decreased INA-induced reporter gene activities in *LS4/5/7*_{mut} plants. In conclusion, two alternate pathways are merging on the *LS*-region: The *as-1*-regulated pathway, which is slightly negatively influenced by *LS4* (see *LS4_{mut}*; Figure **4.3**), and the *as-1*-independent pathway, which is strongly dependent on *LS4*.

In the absence of *LS5*, the promoter is inducible to the same extent as *PR-1_{1294}* and *LS5/7*_{mut}. The activation is due to the *as-1*-independent pathway, as shown by the lack of activation in *LS4/5*_{mut}, which lacks the W-box that is essential for the *as-1*-independent regulation. Thus *LS5* represses the alternate pathway. The inactivity of the *LS4/5*_{mut} construct also shows that the *as-1*-dependent regulation can only function if *LS5* and *LS7* are present.
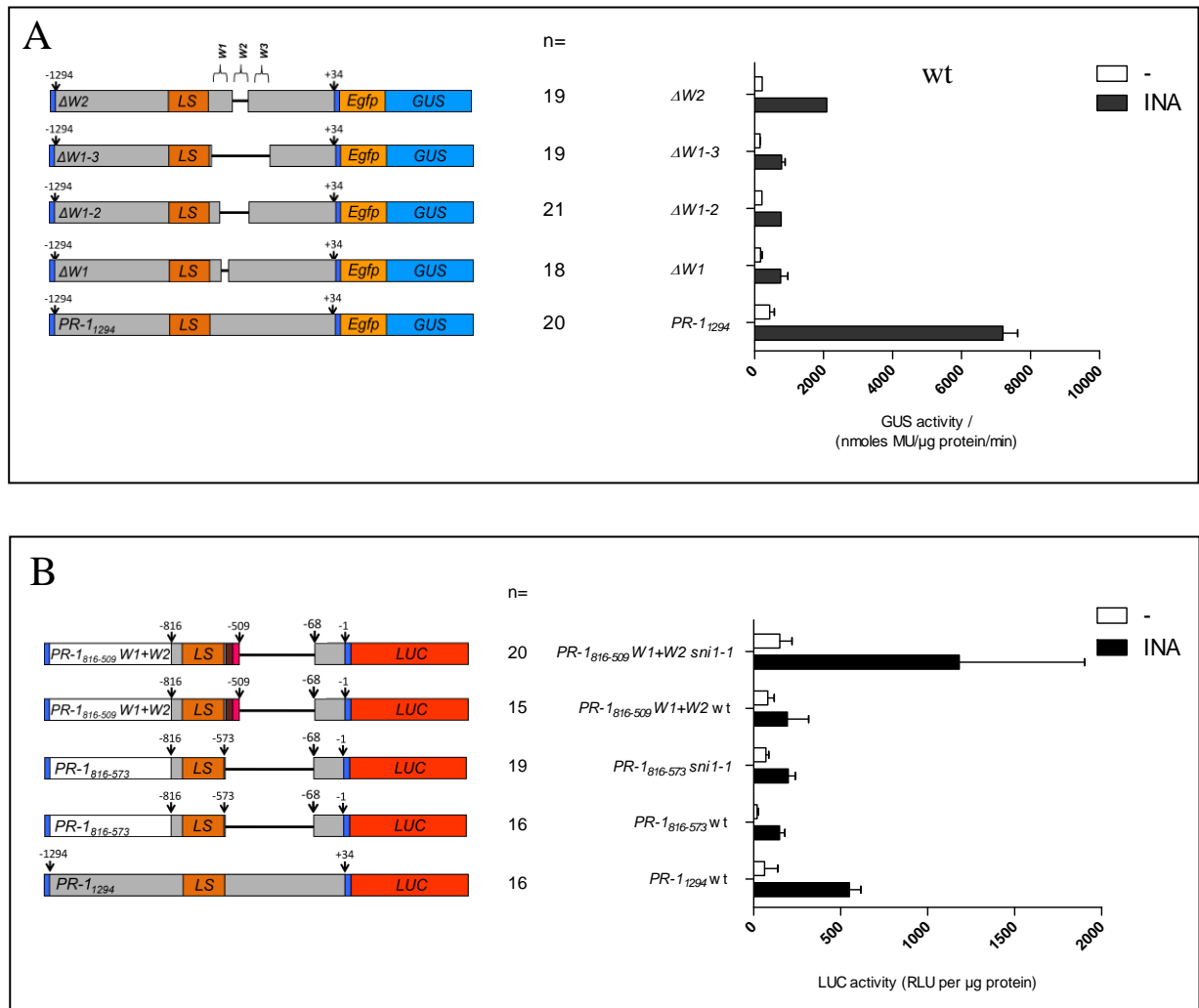
Figure 4.2: Average activities of *PR-1_{1294}* promoter constructs lacking certain W-boxes in wt (A) and of *PR-1_{816-573}* promoter constructs with and without an attached regulatory W-box region in wt and *sni1-1* (B).

Figure **A** shows relative GUS activities of reporter gene lines grown for 18 days on MS-plates supplemented with- or without 30μM INA. The lines are carrying distinct mutations in the W-box cluster. In **B**, the relative LUC activities of the *PR-1_{816-509} W1+W2* reporter plants are displayed in comparison to the *PR-1_{816-573}* promoter derivative. The numbers of individual lines tested in each experiment are indicated (n=), the error bars represent the standard deviation of two independent experiments. The promoter derivative illustrations show *PR-1* promoter sequences fused to an *Egfp/GUS* (**A**) or a *LUC* (**B**) reporter gene. The blue boxes display the gateway® cloning sites, grey shaded regions illustrate *PR-1 cis*-regulatory regions and the white box shows sequence substitutions against the coding sequence of the *CAT* gene. The orange *LS*-labeled box in the center of the constructs displays the location of the *LS*-region. The two attached W-boxes to the modified promoter construct are labeled red and pink. The arrows and the numbers show relative base pair positions, gaps illustrate deleted sequences.

In the absence of *LS7*, the promoter is only weakly inducible, showing that both pathways do not work efficiently. The residual induction is dependent on the *as-1*-independent pathway as revealed by the further reduction when mutating *LS4*. In conclusion, the *as-1*-regulated pathway is compromised upon mutation of either *LS5* or *LS7* or both, and the *as-1*-independent pathway is efficiently repressed by *LS5* and less efficiently by *LS7* and functions best when both elements are missing.
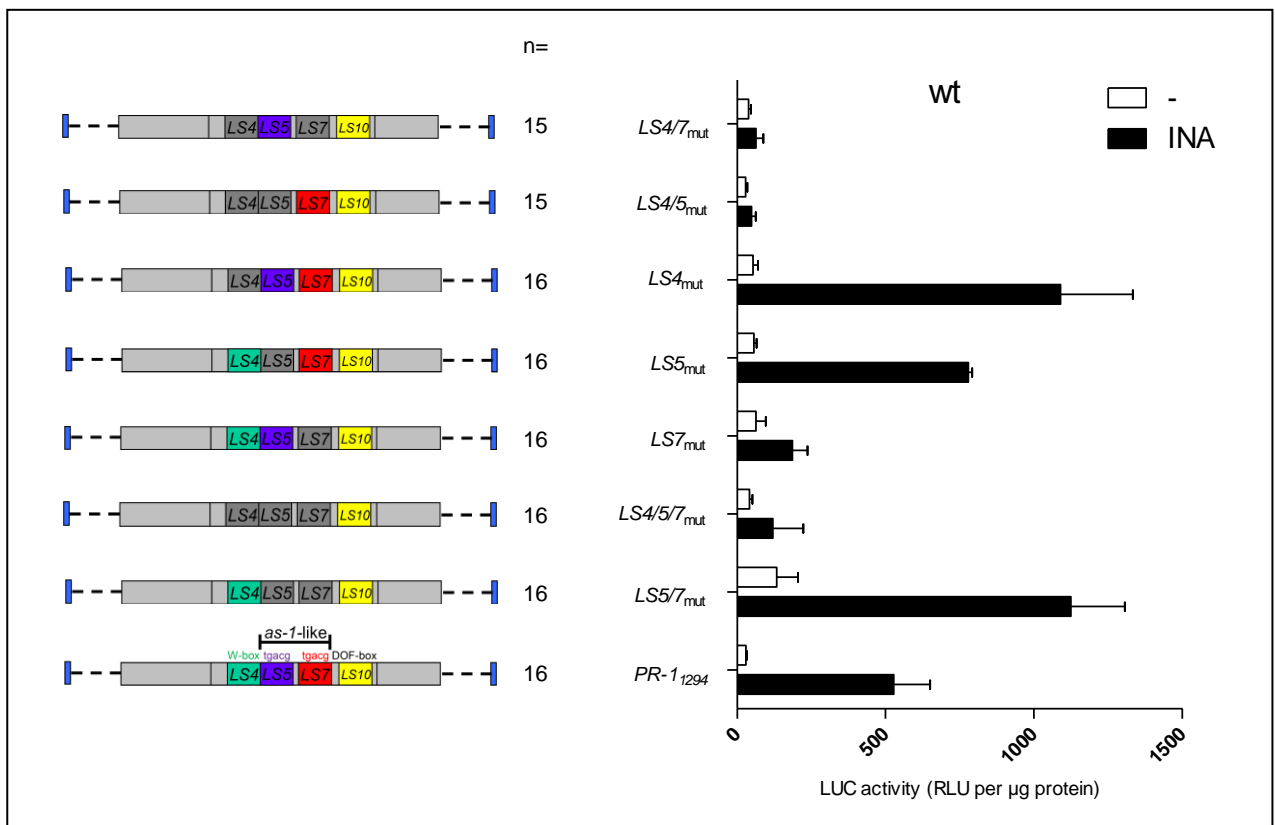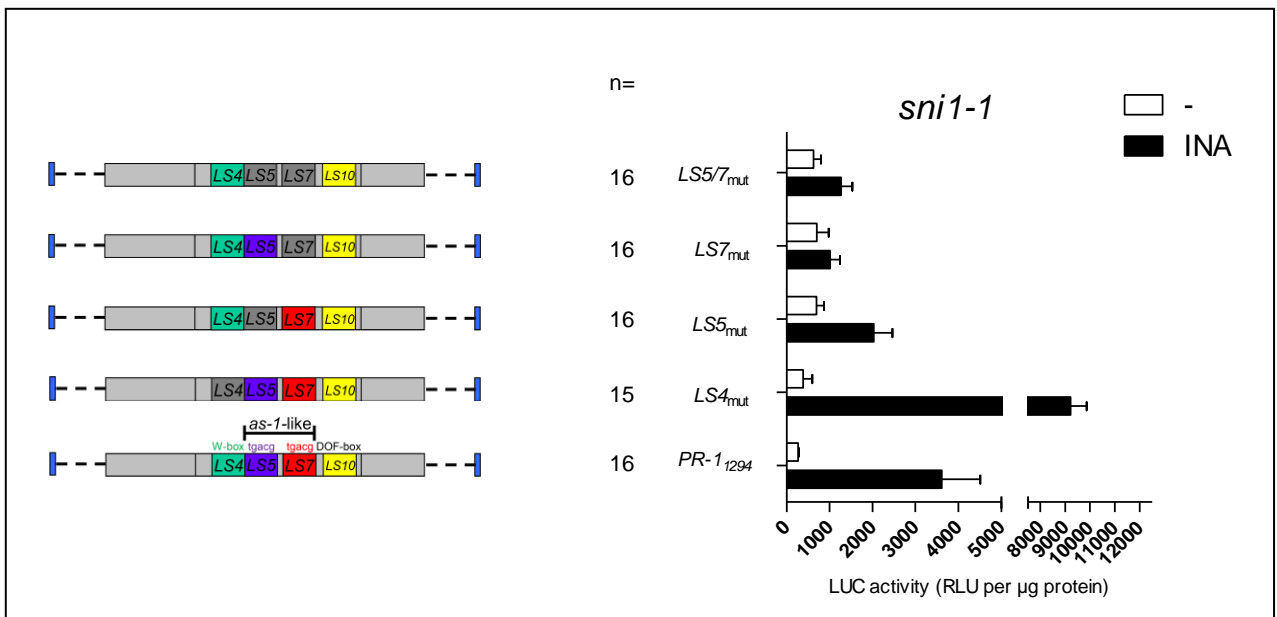

Figure 4.3: Average LUC activities of promoter derivatives carrying distinct mutations in the *as-1*-like element and the adjacent W-box *LS4* in wildtype background.

The figure shows the basal- and induced expression levels of 18-day-old transgenic reporter gene lines grown axenically on MS-plates supplemented with- or without 30 µM INA. The number of individual lines tested for each construct is indicated (n=) and the error bars illustrate the standard deviation of two independent experiments. The promoter figures on the left show a magnified view on the *LS*-region. The grey shaded boxes indicate 10bp sequence substitutions in the corresponding *cis*-elements (for details see supplemental data).

The influence of the repressor SNI1 on the *as-1*-independent regulatory pathway was further examined by analysis of the promoter constructs *LS4*mut, *LS5*mut, *LS7*mut and *LS5/7*mut in *sni1-1* (figure **4.4**). While mutation of *LS4* leads to a 2-fold increased

expression in relation to the *PR-1$_{1294}$* promoter in *sni1-1*, the constructs harboring mutations in one or both TGACG motifs displayed decreased induced expression levels, which were similar to the values obtained in the wildtype (ca. 1000 U, see Figure **4.3**). This illustrates that the *as-1*-independent pathway is not influenced by SNI1 in the induced state and that the *as-1* element is required for the hyperinduction exhibited by the *PR-1$_{1294}$* promoter in *sni1-1*. It seems plausible that NPR1, which is recruited to the TGA factors bound to the *as-1*-like element, is responsible for this hyperinduction. This notion is consistent with the decreased induced expression of the endogenous promoter in *sni1 npr1* when compared to the hyperinduction detected in *sni1-1* (see figure **4.1**). Furthermore, mutation of TGA recruitment sites considerably increased the basal expression of the reporter lines revealing a negative impact of TGA factors on *PR-1* transcription when SNI1 is absent, a phenomenon previously observed when analyzing endogenous *PR-1* expression in a *tga2 sni1* double mutant (Kesarwani et al. 2007). As background levels rise and induction is not altered the constructs only show weak (*LS5$_{mut}$* and *LS5/7$_{mut}$*) or no (*LS7$_{mut}$*) induction at all.

Figure 4.4: Average LUC activities of promoter constructs carrying sequence alterations in the TGACG motifs or the W-box element *LS4* in *sni1-1*.

The figure shows the expression of 18-day-old transgenic reporter gene lines axenically grown under inducing- and non-inducing conditions. The number of transgenic lines examined is indicated (n=), the error bars indicate the standard deviation of at least two independent experiments. The promoter illustrations on the left show the elements *LS4*, *LS5*, *LS7* and *LS10* located within the *LS*-region. The grey shaded boxes indicate 10bp sequence substitutions in the corresponding *cis*-elements (for details see supplemental data).

The inducibility of the *LS5/7*mut construct indicates that the *PR-1* promoter can be activated in the absence of the *as-1*-like element, which is the recruitment site for the NPR1/TGA complex. Next, we asked the question, whether the *as-1*-independent regulatory pathway is also independent from NPR1 and transformed the *LS5/7*mut construct into the *npr1-1* mutant. Analysis of the resulting transgenic lines indicated that the promoter was severely compromised, indicating that NPR1 is needed for this pathway. It seems likely that transcription of WRKY transcription factors recruited to either *LS4* or the W-box cluster downstream of the *LS*-region depends on NPR1.
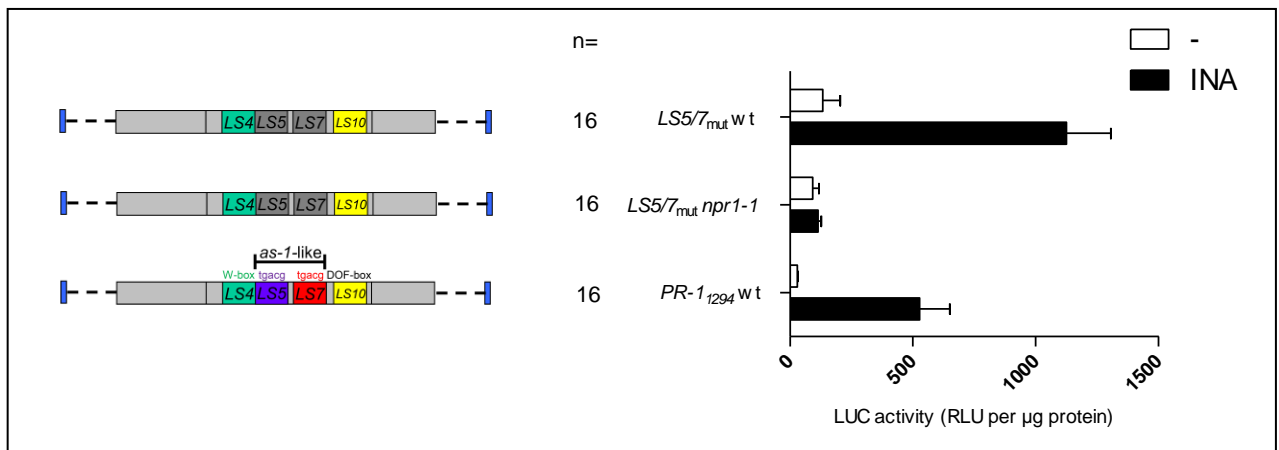

Figure 4.5: Average LUC intensities of *LS5/7*mut reporter gene lines in wt and *npr1-1* mutant background.

18-day-old plants axenically grown on 30µM INA- or control MS-plates were used for protein extraction and subsequent LUC assays. The error bars show the standard deviation of two experiments, the number of transgenic lines examined for each promoter construct is indicated (n=). The dark grey shaded boxes in the promoter illustrations on the left indicate sequence substitutions in the TGACG motif-containing elements *LS5* and *LS7*.

Further experiments in the *sni1-1* mutant have been performed with the loss-of-induction constructs *LS4/5*mut and *LS4/7*mut to evaluate the influence of SNI1 on these promoter derivatives.

Interestingly, *LS4/5*mut and *LS4/7*mut act different in *sni1-1* as inducibility of *LS4/5*mut is partially re-established while *LS4/7*mut even shows decreased expression when treated with INA (figure **4.6**). This implies that a non-mutated *LS7* element is essential for the induction of the *LS4*/5mut promoter construct in *sni1-1*, possibly due to a synergistic

activation mediated by a TGA/NPR1 complex at *LS7* and deregulated WRKY transcription factors at the W-box cluster. In addition, *LS7* maybe recruits SNI1 in case of a mutated *LS5* site and thereby inhibits induction of the $LS4/5_{mut}$ derivative in wildtype plants. Further evidence for the implication of the W-box cluster in the activation of $LS4/5_{mut}$ was gained by analysis of the $LS4/5_{mut}$ *ΔW1-3* construct in *sni1-1* mutant background (figure **4.7**). In contrast to $LS4/5_{mut}$ this promoter derivative totally lacks sensitivity to INA in *sni1-1*. Figure **4.7** also displays once more that the W-box *W1* is involved in *sni1-1* entailed hyperinduction as the induced expression levels of *ΔW1* lines are considerably lower than the levels monitored with the $PR-1_{1294}$ control promoter.
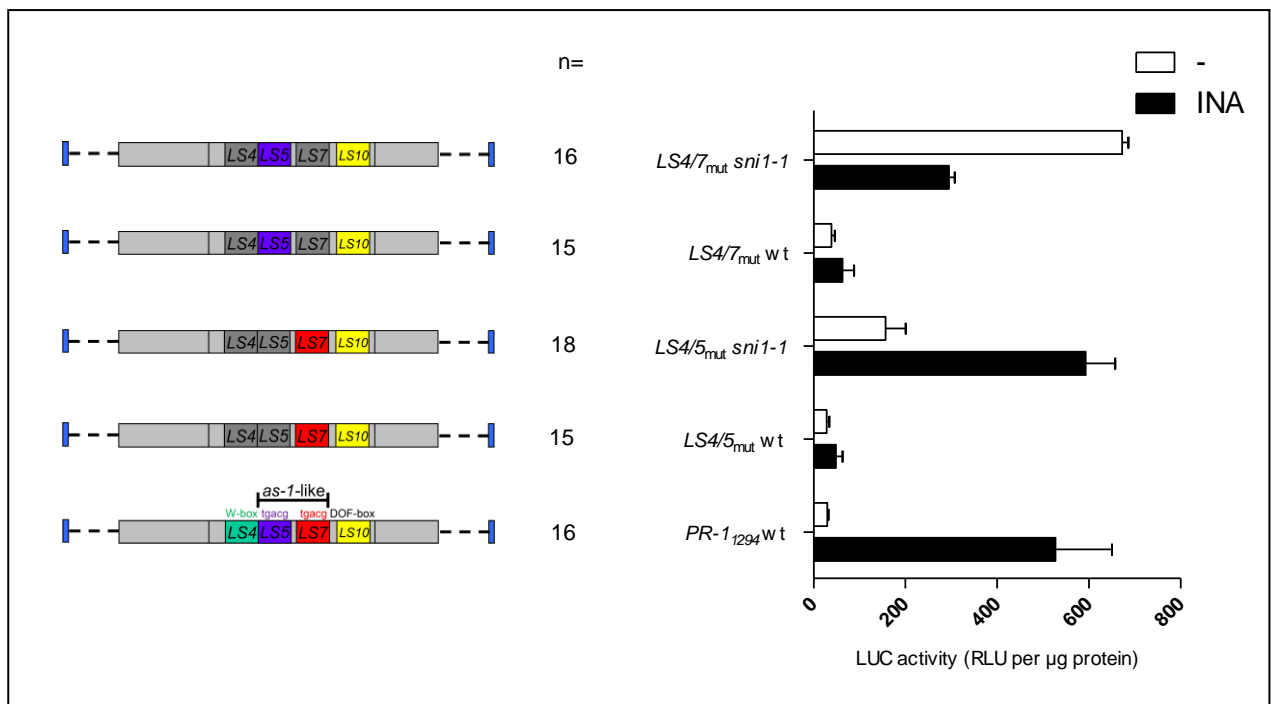
Figure 4.6: Average LUC activities of lines carrying differing sequence mutations in the elements *LS4*, *LS5* and *LS7* in wildtype and *sni1-1* mutant background.

The LUC activities depicted in the figure were obtained from 18-day-old plants axenically grown on MS-medium supplemented with- or without 30 μM INA. The numbers (n=) indicate measured transgenic lines and the error bars represent two independent experiments. The dark grey shaded boxes in the promoter illustrations on the left indicate sequence substitutions in the TGACG motif-containing elements *LS5* and *LS7* and the putative W-box *LS4*.
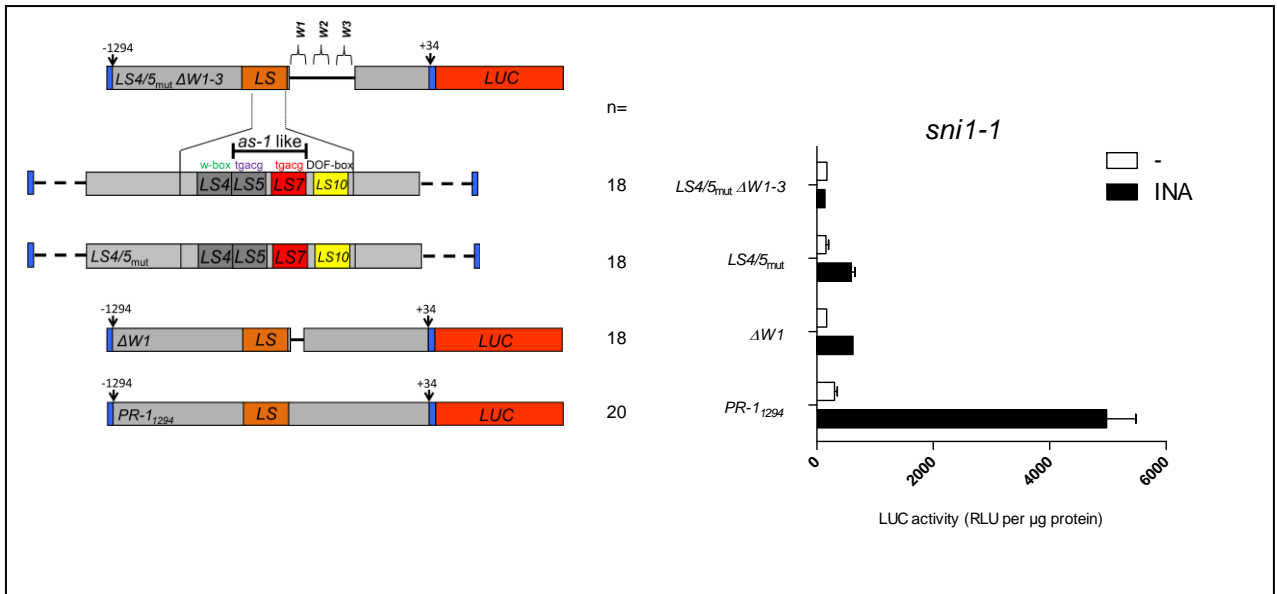
Figure 4.7: Average relative LUC activities of constructs harboring distinct mutations or deletions in the elements *LS4*, *LS5*, *ΔW1* and *ΔW1-3*.
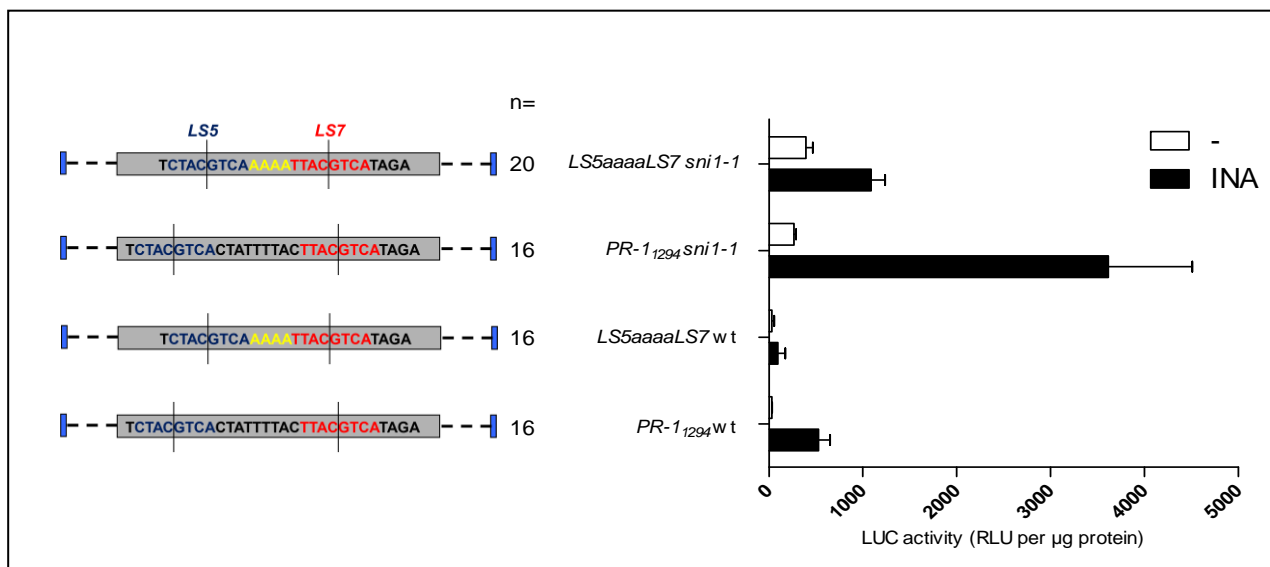
The 18-day-old plants were grown on MS-plates supplemented with- or without 30μM INA. The error bars illustrate the standard deviation of two experiments, the number of investigated transgenic lines is indicated (n=). Dark grey shaded boxes in the promoter illustrations on the left indicate sequence substitutions in the elements *LS4* and *LS5*, gaps show sequence deletions of *W1* and *W1-3* respectively. Blue boxes display the gateway® cloning sites, grey shaded regions illustrate *PR-1* promoter sequence. The orange *LS*-labeled box in the center of the constructs displays the location of the *LS*-region.

4.4 Alteration of the distance between the palindromic repeats of the *as-1*-like element interferes with INA induced expression

The experiments presented so far show that the *as-1*-like element within the *PR-1* promoter contains putative target sites for TGA factor-mediated NPR1 recruitment (*LS7* and *LS5*) and a target site (*LS5*) for a repressor, which suppresses the *as-1*-independent activation pathway. Data published previously indicate that the distance between the two palindromic repeats of the *as-1* element is critical for the NPR1 dependency of SA-inducible promoters (Blanco et al. 2005; Krawczyk et al. 2002). The classical distance between the TGA recruitment sites is 4bp as displayed by the *35S*, *GST6*, and *CYP81D11* promoters whereas the distance in the NPR1-dependent *PR-1* promoter is 9bp.

Therefore, it was tested whether a changed spacing between *LS5* and *LS7* somehow influences the expression of the *PR-1* promoter. For this purpose, the sequence *ctattttac*

was substituted against the sequence *aaaa* and the corresponding construct was termed *LS5aaaaLS7* (figure **4.8**). The promoter derivative shows a substantial reduction in inducible gene expression illustrating an impaired NPR1 regulation at the mutated *as-1*-like element. In *sni1-1*, the INA-induced activity is as high as in the construct lacking the *as-1*-like element, indicating that the *as-1*-independent pathway is operational. This can be taken as evidence that SNI1 represses the *as-1*-independent pathway through *LS5*. As *LS5aaaaLS7* cannot be efficiently activated by NPR1, the promoter is inactive. In the *sni1-1* mutant background, repression of the *as-1*-independent pathway does not occur, allowing high expression levels after INA treatment. The basal expression of *LS5aaaaLS7* in *sni1-1* is slightly enhanced when compared to the expression levels of *PR-1$_{1294}$* and slightly decreased when compared to the expression levels of *LS5/7$_{mut}$*. Thus, TGA factors are still recruited to the *LS5aaaaLS7 as-1*-like element and partially suppress the basal promoter activity in *sni1-1*. Consistently, the induced- and non-induced expression levels of *LS5aaaaLS7* in *sni1-1* look similar to the expression levels obtained with the *PR-1$_{1294}$* construct in *sni1 npr1* (figure **4.1**).


Figure 4.8: Relative LUC activities of a promoter derivative which harbors a mutated linker sequence between *LS5* and *LS7* (*LS5aaaaLS7*) and thereby simulates the distance between the palindromic repeats known from NPR1-independent promoters.

18-day-old transgenic F2 plants grown on MS-plates supplemented with- or without 30μM INA were used for the experiment. The number of investigated lines per construct is indicated (n=), error bars represent the standard deviation of two independent induction treatments. The inserted *aaaa* sequence is labeled yellow in the promoter illustrations on the left. *LS5* (blue) and *LS7* (red) mark the two TGACG motifs.

4.5 The *CaMV 35S as-1* element confers deregulated high expression in the *PR-1* promoter context

So far, our analysis suggests that *LS5* and SNI1 repress the *as-1*-independent SA-induced pathway and that recruitment of NPR1 to the *as-1*-like element is therefore required for activation. However, this recruitment seems to be impaired when the distance between *LS5* and *LS7* is reduced by 5 bps. Next we asked the question, if the *as-1*-element of the *CaMV 35S* promoter, which contains two TGACG motifs in a distance that does not allow NPR1 action, would also be able to recruit SNI1. Therefore, the *as-1*-like element of the *PR-1* promoter was replaced by the *35S as-1* element. Figure **4.9** displays the relative LUC activities of the substituted *as-1* element lines transformed in wt, *sni1-1* and *npr1-1*. The replacement of this regulatory sequence causes a substantial 400-fold increase of basal- and 50-fold increase of induced gene expression in relation to the *PR-1$_{1294}$* wt control lines. This indicates that the *35S as-1* element does not confer repession as observed for the *LS5aaaaLS7* element, but rather recruits a strong activator. As the *35S as-1* element itself confers only weak SA- and auxin-induced gene expression, strong synergistic interactions with *cis*-elements of the *PR-1* promoter seem to occur. These interactions are less strong in the *npr1-1* mutant, indicating that some of these *trans*-factors are expressed in an NPR1-dependent manner. INA treatment leads to an approximately twofold induction of the reporter gene activity independent of the genetic background. Putative recruitment sites for additional NPR1-dependent regulators are once again the W-boxes downstream of the *LS*-region. This hypothesis was tested by integrating the *35S as-1* element in the W-box cluster-lacking *PR-1$_{816-573}$* promoter derivative.
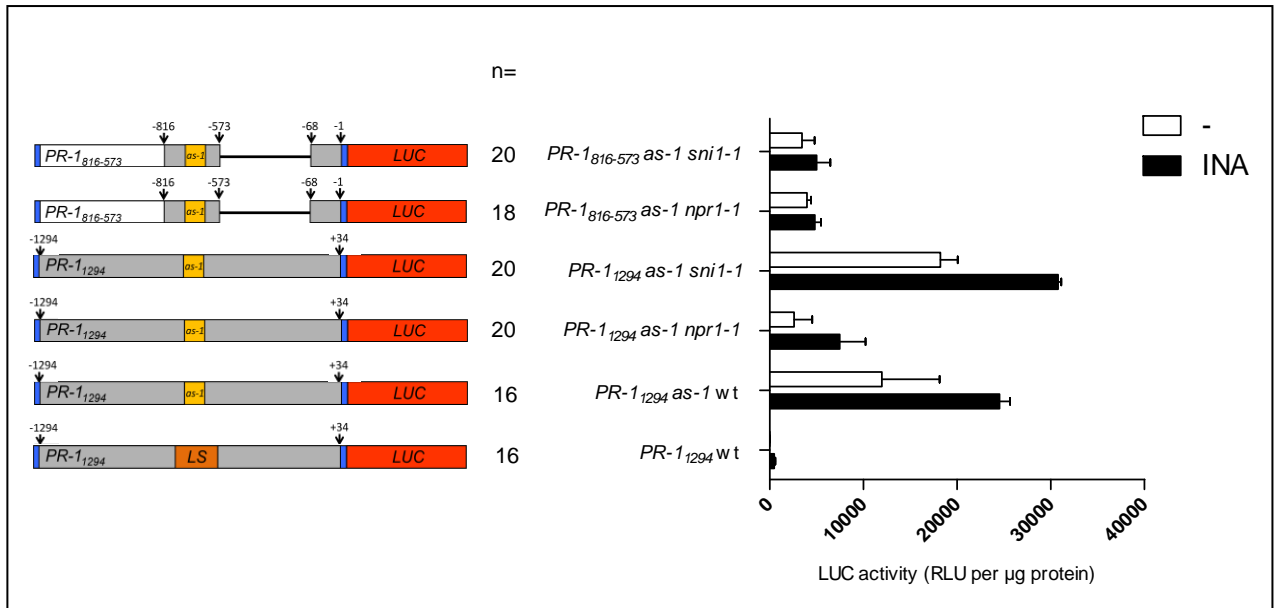
Figure 4.9: Average LUC activities of *PR-1$_{1294}$ as-1*- and *PR-1$_{816-573}$ as-1* promoter constructs carrying substituted *as-1* elements derived from *CaMV 35S*.

The figure shows the LUC activities of 18-day-old plants axenically grown on MS-plates supplemented with- or without 30µM INA. The influence of the genetic background on *PR-1$_{1294}$ as-1*- and *PR-1$_{816-573}$ as-1* promoter activity is displayed, error bars indicate the standard deviation of two experiments while the numbers (n=) illustrate the amount of transgenic lines tested. The gaps in the promoter illustrations on the left denote deletions, the white boxes within the *PR-1$_{816-573}$ as-1* constructs display sequence substitutions. The substitution of the *as-1*-like element against the *35S as-1* element is indicated by the yellow box in the center of the constructs, the *LS*-region in the *PR-1$_{1294}$* promoter is colored orange. The blue boxes display the gateway® cloning sites, grey shaded regions illustrate *PR-1 cis*-regulatory regions and the white boxes show sequence substitutions against the coding sequence of the *CAT* gene. The arrows and numbers show base pair positions.

As illustrated in figure **4.9**, the different reporter gene activities in *npr-1-1* and *sni1-1* are gone when the *PR-1$_{816-573}$ as-1* construct is integrated in these mutants. The loss of NPR1 dependency is accompanied with a lower reporter gene activity in comparison to the *PR-1$_{1294}$ as-1* promoter and a general loss of inducibility. The experiments presented here show that recruitment sites for NPR1-dependent transcription factors are missing in the *PR-1$_{816-573}$ as-1*construct. Candidate sites are the W-boxes downstream of the *LS*-region since it is reported that the induction of many WRKY proteins is dependent on NPR1 (Dong et al. 2003; Wang et al. 2006).

4.6 The sequence between the TGA binding sites of the *as-1* element is critical for the increased constitutive expression observed in the *as-1* reporter plants

The strong expession levels conferred by the *35S as-1* element but not by the *PR-1*-derived *as-1*-like element evoke the question, which sequence within this regulatory unit is responsible for this difference. Common features are, that both elements contain two variants of the TGACGTAC palindromes spaced by a "linker" sequence. We generated a number of chimeric *as-1* elements containing TGA-recruiting sequences and sequences between these motifs either derived from the *PR-1*- or the *35S as-1* element.

The respective constructs were fused to the GUS reporter gene and analyzed in transgenic *Arabidopsis* plants. Surprisingly, the *35S as-1* element conferred increased basal levels, which reached 50% of the induced levels of *PR-1$_{1294}$*, but were not 400-fold higher as observed with the LUC reporter gene (Figure **4.10 A**). Induced levels were similar to *PR-1$_{1294}$*, which is in contrast to the 50-fold higher expression levels observed with the *LUC* construct. An explanation for this discrepancy could be that strong silencing effects occur when the rate of transcription is severely increased in the *GUS* reporter plants leading to attenuated signal intensities. Though the overall expression is weaker, the constitutive activation mediated by the substituted *as-1* element can still be taken as a read-out for addressing the above mentioned questions.

Constitutive activation of the promoter occurs when the *PR-1* linker sequence is substituted against the linker derived from the *35S as-1* element as displayed by the *LS5gggaLS7* construct. Activation is not influenced by NPR1, supporting our previous assumption that NPR1 cannot be recruited to promoters that contain *as-1*-like elements that are spaced by only 4 bps. Whereas *LS5aaaaLS7* construct (see **4.8**) is repressed presumably by SNI1, *LS5gggaLS7* is active. It is concluded that the GGGA-linker sequence of the *as-1* element interferes with repression of the promoter by SNI1.
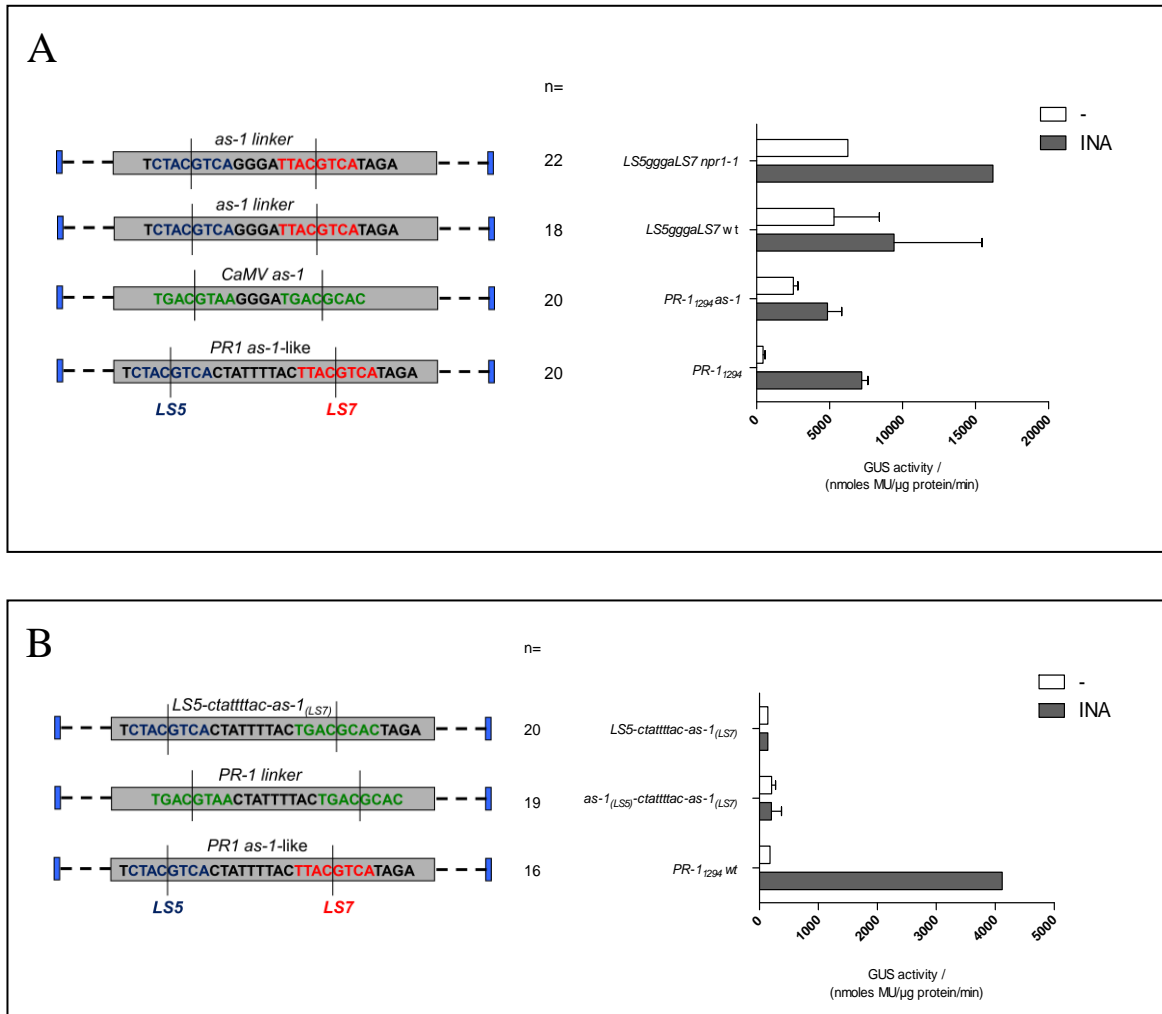
Figure 4.10: GUS activity measurements of distinct promoter derivatives containing alterations in the composition of the *as-1* element.

The MUG assays were performed with plants axenically grown on 30 µM INA- or control MS-plates. The error bars in figure **A** and **B** represent the standard deviation of two independent experiments, in case of lacking error bars the lines were examined just one time. The numbers of single lines tested in each experiment are indicated (n=). *as-1* sequence alterations are illustrated in the promoter diagrams, all mutations were integrated in the *PR-1$_{1294}$* promoter context.

The integration of the 9bp *PR-1* linker sequence within the substituted *as-1* element (*as-1$_{(LS5)}$-ctattttac-as-1$_{(LS7)}$*) leads to loss of basal expression and INA sensitivity indicating that the TGACG motifs of the *as-1* element cannot complement the function of the TGA recruitment sites of the *PR-1* promoter. The failure of the promoter construct to induce gene expression is probably due to the sequence of the TGACG motif in the *LS7* position of the *as-1* element. Whereas *LS7* of the *PR-1* promoter contains the central ACGT palindrome, the downstream TGACG motif of the *35S as-1* sequence (*as-1$_{(LS7)}$*) misses the T and might therefore constitute a weak binding site, that can only be occupied when another binding site is present at a distance (4 bps) allowing cooperative

interactions (Krawczyk et al. 2002). Consistently, INA induction also fails in the *LS5-ctattttac-as-1$_{(LS7)}$* promoter derivative (figure **4.10 B**).

4.7   The first base within the *as-1* linker region determines the overexpression capacity of the *as-1* promoter construct

The experiments conducted with the transgenic reporter gene lines revealed a central role of the *35S as-1* linker sequence for the increased promoter activity. A more detailed analysis of this 4bp spanning sequence was performed by transient protoplast transformation experiments using *PR-1$_{1294}$ as-1* constructs carrying distinct sequence alterations in the critical linker region.

In our hands, transiently transformed protoplasts are not an optimal system to investigate *PR-1* expression due to a high background activity of transiently introduced promoter constructs and the failure of SA- and INA-mediated induction. Nevertheless, *PR-1* promoter constructs containing the *35S as-1* element instead of the *L5-LS7* region show increased expression known from the results obtained with transgenic plants (figure **4.11 A**). Thus, the sequence requirements of the *35S as-1* element can be studied in this system. The *PR-1$_{816-573}$*- and *PR-1$_{816-573}$ as-1* promoter derivatives show weaker expression levels than the corresponding *PR-1$_{1294}$* constructs. Thus, the constitutive activity of the promoter might be due to de-repressed WRKY-transcription factors binding to this region. Consistently, the expression of the *PR-1$_{1294}$* promoter fragment in protoplasts was essentially abolished when the W-box cluster was deleted (figure **4.11 B**). Interestingly, the constructs *as-1$_{(LS5)}$-ctattttac-as-1$_{(LS7)}$* and *LS5aaaaLS7*, which are inactive in the transgenic situation, are as strongly expressed as *PR-1$_{1294}$*, indicating that the repressive effect of LS5/SNI1 may not be operational any more.
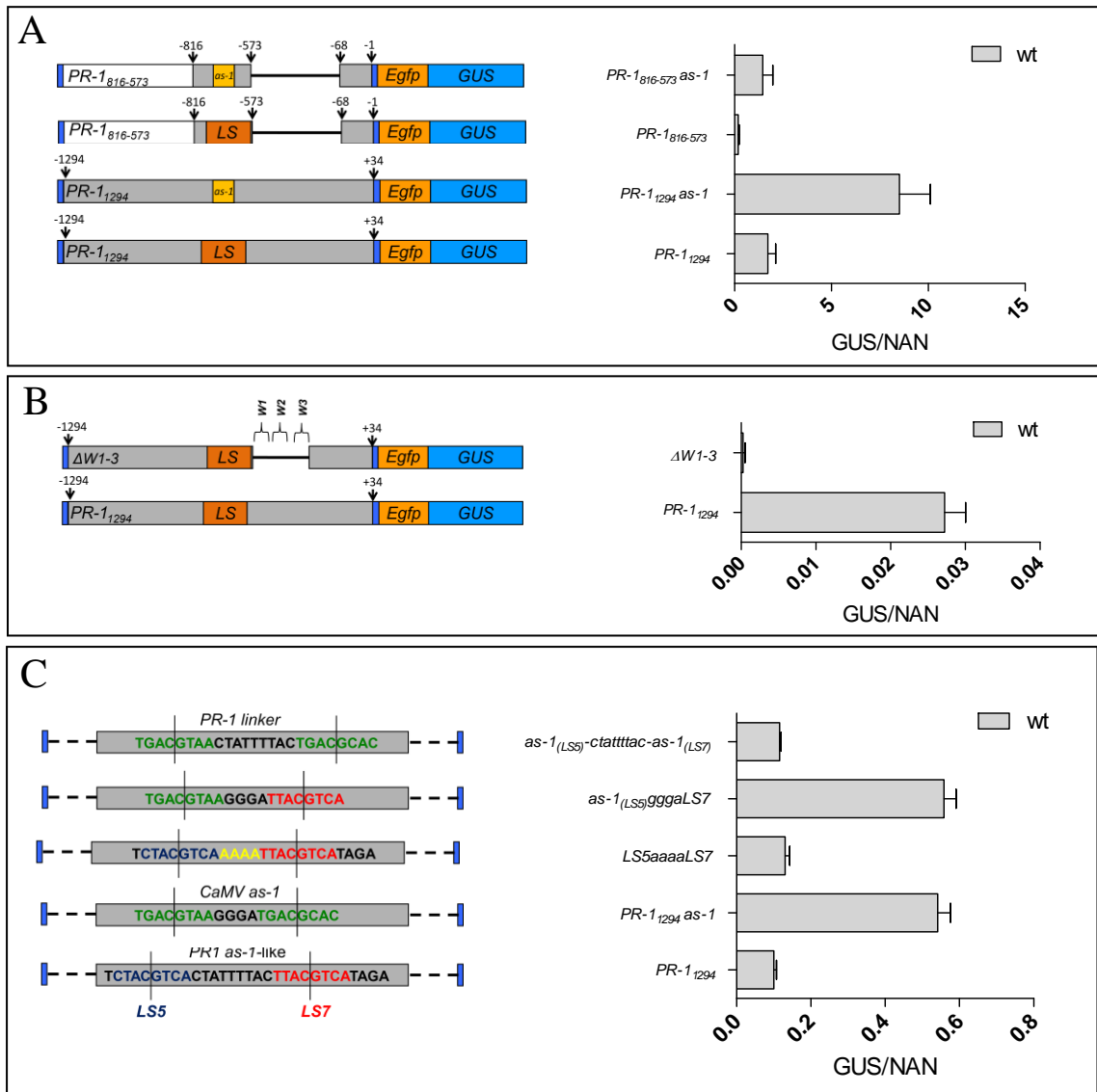
Figure 4.11: Transient expression analysis of distinct *PR-1* promoter constructs carrying sequence alterations at the site of the *as-1*-like element (A+C) or a W-box cluster deletion (B).

The figures show GUS activities obtained from transient transformations of distinct promoter:*GUS* constructs in *A. thaliana* protoplasts. Leaves of 3-week-old soil grown and non-induced wt plants were used for protoplast isolation. The y-coordinates demonstrate the ratio of GUS activity to the internal NAN standard. Figure **A** shows the impact of the *35S as-1* element in the *PR-1$_{1294}$*- or *PR-1$_{816-573}$* promoter context on increased reporter gene activity, while **B** displays the effect of a W-box cluster deletion (between -546 to -484) on constitutive expression. In **C** the increased reporter activities of constructs with integrated GGGA linker sequence are illustrated in combination with *as-1*-mutated constructs which do not cause enhanced expression. The error bars represent the standard deviation derived from 4 replicates. The orange *LS*-labeled boxes in the promoter diagrams in **A** and **B** illustrate the *LS*-region; the smaller yellow boxes display *as-1* element substitutions against *35S as-1*. Blue boxes display the gateway® cloning sites, grey shaded regions illustrate *PR-1 cis*-regulatory regions and the white box shows sequence substitutions against the coding sequence of the *CAT* gene. Arrows and numbers show base pair positions. The sequences of the *as-1* element mutations examined in **C** are indicated in the promoter illustrations. *LS5* is labeled blue, *LS7* red, and the TGACG motifs of the *35S* element are labeled green. The bars indicate the palindromic centers of the TGACG motifs.
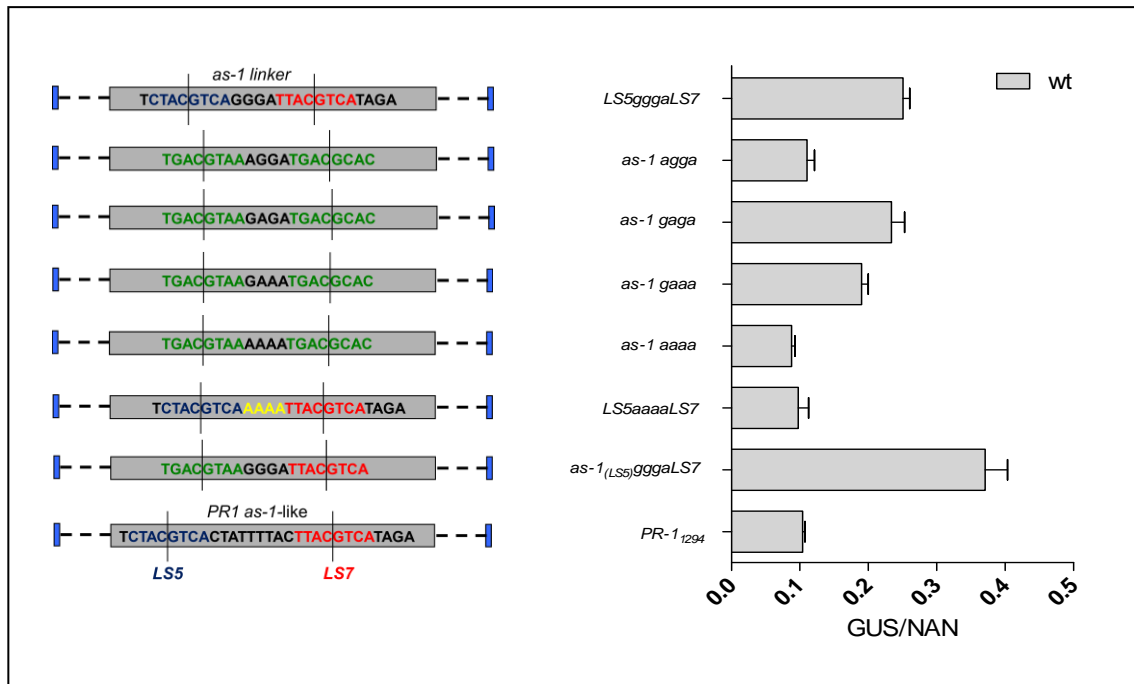
Figure 4.12: Analysis of the *35S as-1* linker sequence with regard to transcriptional activation.

Leaves of 3-week-old soil grown and non-induced wt plants were used for protoplast isolation and subsequent transformation. The influence of the *as-1* linker sequence on increased promoter activity is displayed. Error bars represent the standard deviation of 4 replicates. The TGACG sequences derived from the *PR-1* promoter are dyed blue (*LS5*) and red (*LS7*), while the TGA recruitment sites of the *35S as-1* element are colored green. Bars indicate the palindromic centers of the TGACG motifs, all mutations are integrated in the $PR-1_{1294}$ promoter context.

In figure **4.12** the GUS activities of $PR-1_{1294}$ *as-1* constructs harboring different linker sequences are displayed. The first base within the *35S* linker seems to be critical for *as-1*-mediated activation as revealed by the $PR-1_{1294}$ *as-1 gaaa* construct, which shows increased reporter gene activity as compared to $PR-1_{1294}$ *as-1 aaaa*. The $PR-1_{1294}$ *as-1 agga* derivative indicates wildtype background expression, again supporting the relevance of the first guanine.

4.8   Substitution of the *as-1* element within the *PR-1* promoter changes the influence of subgroup II TGA factors on expression

The analysis of the function of the *35S as-1* element in the *PR-1* promoter context implies that the linker sequence between the palindromes determines whether an activating protein can be recruited. This activator might interfere with the repression of the promoter through *LS5*. Class II TGA factors (TGA2, TGA5 and TGA6) have been shown previously to be essential for the activation of promoters containing *35S as-1-like* elements (Fode et al. 2008). These factors are also responsible for the strong activation of *PR-1$_{1294}$ as-1* as revealed by complete loss of promoter activity in the *tga2 tga5 tga6* triple mutant (Figure **4.13 A**). Thus, it seems likely, that these factors are necessary for the recruitment of a yet unknown factor that requires the g in the first position. Nevertheless, in the absence of the subclass II members, the *35S as-1* element can still repress the *LS4*-dependent activation pathway, which is activated when *LS5* is mutated. It is concluded that other TGA factors can recruit the repressor (SNI1?) to *LS5*. Whereas class II TGA factors are essential for the induction of promoters that are activated by INA in an NPR1-independent way (Blanco et al. 2005; Fode et al. 2008), conflicting data have been reported concerning their role for *PR-1* expression. The *PR-1$_{1294}$* promoter transformed in *tga2 tga5 tga6* protoplasts shows an increased constitutive expression in relation to the wildtype control (figure **4.13 B**), indicating that the subgroup II members are at least partially acting as transcriptional repressors in the *PR-1* promoter context. This conclusion is consistent with increased basal *PR-1* expression measured previously in the triple mutant (Blanco et al. 2009; Zhang et al. 2003).
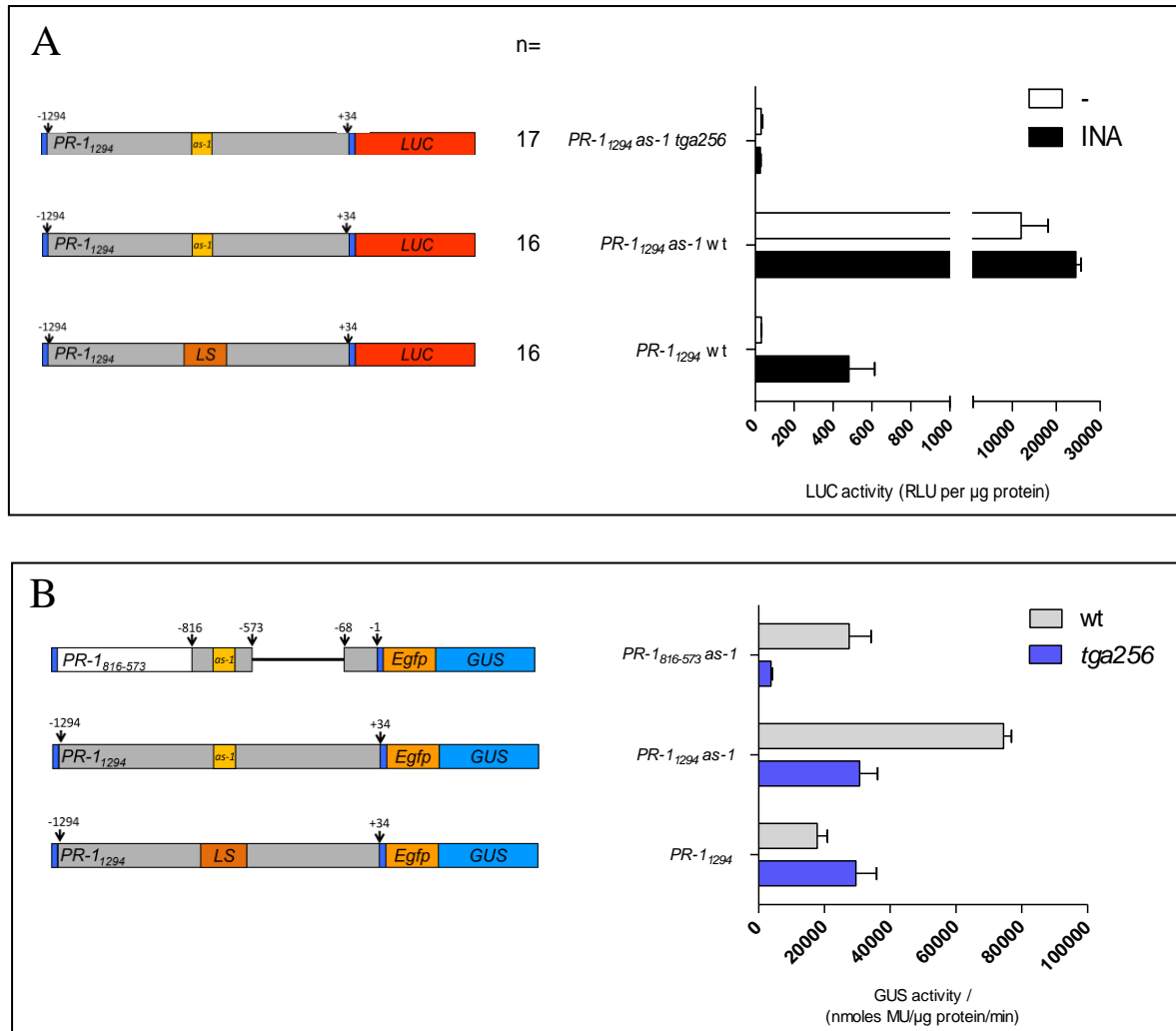
Figure 4.13: Transgenic- (A) and transient (B) expression analysis of the *PR-1$_{1294}$ as-1* promoter derivative in wt and *tga2 tga5 tga6*.

Leaves of 3-week-old soil grown and non-induced wt and *tga256* plants were used for protoplast isolation and subsequent transformation. **A** illustrates the activity of the *PR-1$_{1294}$ as-1* reporter constructs in wt and *tga2 tga5 tga6*. The number of investigated lines is indicated (n=) and the error bars show the standard deviation of two independent experiments. Figure **B** shows the promoter activities of the *PR-1$_{1294}$*, *PR-1$_{1294}$ as-1* and *PR-1$_{816-573}$ as-1* constructs gained from wt- and *tga2 tga5 tga6* protoplast transformations. The ordinate displays relative GUS activity and the error bars indicate the standard deviation of four replicates. The grey columns illustrate expression levels in wt protoplasts while the blue columns correspond to the *tga2 tga5 tga6* mutant. The promoter illustrations on the left display the *LS*-region (orange) or a substituted *as-1* element (yellow). Blue boxes display the gateway® cloning sites, grey shaded regions illustrate *PR-1 cis*-regulatory regions and the white box shows sequence substitution against the coding sequence of the *CAT* gene. Arrows and numbers indicate base pair positions.

4.9 *In vitro* binding studies reveal a similar recruitment of TGA factors to the *35S as-1-* and *as-1*-like element

The *as-1* element substitution experiments presented so far illustrate that the composition of this regulatory unit has a severe impact on the activity of the *PR-1* promoter. A possible explanation for the differences in promoter activity is a differential recruitment of TGA factors to the distinct types of *as-1* elements. Therefore EMSA experiments were performed to monitor the recruitment of TGA factors to the *35S* as-*1* element and the *as-1*-like element derived from the *PR-1* promoter under inducing- and non-inducing conditions.
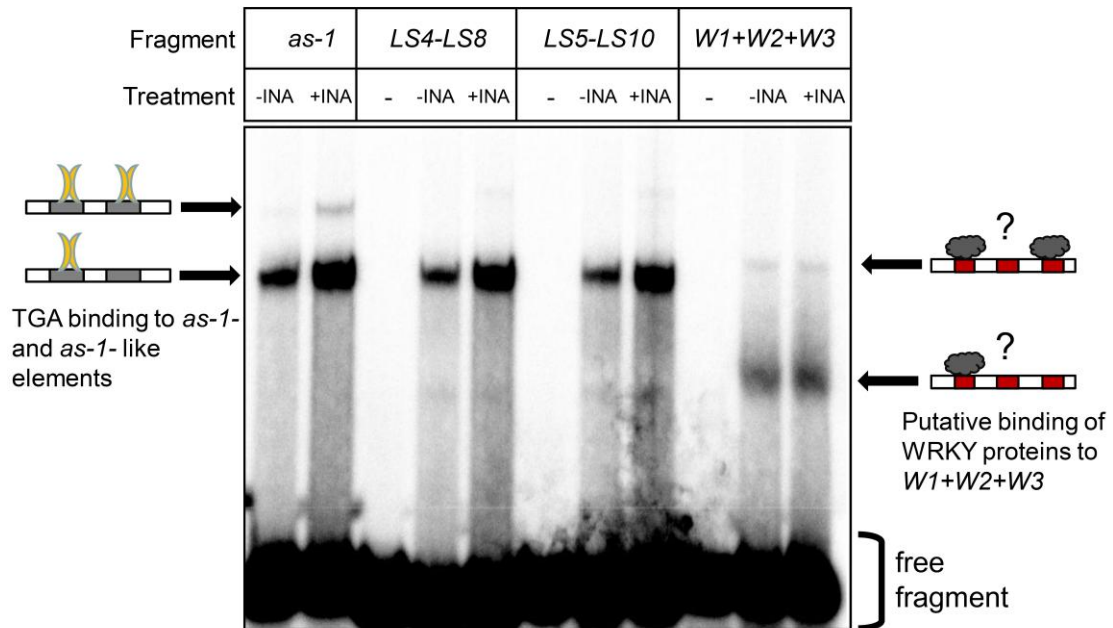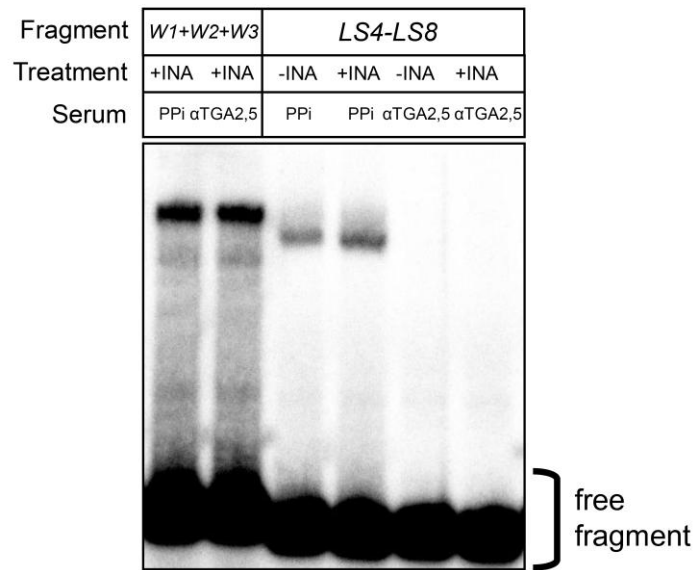

Figure 4.14: *In vitro* **binding study of TGA factor recruitment to the** *35S as-1-* **and the** *PR-1 as-1-***like element.**

The nuclear extracts used in this EMSA were obtained from 14-day-old wt plants grown on MS-medium supplemented with- or without 30μM INA. The *as-1* fragment is derived from the *35S* promoter while *LS4-LS8, LS5-LS10* and *W1+W2+W3* contain regulatory sequences of the *PR-1* promoter. The bolt arrows indicate single- or double occupancy of the fragments while the question marks illustrate a speculated binding of unknown proteins.

INA treated samples show an increased TGA binding affinity to the two different types of *as-1* elements (figure **4.14**). In case of the *35S as-1* fragment, an additional band appears above the solid band illustrating recruitment of TGA homo- or heterodimers to both TGACG motifs while the *as-1*-like element only shows a single occupancy (see

black arrows in figure **4.14**). The rightmost fragment displayed on the EMSA contains the *PR-1* W-box cluster localized between -546 and -484. Contrary to expectation, this fragment shows constitutive protein recruitment and no alteration when the plants were grown on INA plates. Many WRKY genes exhibit increased transcription after SA- or INA treatment; hence, a strengthened WRKY recruitment to the *W1+W2+W3* fragment would be reasonable.

The specific binding of TGA factors to the *as-1* element was proven by supershift experiments conducted with whole cell protein extracts as displayed in figure **4.15**.

Fragment	*W1+W2+W3*		*LS4-LS8*			
Treatment	+INA	+INA	-INA	+INA	-INA	+INA
Serum	PPi	αTGA2,5	PPi	PPi	αTGA2,5	αTGA2,5

**Figure 4.15: Supershift experiments performed with αTGA2,5 antiserum on the *W1+W2+W3* and *LS4-LS8* promoter fragments.**

The whole cell protein extracts were obtained from 14-day-old wt plants grown axenically on 30μM INA- or control medium plates. The *W1+W2+W3* construct serves as a negative control to show the specific effect of the serum on *as-1*-like element binding proteins. PPI designates addition of preimmune serum while antiserum treatment is indicated by αTGA2,5.

The recognition of TGA transcription factors by the αTGA2,5 antiserum seems to inhibit the DNA binding of the bZIP proteins as no bands appear on the EMSA when the antibody is added to the samples. This loss of recruitment is specific for the *as-1*-like element, since the W-box cluster fragment does not show an influence of the serum on general protein binding properties. Surprisingly the W-box control shows a different band pattern than observed in EMSAs performed with nuclear extracts (see figure **4.14**). Though TGA association to the two different *as-1*-like elements occurs in a similar way it may be that differing compositions of TGA family members recruited to the regulatory sequence are responsible for the distinct impacts on global promoter activity.

This idea was pursued by investigating TGA recruitment in several *tga* mutant plants (figure **4.16**).

The two EMSA experiments imaged in figure **4.16** show a similar pattern of TGA factor recruitment when distinct members of the subgroup II TGA family are mutated. The *tga2 tga5 tga6* triple mutant reveals that mainly the subclass II members contribute to the shifts observed in the EMSAs as just faint background signals are detectable in the SA-induced samples when all three proteins are missing. The *tga6* mutant does not influence binding to the fragments while the *tga2 tga5* double mutant displays a more severe effect as the protein recruitment seems to be reduced illustrated by the absence of an upper band in case of the *as-1* fragment and by general weaker signal intensities. Though there is no more *in vitro* TGA factor recruitment in the triple mutant, the TGACG motifs are capable to recruit other proteins or TGA family members in this mutant *in vivo*. As the *PR-1$_{1294}$ as-1* construct transformed in *tga2 tga5 tga6* shows no expression at all the *LS5*-mediated suppression of the *as-1*-independent induction pathway is still operational.
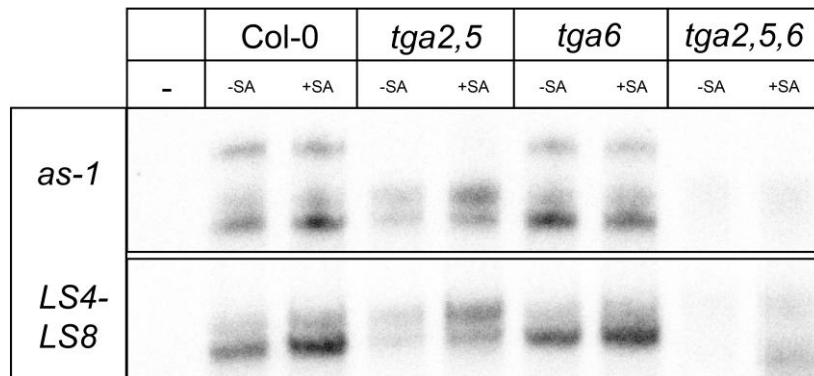
Figure 4.16: *In vitro* **binding study of TGA factor recruitment to the two types of *as-1* elements in subgroup II mutants.**

The 14-day-old plants used for whole cell protein extraction were grown on MS-medium and treated with 1mM SA solution or H$_2$O 24h before harvesting the material. The figure shows two EMSA experiments exhibiting TGA recruitment to the two examined types of *as-1* elements in subgroup II *tga* mutants.
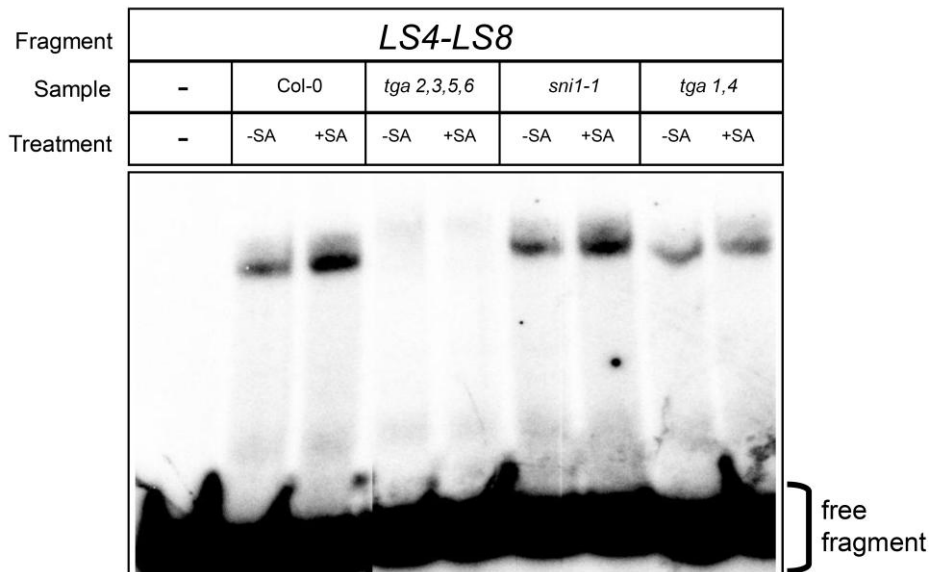
Figure 4.17: *In vitro* **binding studies of TGA factor recruitment to the PR-1 *as-1*-like element in different mutant backgrounds.**

Whole cell protein extracts were obtained from 14-day-old plants axenically grown on MS-plates and sprayed with 1mM SA or H$_2$O 24h prior to harvest. The figure displays the TGA factor association to the *LS4-LS8* fragment in *tga2,3,5,6*, *sni1-1* and *tga1,4*.

The gel shift experiments clearly show the redundant character of subgroup II members. The recruitment of TGA factors to *as-1* elements can at least be complemented partially when other subgroup members are absent as illustrated by the *tga2 tga5* and the *tga6* mutants.

Figure **4.17** shows an EMSA performed with the *LS4-LS8* fragment and whole cell protein extracts from *tga2 tga3 tga5 tga6*, *sni1-1* and *tga1 tga4*. While the *tga* quadruple mutant extracts demonstrate no TGA binding there seems to be no impact of SNI1 and the TGA subgroup I members on *in vitro* TGA recruitment to the *as-1*-like element. Thus, it seems that SNI1 does not influence promoter activity by modification of TGA factor DNA-binding affinities.

4.10   *LS10* contributes to *PR-1* induction in wt and *sni1-1*

It was reported previously that beside *LS7* the DOF (<u>D</u>NA-binding with <u>o</u>ne <u>f</u>inger) binding motif containing element *LS10* shows a positive effect on *PR-1* expression (Lebel et al. 1998). The function of *LS10* in SNI1-dependent- and independent regulation was evaluated in more detail by investigating reporter gene activities of *LS10*mut and *LS7/10*mut constructs in wt and *sni1-1* mutants. The general inducibility of the *LS10*mut promoter derivative is reduced but not abolished in wt plants (figure **4.18 A**), illustrating that *LS10* fulfills positive functions in *PR-1* induction. This is supported by the decreased basal- and induced expression of *LS10*mut in *sni1-1*. A combination of *LS7* and *LS10 cis*-element mutations determines a complete loss of induction in both genotypes (figure **4.18 B**). Since it has been reported that DOF transcription factors can stimulate TGA factor binding to *as*-1 elements (Chen et al. 1996) it is reasonable that *LS10* is functioning by modulating TGA factor recruitment to *LS7*. Taken together, *LS7* and *LS10* display additive effects as *LS7/10*mut shows a more severe impact on *PR-1* expression than the single-mutated promoters and are therefore of universal importance for the induction of *PR-1*.
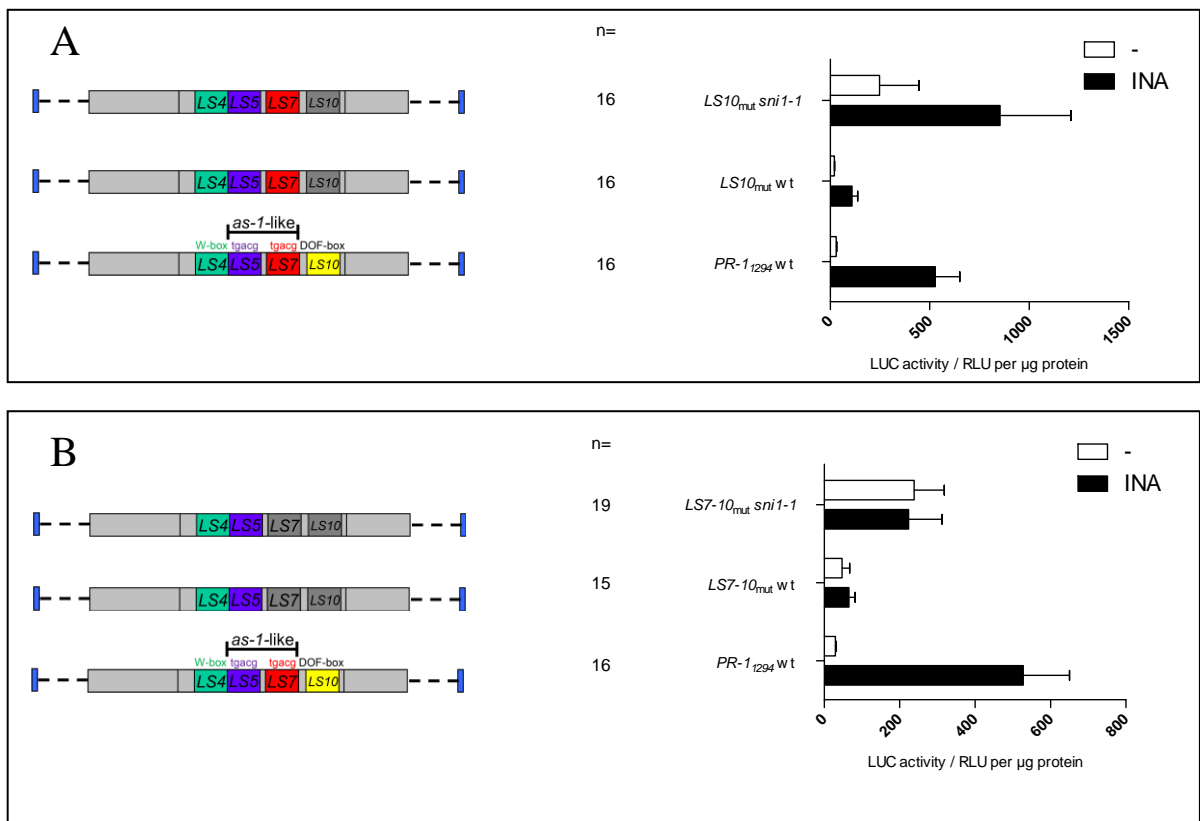
Figure 4.18: Relative average LUC activities of the promoter constructs *LS10*mut and *LS7/10*mut in wt and *sni1-1*.

The figure **4.18** illustrates the LUC activities of 18-day-old plants axenically grown on MS-plates supplemented with- or without 30µM INA. Figure **A** shows the expression of *LS10*mut in the different genotypes while **B** displays the combination of both promoter element substitutions (*LS7/10*mut) in wt and *sni1-1*. The number of lines investigated for calculation of the average LUC activities is indicated (n=), the error bars represent two independent experiments. The dark grey shaded boxes in the promoter illustrations display 10bp mutations in the corresponding *cis*-elements.

4.11 The *cis*-elements *LS4* and *LS10* contribute to the overall overexpression competence of the substituted *35S as-1* element

The constitutive activation of the *PR-1$_{1294}$ as-1* promoter is obviously dependent on the recruitment of an additional activator which may localize to the linker sequence of the substituted *as-1* element via interaction with subgroup II TGA factors. The conclusion that the *as-1* element enables constitutive expression is not consistent with previously published data as an *as-1* element containing minimal *35S* promoter does not show increased basal activity (Redman et al. 2002). Instead the *as-1* element is thought to be critical for SA- and auxin sensitivity. It seems that the strong overexpression determined by the *35S as-1* element in the *PR-1* promoter context is dependent on surrounding regulatory sequences which are capable to boost transcriptional activity synergistically. For instance the overexpression capacity as well as the inducibility of *35S as-1* substituted *PR-1* promoter constructs is partially dependent on the W-box cluster downstream of the *LS*-region (figure **4.9**). The influence of regulatory elements adjacent to the *as-1* element was examined by combining *LS4-* and *LS10* mutations with *35S as-1* element substitutions (figure **4.19**). Mutations in the W-box (*LS4*$_{mut}$) or the putative DOF transcription factor recruitment site (*LS10*$_{mut}$) cause decreased *35S as-1* element mediated reporter gene activities. The reduction in activity is not very strong and the general character of the overexpression inducing *35S as-1* element is not changed. In conclusion, it seems that the *cis*-elements *LS4* and *LS10* act as positive enhancers for the constitutive expression conditioned by the *35S as-1* element, though there are maybe other regulatory sequences which contribute to the increased transcriptional activity.
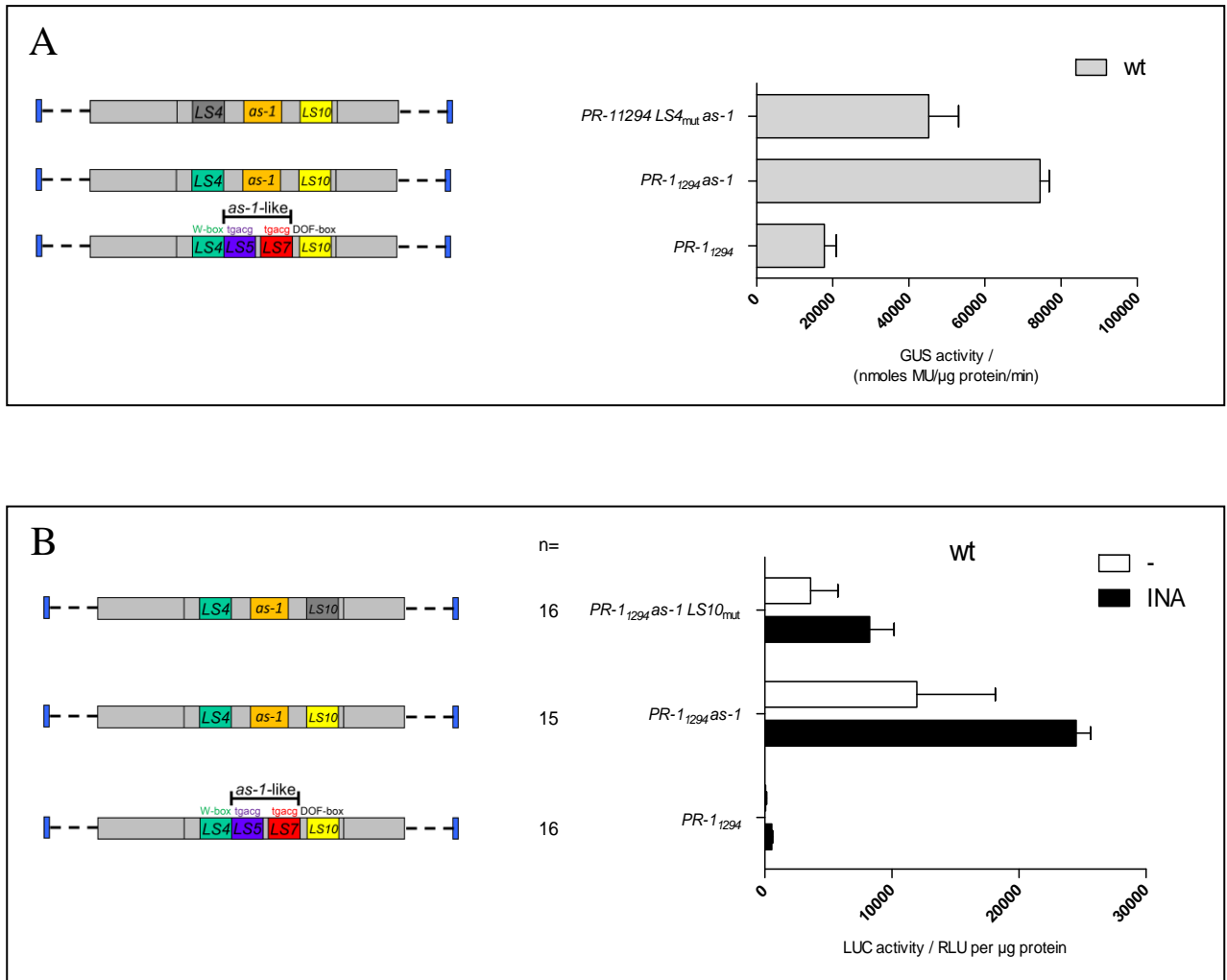
Figure 4.19: Transient- (A) and transgenic (B) expression analysis of the *PR-1$_{1294}$ LS4$_{mut}$ as-1* and *PR-1$_{1294}$ as-1 LS10$_{mut}$* promoter derivatives.

Transient transformations were performed with isolated leaf protoplasts from 3-week-old soil grown and non-induced wt and *tga256* plants (**A**). Each error bar represents the standard deviation of 4 replicates. The transgenic lines illustrated in (**B**) were grown on MS-plates supplemented with- or without 30µM INA. The number of investigated single lines is indicated (n=), the error bars show the standard deviation of two independent experiments. A magnified view on the LS-region is shown in the promoter illustrations. The PR-1 promoter illustrations show the different sequence alterations tested in tThe *35S as-1* element is colored yellow, 10bp linker scan mutations are indicated by the dark grey shaded boxes.

5   Discussion

The salicylic acid-inducible *PR-1* gene serves as a marker gene that is subject to the same regulatory mechanisms that control systemic acquired resistance. Therefore, analysis of the molecular mechanisms regulating this gene is of major importance for understanding plant defense responses. Classical promoter deletion and linker scanning analysis have previously identified critical *cis* regulatory sequences within the so called *LS*-region of the promoter (Lebel et al. 1998). Complementary genetic approaches have led to the identification of the *trans*-regulatory factors NPR1, SNI1 and TGA (Cao et al. 1994; Kesarwani et al. 2007; Mosher et al. 2006; Zhang et al. 2003) The study presented here combines both approaches by analyzing distinct *PR-1* promoter derivatives in different mutant backgrounds implicated in SA signaling and *PR-1* regulation. This strategy yielded the following novel information: (1) The promoter contains a W-box cluster downstream of the *LS*-region which contributes to *PR-1* induction and hyperinducibility in *sni1-1*. (2) The corresponding WRKY transcription factors are regulated in an NPR1/SNI1-dependent manner (3) The *as-1*-like element presumably serves as direct target site for TGA/NPR1 mediated regulation. (4) NPR1-dependent regulation at *as-1* elements is only operational when the distance between the TGACG-motifs is not too close. (5) The *as-1*-like element is not essential for *PR-1* induction, as an alternative pathway can be activated in the absence of the TGACG motif *LS5*. (5) A TGA/SNI1 complex is potentially formed at the *LS5* site to suppress the *as-1*-independent pathway. (6) Induction of the *as-1*-independent mechanism is influenced by WRKY factors whose regulation is dependent on NPR1 and SNI1. (7) The sequence between the palindromic repeats determines the recruitment of transcriptional activators or repressors. The data obtained in this study give rise to detailed models of *PR-1* regulation.

One of the most unexpected results of this work is the finding that the *as-1*-like element, which has been considered as the crucial binding site for NPR1-dependent regulation of the promoter, is dispensable for induction (*LS5/7* mut, figure **4.3**). In the absence of this element, an alternative pathway can be activated. The function of the different *cis* elements of the *LS*-region, which has been found previously to contain critical *cis*

elements (Lebel et al. 1998), and the W-box cluster between -573 and -486, is discussed with respect to their functional relevance for the two pathways.

5.1   A W-box cluster enhances *PR-1* expression and integrates signals of SNI1-repressed activators

A novel regulatory region identified in this study is the W-box cluster located between -573 and -486 relative to the transcriptional initiation site. When compared to the wildtype promoter, the W-box cluster-lacking *PR-1$_{816-573}$* construct shows reduced transcription after INA treatment, indicating that it contributes to maximal expression, but that it is not essential. However, these W-boxes are required for the strong hyperinduction of the promoter in *sni1-1* (figure **4.2 B** and **4.7**). As hyperinducibility depends on the *as-1*-element in the *LS*-region (see figure **4.4** and below), we speculate that the TGA/NPR1 complex synergistically interacts with WRKY factors at the downstream W-boxes.

WRKY proteins are members of a large class of over 70 DNA-binding proteins in *Arabidopsis* (Eulgem and Somssich 2007) that can either positively or negatively affect transcription of either pathogen- or senescence-regulated genes. The function of individual members of this family has been addressed by analyzing knock-out mutants and plants with ectopic expression. Ectopic expression of *WRKY70* yields constitutive activation of *PR-2* and *PR-5* (Li et al. 2004). In the case of *PR-1*, an aberrantly long mRNA appears upon mock treatment, but the SA-regulated accumulation of the wildtype transcript is not altered. All three genes usually show a similar expression pattern during SAR or after SA treatment and are therefore grouped in the same regulon (Maleck et al. 2000). One main difference between the *cis*-regulatory sequences of these genes is the absence of an *as-1*-like element in case of *PR-2* and *PR-5*. As a number of evidences of this study point at the *as-1*-like element functioning as an indirect target site for SNI1, we speculate that the *PR-1* promoter is in a "locked" state, where WRKY factors cannot achieve any activation, unless SNI1 is counteracted by activated NPR1. In contrast, *PR-2* and *PR-5*, which are presumably not under the direct control of SNI1, can be activated by ectopically expressed WRKY factors.

Interestingly, ectopic expression of *WRKY18* can lead to constitutive *PR-1* expression, which is dependent on NPR1. However, this constitutive expression only occurs in at least 4 week-old plants. Nuclear localized NPR1 may accumulates in older leaves and this accumulation could be sufficient to interfere with SNI1 action so that activation of *PR-1* by *WRKY18* can occur in the absence of INA.

One approach to investigate regulated events at the W-box cluster was undertaken by EMSA experiments to monitor the protein recruitment to the *PR-1* W-box cluster under non-inducing and inducing conditions (figure **4.14** and **4.15**). Surprisingly, the *W1+W2+W3* fragment showed constitutive protein binding instead of an inducible shift, though other groups reported altered protein recruitment to similar W-box fragments under non- and SA-induced conditions (Dong et al. 2003; Knoth and Eulgem 2008). An explanation for the lacking inducible shift may be that our EMSA conditions or the protocol used for protein extraction are not capable to reveal the protein recruitment occurring at the W-boxes *in planta*. Additionally, the gel shifts revealed that different proteins are recruited to *W1+W2+W3* as EMSAs conducted with nuclear extracts show a distinct band pattern than EMSAs performed with whole cell extracts (compare figure **4.14** and **4.15**).

5.2   The *PR-1* promoter is regulated by an *as-1*-dependent- and *as-1*-independent induction pathway

In previous studies, the 1294 bp *PR-1* promoter with a mutated *LS5* element showed slightly increased basal and induced expression levels (Lebel et al. 1998). Therefore, it was concluded that the *LS5* element is a negative-acting TGA recruitment. Increased expression levels in $LS5_{mut}$ lines could not be detected in our experiments, but the negative function of this element became evident after analysis of the $LS5/7_{mut}$ construct (figure **4.3**): While mutation of the more downstream located TGACG motif (*LS7*) causes a considerable decrease in reporter activity, the additional mutation of *LS5* restores induction. It is concluded that *LS5* represses a pathway that is independent from *LS5* and *LS7* and which we call the *as-1*-independent pathway. Mutation of *LS5* and the adjacent W-box *LS4* ($LS4/5_{mut}$) leads to an abolished expression under inducing conditions (figure **4.3**). This result indicates that the W-box *LS4* is essential for the *as-1*-

independent pathway. As *LS4* is dispensable for the *as-1*-dependent pathway (figure **4.3**), this construct reveals that *LS5* is required for the *as-1*-dependent pathway.

5.3   The *as-1*-independent pathway requires NPR1

Analysis of the transgenic *LS5/7*$_{mut}$ lines revealed that NPR1 is still essential for the induction of this promoter construct. At first glance, this seemed unexpected, as the *as-1*-like element has been hypothesized before to function as the recruitment site for NPR1. However, a number of NPR1-dependent genes, like for instance *PR-2* and *PR-5* do not contain a TGACG motif, indicating that NPR1 might either control gene activity indirectly through the regulation of e.g. WRKY transcription factors, which contain TGACG motifs and are therefore under direct control of NPR1 (Dong et al. 2003; Wang et al. 2006). This seems likely, as the *as-1*-independent pathway depends on *LS4*, which might recruit such SA-inducible, NPR1-dependent WRKY transcription factors. Alternatively other proteins might be able to recruit NPR1 to the *LS5/7*$_{mut}$ promoter. Recruitment of NPR1 to the *PR-1* promoter has been detected by chromatin immunoprecipitation experiments in the *tga2 tga5 tga6* mutant, which lacks the so called subclass II TGA factors (Rochon et al. 2006). However, other TGA factors may complement the function of the subclass II members. In addition, no conserved *TL1* motifs, associated with NPR1-mediated regulation of genes involved in the secretory pathway (Wang et al. 2005), are present in the *PR-1* promoter. We consider it most likely that INA-activated NPR1 induces transcription of WRKY factors, which are in turn binding to *LS4* and possibly the W-box cluster, thus leading to the up-regulation of *PR-1*. *PR-1* is an unusually complex promoter as it can be under direct and indirect regulation of NPR1. The *as-1*-element provides the promoter with the advantage of a tighter regulation as revealed by the decreases background levels of the *PR1*$_{1294}$ promoter as compared to the *LS5/7*$_{mut}$ promoter.
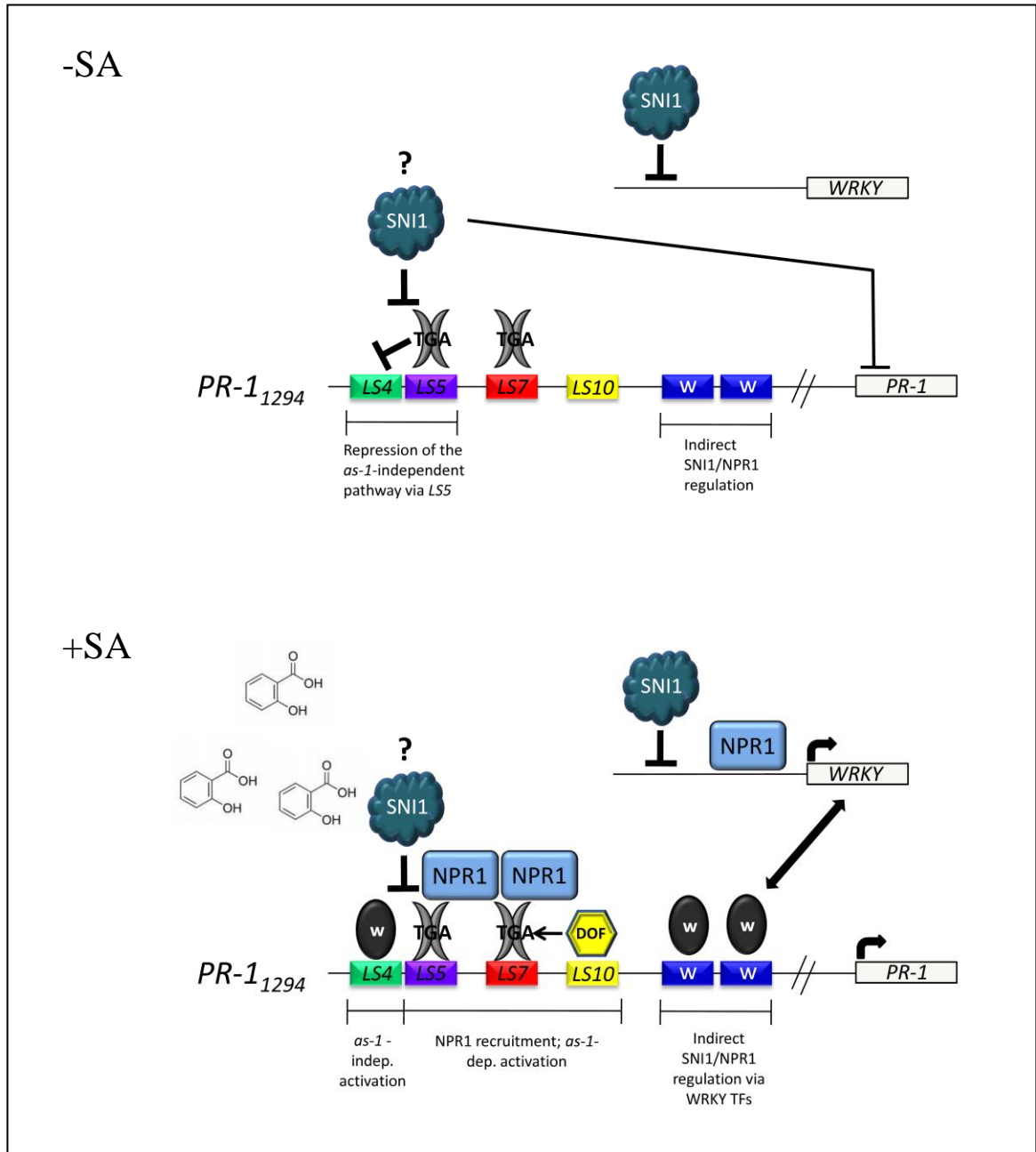
Figure 5.1: Model of *PR-1* regulation under non-inducing- and inducing condition in wt.

SNI1 represses the *PR-1* promoter via the *as-1*-like element and indirectly via repression of WRKY TFs. When SA levels rise, NPR1 is shuttled to the nucleus and interacts with TGA TFs whose DNA binding affinity is increased under these conditions. NPR1 also induces WRKY TFs which serve as additional activators for *PR-1* induction. The *as-1*-independent induction pathway (*LS4*) is constitutively repressed via *LS5*. A putative SA-inducible DOF transcription factor enhances TGA binding to the promoter.

5.4 SNI1 might be the repressor acting at *LS5* to repress the *as-1*-independent pathway

One hypothesis to explain the negative effect of *LS5* on the *as-1*-independent transcription is that SNI1 is responsible for this effect. Consistent with this idea is that the *as-1*-independent pathway shows increased basal expression levels, a phenomenon also observed in *sni1-1*. Supporting data for this assumption were obtained from reporter gene constructs harboring the two original TGACG motifs *LS5* and *LS7* but an altered distance between the centers of the palindromic repeats (*LS5aaaaLS7*). This promoter showed only very weak sensitivity to INA, which indicates that repression of that *as-1*-independent pathway was operational. In contrast, the *as-1*-dependent pathway, which depends on the direct recruitment of NPR1 to the TGA factors at the *as-1*-like element, did not seem to function (figure **4.8**). Intriguingly, the *LS5aaaaLS7* construct is active in *sni1-1* illustrating that SNI1 could be the repressor of this construct. The induction of *LS5aaaaLS7* is not as strong as the *PR-1$_{1294}$* control in *sni1-1*. This is consistent with the observation made with the *LS5/7*$_{mut}$ promoter (figure **4.4**) that a functional *as-1*-element is required for hyperinduction of the *PR-1* promoter in the presence of INA.

5.5 *LS4* is a positive element in the absence, and a negative element in the presence of *LS5*

The W-box *LS4* was previously reported to be a negatively acting *cis*-element as mutation of this box yielded an approximately 4-fold increased reporter gene expression in wt after INA induction (Lebel et al. 1998). This result led to the hypothesis that SNI1 regulation may functions via WRKY transcription factor interaction to suppress the activity of the promoter (Kesarwani et al. 2007). Though experiments conducted with *LS4*$_{mut}$ constructs presented in this work also showed an approximately 2-fold increased average expression level as compared to *PR-1$_{1294}$* control plants, combinatorial promoter sequence mutations (*LS4/5*$_{mut}$ and *LS4/7*$_{mut}$; figure **4.6**) clearly revealed an additional positive function of this element concerning *PR-1* induction by providing an *as-1* independent activation mechanism. The factor recruited to *LS4* usually mediates a weak suppression on the *as-1*-dependent pathway, but mutation of the TGACG motifs

turns the character of *LS4* from a weak silencing- to a critical enhancing *cis*-element. Since WRKY transcription factors can act as transcriptional activators or repressors (Eulgem and Somssich 2007), the conversion of *LS4* function maybe associated with the recruitment of different WRKY transcription factors in case of a present- or lacking *as-1*-like element. For instance the endogenous *PR-1* promoter may recruits WRKY38 and/or WRKY62 to *LS4*, two SA-inducible WRKY TFs known to be negative regulators of *PR-1* expression (Kim et al. 2008). When the TGA binding sites are mutated (*LS5/7*$_\text{mut}$) binding affinity for a positive-acting WRKY to *LS4* could be increased (e.g. WRKY18 or WRKY70) and the *as-1*-independent induction pathway is established. In addition, the *LS4*$_\text{mut}$ construct was examined in *npr1-1* (see supplemental data; table **6.3.1**) to rule out the possibility that mutation of the putative SNI1 recruitment site leads to NPR1-independence, similar to the events in the *sn1 npr1* double mutant. As expected, no increased reporter gene activities were detected when these lines were grown on INA plates. In summary, it is unlikely that *LS4* is the recruitment site for SNI1 but it contains a putative binding site for a positive regulator which mediates *as-1*-independent induction if repression through LS5 is released.

5.6 The LS7 element is essential for *PR-1* induction in *sni1-1*

In contrast to constructs lacking a functional *LS7* sequence (*LS4/7*$_\text{mut}$, *LS7*$_\text{mut}$ or *LS7/10*$_\text{mut}$) the inducibility of *LS4/5*$_\text{mut}$ was restored in *sni1-1* illustrating that a non-mutated *LS7* is needed for induction in this mutant. However, two issues have to be considered: (1) Mutation of one or both TGACG motifs is always associated with an increased basal expression in *sni1-1* similar to endogenous *PR-1* expression in the *tga2 sni1-1* double mutant (Kesarwani et al. 2007). (2) This background expression makes it hard to distinguish if a promoter is "not inducible" or "completely de-repressed". For instance, the *LS7*$_\text{mut}$ construct shows a very strong constitutive expression in *sni1-1* (figure **4.4**) but hyperinduction cannot occur as NPR1-dependent regulation is disturbed; hence, the promoter shows no inducibility. In case of the *LS4/5*$_\text{mut}$ construct the basal activity in *sni1-1* is rather moderate (figure **4.6**) and the functional *LS7* element is sufficient for a 4-fold induction. The activation of the *LS4/5*$_\text{mut}$ construct is maybe also repressed as SNI1 is constitutively repressing the promoter via *LS7*. Interestingly, *LS7* seems to mediate activation of this construct in *sni1-1* though *LS5* is mutated and the *as-1*-dependent pathway should be dysfunctional. Thus, it is concluded

that induction of the *LS4/5*~mut~ construct in *sni1-1* is achieved by a synergistic activation process mediated by a putative TGA/NPR1 complex at *LS7* and deregulated WRKY factors functioning via the W-box cluster. The importance of the W-box cluster has been shown by analysis of the *LS4/5*~mut~ *ΔW1-3* construct in this context, which did not display sensitivity to INA in *sni1-1*(figure **4.7**).

5.7   *LS7* and *LS10* are potentially both involved in TGA factor recruitment and NPR1 mediated activation

The TGACG motif containing element *LS7* shows a general positive effect on *PR-1* regulation in wt since *LS7*~mut~ causes a severe loss of induced expression. The influence of *LS7* on promoter activity is not as strong in our experiments as previously described by Lebel et al. who reported a completely abolished sensitivity to INA when *LS7* was mutated. The same holds true for a construct harboring a mutated DOF transcription factor binding site (*LS10*~mut~) which still exhibits weak inducibility in wt plants in our hands. Mutation of both elements (*LS7/10*~mut~) finally provokes an absolute insensitivity to INA treatment in wt and *sni1-1*. These results show that *LS7* and *LS10* are implicated in general activation processes as induction also fails when the repressor SNI1 is absent. Both elements probably influence expression in a synergistic way as it is reported that the DOF transcription factor OBP1 can enhance TGA4 recruitment to the sequences of *as-1* elements *in vitro* (Chen et al. 1996). The supporting function of OBP1 is dependent on DOF recruitment sites adjacent to the *as-1* elements of the *35S-* and *GST6* promoters. In case of the *PR-1* promoter this increased binding might enables efficient NPR1 recruitment. The weak inducibility of *LS7*~mut~- and *LS10*~mut~ lines potentially originates from a slightly derepressed *as-1*-independent pathway, caused by declined repressor recruitment to the *as-1*-like element or *LS5* respectively. As *LS7* only has a presumably moderate impact on the repression of the *as-1*-independent pathway, the induction is considerably decreased in relation to the *LS5/7*~mut~ construct. However, the mechanism occurring at the *LS10* element to increase *PR-1* expression remains elusive as a proof for an *in vivo* implication of DOF/TGA-interactions regarding gene expression has not been reported yet.
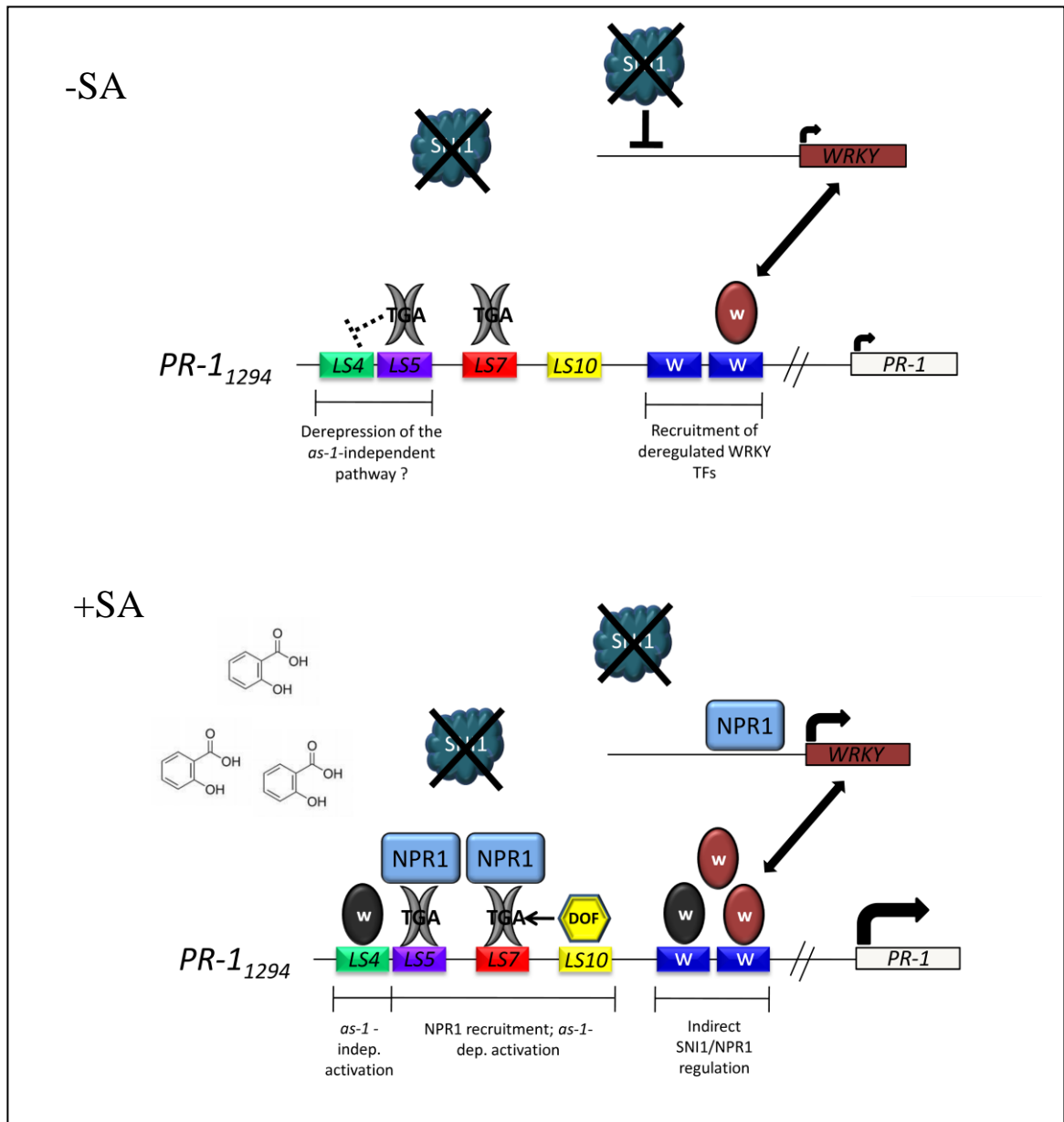
Figure 5.2: *PR-1* regulation under inducing- and non-inducing conditions in *sni1-1*.

Due to the absence of the repressor SNI1 the expression of WRKY genes is deregulated and the putative direct suppression via the *as-1*-like element is reduced leading to an increased basal expression. After induction treatment the NPR1-mediated regulation at the *as-1*-like element and the regulation at the more downstream located W-box cluster act synergistically to induce hyperinduction of *PR-1*.

5.8   *PR-1* regulation in *sni1-1* and *sni1 npr1* mutant background

Figure **5.2** displays our model for *PR-1* regulation in *sni1-1* mutant background. Mutation of SNI1 leads to derepression of positive regulators involved in defense-related processes, e.g. distinct WRKY transcription factors (Mosher et al. 2006). These de-regulated WRKY factors induce the strong expression levels monitored in *sni1-1* via the W-box cluster between -573 and -486 as revealed by the analysis of the *PR-1$_{816-573}$* construct in comparison to *PR-1$_{816-509}$ W1+W2* (figure **4.2 B**). The W-box cluster does not only boost signal intensities in *sni1-1*, it is also essential for NPR1-independent activation of the promoter as the W-box cluster lacking *PR-1$_{816-573}$* constructs shows no induction in the *sni1 npr1* double mutant (figure **4.1 B**). Beside the W-box cluster direct NPR1-dependent regulation via the *as-1*-like element contributes to the strong induction in *sni1-1*, since mutations in *LS5* and/or *LS7* lead to weaker reporter gene activities. Furthermore, endogenous *PR-1* induction in *sni1 npr1* is decreased in relation to the *sni1-1* mutant. At the genetic level, *PR-1* induction in *sni1 npr1* is dependent on RAD51D, a recombination factor identified in a mutagenesis screen for a rescued *sni1 npr1* leaf phenotype (Durrant et al. 2007). Mutation of *RAD51D* completely abolishes *PR-1* induction in the *rad51d sni1 npr1* triple mutant while the *rad51d sni1* double mutant lacks the increased *PR-1* background expression and hyperinducibility. The wrinkled leaf phenotype conditioned by the *sni1-1* mutation is reverted to wildtype leaf morphology in both mutants. These results indicate that SNI1-repressed genes are positively regulated by RAD51D, but this positive effect can only be monitored in *sni1-1* mutant plants, as *rad51d* single mutants neither show an impact on *PR-1* expression nor on the leaf phenotype. Concerning our model RAD51D maybe induces expression of the deregulated WRKY transcription factors in *sni1-1* and thereby contributes to the hyperinduction of *PR-1* in this mutant and to the general inducibility of the *sni1 npr1* double mutant via W-box cluster-mediated activation.

5.9   An activator is recruited to the *35S as-1* element in the *PR-1* promoter context

Substitution of the *as-1*-like- against the *35S as-1* element yields a substantial increase of gene expression under uninduced- (~400-fold) and induced (~50-fold) conditions in wildtype plants (figure **4.8**). The strong activation potential of the *35S as-1* element is strictly dependent upon subclass II TGA factors (figure **4.13 A**) and the linker sequence "ggga" between the TGACG motifs (figure **4.12**). NPR1 is no longer required to activate the promoter but it is still able to modulate transcription as overall expression levels are lower in *npr1-1* than in wt and *sni1-1* mutant plants (figure **4.8**). The influence of the regulator NPR1 and the inducibility are vanished in the *PR-1$_{816-573}$ as-1* construct illustrating that processes at the W-box cluster are maybe responsible for the induction and increased background expression detected with the full length *PR-1$_{1294}$ as-1* promoter derivative.

In conclusion, the *35S as-1* element recruits a strong constitutive activator to its linker sequence via TGA subclass II transcription factors but does not assure autonomous INA-sensitivity in the *PR-1* promoter context (figure **5.3**).

Potential additional proteins recruited to the *35S as-1* element belong to the classes of Myb- and NAC transcription factors, as putative binding sites for these proteins are encoded in the sequence (Baranowskij et al. 1994; Duval et al. 2002). It has been reported that the potato Myb-like protein MybSt1 specifically binds to the "ggatg" sequence of the *as-1* element and activates transcription (Baranowskij et al. 1994). Though the "ggatg" motif is changed to "ggatt" in the constitutive active *LS5gggaLS7* derivative (figure **4.10 A**) recruitment of a Myb factor cannot be excluded as Baranowskij et al. showed that a perfect binding site for MybSt1 ("ggata") displayed 20-30 fold higher binding affinity than the *as-1* sequence. This result shows that the most downstream located guanine in the "ggatg" binding site does not necessarily increase Myb recruitment. BLAST search revealed that there are several *Arabidopsis* proteins (~16) sharing a high protein sequence homology to *MybSt1*. The gene with the highest sequence similarity (At5g47390) shows a constitutive expression in leaf tissue and additionally no sensitivity to the plant hormones SA, JA, GA and ABA (Yanhui et al. 2006). Therefore, this so far non-characterized Myb factor is a good candidate for

mediating constitutive expression of the *PR-1~1294~ as-1* construct via interaction with subclass II TGA factors.

Another transcriptional activator known to bind to the *35S as-1* element linker sequence is the NAC transcription factor AtNAM as revealed by DNase I footprinting (Duval et al. 2002). The region protected from DNase I digestion in this assay included the nucleotide sequence "gggatg" and thereby contains the putative MybSt1 binding site. In contrast to the closest *MybSt1* ortholog in *Arabidopsis* (At5g47390) *AtNAM* is exclusively expressed in developing- and mature seeds. Therefore, it is questionable if AtNAM could induce such strong expression levels monitored with *PR-1~1294~ as-1* promoter constructs in leaf tissue. However, the NAC family of transcription factors consists of 106 members (Qu and Zhu 2006) and it cannot be ruled out that other NAC factors can bind to the linker sequence of the *as-1* element and activate expression. Regardless to the question if a NAC- or a Myb transcription factor is recruited to the *35S as-1* element the first guanine in the linker sequence ("ggga") is critical for the binding of the constitutive activator as revealed by transient protoplast transformations with *PR-1~1294~ as-1* promoter constructs harboring mutations in the linker sequences (figure **4.12**). Furthermore it could be shown that the elements *LS4* and *LS10* positively influence constitutive induction mediated by a substituted *35S as-1* element, but the effect is only moderate (figure **4.19**).
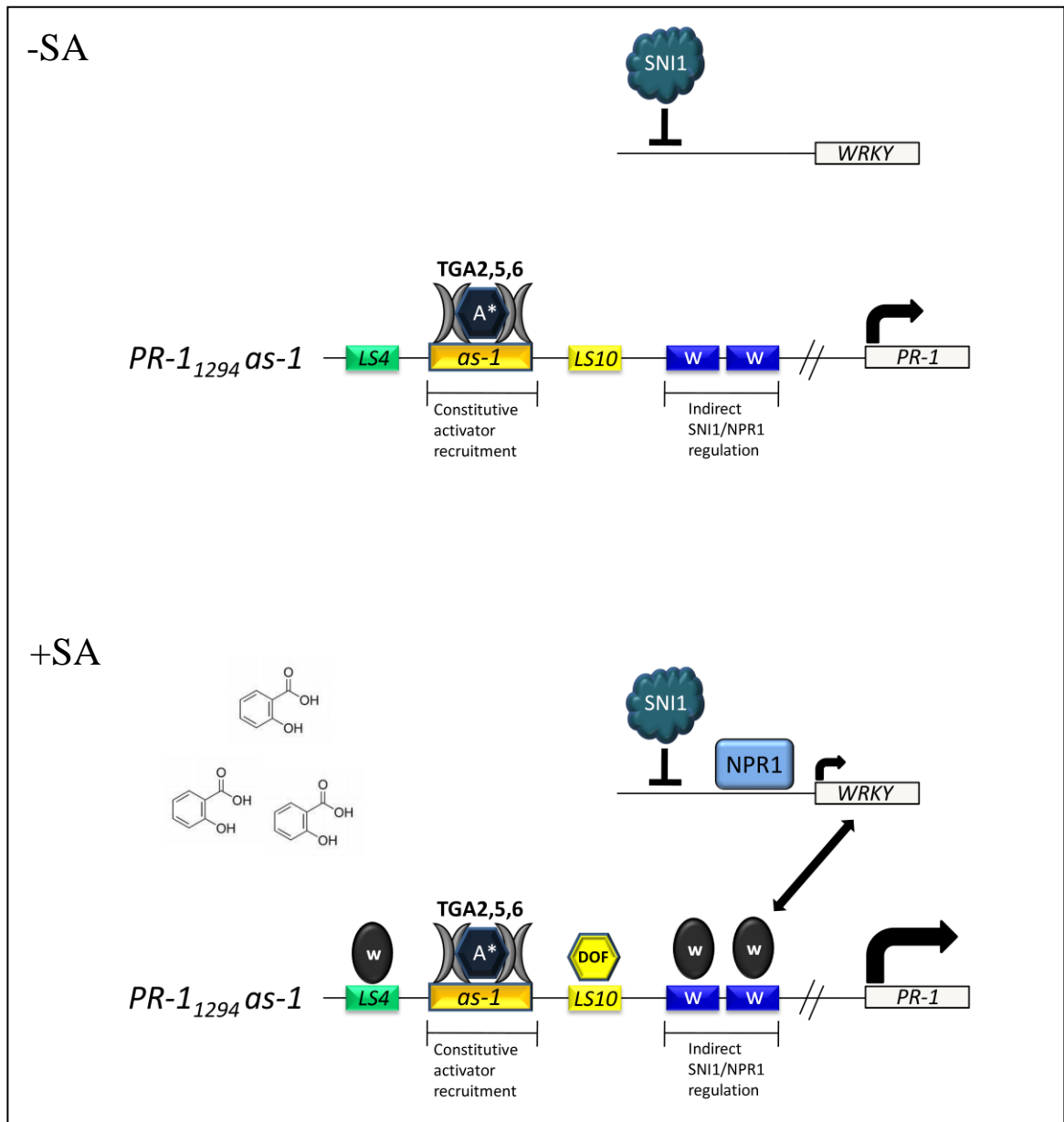
Figure 5.3: Model showing the constitutive activation of the *PR-1* promoter by a substituted *as-1* element derived from the *CaMV 35S* promoter in wildtype plants.

The mutated *as-1* element recruits an activating protein which strongly increases basal expression of the promoter. After SA-treatment other regulatory sequences, presumably the W-box cluster, contribute to an approximately twofold synergistic induction. The recruitment of the unknown activator is strictly dependent on subclass II TGA factors.

5.10 In the absence of subclass II TGA factors a negative regulator is recruited to the *35S as-1* element

As mentioned above, mutation of the subclass II TGA factors leads to a completely abolished expression of the *PR-1$_{1294}$ as-1* reporter constructs under non-inducing- and inducing conditions (figure **4.13 A**). This result illustrates two remarkable issues: (1) The unknown constitutive activator requires these TGA factors for *as-1* element binding and (2) a constitutive repressor must be recruited to the *35S as-1* element in *tga2 tga5 tga6* since the endogenous *PR-1* promoter shows increased basal expression and inducibility in this mutant (Blanco et al. 2009). The binding of the repressor obviously depends on other TGA transcription factor family members as deletion of both TGACG motifs (*LS5/7$_{mut}$*) regains inducibility (figure **4.3**). The recruitment of the suppressor complex to *35S as-1* only occurs in the absence of TGA2, TGA5 and TGA6, likely because the subclass II TGA factor-mediated binding of the activator complex is disturbed. Induction of the *PR-1$_{1294}$ as-1* construct is dysfunctional in the triple mutant as direct NPR1 regulation cannot occur at the *35S as-1* element due to the shortened distance (4bp) between the TGACG motifs. Taking into account the results obtained with the *LS5aaaaLS7* construct in wt and *sni1-1* (figure **4.8**) it is reasonable that SNI1 suppresses the *as-1*-independent induction pathway via the *35S as-1* element in *tga2 tga5 tga6*. Unfortunately, a *tga2 tga5 tga6 sni1-1* quadruple mutant is not available so far to further verify the hypothesis of SNI1 recruitment to the *35S as-1* element.

The presence of a suppressor complex at the *35S as-1* element in the triple mutant further indicates that EMSA binding studies do not reflect the protein recruitment to the *as-1* element occuring *in planta* as no gel shifts appeared when whole cell extracts from *tga2 tga5 tga6* plants were used (figure **4.16**).

In conclusion, the experiments conducted with the *PR-1$_{1294}$ as-1* construct in *tga2 tga5 tga6* mutant plants illustrate that the *35S as-1* element is a potential recruitment site for a transcriptional activator or repressor, dependent on the TGA factors available in the plant. In the presence of subclass II TGA factors activator binding to the *as-1* element is mediated whereas other TGA family members are able to recruit a repressor, presumably SNI1, in the absence of TGA2, TGA5 and TGA6.
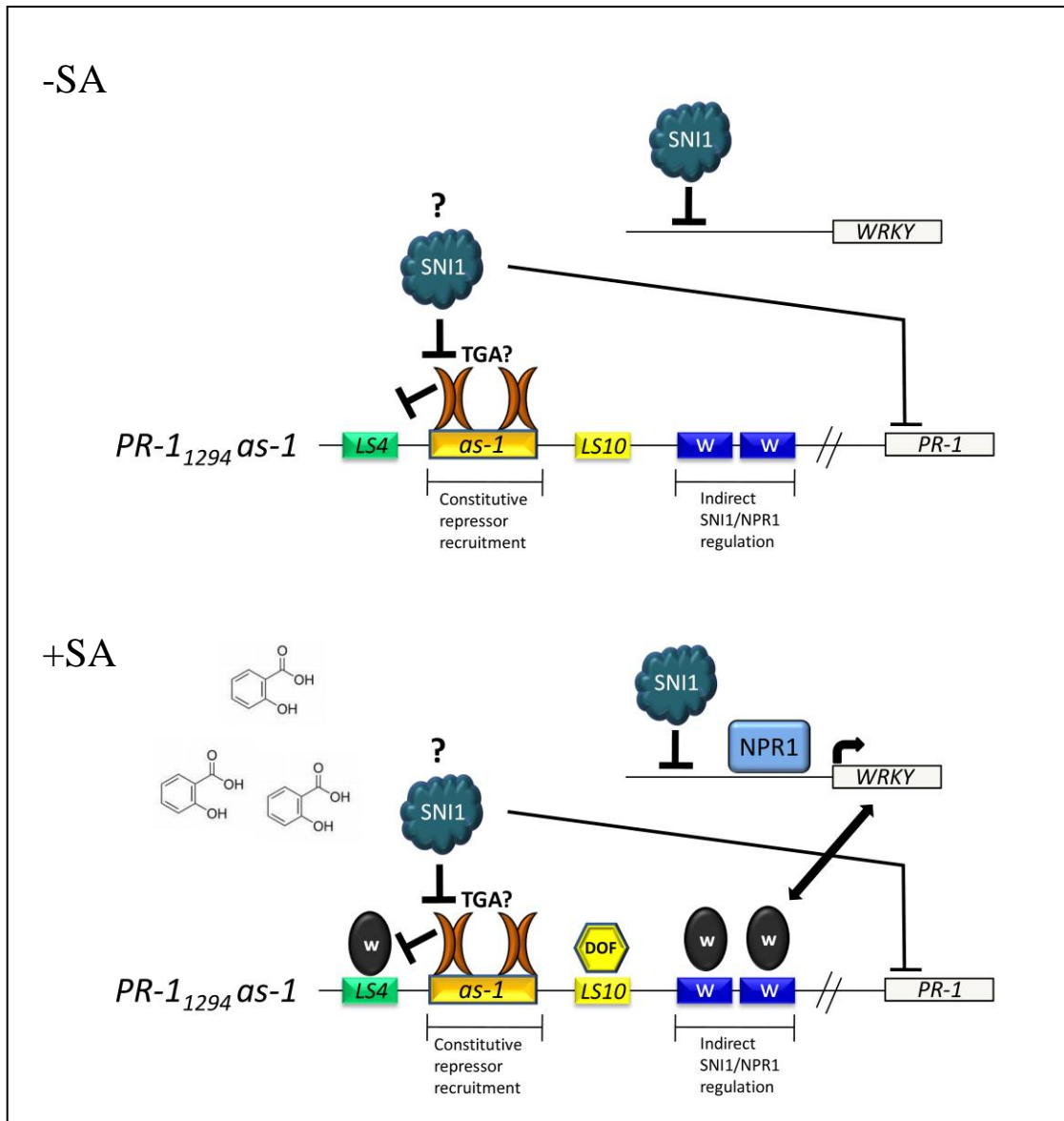
Figure 5.4: Model explaining the abolished expression of the _PR-1_1294 _as-1_ construct in _tga2 tga5 tga6_ mutant background.

In the absence of subclass II TGA factors other TGA factors are recruited to the promoter which do not enhance activator- but repressor recruitment. A potential repressor recruited to the _as-1_ element is SNI1 which suppresses the activation of the _as-1_-independent pathway efficiently. As the distance between the TGACG motifs is altered NPR1 can no longer be recruited to the _as-1_ element and induction fails.

6   Supplemental data

6.1   Promoter constructs
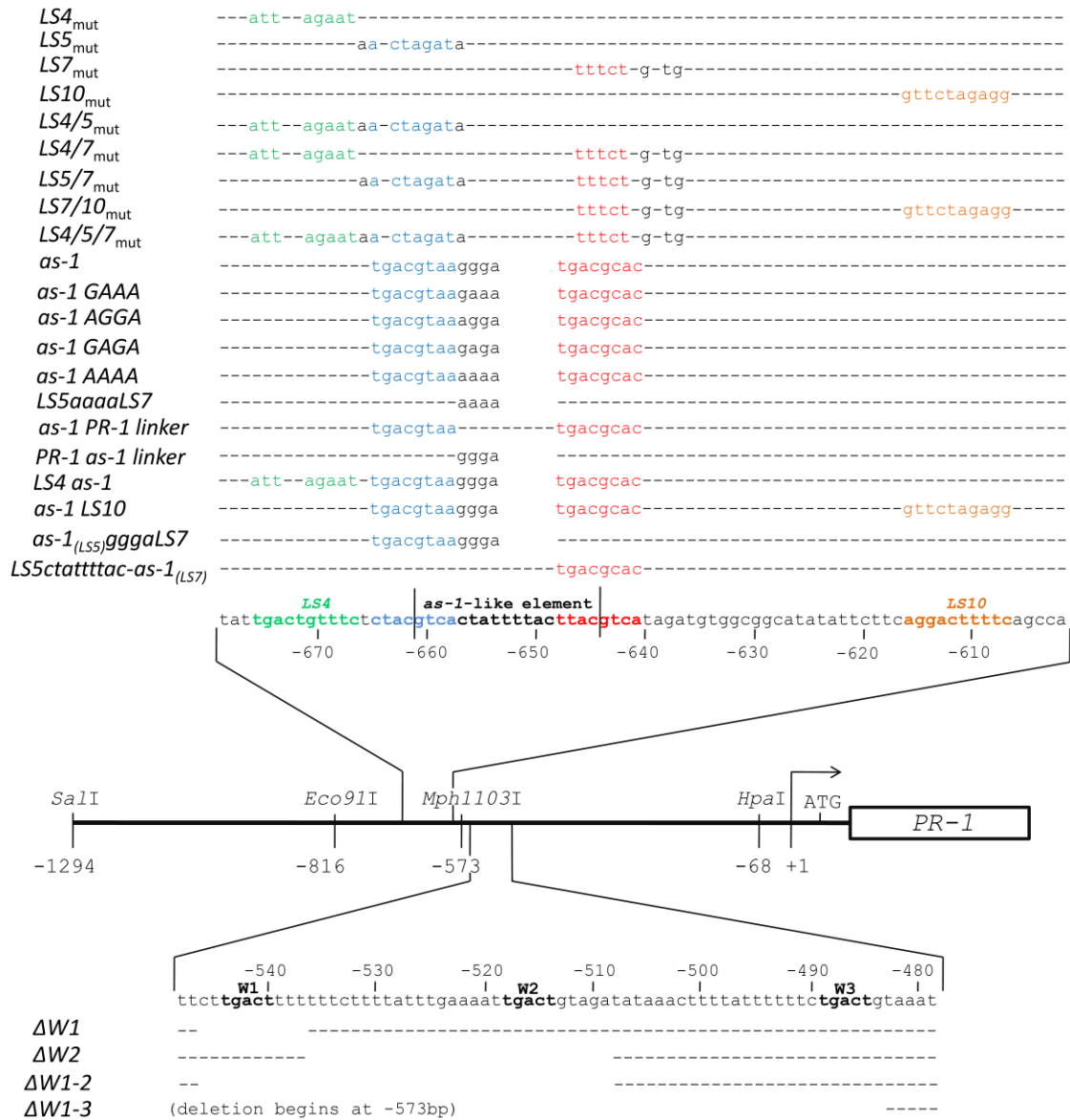
**Figure 6.1: Composition of the different *PR-1* promoter derivatives presented in this work.**

On the top, distinct substitution constructs within the linker scanning region between -602bp and -679bp are illustrated, while the lower panel shows promoter deletions in a regulatory section containing W-box elements between -479bp and -549bp. Central sequences of the *PR-1* promoter are highlighted by individual colors and bolt letters respectively.
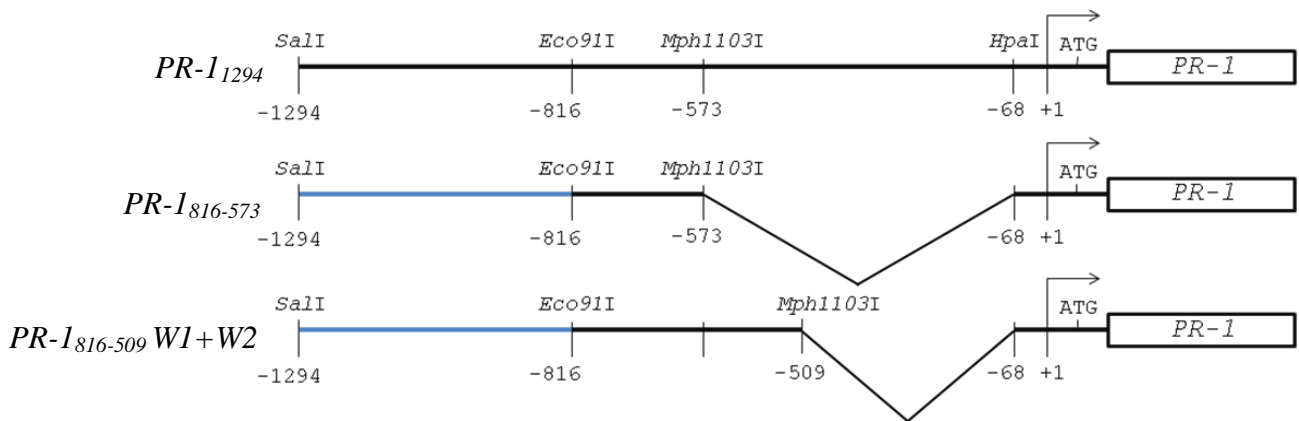
Figure 6.2: Design of *PR-1* promoter deletion constructs.

The blue labeled areas illustrate 478bp substitutions against a stretch of coding sequence derived from the CAT gene, while the deletions in the downstream section of the *3`D* promoter constructs differ in respect to their size.

6.2   Binary vectors used for transgenic- and transient reporter gene studies
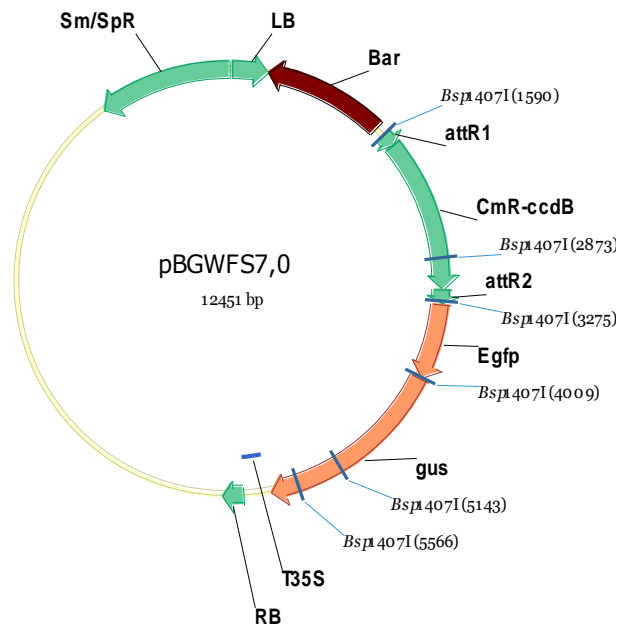
Figure 6.3: Plasmid map of the binary reporter gene (Egfp-GUS fusion) vector pBGWFS7 used for transient and stable transformations.
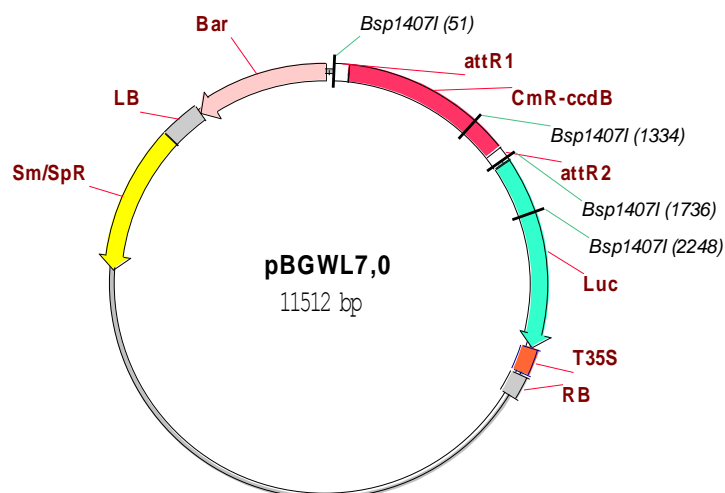
Figure 6.4: Plasmid map of the binary reporter gene vector (LUC) pBGWL7 used for *A. tumefaciens* mediated gene transfer.

6.3 Overview of relative promoter activities

6.3.1 *LS*-constructs (pBGWL7)

Promoter derivative	plant	Uninduced expression	Induced expression
PR-1$_{1294}$	wt	-	++
	npr1-1	-	-
	sni1-1	+	+++
	sni1-1 npr1-1	+	++
LS4$_{mut}$	wt	-	++
	npr1-1	-	-
	sni1-1	+	+++
LS5$_{mut}$	wt	-	++
	npr1-1	-	-
	sni1-1	+	++
LS7$_{mut}$	wt	-	+
	sni1-1	++	++
LS10$_{mut}$	wt	-	+

Promoter derivative	plant	Uninduced expression	Induced expression
	sni1-1	+	++
*LS4/5*mut	wt	-	-
	sni1-1	+	++
*LS4/7*mut	wt	-	-
	sni1-1	++	+
*LS5/7*mut	wt	+	++
	sni1-1	++	+++
*LS7-LS10*mut	wt	-	-
	sni1-1	++	++
*LS4/5/7*mut	wt	-	-
	sni1-1	+	+

6.3.2   *as-1* element constructs (pBGWL7)

Promoter derivative	plant	Uninduced expression	Induced expression
PR-1$_{1294}$	wt	-	++
	npr1-1	-	-
	sni1-1	+	+++
PR-1$_{1294}$ *as-1*	wt	++++	+++++
	sni1-1	++++	+++++
	npr1-1	+++	++++
PR-1$_{816-573}$ *as-1*	wt	+++	+++
	sni1-1	+++	+++
	npr1-1	+++	+++
PR-1$_{1294}$ *as-1 LS10*	wt	+++	++++
LS5aaaaLS7	wt	-	-
	sni1-1	+	+++

6.3.3   *as-1* element constructs (pBGWFS7)

Promoter derivative	plant	Uninduced expression	Induced expression
PR-1$_{1294}$	wt	-	++
	npr1-1	-	-
PR-1$_{1294}$ as-1	wt	++	+++
	npr1-1	++	+++
PR-1$_{816-573}$ as-1	wt	++	++
	npr1-1	++	++
PR-1$_{816-573}$ as-1 linker	wt	-	-
PR-1$_{1294}$ as-1linker	wt	++	+++
	npr1-1	++	+++
PR-1$_{1294}$ as-1PR-1 linker	wt	-	-
PR-1$_{1294}$ (as-1/as-1/PR1)	wt	++	+++
	npr1-1	++	+++
PR-1$_{1294}$ (PR-1/PR-1/as-1)	wt	-	-

6.3.4   W-box constructs (pBGWFS7)

Promoter derivative	plant	Uninduced expression	Induced expression
PR-1$_{1294}$	wt	-	++
ΔW1	wt	-	(+)
ΔW1-2	wt	-	(+)
ΔW1-3	wt	-	(+)
ΔW2	wt	-	+
3`D + 23bp	wt	-	-
3`D + W1	wt	-	+
3`D + W1 + W2 + W3	wt	-	+(+)

6.3.5   W-box constructs (pBGWL7)

Promoter derivative	plant	Uninduced expression	Induced expression
PR-1$_{1294}$	wt	-	++
	sni1-1	+	+++
ΔW1	*sni1-1*	+	++
LS4/5 ΔW1-3	*sni1-1*	+	+

6.4   Overview of the LS-construct promoter activities in wt and *sni1-1*

The experiments presented above show a complex mode of regulation occurring on *PR-1* promoter level. For a better overview of the different promoter constructs in wt and *sni1-1* the relative LUC activities are compared in a single diagram. Figure **6.5** shows the expression levels of all introduced *LS*-constructs as well as the $PR-1_{816-573}$ promoter derivative in wt and *sni1-1*.
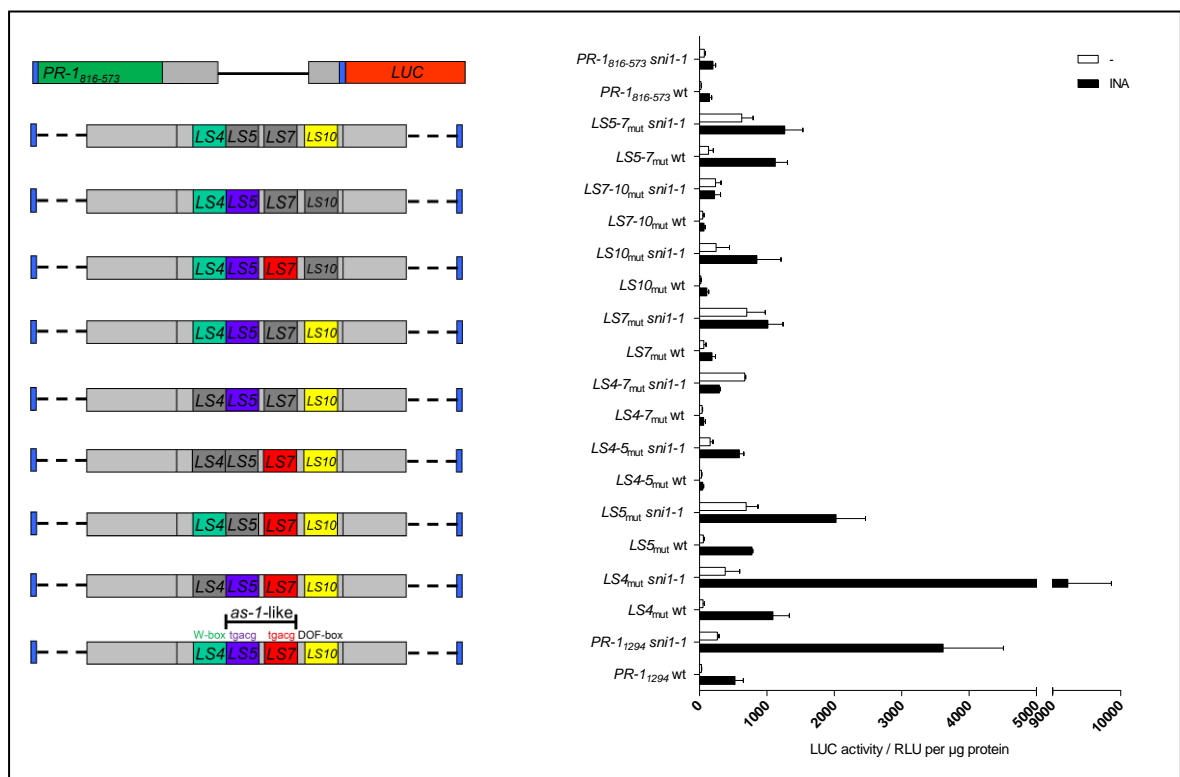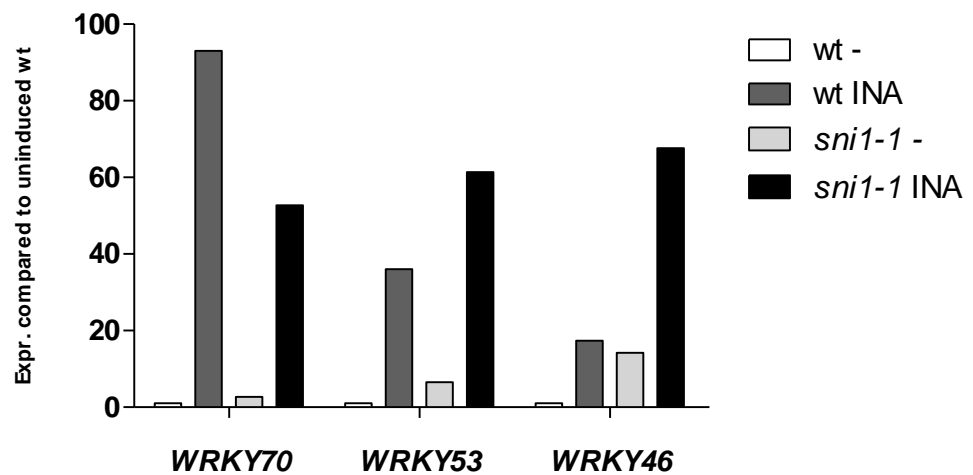

Figure 6.5: Overview of the different *LS*-promoter constructs in wt and *sni1-1* mutant background.

6.5   Expression of *WRKY46*, *WRKY53* and *WRKY70* in wt and *sni1-1* under inducing- and non-inducing conditions

Figure 6.6: RT-PCR analysis of *WRKY70, WRKY53* and *WRKY46* expression in wildtype and *sni1-1*.

Plants were grown for 18 days on MS-plates supplemented with- or without 30μM INA. White columns show uninduced - whereas dark grey columns display induced expression levels in wt. The transcript levels in *sni1-1* are displayed by light grey (non-induced) and black columns (INA treated). Expression of *WRKY46* is strongly influenced in *sni1-1* whereas the other two *WRKY* TFs only show weak (*WRKY53*) or no de-repression (WRKY70) in this mutant.

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8   Acknowledgement

9   Curriculum Vitae

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Göttingen, den 26. Mai 2009