Defining WRKY factors involved in plant defense: characterization of *Arabidopsis thaliana WRKY27*, a gene affecting *Ralstonia solanacearum* disease development

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For my Mom Razia Begum

and

Dad Mukhtar Ahmad

امی رضیہ بیگم

اور ابو مختار احمد کیلئے

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Abbreviations/acronyms

(v/v)	volume per volume
(w/v)	weight per volume
µ	micro
ACC	1-aminocyclopropane-1-carboxylic acid
Amp	Ampicillin
AOS	Allene Oxide Synthase
At	*Arabidopsis thaliana*
ATP	adenosine 5-triphosphate
avr	avirulence
Bgh	*Blumeria graminis* f.sp. *hordei*
bp	base pair(s)
C	carboxy
c	centi
CC	coiled-coil
cDNA	complementary DNA
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
coi1	*coronatine insensitive 1*
cpr1	*constitutive PR 1*
CT	carboxy-terminal
cv.	cultivar
d	deoxy
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
dd	dideoxy
ddH_2O	deionised and distilled water
DEPC	diethylpolycarbonate
dGTP	deoxyguanosinetriphosphate

dicot	dicotyledonous
DMF	Dimethyl Formamide
DMSO	dimythysulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dnd1	*defense no death 1*
dNTP	deoxynucleosidetriphosphate
dpi	days post inoculation
dth9	*detachment 9*
DTT	dithiothreitol
dTTP	dioxythimydinetriphosphate
EDS1	*Enhanced Disease Susceptibility 1*
EDTA	ethylenediaminetetraacetic acid
ein2	*ethylene insensitive2*
ER	endoplasmic reticulum
EST	expressed sequence tag
ET	ethylene
EtBr	ethidium bromide
EtOH	ethanol
f.sp.	*forma specialis*
Flg	Flagellin
FLS2	Flagellin Sensitive 2
FRET	Fluorescence Resonance Energy Transfer
g	gram
g	gravity constant
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hour
H^+	hydrogen proton
hpi	hours post inoculation
HR	hypersensitive response

Hv	*Hordeum vulgare*
ISR	induced systemic resistance
JA	jasmonic acid
jar1	*jasmonic acid resistant 1*
Kan	Kanamycin
kb	kilobase(s)
kDa	kiloDalton(s)
l	litre
LPS	Lipopolysaccharide
LRR	leucine-rich repeats
LZ	leucine-zipper
m	milli
M	Molar
MAPK	Mitogen-Activated Protein Kinase
min	Minute(s)
mmol	millimolar
monocot	monocotyledonous
mRNA	messenger ribonucleic acid
N	amino
nahG	salicylcate hydroxylase
NBS	nucleotide binding site
NDR1	*Non-race-specific Disease Resistance 1*
ng	nanogram
NPR1	*Non-expressor of PR1*
Nt	*Nicotiana tabacum*
ORF	open reading frame
Os	*Oryza sativa*
p	pico
PAD4	*Phytoalexin Deficient 4*
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern

PBS1	*avrPphB Susceptible 1*
PCR	polymerase chain reaction
PDF1.2	*Plant Defensin1.2*
PEG	polyethylene glycol
pen	penetration
pg	picogram
PGT	primary germ tube
pH	negative decimal logarithm of the H^+ concentration
PIP_2	phosphatidyl-inositol 4, 5-bisphosphat
pmol	picomolar
pmr	powdery mildew resistance
PR 1	pathogenesis related protein 1
PR 5	pathogenesis related protein 5
pv.	pathovar
R	resistance
RFP	red fluorescent protein
Rho	ras homolog
RLK	receptor like kinases
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	second(s)
ssp.	species
Ta	*Triticum aestivum*
T-DNA	transfer DNA

TIR	*Drosophila* Toll and human interleukin-1 receptor
TLR	Toll-like receptor
TM	transmembrane
TRIS	Tris-(hydroxymethyl)-aminomethane
U	unit
UTR	untranslated region
UV	ultraviolet
V	Volt
VIGS	virus induced gene silencing
vir	virulence
Wt	wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

1. Introduction

1.1. The various layers of plant defense

In any natural habitat, there is unremitting antagonism between pathogenic microbes and plants (Glazebrook, 2001; Gfeller and Farmer, 2004; Glazebrook, 2005). In plants, biotic stresses often result by the detrimental effects of diverse potential pathogens like fungi, bacteria, viruses, nematodes and insects (Downward, 2001; Nürnberger et al., 2004; Rinkevich, 2004; Bais et al., 2005; Bostock, 2005; Glazebrook, 2005). In order to protect themselves against pathogenic infection, plants have acquired a vast array of defense mechanisms (Dangl and Jones 2001; Gfeller and Farmer, 2004; van Doorn and Woltering, 2005). These mechanisms encompass physical barriers and the production of antimicrobial compounds both in constitutively or in an inducible dependent manner. By providing a barrier to entry, the epidermis and cuticle of plants, together with pre-formed antimicrobial enzymes and other secondary metabolites operate as the first layer of defense against invaders (Heath, 2000; Dangl and Jones 2001; Dixon, 2001; Kamoun, 2001; Nürnberger et al., 2004; Nürnberger and Lipka, 2005). Plants produce a remarkably diverse array of secondary metabolites, many of which are antimicrobial (Dixon, 2001; Sirvent et al., 2003; Thoma et al., 2004). The majority of the antimicrobial compounds have evolved to confer selective reinforcement against microbial attack (Dangl and Jones 2001; Dixon, 2001;; Nürnberger and Lipka, 2005; Zhu-Salzman et al., 2005). These compounds may be present constitutively in healthy plants (pre-formed antimicrobial compounds, or phytoanticipins such as saponins, tannins, terpenes, flavonoids and other phenolics) or alternatively may be synthesized in response to pathogen attack or stress (phytoalexins) (Morassutti et al., 2002; Raj and Dentino, 2002; Ganz, 2004). In many plants, the epidermis contains hairs (loaded with defense metabolites) or spines acting also as the earliest barrier (s) against herbivores as well as certain pathogens (Nürnberger et al., 2004; Nürnberger and Lipka, 2005). On the other hand, some fungi/oomycetes succeed to traverse this barrier by secreting several enzymes, including cutinase, that cleave some of the molecules found in the cuticle (Green et al., 2002; Hückelhoven 2005). Bacteria and some fungi circumvent this barrier by entering tissues through open stomata or wound sites. In addition, some pathogens like *Gaeumannomyces graminis*

combat the antimicrobial compounds of their host plants by producing enzymes such as avenacinase that detoxify these phytoprotectants.

Rapid induced defenses form the second layer of plant defense. These include various mechanisms that reorganize the cell wall and cell membranes after pathogen attack and recognition (Schilmiller and Howe, 2005). Preparations for the intensification of the cell wall, which can enhance host resistance, are activated very promptly after a pathogen attempts to penetrate a host cell. This is characterized by an increase in cytoplasmic streaming and translocation of cytoplasm and of the cell nucleus to the fungal attempted penetration site (Green et al., 2002; Schmelzer; 2002**;** Hückelhoven 2005). The cytoplasmic aggregates are thought to contain the cellular apparatus for the synthesis of cell wall fortifications. If the host cell can repair and reinforce its cell walls quickly enough, it might dampen the penetration probability of the pathogen. Several types of reinforcements are produced by host cells including callose deposition at the site of attempted penetration, lignified callose reinforcements and secondary cell wall thickening (Zeyen et al., 2002; Hückelhoven 2005).

The host plasma membrane appears to be involved in the earliest stages of pathogen recognition and signal transduction. A change in membrane permeability after exposure to a pathogen causes major ions fluxes, such as K^+, H^+ and Ca^{2+}, that are essential for the subsequent changes in gene activation and the triggering of the defense responses (Laloi et al., 2004; Karyotou and Donaldson, 2005; Torres and Dangl, 2005). Also at the membrane, the 'oxidative burst', which involves the generation of reactive oxygen species, such as hydrogen peroxide, triggers signals that affect gene expression, cross-linking in the host cell wall and initiation of later defense responses (Garcia-Olmedo et al., 2001; Wendehenne et al., 2004; Delledonne, 2005; Torres and Dangl, 2005). The reactive oxygen species at the site of infection are also produced in quantities capable of directly killing micro-organisms.

In addition, *de novo* synthesized antimicrobial compounds like phytoalexins, also function as a second line of defense by interfering with pathogen nutrition and consequently retarding their development (Raj and Dentino, 2001; Morassutti et al., 2002; Ganz, 2004).

At the early infection process stage, certain bacterial pathogens bypass the two earliest plant defense layers by injecting effector molecules via a type III secretion system (TTSS) directly into the host cytoplasm. Once inside, these molecules target host proteins, that control various plant processes (like defense responses or metabolism) in order to block/suppress plant defense mechanisms with the aim to enhance virulence activities in the disease establishment process (compatible plant microbe interaction) (Abramovitch and Martin, 2005; Kim et al., 2005; Nomura et al., 2005; Normark et al., 2005).

Surveillance systems, constituting the third but crucial layer of plant defense, are the essential components in the plant defense machinery which are employed to recognize such pathogenic events (Hotson and Mudgett, 2004; Mota and Cornelis, 2005; Mota et al., 2005; Mudgett, 2005; Schulze-Lefert and Bieri, 2005). These specialized defense mechanisms are comprised of diverse plant disease resistance genes (*R*-genes) encoding proteins that recognize directly or indirectly these pathogenic effector molecules termed avirulence products. Thus, *R*-gene mediated resistance forms an allele specific genetic interaction between a host *R* gene and a pathogen avirulence gene *avr* (Flor, 1971; McDowell and Woffenden, 2003) known as incompatible plant microbe interaction. Consequently, this interaction leads to the activation of plant defense responses often leading to rapid local cell death termed hypersensitive response (HR) (Heath, 2000; Dangl and Jones, 2001; Mackey et al., 2002; Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003; Belkhadir et al., 2004; Kim et al., 2005). Moreover, identification of mutants affected in both compatible and incompatible plant microbe interactions, such as *rin4*, *pmr*, *ebr* mutants and many others, has enabled a comprehensive characterization of host target genes (Vogel et al., 2002; Nishimura et al., 2003; Vogel et al., 2004; Campbell and Ronald, 2005; Nandi et al., 2005).

In the absence of such a specific *R*-gene type recognition, a basal defense response still occurs by detecting microbial cell surface molecules. These invariant structures are referred to as pathogen-associated molecular patterns (PAMPS) like flagellin and lipopolysaccharides (LPS), although they are not unique to phytopathogens and are produced by all microorganisms, pathogenic and non pathogenic (Gomez-Gomez and

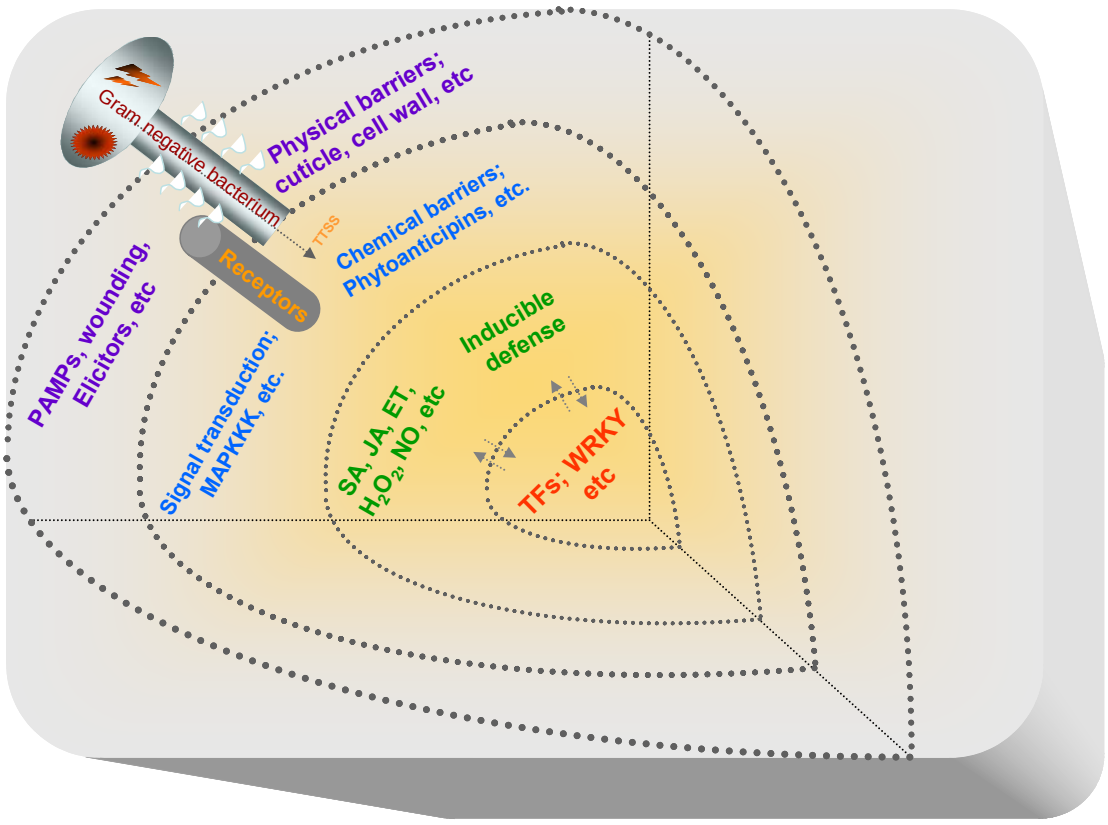


Fig.1. The various layers of plant defense. Physical barriers (cuticle and cell wall) and pre-formed antimicrobial compounds (phytoanticipins) form the first line of plant defense. Various re-arrangements in the cell wall and cell membranes, the generation of reactive oxygen species (H_2O_2), and the synthesis of secondary metabolites are allocated in second layer of plant defense which are part of rapid early defense response. The third layer of plant defense includes recognition of effector molecules delivered into the host plant via type III secretion systems (TTSS) and pathogen associated molecular patterns (PAMPs) leading to the activation of plant defense responses. Activation of various defense signaling cascades involving MAP kinases , SA, JA or ET are part of induced plant defense forming the fourth layer of plant defense. Delayed active defenses are the fifth layer of plant defense. The core of both early and delayed active defense responses depends upon transcriptional reprogramming of gene expression. Thick dotted black circles represent various layers of plant defense. Grey arrows at the innermost layer represent the in and/or out of signals. TTSS: type III secretion systems, PAMPs: pathogen associated molecular patterns, MAPKKK: mitogen-activated protein kinase

kinase kinase, SA: salicylic acid, JA: jasmonic acid, ET: ethylene, NO: nitric oxide, H_2O_2: hydrogen per oxide, TFs: transcription factor (s).

Boller, 2002; Belkhadir et al., 2004, Kunze et al., 2004; Boller, 2005; Zipfel and Felix, 2005). However, the basal defense is temporally slower and of a lower amplitude. Basal defense often does not prohibit pathogen colonization but does limit the extent of its spread (Glazebrook et al., 1997; Belkhadir et al., 2004). Recently, the laboratory of J. Dangl experimentally demonstrated that both of these above mentioned pathways overlap significantly and are linked. They found that two *Pseudomonas syringae* type III effectors, AvrRpt2 and AvrRpm1, inhibit PAMP-induced signaling and thus compromise the host's basal defense system (Kim et al., 2005). Nevertheless, basal plant defense is proven to be a powerful shield against numerous non-host pathogens (Thordal-Christensen, 2003; Jones and Takemoto, 2004; Mysore and Ryu, 2004). Primarily, microbial invaders incapable of infecting a certain plant species are phrased as non-host pathogens, while plants exhibiting full resistance to various members of a particular class of pathogens are called non-host plants (Gabriel and Rolfe, 1990; Prell and Day, 2000; Holub and Cooper, 2004).

Certain defense responses require specific endogenous signal molecules like salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) to activate defense signaling cascades that lead to the expression of certain subsets of genes which are thought to contribute to resistance and thereby constitute a fourth layer of plant defense (Glazebrook, 2001; Kunkel and Brooks, 2002; Hammond-Kosack and Parker, 2003; Katagiri, 2004; Bostock, 2005; Glazebrook, 2005; Wiermer et al., 2005). These include defense against abiotic stresses, such as wounding and exposure to ozone, as well as defense against insect and microbial attack (Ecker, 1995; Creelman 1998; Kunkel and Brooks, 2002). Numerous *Arabidopsis thaliana* (*A. thaliana*) mutants, impaired in resistance, have been identified and genetically well characterized at various levels of these three known defense signaling pathways. An increasing number of reports elaborated that the SA, JA, and ET defense signaling pathways do not seem to function in a linear, independent fashion. These pathways appear to be involved in a complex signaling network instead, in which

each can influence the other (s) through positive and negative regulatory interactions (Glazebrook, 2001; Hammond-Kosack and Parker, 2003; Glazebrook, 2005).

Delayed active defenses, the fifth layer of plant defense, comprise pathogen restraint, wound repair, and the acquisition of systemic acquired resistance. These defense responses limit pathogen spread after the establishment of infection and restrict host tissue damage (Bostock, 2005; van-Bel and Gaupels, 2005).

A common feature of inducible defense responses upon pathogenic infection is the transcriptional regulation of a large number of plant genes triggered during *R*-mediated resistance, basal defense and systemic acquired resistance (Yang et al., 1997; Rushton and Somssich, 1998; Uelker and Somssich, 2004; Eulgem, 2005). The core of both constitutive and induced (early and delayed) defense responses depends upon transcriptional expression of genes (Fig. 1). This comprehensive transcriptional re-programming is thought to function under a systematic regulatory network (Eulgem, 2005).

1.2. Transcriptional regulatory network

Transcription factors (TFs) modulate the expression patterns of both constitutively or stimuli-specific expressed genes (Guilfoyle, 1997). TFs are thought to function in networks, in which a regulatory protein can influence the functions of another to control directly and/or indirectly the temporal or spatial expression of a particular gene (Wyrick and Young, 2002). The full Arabidopsis genome sequence provides a remarkable opportunity to identify various specific TF's and to explore the regulatory networks (The Arabidopsis Genome Initiative, 2000). A typical plant transcription factor contains, with few exceptions, a DNA-binding region, an oligomerization site, a transcription-regulation domain, and a nuclear localization signal (Liu et al., 1999). Generally, TFs are classified according to their DNA binding domains (Pabo and Sauer, 1992). At least, 1500 TFs were identified and clustered into 34 families (Reichmann et al., 2000). Only a few of them are genetically characterized and have been shown to bind directly to their specific *cis*-regulatory DNA sequences *in vivo*. Several members of various transcription factor

families such as TGA-bZIP, ERF, Myb, Whirly and WRKY, are shown to be linked with plant defense responses and specific gene regulation (Rushton et al., 1996; Yang and Klessig, 1996 ; Zhou et al., 1997; Rushton and Somssich, 1998; Eulgem et al., 1999; Yang et al., 1999; Eulgem et al., 2000; Jakoby et al., 2002; Rushton et al., 2002; Desveaux et al., 2004 ; Uelker and Somssich, 2004; Turck et al., 2004; Desveaux et al., 2005).

Energy cost-effective plant defenses depend upon an alert transcriptional circuit. In Arabidopsis, various TF family representatives have been shown to bind to promoter elements of defense-related genes and to regulate their expression (Eulgem, 2005). TGA factors constitute a conserved plant sub-family of basic domain/Leucine zipper (bZIP) transcriptional regulators, whose genomic targets are thought to include *glutathione S-transferase* and *pathogenesis-related* (*PR*) genes that are associated with detoxification and defense (Klinedinst et al., 2000; Niggeweg et al., 2000a; Johnson et al., 2001; Pontier et al., 2001). Using a leaf tissue chromatin immunoprecipitation (ChIP) assay, it was demonstrated that Arabidopsis TGA2 and TGA3 are recruited to the *PR-1* promoter *in planta* in response to a stimulus and subsequent activation of a pathway involving SA and NPR1 (Johnson *et al.* 2003). NPR1 is a key regulator of the pathway acting as a modulator within the cell nucleus. Ethylene-Responsive-Element-Binding Factor (ERF) proteins are a sub-family of the plant-specific APETALA2 (AP2)/EREBP (ethylene responsive element binding protein) TFs and consist of about 124 members in Arabidopsis (Reichmann et al., 2000). The ERF domain, a conserved peptide stretch of 58 to 68 amino acids can bind to GCC box DNA elements present in several promoters of the *PR*-gene family (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997; Rushton and Somssich, 1998; Eulgem, 1999; Fujimoto et al., 2000; Kirsch et al., 2000; Rushton et al., 2002; Chakravarthy et al., 2003). Several members of the Myb plant transcription factor family are up-regulated upon pathogen challenge or by pathogen related stimuli suggesting a role in plant defense (Kranz et al., 1998; Stracke et al., 2001). Plants lacking *BOS1*, a Myb family member, show enhanced disease symptoms towards several biotrophic and necrotrophic pathogens (Mengiste et al., 2003). *At*Why1, a member of the recently identified Whirly transcription factor family, is proposed to play a role in both basal and induced gene-for-gene defense responses, as the loss of function mutant of

AtWhy1 shows enhanced susceptibility to the incompatible *P. parasitica* isolate Emoy2 (Desveaux et al., 2004 ; Desveaux et al., 2005). Interestingly, *Atwhy1* plants exhibit reduced *PR1* transcript levels upon SA treatment, suggesting a role in combination with NPR1 to promote the salicylic acid signal (Desveaux et al., 2005). NPR1 lacks a DNA-binding domain, but through interaction with TGA transcription factors regulates *PR* gene expression (Dong, 2004; Wang et al., 2005). Microarray data suggest that WRKY proteins (see 1.3.) also regulate the expression of defence-related genes including NPRI (Dong et al., 2003). The *AtWRKY70* transcription factor was recently found to be a principal mediator of SA–JA crosstalk (Li et al., 2004). Intriguingly, the presence of one conserved Myb-binding motif (type I, GG/TTA/TGG/TT) in the promoters of *WRKY* genes suggests their regulation also by Myb factors (Dong et al., 2003). These various lines of evidence support the existence of a sophisticated transcriptional regulatory network.

1.3. The WRKY superfamily of transcription factors

The first cDNA encoding a WRKY protein, Sweet Potato Factor 1 (SPF1), was cloned from sweet potato (*Ipomoea batatas*) (Ishiguro and Nakamura, 1994). Subsequently, a large number of genes encoding WRKY proteins were identified and cloned from more than 10 other plant species, including *Arabidopsis thaliana*, wild oats (*Avena fatua*) ,orchardgrass (*Dactylis glomerata*), barley (*Hordeum vulgare*), tobacco (*Nicotiana tabacum*), chamomile (*Matricaria chamomilla*), rice (*Oryza sativa*), parsley (*Petroselinum crispum*), a desert legume (*Retama raetam*), sugarcane (*Saccharum* hybrid cultivar) , bittersweet nightshade (*Solanum dulcamara*) , potato (*Solanum tuberosum*), and wheat (*Triticum aestivum*) (Eulgem *et al*., 2000; Uelker and Somssich, 2004; Zhang and Wang 2005). Before the identification of two ESTs homologues to WRKY proteins, one each from *Giardia lamblia*, a primitive protozoan, and *Dictyostelium discoideum*, a slime mold, the WRKY superfamily was considered to be restricted to the plant kingdom only (Eulgem *et al*., 2000; Uelker and Somssich, 2004). On the basis of available information, it is hypothesized that the early origin of WRKY genes was in Eukaryota, which immensely amplified afterwards in the plant lineage (Uelker and Somssich, 2004).

In *A. thaliana*, WRKY proteins are classified into one of the major families of transcription factors comprising 74 members (Eulgem *et al*., 2000). WRKY proteins contain one or two domains with a conserved peptide stretch of about 60 amino acids (WRKY domain) encompassing a novel Zn-finger motif. The WRKY domain shows a high binding affinity to a distinct *cis*-acting DNA element termed the W Box (TTGACC/T). However, Sun et al. (2003) reported the binding of a barley WRKY factor, SUSIBA2 (sugar signaling in barley), to a newly identified sugar responsive *cis* element named SURE. Due to structural features, this multigene family can be further subdivided into 3 different groups in Arabidopsis. This classification is based on the number of WRKY domains and certain features of the Zn finger-like motif. *At*WRKY proteins with two WRKY domains belong to group I, whereas most proteins with one WRKY domain belong to group II. WRKY domains of group I and group II have the same type of Zn finger motif whose pattern of potential zinc ligand is C-X_{4-5}-C-X_{22-23}-H-X-H. The pattern of *At*WRKY group III is C-X_7-C-X_{23}-H-X-C (Eulgem *et al*., 2000). The recently reported NMR solution structure of the C-terminal WRKY DNA binding domain of *At*WRKY4 reveals that the novel domain consists of a four-stranded β-sheet (Yamasaki et al., 2005). The zinc-binding pocket of the WRKY domain is located at one end of the β-sheet and the WRKYGQK residues correspond to the most N-terminal β-strand that most likely enters the DNA groove and forms contact with the DNA bases (Yamasaki et al., 2005).

A number of studies have shown that WRKY factors are key regulators in certain developmental programs and are also involved in plant defense. *At*WRKY44 is required for proper trichome development (Johnson *et al*., 2002). High levels of expression of *AtWRKY6* or *AtWRKY18* led to growth retardation and other stress-related phenotypes (Robatzek and Somssich, 2002; Chen and Chen, 2002). Additionally, the recent identification of WRKY factors from plants other than Arabidopsis further supports their involvement in various cellular processes. The rice WRKY protein, *Os*WRKY71, acts as a transcriptional repressor of gibberellin-responsive genes (Zhang et al., 2004). Barley *Hv*WRKY38, an orthologue of *At*WRKY40, oat ABF2 and rice *Os*WRKY71, was shown to be involved in cold and drought stress responses (Mare et al., 2004). *Sc*WRKY1 (*Solanum chacoense*), an orthologue of *At*WRKY33 is shown to express strongly but transiently in fertilized ovules bearing late torpedo-staged embryos, suggesting a specific

role during embryogenesis (Lagace and Matton, 2004). Based on a study in a heterologous system, it was proposed that *Ga*WRKY1 (*Gossypium arboreum*), an orthologue of *At*WRKY18, participates in regulation of sesquiterpene biosynthesis in cotton (Xu et al., 2004).

Specific WRKY family members also show enhanced expression upon induction by a wide range of pathogens and upon wounding (Eulgem *et al*., 2000; Uelker and Somssich, 2004). Expression analysis of group-III *At*WRKY factors showed strong transcript accumulation of 11 out of 13 *AtWRKY* genes upon inoculation with compatible, incompatible and non-host pathogens (Kalde et al., 2003). A similar study revealed that 49 out of 72 tested *AtWRKY* genes exhibited altered expression patterns upon pathogen infection or SA treatment (Dong et al., 2003). Transient silencing of the *HvWRKY1* gene showed increased resistance to *Blumeria graminis* f.sp. *hordei* (Eckey et al., 2004). Plants ectopically expressing either *AtWRKY18* or *AtWRKY70* display enhanced resistance towards certain virulent pathogens (Chen and Chen, 2002; Li et al., 2004). Overexpression, RNAi and knock-out lines of *AtWRKY53* showed accelerated and delayed senescence phenotypes, respectively (Miao et al., 2004). WRKY proteins bind to W boxes which are found in the promoters of many plant defense genes including the well studied *PR* genes (Rushton et al., 1996; Eulgem. et al., 1999; Yang et al., 1999; Du and Chen, 2000; Dong et al., 2003; Kalde et al., 2003; Eckey et al., 2004; Kim and Zhang, 2004; Uelker and Somssich, 2004). Regulation of the *PR1* promoter by *At*WRKY proteins appears to be complex and may involve both activation and repression functions (Turck et al., 2004; Rocher et al., 2005). In addition W boxes are present in clusters within short promoter stretches (Eulgem *et al*., 2000; Maleck *et al*., 2000) suggesting that WRKY proteins may act synergistically with other family members or other classes of transcription factors. *At*WRKY6 positively influenced pathogen defense-associated *PR1* promoter activity. Target gene analyses for At*WRKY6* using cDNA-AFLP identified a gene designated senescence induced receptor kinase (*SIRK*). Interestingly, *At*WRKY6 activates the expression of *SIRK* that contains W boxes in its promoter but represses its own expression (Robatzek and Somssich, 2002), although the mechanism by which it acts as a repressor is not yet known. Expression profiling revealed that *AtWRKY70* also influences the expression of other *At*WRKY factors including *AtWRKY53* (Dong et al.,

2003; Li et al., 2004; Miao et al., 2004). Recent reports suggested the involvement of specific WRKY factors associated with defense-induced mitogen-activated protein kinase (MAPK) signaling cascades (Asai et al., 2002; Wan et al., 2004). A complete plant MAPK cascade (*At*MEKK1, *At*MKK4/*At*MKK5 and *At*MPK3/*At*MPK6) was identified that functions downstream of the bacterial flagellin receptor FLS2. The FLS2 pathway is controlled by *At*FLS2, a receptor-like leucine-rich repeat serine/threonine kinase (a LRR receptor kinsae) which is involved in perception of flagellin. Transient overexpression of truncated *At*MEKK1, constitutively active *At*MKK4 and *At*MKK5, or *At*WRKY22/*At*WRKY29 was shown to confer resistance to the bacterial pathogen *Pseudomonas syringae* or the fungal pathogen *Botrytis cinerea* (Asai et al., 2002). *At*WRKY22 and *At*WRKY29 were also able to activate flagellin induced receptor kinase1 (*FRK1*) expression. FRK1 is identical to *SIRK*. In tobacco, virus-induced silencing of genes encoding a MAPK cascade resulted in the transcript reduction of three *WRKY* genes. Moreover, silencing of these three *WRKY* genes compromised *N* gene mediated resistance to Tobacco Mosaic Virus (Liu et al., 2004). *Nt*WRKY1 is phosphorylated by salicylic acid-induced protein kinase (SIPK). Co-expression of *SIPK* and *Nt*WRKY1 in *N. benthamiana* resulted in a more rapid cell death than expression of SIPK alone, suggesting that *Nt*WRKY1 is involved in the development of an HR-like cell death (Menke et al 2005). More recently, in an elegant experiment using chromatin immunoprecipitation, it has been shown in cultured parsley cells, that the promoter sites of elicitor-induced genes such as *PcWRKY1* and *PcPR1-1* are constitutively occupied by certain WRKY proteins in the non-induced state but replaced by other WRKY proteins in a stimulus dependent manner *in vivo* (Turck et al., 2004; Uelker and Somssich, 2004).

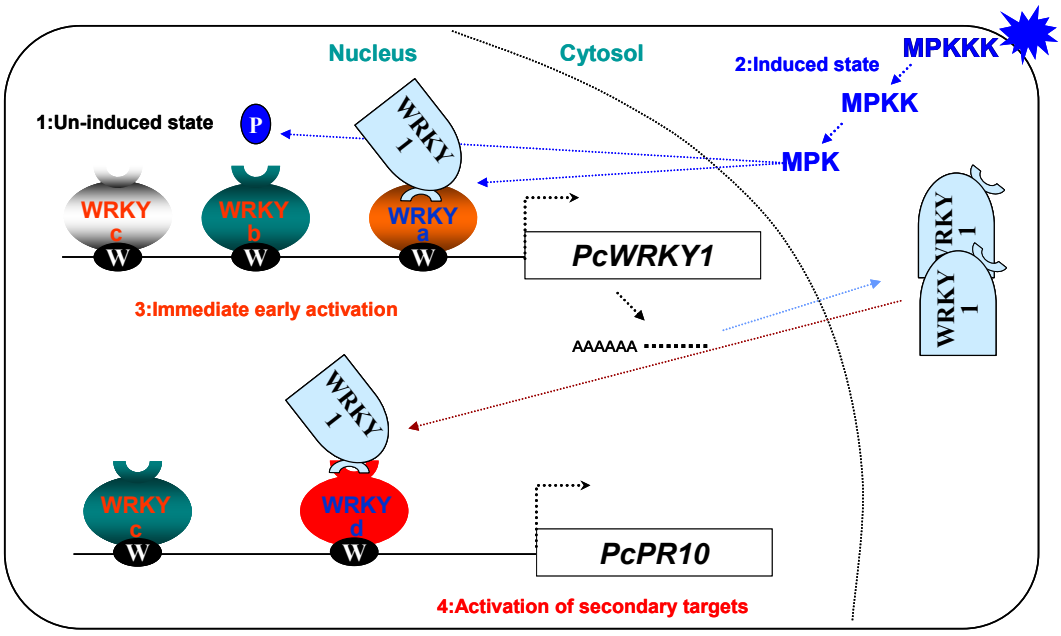


Fig.2. Hypothetical model for WRKY/W-box-mediated transcriptional gene regulation in parsley. 1: In un-induced parsley cells, W box promoter elements of direct target genes are already bound by a set of WRKY factors (shown in black box) that are inactive or participate in actively repressing basal gene expression. 2: Upon receptor-mediated recognition of a pathogen, a MAPK cascade (MAPK kinase kinase [MAPKKK]—MAPK kinase [MKK]—MAPK [MPK]) (shown in blue) is rapidly activated and ultimately results in the translocation of the protein kinase (MPK) to the nucleus. 3: The activity of this kinase directly modifies certain WRKY factors at the promoter of immediate-early-type genes such as *PcWRKY1* (shown in orange box), and thereby derepressing/activating the expression of these genes by positive feedback loop. 4: Consequently, WRKY1 protein levels in the cell increase, resulting in the autoregulation of *PcWRKY1* (shown in red box) and in the activation of secondary target genes such as *PcPR10*. Blue arrows show the activation of MAPK pathway and translocation of MAPK from cytoplasm to nucleus. Green arrow shows synthesis of *Pc*WRKY1 (immediate-early-type genes products). Red arrow shows the activation of *Pc*PR10 by *Pc*WRKY1. Adapted from Uelker and Somssich (2004).

These experimental observations revealed the involvement of a dynamic mechanism in which the various regulatory WRKY proteins are replaced by other WRKY factors upon pathogen infection or elicitor treatment in a mutual competing manner rather than by recruitment of WRKY factors to specific unoccupied *cis*-acting elements (Fig. 2).
Genetic analysis between Arabidopsis and *Ralstonia solanacearum* (*R. solanacearum*) interactions yielded in the cloning of *Resistance* to *Ralstonia solanacearum* (*RRS1*) gene, also named *AtWRKY52*. It encodes a protein that exhibits structural motifs similar to those of other resistance proteins (TIR-NBS-LRR). RRS1 has a C-terminal extension with a putative nuclear localization signal (NLS) and a WRKY domain (Deslandes et al., 2002). RRS1-GFP localized to nucleus upon pathogen challenge (Deslandes et al., 2003).

1.4. Bacterial wilt disease

R. solanacearum is a soil borne bacterium causing lethal wilting disease of more than 200 plant species representing over 50 families (Hayward, 1991). This broad host range covers *Solanaceous* plants (e.g tomato, potato, tobacco, and eggplant), various *Leguminous* plants (peanut, French bean etc), diverse monocots (like banana, ginger) and extends from annual plants to trees and shrubs (mulberry olive, cassava, and eucalyptus). Recently, Deslandes and co-workers in 1998, 2002 and 2003 showed that certain Arabidopsis ecotypes are also susceptible to several *R. solanacearum* strains (Fig. 3). Beside the broad and diverse host range of *R. solanacearum*, this devastating pathogen adapted a wide geographic distribution mainly in warm and tropical climates (Hayward, 1994). However, an increasing number of reports elaborate on the expansion of regional spectra more towards temperate countries in Europe and North America as the consequence of faster adaptation of certain strains to cooler environmental conditions (Janse, 1996), demonstrating the versatile and aggressive nature of the pathogen.

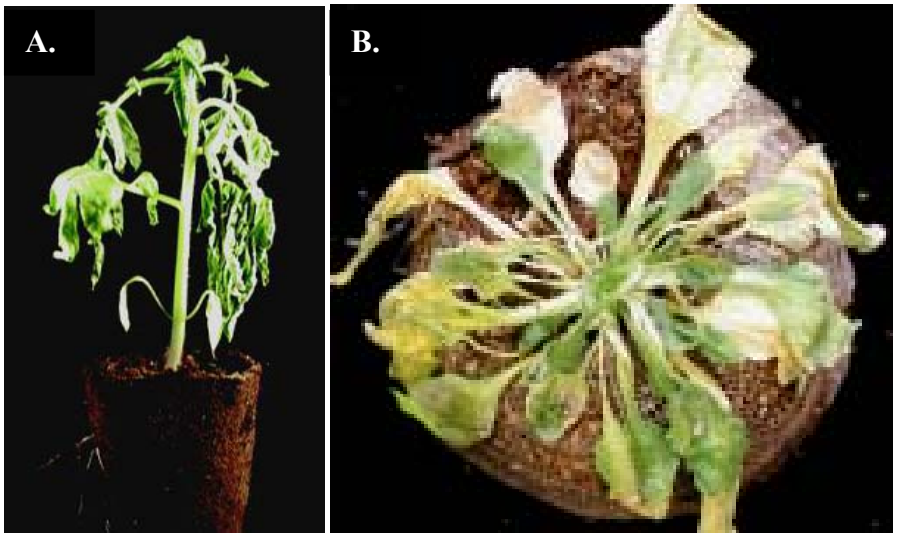


Fig. 3. A: Symptoms caused by *Ralstonia solanacearum* on tomato. B: Phenotype of susceptible *Arabidopsis thaliana* Col-5 plants, 10 days after root inoculation by the *Ralstonia solanacearum* GMI1000 strain. Photo courtesy of Stephane Genin, Christian Boucher and Laurent Deslandes, Toulose, France

1.5. *R. solanacearum*

R. solanacearum, previously known as *Pseudomonas solanacearum*, is a Gram-negative bacterium (Palleroni and Doudoroff, 1971; Palleroni, 1984; Stackebrandt et al., 1988). The closest members of *R. solanacearum* are *R. pickettii*, and *R. eutropha*. Surprisingly, both these species are not pathogens of plants. However, they are able to metabolize a wide range of aromatic compounds. Additionally, *R. pickettii* is an opportunistic human pathogen (Palleroni, 1984).

R. solanacearum strains show a high degree of ecological and physiological dissimilarities and are divided into five races as a result of their host preferences (Buddenhagen et al., 1962). By contrast, the six biovars were classified based on their ability to metabolize disaccharides (cellobiose, lactose, and maltose) and hexose alcohols (mannitol, sorbitol, and dulcitol) (Hayward, 1991 and 1994). For instance, bv-1 can't oxidize any of the disaccharides and hexose alcohols, while bv-3 can oxidize all. On the contrary, bv-2 can only oxidize the disaccharides, whereas bv-4 oxidizes only the hexose alcohols (Buddenhagen et al., 1962). In spite of these biological and biochemical

differences, *R. solanacearum* has clearly delineated into two evolutionary/geographic conserved divisions on the basis of restriction fragment length polymorphism (RFLP) analyses (Cook et al., 1989 and 1991), along with DNA sequence analyses of 16s rRNA, *egl* (extracellular glucanases), and *pglA* (polygalacturonaseA) (Fegan et al., 1998). Division 1 strains are primarily from Asiatic origin, and belong to bv-3, bv-4, or bv-5; division 2 strains belong to mostly bv-1, and are from the Americas. Recently, isolates belong to a third group have also been identified and known to be of African origin (Poussier et al., 2000).

In order to understand the molecular mechanism of bacterial pathogenicity, three strains, namely K60 (Kelman, 1954), GMI1000 (Boucher et al., 1986) and AW (Schell, 1987) have been intensively studied. Particularly, the whole genome sequence availability of strain GMI1000 (Salanoubat et al., 2002) facilitated a deep insight into bacterial virulence by developing numerous genetic and biochemical tools.

1.6. Molecular mechanism for disease establishment

Primarily *R. solanacearum* behaves as a saprophytic bacterium which can survive over a long period in various natural habitats like water surfaces, humid soil, and even among the roots of non-susceptible plant hosts (Hayward, 1991). Thus, the bacterium developed sophisticated and versatile metabolic mechanisms to detoxify deleterious compounds as well as increased adhesion for efficient colonization and maintenance in specific ecological zones (Genin and Boucher, 2002 and 2004).

Under favorable conditions, the early events in the natural infection process involve the bacterial attachment to the root surface, motility and microcolony formation, especially at the root elongation zones (Kang et al., 2002; Tans-Kersten et al., 2001). *R. solanacearum* strain GMI1000, an adherent pathogen, contains a large set of genes encoding attachment factors and extracellular molecules/structure that interact with

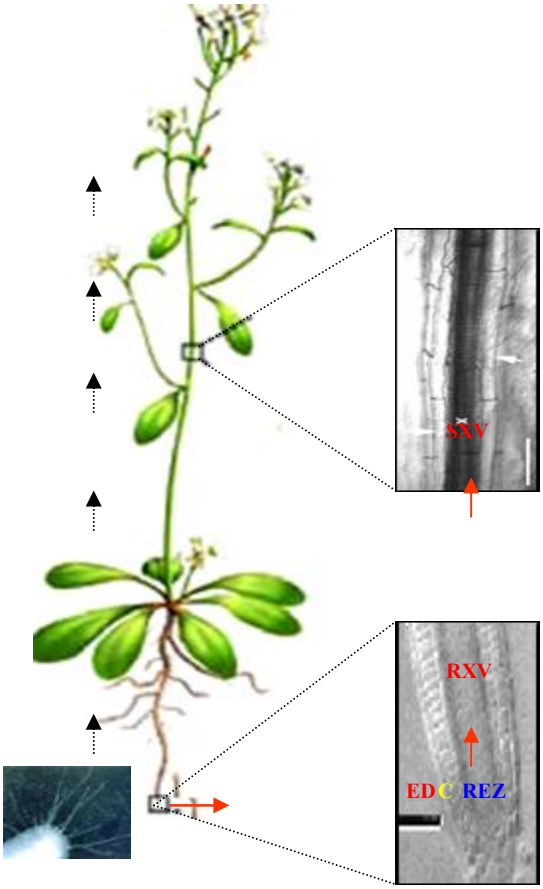


Fig. 4. Hypothetical scheme of *Ralstonia solanacearum* strain GMI1000 natural infection process in *Arabidopsis thaliana*. A magnified view of Arabidopsis root (lower picture) and stem vasculature (upper picture.). Directions of black and red arrows show the bacterial disease development path after the adhesion of bacterium at root elongation zone. REZ (in blue): root elongation zone (bacterial attachment site), ED (in red): endodermis (bacterial entry port into plant root). C: root cortex (bacterial path towards root vasculature), RXV (in red): root xylem vessels (bacterial free movement space), SVX (in red): stem xylem vessels (bacterial multiplication site).

diverse surfaces. The well known adherence structures are the filamentous appendages called pili. From here, bacteria invade the intercellular spaces of the root cortex by natural wounding sites of lateral roots and after crossing the endodermal barrier enter into

the vascular parenchyma (Schmit., 1978; Vasse et al., 1995; Saile et al., 1997; McGarvey. 1999). Pectic polymers of the middle lamella are disrupted by the pectinolytic enzymes, providing the nutrients to the bacteria and also allowing them to disperse throughout the tissue (Schell, 2000). Finally, bacteria break the xylem vessels probably by their cellulolytic enzymes and can freely move upwards throughout the stem into the aerial parts of the plants (Fig. 4).

In vitro studies showed the production of two extracellular glucanases (Egl1and CbhA), which are capable of breaking β-1-4 glycosidic linkages of cellulose (Schell., 1987 and 2000; McGarvey., 1999). In xylem vessels, bacteria rapidly multiply and reach high titer levels of > 10^{10}cells/cm of stem (in tomato). Eventually, plants wilt owing to the reduced sap flow caused by the high accumulation of bacteria and their exopolysaccharide (EPS) slime in the xylem (Saile et al., 1997; McGarvey et al., 1999). The bacteria released from the collapsed stems and infected roots then return to soil again surviving as saprophytic organisms.

1.7. Pathogenicity determinants and protein secretion systems in *Ralstonia solanacearum* strain GMI1000

Extracytoplasmic protein secretion trafficking is an essential characteristic of almost all bacteria. It facilitates many biological processes including bacterial host pathogenic/symbiotic interactions (Ma et al., 2003). Unlike nutrient molecules, extracellular molecules are comparatively much larger and are unable to diffuse from the outer membranes (Pallen et al., 2003). Thus, secreted proteins in gram-negative bacteria are translocated through the lipid bilayer of the cell envelope by the general secretion pathway (Sec-pathway) in two independent steps (de Keyzer et al., 2003). In gram negative bacteria, at last six major secretion systems (the type I, II, III, IV, V and the chaperone/usher pathway) are operated for protein translocation through the outer membranes (Cornelis et al., 2000; Henderson and Nataro, 2001; Jacob-Dubuisson et al., 2001; Sandkvist et al., 2001; Ding et al., 2003; Desvaux et al., 2005). Noticeably, *R. solanacearum* strain GMI1000 contains genetic information for all of the known

secretion pathways (Salanoubat et al., 2002; Genin and Boucher, 2004). However, the well studied Type III secretion systems (TTSS) plays a major role in the pathogenicity of several important pathogens that differ in host range and lifestyle including *R. solanacearum*. Type III secretion toxins, also known as effectors, can be delivered directly into the host cell cytosol by an injection mechanism (Abe et al., 2005; Mota and Cornelis, 2005). TTSS are encoded by *hypersensitive response and pathogenicity* (*hrp*) genes (Van Gijsegem et al., 1995). The *hrp* genes are required for bacteria to trigger the hypersensitive response (HR, a plant defense response) in resistant plants and to contribute to disease in susceptible plants (He et al., 2004). Christian Boucher and co-workers in 2004 predicted the presence of at least 60-80 TTSS-effector molecules in the genome of *R. solanacearum* strain GMI1000. Among them, PopP2, RipA, RipB, RipG, and RipT effectors were shown to transit through the TTSS (Sory et al., 1994; Casper-Lindley et al., 2002; Cunnac et al., 2004; Genin et al., 2005).

1.8. Recognition of TTSS-effectors by plant surveillance system

R. solanacearum, PopP2 and RipT, although unrelated in sequence, possess the catalytic triad characteristic of classes of cysteine proteases related to *Yersinia* sp. YopP/J and YopT, respectively (Deslandes et al., 2002; Deslandes et al., 2003). Some plants have evolved resistance proteins to recognize YopJ-like effectors. *Arabidopsis RRS1* confers resistance to *R. solanacearum* strain GMI1000 expressing PopP2 (Deslandes et al., 2002). RRS1 has been shown to interact with PopP2 of *R. solanacearum* in a yeast split ubiquitin yeast two-hybrid assay (Deslandes et al., 2003). Furthermore, RRS1 and PopP2 co-localize to the nucleus when transiently coexpressed in Arabidopsis protoplasts and nuclear translocation of RRS1 is dependent upon the nuclear localization signal present in PopP2. The presence of a WRKY transcriptional activator domain on the C terminus of RRS1 and the putative SUMO-protease activity of PopP2, might suggest that RRS1 is a target of PopP2, rather than being a specific receptor for PopP2 (Lahaye, 2004).

1.9. Objectives of this study

Very little is known about the transcriptional involvement of *At*WRKY factors activating defense responses. The primary goal of this project was to define the biological function of a specific *At*WRKY factor involved in plant defense. Functional redundancy is the common problem often associated with transcription factor families. Therefore, initially there was a need to have a deep insight into the whole family, and then further characterize a specific *At*WRKY family member. In order to achieve the primary objective, the following topics were studied:

1. Analysis of *At*WRKY expression profiles in *A. thaliana* mutants impaired in different signaling pathways
2. Phenotypical responses of WRKY-KO mutants to plant pathogens.

On the basis of results obtained from the above analysis, a specific candidate was chosen. In order to further characterize it, the following questions were addressed:

1. What role does the *At*WRKY factor play in plant defense?
2. What are the direct/indirect target genes of the chosen *At*WRKY factor?

2. Materials and Methods

2.1. Materials

2.1.1. Bacteria / fungi / oomycetes

***E coli* strains:**

DH5α: Genotype: supE44 DlacU169 hsdR17, recA1, endA1, gyrA96, thi-1, relA1, F (Hanahan, 1983).

DB3.1: Genotype: F- *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(rB-, mB-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Smr) *xyl*5 Δ*leu mtl*1 (Invitrogen)

Agrobacterium tumefaciens:

GV3101 pMP90RK (Koncz et al., 1990)

Pathogens

Bacterial pathogens

***P seudomonas syringae* pv. *tomato* DC3000** (Whalen et al., 1991)

***Ralstonia solanacearum* Strain GMI1000** (Deslandes et al., 1998)

Fungal Pathogens

Barley powdery mildew *Blumeria graminis f. sp. hordei (Bgh)* (kindly provided by Ralph Panstruga MPIZ-Koeln

Pea powdery mildew *Erysiphe pisi* (kindly provided by Ralph Panstruga MPIZ-Koeln)

A. thaliana powdery mildew *Golovinomyces orontii*(kindly provided by Ralph Panstruga MPIZ-Koeln)

Oomycete pathogen

Peronospora parasitica isolate Noco2 (McDowell et al., 2000; Dangl et al., 1992).

2.1.2. Plant Material

All *A. thaliana* plants used in this study belong to Col-0, Col-1, Col-6, Ler, Ws0 and Nd-1 genetic background. Plants were grown at 20° C and and PFD of 80 mmol photons m^{-2} s^{-1} in pathogens free chambers either in long day conditions (16h light/8h darkness) or short day conditions (8h light/16h darkness) with the exception mentioned specifically. For pathogen test, plants were grown under a 10 h light/ 14 h darkness cycle. Below, here is the list of all mutants and transgenic/non-transgenic lines used in this study.

Mutants:

Mutant names	**Abbreviations**	**Backgrounds**
allene oxide synthase	*(aos)*	Col-6
constitutive expressor of PR genes	*(cpr5-2)*	Col-0
constitutive triple response1	*(ctr1)*	Col-0
defense no death 1	*(dnd1)*	Col-0
enhanced disease resistance1	*(edr1)*	Col-0
enhanced disease susceptibility1	*(eds1)*	Ws0
ethylene insensitive2	*(ein2-1)*	Col-0
ethylene-related1	*(etr1)*	Col-0
jasmonic acid resistant1	*(jar1)*	Col-0
non-race–specific disease resistance1	*(ndr1)*	Col-0
non-expressor of PR 1	*(npr1)*	Col-0
phytoalexin-deficient 4	*(pad4-1)*	Col-0
Atwrky27ETL	*27ETL*	Col-1
Atwrky27-90-1(SALK_109290)	*27-90-1*	Col-0

Transgenic Lines:

Transgenic designation	**Description**
GFP lines	
AtWRKY27p::sm-GFP::Term	Plants expressing a 2kb *AtWRKY27* promoter driven coding sequence of sm-GFP and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
AtWRKY27p::AtWRKY27::GFP-Term	Plants expressing a 2kb *AtWRKY27* promoter driven *AtWRKY27* cDNA, coding sequences of GFP and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
Complementation lines	
AtWRKY27p::AtWRKY27::StrepII-Term	Plants expressing a 2kb *AtWRKY27* promoter driven *AtWRKY27* cDNA, coding sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
At4CL-2p::AtWRKY27::StrepII-Term	Plants expressing a 1550 bps *At4CL-2* promoter driven *AtWRKY27* cDNA, coding sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
AtSUC2p::AtWRKY27::StrepII-Term	Plants expressing a 2067 bps *AtSUC2* promoter driven *AtWRKY27* cDNA, coding sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
AtCel1p::AtWRKY27::StrepII-Term	Plants expressing a 1649 bps *AtCel1* promoter driven *AtWRKY27* cDNA, coding

	sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
Promoter GUS lines	
AtWRKY27p::GUS::StrepII-Term	Plants expressing a 2kb *AtWRKY27* promoter driven GUS reporter gene, coding sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
At4CL-2p::GUS::StrepII-Term	Plants expressing a 1550 bps *At4CL-2* promoter driven GUS reporter gene, coding sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
AtSUC2p::GUS::StrepII-Term	Plants expressing a 2067 bps *AtSUC2* promoter driven GUS reporter gene, coding sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
AtCel1p::GUS::StrepII-Term	Plants expressing a 1649 bps *AtCel1* promoter driven GUS reporter gene, coding sequences of StrepII and terminator sequences using MultiSite Gateway ® Three-Fragment Vector System
Ectopic overexpressor lines	
2x35S::AtWRKY27-GFP	Plants expressing double *35S* promoter driven *AtWRKY27* cDNA, coding sequences of GFP and terminator sequences

2x35S::AtWRKY27-Term	Plants expressing double *35S* promoter driven *AtWRKY27* cDNA and terminator sequences
2x35S::AtWRKY27-myc-Term	Plants expressing double *35S* promoter driven *AtWRKY27* cDNA, coding sequences of myc and terminator sequences
2x35S::AtWRKY27-StrepII-Term	Plants expressing double *35S* promoter driven *AtWRKY27* cDNA, coding sequences of StrepII and terminator sequences
Inducible Overexpressor lines	
RGRBD::GFP→SIRKp::GUS line	*SIRKp::GUS* transgenic plants expressing a strong Dex inducible $P_{G10\text{-}90}$ promoter driven coding sequence of GFP and terminator sequences
RGRBD::27→SIRKp::GUS line	*SIRKp::GUS* transgenic plants expressing a strong Dex inducible $P_{G10\text{-}90}$ promoter driven *AtWRKY27* cDNA and terminator sequences
pMD::vector→ Atwrky27-90-1	*Atwrky27-90-1* mutant plants expressing a strong β-estradiol inducible $P_{G10\text{-}90}$ promoter driven vector sequences
pMD::vector→ Atwrky27ETL	*Atwrky27ETL* mutant plants expressing a strong β-estradiol inducible $P_{G10\text{-}90}$ promoter driven vector sequences
pMD::27→ Atwrky27-90-1	*Atwrky27-90-1* mutant plants expressing a strong β-estradiol inducible $P_{G10\text{-}90}$ promoter driven *AtWRKY27* cDNA and terminator sequences

pMD::27→Atwrky27ETL	*Atwrky27ETL* mutant plants expressing a strong β-estradiol inducible $P_{G10\text{-}90}$ promoter driven *AtWRKY27* cDNA and terminator sequences

2.1.3. Media and Additives

Media for Bacteria

LB medium (Sambrook) 5g yeast extract, 10g trypton, 10g N, pH=7.5.
SOC medium (Sambrook) 5g yeast extract, 20g trypton, 20 mM glucose,
0.5g NaCl, 2.5 mM $CaCl_2$, pH=7.5.
YEB medium 10g yeast extract, 10g peptone, 5g NaCl.
When required, antibiotics were supplemented to the following final concentration:
Ampicillin 100 mg/l
Carbenicillin 100 mg/l
Gentamycin 10 mg/l
Rifampicin 100 mg/l
Kanamycin 50 mg/l

Media for plants

Media were diluted in 1l deionized H_2O.
MS-medium: 4.7g MS salt supplemented with vitamins, 5-10 g glucose, pH 5.7-5.8
Media were diluted in deionized 1l H_2O. For solid media 15 g of agar was added.

Additives

Antibiotics:

Ampicillin (1000x):	100 mg/ml in H_2O
Carbenicillin (1000x):	50mg/ml in Ethanol
Chloramphenicol (1000x):	25 mg/ml in Ethanol

Gentamycin (1000x): 25mg/ml in DMF

Kanamycin (1000x): 50 mg/ml in H_2O

Rifampicin (1000x): 100mg/ml in DMSO

Streptomycin (1000x): 20mg/ml in H_2O

Spectinomycin (1000x): 100mg/ml in H_2O

Stock solution stored at –20 ^oC

2.1.4. Nucleic Acids

Plasmids

Plasmids used for the generation of constructs described in this thesis are listed below:

pCR TOPO2.1 (Invitrogen, Heidelberg) amp^R kan^R

pDONR™ 201 (Invitrogen, Heidelberg) kan^R

pDONR™P4-P1R (Invitrogen, Heidelberg) kan^R

pDONR™P2-RP3 Terminator (Laurent Deslandes and Imre E Somssich) kan^R

pDONR™P2-RP3 StrepII-Terminator (Laurent Deslandes and Imre E Somssich) kan^R

pDONR™P2-RP3 GFP-Terminator (Laurent Deslandes and Imre E Somssich) kan^R

pAM-PAT 35S GW Terminator (Bekir Uelker and Imre E Somssich) amp^R

pAM-PAT 35S GW myc-Terminator (kindly provided by Imre E Somssich) amp^R

pAM-PAT 35S GW StrepII-Terminator (Laurent Noel, Laurent Deslandes and Imre E Somssich) amp^R

pAM-Kan 35S GW GFP (Franziska Turck and Imre E Somssich) amp^R

pAM-PAT Mult based on pDEST™R4-R3 ((Laurent Deslandes and Imre E Somssich) amp^R

pAM-PAT $P_{G10\text{-}90}$ *RGRBD GW* ((Laurent Deslandes and Imre E Somssich) amp^R

*pMD-pER8-LexA-GUS-stop-LexA-GW-StrepII (*M S Mukhtar, Laurent Deslandes and Imre E Somssich) $Spectinomycin^R$

Oligonucleotides

Table 1. Listed below are primers used in the present study and were synthesized by Invitrogen.

Primers	**Primer sequence 5' → 3'**
ASN2Qt-R	GCTTTCGCGGTACTGCAAGCTAT
LTP-fam-R	GAGAAGAAGACTGAGAGAGAGT
LTP-fam-F	CGGTAACTGCGGTTGCCCTTCTC
Dof-znc-R	CGCTCTGTTGATCACAGTTGTTC
Dof-znc-F	GTGAATTGTTATGATCCGTCGTC
GH3-R	GCTTAAGACCACGTTCTTGCGGC
GH3-F	GTGGAGTTACTAGCTCTATCAGT
ARD-fam-R	CTATGTTACGGTCCCCAATCACC
ARD-fam-F	CTTATGTGAGGTGTGTCCAGAG
SAM-R	GGGTTTAGATCCCAAAGCAAGAAG
SAM-F	CAGTCAAGCTACCAGGAAGAAATC
NR2-R	CCAGATTCATTACCAGGAAGCG
NR2-F	CCATGTCACGAGGAGATTCTTC
NR1-R	CATCCACCCACCCGACTGGTTTC
NR1-F	GTGTTTGTGAGCTTGACCACCAG
Exp-prot-R	GCTGGGTCCAGACCCCGGACGA
Exp-prot-F	CCGGAGAGATCCCTCCTGTTTCT
DSS1-SEM1-R	GTACCATTCTCAAGCTCCTTCC
DSS1-SEM1-F	ATGGCGGCAGAACCGAAGGCAGC
ASN2-R	GTTCCATTCTTAGGAAGAGGATC
ASN2-F	GTATAGCTGGATAGATGGTCTG
UBQ-Exon4-Rev	GCAGTTGACAGCTCTTGGGTG
UBQ-Exon1-Frw	CCGGCAAGACCATCACTCTCG
SIRK-Exon3-Rev	TTCTGAACTCACTATACGCGGTGTC
SIRK-Exon1-Frw	CATCGATTTTATTCACAAGCTTTGC
W27Exon3_684R-2	CCGGTGAGTAGGACGTGGATGAGT

W27-exon1-F-2nd	GAGGTCAACGCGACCAGCTTCTTCA
SALK_LBb1_R489s	GCGTGGACCGCTTGCTGCAACT
SUC2-int1700b-R	CTGATATGGACGTTCGATTCTC
SUC2-int1700b-F	GAGAATCGAACGTCCATATCAG
SUC2-int1400b-R	GTTTTATTTTCAGACGAAAGCG
SUC2-int1264b-F	CAAATAAACAGTGGGAGAGGTG
SUC2-int-849b-R	CGATCTCTTCCGTTATCTCTG
SUC2-int-803b-F	GATTACAAGTGTCAAGTCCATGAC
SUC2-int-468b-R	CTTTATGGATGAGACAACATAAC
SUC2-int-359b-F	GGTTGTTCGTAAATGGTGC
Cel1-int1240b-F	TAGGGCGGTGGATCACATGGT
Cel1-int-985b-F	CACAACCACAACCACAAGTTTG
Cel1-int-960b-R	CTCGATGTATATAGTAACGT
Cel1-int-750b-F	GGCACACAAAGAACAGAACAGG
Cel1-int-450b-R	CTAGTAGACCAAATCAGAGAC
Cel1-int-340b-F	CGTGTATGACATAATGATTAGCA
4CL-int1150bp-F	GGGCTAGTTGCAGAGGAAACTC
4CL-int-800bp-F	GTTCTGTCTTGGAAGAGTACTG
4CL-int-600bp-R	GCTGACACTTCTCTAGAGCCTGC
4CL-int-400bp-F	GGCCTTAAAGACCTGAGAATG
SUC2-pr-mltis-R	GGGGACTGCTTTTTTGTACAAACTTGT ATTTGACAAACCAAGAAAGTAAG
SUC2-pr-mltis-F	GGGGACAACTTTGTATAGAAAAGTTGG GGGACCATGAAATCATTTGCATATG
Cel1-pr-mltis-R	GGGGACTGCTTTTTTGTACAAACTTGTT CTTTTGTCTCTGTTTTTTGTGC
Cel1-pr-mltis-F	GGGGACAACTTTGTATAGAAAAGTTGG GGAGGATCACATGCATCAGCACT
4CL2-pr-mltis-R	GGGGACTGCTTTTTTGTACAAACTTGT GAATCAGAAGTTAATATCAAAT
4CL2-pr-mltis-F	GGGGACAACTTTGTATAGAAAAGTTGG

	GGAATTCCATCATTTCAGTAGAG
Glutathione-R	GTAACACTTCCAAACATGGCC
Glutathione-F	GGTAGATCGATAGCCGAATCA
GAD1-R	CCAGCCAGTGATGATATCTCTC
GAD1-F	GACCAACCCACCTTTACTCTC
AttB2-smGFPstpR	(GWR)CTTATTTGTATAGTTCATCCATG CC
AttB1-smGFPstpF	(GWF)TAATGAGTAAAGGAGAAGAACT TTTC
EIN2_CAPS_318_R	CTTCTCCACAGGACTCATTGG
EIN2_CAPS_318_F	CAATAGTGCGGTTGGAAAGCAGGG
GUS-Rev-375nstd	CCCGGCAATAACATACGGCGTGA
W27-Exon3_FRW	CTCATCCACGTCCTACTCACCGGA
SelB_Rev	GTAACATCAGAGATTTTGAGACAC
SelA_FRW	TCGCGTTAACGCTAGCATGGATCTC
THI2.1_Rev	CAACAGTTTAGGCGGCCCAG
THI2.1Frw	TGGTCATGGCACAAGTTCAAGTAG
PDF1.2_rev	CAGATACACTTGTGTGCTGGG
PDF1.2_Frw	CTTATCTTCGCTGCTCTTGTTCTC
PR5_Rev	CCGGATGGTCTTATCCCCAGC
PR5_Frw	CTCTTCCTCGTGTTCATCAC
PR1_Rev	GACCGATGTAATTCCCCGGAGGATC
PR1_Frw	CGTCACACTCCCGCTCAACCGCC
VSP1_attB2	(GWR)CAGAAGGTACGTAGTAGAGTGG
VSP1_attB1	(GWF)TAATGAAAATCCTCTCACTTTCA
Strep_Rev19bp	CAAATTGAGGATGAGACCA
LBa1-Salk-Left	TGGTTCACGTAGTGGGCCATCG
W27-exon1-FRW	GAGGTCAACGCGACCAGCTTCTTCA
GUSFRW817	CCCGCTTCGCGTCGGCATCCGGT
GUSrev556	CGTCCACCCAGGTGTTCGGCGT
GUSFRW898bps	GGCTTTGGTCGTCATGAAGATGCGGA

GUSRev1001bp	GCGTAAGGGTAATGCGAGGTACGGTA
AttB2-SpeI-GUS	(GWR)CACTAGTTCATTGTTTGCCTCCC TGCTGCGGT
AttB1-XhoI-GUS	(GWF)TACTCGAGATGTTACGTCCTGTA GAAACCCCA
W27Exon3_684Rev	CCGGTGAGTAGGACGTGGATGAGT
SALK_LBb1_Rev	GCGTGGACCGCTTGCTGCAACT
W27exon3_890FRW	CATGGAAGAGGAAGAGGAGGA
W27exon1_245Rev	GGAGGAAGTAGAGGAGGAGGAGACCA
W27_pr_intF1027	CTTGTTTTCGCAAGAAAATCTGATA
W27_pr_intR1136	CGACCACTAGCCCTCAAAAATCTA
W27pr_msiteGW-R	TTTGTACAAACTTGTGGCGATCTTGAA TTCGTGAGGAAAGCT
W27pr_msiteGW-F	GTATAGAAAAGTTGGGATACGGAAGC AGACAATCATTACCT
Tublin_reverse	ATATCGTAGAGAGCCTCATTGTCC
Tubilin_forward	ACGTATCGATGTCTATTTCAACGA
M13reverse	GGAAACAGCTATGACCATG
M13forward	GTAAAACGACGGCCAGT

The universal Gateway – compatible extensions for the BP recombination reactions (between an attB-flanked PCR product and a donor vector containing attP sites to create an entry clone) were:

GWF (*att*B1) 5′ *ggggacaagtttgtacaaaaaagcaggctta*3′

GWR (*att*B2) 5′*ggggaccactttgtacaagaaagctgggtc*3′

2.1.5. Proteins

Antibodies:

2 mol AP / mol *Strep*-Tactin conjugate (0.125 ml) is used in this study.

Enzymes

If not indicated otherwise, enzymes used for experiments in this thesis were obtained from Roche and New England Biolabs. 10 x buffers for restriction enzymes were companied with the enzymes and supplied by manufacturers.

Nucleic acid modifying enzymes

Taq DNA Polymerase Mix Roch

Pfu DNA-Polymerase Stratagene (Heidelberg)

Pfx DNA-Polymerase Invitrogen (Heidelberg)

TAKARA LA *Taq* polymerase (Takara, Seta 3-4-1, Otsu, Shiga 520-2193, Japan)

Klenow fragment exo- MBI Fermentas

Lysozym Serva

RNase A (DNase-free) MBI Fermentas

T4 DNA ligase MBI Fermentas

Ribonuclease Inhibitor MBI Fermentas

2.1.6. Buffers and Solutions

Buffer/Solution	**Recipe**
Coomassie staining solution	0.01% (w/v) Coomassie brilliant blue G-250 10% (v/v) Glacial acetic acid
C-TAB solution	CTAB (20grams), β-mercaptoethanol 10ml) dH_20 (990ml)
DNA loading buffer	67% (w/v) Sucrose 50 mM EDTA pH 8.0 0.42% (w/v) Bromphenolblue 0.42% (w/v) Xylenecyanol 0.42% (w/v) Orange G

DNA extraction buffer	0.1 M NaCl 0.01 M Tris-HCl, pH 7.5 1 mM EDTA 1% SDS
Denaturation extraction buffer for total protein	4 M Urea 16.6% (v/v) Glycerol 5% (v/v) ß-ME (fresh) 5% (w/v) SDS 0.5% (w/v) Bromphenolblue
GUS De-staining solution	50% glycerol 25% lactic acid H_2O
GUS staining solution	Na_2HPO_4 (1M) 57.7 ml NaH_2PO_4 (1M) 42.3 ml Na_2EDTA (0.5 M) 20.0 ml $K_4Fe[CN]_6$ 2.11 g $K_3Fe[CN]_6$ 1.65 g Triton X-100 0.1 % (v/v) Methanol 20.0 % (v/v) X-Gluc 1.0 g X-Gluc: 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, cyclohexylammonium salt (Roth).
Hybridization buffer	1 M NaCl 10% Dextran Sulphate 1 % SDS
1x PBS buffer	68 mM NaCl 58 mM Na_2HPO_4 17 mM NaH_2PO_4 pH 7.4 (NaOH)
1x PBS-T	1x PBS 0.1% (v/v) Tween 20
2x Protein Loading buffer (40ml)	H_2O 5ml Tris pH 6.8 (1M) 5ml SDS (10%) 20ml glycerol 10ml Bromphenol blue 0.01g Prior to use, add DTT (20µl DTT (1M) to 80µl loading buffer)
Resolving gel components10%	H_2O 4ml 30% acrylamide mix 5ml 1M Tris-HCl (pH8.8) 5.7ml 10% SDS 0.15ml 10% ammonium persulfate 0.15ml TEMED 0.006ml
10x RNA Gel running buffer	200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid) 50 mM sodium acetate 10 mM EDTA pH to 7.0 with NaOH
1.2% RNA gel preparation	FA gel (1.2% agarose) of size 10 x 14 x 0.7 cm, mix 1.2 g agarose 10 ml 10x RNA gel buffer Add RNase-free water to 100 ml
5x RNA Loading Buffer	16 µl saturated aqueous bromophenol blue solution 80 µl 500 mM EDTA, pH 8.0 720 µl 37% (12.3 M) formaldehyde 2 ml 100% glycerol 3084 µl

	formamide 4 ml 10 x FA gel buffer RNase-free water to 10 ml Stability: Approximately 3 months at 4°C
Spermidine solution (0.1 M)	Spermidine 73.0 mg ddH_2O ad 50.0 ml
10x SDS-PAGE running buffer	25 mM Tris-HCl, pH 8.3 200 mM Glycine 0.1% (w/v) SDS
20x SSC	2 M NaCl 0.3 M Sodium citrate Adjust pH to 7.0 with HCl
Stacking gel components (5ml)	H_2O 2.7ml, 30% acrylamide mixb 0.67ml, 1.5M Tris-HCl (pH8.8) 0.5ml, 10% SDS 0.04ml, 10% ammonium persulfatec 0.04ml, TEMED 0.004ml
StrepII Extraction buffer	100 mM Tris, pH 8.0; 5 mM EGTA; 5 mM EDTA; 150 mM NaCl; 10 mM DTT; 0.5 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride); 5 lg/ml aprotinin; 5 lg/ml leupeptin; plant protease inhibitor cocktail (Sigma P9599, Taufkirchen, Germany); diluted 1:200; 0.5% Triton X-100; and 100 lg/ml avidin
20x TAE	800 mM Tris 20 mM EDTA 2.3% (v/v) Glacial acetic acid
TE Buffer	10mM Tris, 0.1mM EDTA
TFB I	for 150 ml for 300 ml 30 mM KaC 0,44 g 0,883 g 0 mM MnCl2x4H2O 1,48 g 2,969 g 100mM RbCl2 1,81 g 3,627 g 10 mM CaCl2x2H2O 0,22 g 0,441 g 15 % Glycerin 22,5 ml 45 ml Adjust pH to 5,8 with HCl
TFB II	for 50 ml for 150 ml 10 mM Mops 0,105 g 0,45 g 75 mM CaCl2x2H20 0,551 g 1,65 g 10 mM RbCl2 0,061 g 0,18 g 15 % Glycerin 7,5 ml 22,5 ml Adjust pH to 7,0 with NaOH

Western-blotting Transfer buffer	NaPO4 pH 7 1M 15ml SDS 10% 5ml Methanol 200ml H_2O add up to 1l. Pre-cool transfer buffer on ice (for 1l)

2.1.7. Chemicals and radiochemicals

If not indicated otherwise, chemicals and radiochemicals used for experiments in this thesis were obtained from Amersham Buchler GmbH & Co KG, J.T. Baker Chemicals, BioRad, Difco Laboratories, Fluka, Merck AG, Serva Feinbiochemica GmbH & Co, Sigma Aldrich GmbH.

2.1.8. Microscopes

Fluorescence microscope: Leica MZ12 with Mercury HBO 50 W/Ac lamp and FITC filter. Confocal laser scanning microscope: Leica DMIRBE, TCS4D, with digital imaging processing, A 530+/-15nm band pass filter for FITC specific detection and a 580 nm band pass filter for autofluorescence detection.

2.1.9. Photographical data processing

Pictures were taken with assistance of Mrs. Maret-Linda Kalda, MPI-Photo Laboratory. Photos were processed using Adobe Photoshop 6.0 (Adobe Systems Inc.).

2.1.10. Online Softwares

BLAST and Bioinformatics	NCBI, MIPS, TAIR and TIGER
Cluster analysis	NASC "EPCLUST"
MultAlin software	Corpet, INRA Toulouse, France, BCM Launcher
Mutant search tools	GABI PoMaMo Database, SALK, NASC
Promoter analysis	PlantCare, PLACE

Protein prediction	ProtParam Tools, nterProScan Sequence Search tool, iPSORT, TargetP, Predotar

2.1.11. Other materials and Kits

Autoradiofilm XOMAT AR - Kodak

BP-Clonase - Invitrogen, Heidelberg

First Strand cDNA Synthesis Kit MBI Fermentas

Gel Extraction Kit - QIAGEN

Hybond N - Amersham Pharmacia Biotech

LR-Clonase - Invitrogen, Heidelberg

LR-Clonase plus- Invitrogen, Heidelberg

Miniprep® Kit - QIAGEN

Parafilm M - American National Can.

Reaction tubes - Eppendorf

Petridishes - Greiner GmbH

Pipette tips - Greiner GmbH

RNAwiz extraction reagent - Ambion

RNeasy Plant Mini® Kit - QIAGEN

Shrimp alkaline phosphatase – Roche, Mannheim

Sterile filtration units - Millipore

Strep-tag® AP Detection Kit - IBA

Whatman 3MM paper – Whatman

2.2. Methods

If not indicated otherwise, the methods employed in this study were taken from Sambrook, J. et al., eds. (1989) *Molecular cloning — a laboratory manual,* 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

2.2.1. Nucleic acids-related methods

DNA isolation:

DNA was isolated by C-TAB method (modified protocol of Shahjahan et al. 1995). 100 mg plant material was ground in liquid N_2 and transferred into 1.5 ml microfuge tube. 300µl hot (pre-heated at 65°C C-TAB was added as extraction buffer and incubated the tubes for 20 minutes. 600µl of Chloroform:Isoamyl-alcohol (24:1) was added and mixed on shaker for 15 minutes. The samples were centrifuged at 13000rpm for 5 minutes. Supernatant was transferred to a new Eppendorf tube containing equal volume (600µl) Isopropanol. The samples were thoroughly mixed and placed at -20°C for 10 minutes. Supernatant was discarded and pellet was washed with 70% cold Ethanol. Finally dry pellet was resuspended in 100µl TE Buffer. The DNA was measured by Eppendorf BioPhotometer with sample concentrations, absorption values, OD260/OD230 and OD260/OD230.

Separation of DNA fragments by agarose gel electrophoresis

DNA fragments were mixed with DNA loading buffer and analyzed by agarose gel electrophoresis. The agarose concentration depended on the size of fragments to be resolved (Sambrook and Fritsch, 1998). Electrophoresis was performed at 5 V/cm using TAE buffer. 1kb ladder DNA size marker (Invitrogen) was used to estimate the size of DNA fragments. After electrophoresis, DNA was visualized on a transilluminator under UV light (254 nm).

Purification of gel-extracted DNA fragments

PCR products were purified using Qiagen PCR Fragments Purification Kit or Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

DNA sequencing

DNA sequences were ascertained by the DNA core facility (ADIS) of Max-Planck Institute for Plant Breeding Research, Cologne on Abi Prism 377, 3100 or 3730 sequencers (Applied Biosystems, Weiterstadt, Germany) using BigDye-terminator v3.1

chemistry. Premixed reagents were from Applied Biosystems. Sequences were analyzed with various softwares an internet tools mentioned above.

RNA isolation and mRNA purification

Total RNA was isolated from 100 mg fresh tissue. The tissue was flash-frozen and ground in liquid nitrogen. Total RNA was extracted with 1 ml RNAwiz extraction reagent (Ambion, Huntingdon, Cambridgeshire, UK) following the supplier's protocol. The RNA was measured by Eppendorf BioPhotometer with sample concentrations, absorption values, OD260/OD230 and OD260/OD230. DNaseII treatment was performed using the DNA-*free* reagent (Ambion) for 20 min at 37ºC, and reaction composition was as suggested by the producer. Poly(A)$^+$ RNA was purified using Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech GmbH, Hamburg, Germany) according to the supplier's instructions. Poly(A)$^+$ RNA was eluted in 20 µl of DEPC-treated water.

PCR

Routine PCRs were carried out according to a standard protocol. Fifty ng DNA template (genomic DNA, plasmid DNA, cDNA, bacterial colony suspended in $dH2_O$/NaOH etc) were amplified in 25/50 µl of 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP and dTTP (Carl Roth & Co. KG, Karlsruhe, Germany), 0.25 µM of each primer (synthesized by Invitrogen), and 0.025/0.05 U/µl of *Taq* DNA polymerase (Invitrogen, Life Technologies, Karlsruhe, Germany). Reaction conditions were as follows: denaturation (2 min, 94°) one cycle, denaturation (15 sec, 94°), primer annealing (30 sec, 57-65 °C) and elongation (1 min per 1 kb, 68/72 °C), terminated by final elongation (72°C, 5-10 min).

RT-PCR

Reverse transcription–polymerase chain reactions (RT-PCR) were carried out with total RNA/mRNA templates, isolated and purified as described above. 3mg RNA or 250 ng mRNA was used as starting template material for first strand cDNA synthesis using SuperScript^{TM} II RNase H$^-$ (Invitrogen) as instructed by manual provided with the SuperScript^{TM} II RNase H$^-$. For subsequent RT-PCR analyses, 2 µl of above mixture as

template was used and reactions were standardized using tubulin primers, specific to tubulin β-subunit of Arabidopsis

Northern and dot blot analyses

Formaldehyde agarose gel preparation, quantification, electrophoresis and samples prepration/loading were done following the RNeasy Plant Mini® Kit QIAGEN protocol.

Plasmid DNA spotting

50ng of gel purified plasmid DNA or PCR products (plasmids were specifically amplified using defined respective two flanking primers) was spotted on Hybond N^+ nylon membrane (Hybond N - Amersham Pharmacia Biotech) by the DNA core facility (ADIS) of Max-Planck Institute for Plant Breeding Research, Cologne on Micro Gid II (Biorobotics).

Transfer the gel on nylon membrane

The gel was rinsed with DEPC-H_20. 4-5 pieces of Whatman 3mm paper and a piece of Hybond N^+ nylon membrane (Hybond N - Amersham Pharmacia Biotech), according to the pattern of the gel, were also wet with DEPC-10X SSC and placed on a platform (made of filter papers in a tray containing 10XSSC solution). Inverted marked gel was transferred on to Nylon membrane such that all of the area containing RNA is touching the nylon membrane. Air bubbles were rolled out during all the procedures. This sandwich was covered with a layer of 4 to 5 Whatman papers. Over the top a bit heavy scientific catalogue (mostly Sigma) was placed. After at least 20 hours, the sandwich was disassembled and RNA was crosslinked on the membrane by UV crosslinker provided by Stratgene by applying 120,000J x cm^{-2} of energy.

RNA hybridization

Pre-hybridization and hybridization were carried out in hybridization solution in glass tubes (30 cm x 4 cm) at 65°C under continuous rotation in a hybridization oven (Bachofer, Reutlingen, Germany). The pre-hybridization was performed overnight. Upon

adding the denatured radio-active probe, the hybridization was performed for at least 16 hrs. After hybridization the filter was washed accordingly:

1. twice 50 ml 2 x SSC + 0.1 % SDS at RT for 10 minutes
2. twice 50 ml 1 x SSC + 0.1 % SDS at 65°C for 10 minutes
3. once 50 ml 0.1 x SSC + 0.1 % SDS at 65°C for 15 minutes

The filter was wrapped in thin plastic foil (Saran wrap) and exposed overnight to a phosphoimager screen (Molecular Dynamics) in a cassette at room temperature.

Radioactively labeled probe preparation for Northern analysis

50-100 ng of gel-purified PCR product for Northern analysis was used in this protocol. Probe was prepared according to Rediprime II Random Prime Labelling System protocol manual provided by Amersham Biosciences. Probe was later on purified on a Sephadex G25 column.

Radioactively-labeled complex-probe preparation for dot-blot analysis

mRNA denaturation

mRNA	250 ng	(Dynabeads)
Oligo dT	1µl	(stock1ug. µl, pd(T)12-18 Amersham, cat. 27-7858-02)
DEPC-dH_2O	final volume of 12.5 µl	

The above 3 components were mixed and incubated at 65°C for 5 minutes and afterwards immediately placed at RT to allowing slow cool down for about 10 min.

Reverse Transcription

RT buffer 5x	6 µl	
dNTP mix	1 µl	(10 mM A+G+T and 0.01mM dCTP)
DTT 100mM	3 µl	
RNAseout	1 µl	
P^{32} α-dCTP	5 µl	(50 uCi. µl)
SuperscriptII	1.5 µl	(10 U.µl)

The above mentioned components were mixed and incubated at 42°C for 90 min. The reaction was stopped by adding 2 µl of 10 mM EDTA. The remaining RNA was

hydrolyzed by adding 2 µl of 0.5 M NaOH. The reaction was inactivated at 70°C for 10 min.

Probe purification by Sephadex column

An I ml blue tip (with a filter) was filled with Sephadex G25 (resuspended in DEPC treated water and autoclaved) and centrifuged at 2000 rpm for 2 min. Subsequently, column was equilibrated with 100 µl dH_2O. Hot probe was loaded onto Sephadex containing blue tipand subsequently centrifuged at 2000 rpm for 2 min. The purified probe was taken into a new Eppendorf tube.

Affymetrix transcriptome analysis

Purified high yielded RNA isolation

For genome-wide microarray analysis samples were prepared according to the instruction available on Nottingham Arabidopsis Stock Centre (NASC) homepage (http://affymetrix.arabidopsis.info/sampleprep/index.html). Total RNA was first isolated from entire aerial parts of 15 days old seedlings by RNAwiz extraction reagent (Ambion, Huntingdon, Cambridgeshire, UK) followed by a clean up step using the Qiagen columns in order to remove most impurities. 40µg (~1µg/1µl) of total RNA of each sample was shipped on dry-ice to NASC. The samples were hybridized with affymetrix chip (Gene Chip) after QC (Quality control) analysis.

Affymetrix transcriptome data analysis

Crude data was normalised using the Affymetrix standard procedure by NASC service by applying a so-called "Scaling Factor" using the Affymetrix software led to the removal of 2% of signal values from top and bottom values. These data were further on subjected to Microsoft excel spread sheet and sorted the normalized signal values in ascending order. A ratio of up/down regulated genes was assorted by dividing the normalized signal values of vector control with their respective partner. Furthermore, Clustering analysis was performed using clustering tool "EPCLUST" available at http://ep.ebi.ac.uk

Cloning strategies

Cloning strategies performed in the course of this thesis are described below. Plasmids and primers used for cloning procedures are listed in materials section.

TOPO reaction

PCR Product, gel-purified (20 ng in 4µl)	4 µl
pCR TOPO2.1® salt solution	1 µl
pCR TOPO2.1® topoisomerase/vector	1 µl

BP reaction

*att*B-PCR Product (50 ng/µl)	1 µl
GATEWAY® BP clonase	1 µl
BP reaction buffer (5x)	1 µl
pDONR™201 vector (50 ng/µl)	1 µl
ddH_2O	1 µl

LR reaction

Entry clone (50 ng/µl)	1 µl
GATEWAY® LR clonase	1 µl
LR reaction buffer (5x)	1 µl
Destination vector (50 ng/µl)	1 µl
ddH_2O	1 µl

LR Plus reaction

5' element p4P1R Entry clone (20 ng/µl)	1.2 µl
Entry clone pENTR201 (20 ng/µl)	1.2 µl
3' element p2RP3 Entry clone (20 ng/µl)	1.2 µl
GATEWAY® LR Plus clonase	2.0 µl
LR Plus reaction buffer (5x)	2.0 µl
Destination vector (20 ng/µl)	1.4 µl
ddH_2O	1.0 µl

Typically, reactions were carried out in 1.5 ml Eppendorf tubes. Reactions were incubated at 25ºC for at least 1 h (for TOPO TA cloning) or 12 h (for Gateway cloning), before completely transformed into *E. coli* strain DH5α.

Plasmid DNA cleavage by digestion with restriction endonucleases

Isolated plasmids were cleaved in diagnostic sites using restriction enzymes in order to confirm the accuracy of obtained construct. For the digestion of plasmid DNA with restriction endonucleases, buffers supplied by manufacturers were used. Cleavage of DNA was performed at recommended optimal temperatures, usually at 37°C. 5-10 U of enzyme were used. Digestion of plasmid DNA and was performed for 1-3 hrs. Enzyme reactions were stopped by heat inactivation of restriction enzymes upon transfer of the restriction mix to 65° for 20 min.

Complementation and promoter GUS fusion using Multisite Gateway® Technology

Three DNA/cDNA sequences of interest were amplified (*i.e.* 5′ element, gene of interest, and 3′ element) using the recommended *att*B primers extension sites by Invitrogen to generate PCR products that are flanked by *att*B sites. Separate BP recombination reactions with three donor vectors (*pDONR.P4-P1R*, *pDONR.221*and *pDONR.P2R-P3*) were done to generate three entry clones containing your DNA sequences of interest. These three entry clones were used in a single MultiSite Gateway® LR plus recombination reaction with a *pAM-PAT* based *pDEST.R4-R3* vector to create a transcriptional fusion among 5′ element (promoter sequence), cDNA (GUS reporter gene/cDNA) and terminator sequence/ epitop tag sequence (*StrepII*-terminator).

2.2.2. Methods for the cultivation of bacteria and transformation of plants

The *Agrobacterium* strain GV3101 was used for all the below described transformations. The strain has a C58C1 chromosomal background marked by a rifampicin resistance mutation, and carries pMP90RK, a helper Ti plasmid encoding virulence functions for T-DNA transfer from *Agrobacterium* to plant cells (Koncz et al., 1990).

Preparation of chemo-competent *E. coli* cells (Hanahan, 1983)

All steps in this experiment were performed at 4°C. 5 ml of an over night culture of *E. coli* strains DH5α or DB3.1 were added to pre-warmed 100 ml of LB and keep on shaking at 37°C to achieve the bacterial growth to an OD_{600} of 0.5-0.6. The bacteria were centrifuged at 5000 x g for 10 minutes at 4°C. After discarding the supernatant, the pellet was gently re-suspended in 30 ml ice-cold TFBI solution. After the second step of centrifugation, the pellet was resuspended in ice-cold TFBII solution. 1.5 ml Eppendorf reaction tubes containing 50 µl aliquots of cells were frozen in liquid nitrogen and stored at -80°C until use.

Transformation of chemo-competent *E. coli* cells

For each transformation, one aliquot of chemo-competent cells was thawed on ice. For Gateway vectors propagation, 20 to 50 ng of plasmid DNA was mixed with the aliquot of *E. coli* DB3.1 cells. In case of TOPO TA cloning and Gateway recombination reactions, entire reaction mixture (6 µl or 5 µl, respectively) were added to an aliquot of *E. coli* DH5α cells. The cells were incubated on ice for 30 minutes. The mixture was heat-shocked for 30 seconds at 42°C and again incubated on ice for 2 minutes. 900 µl of SOC medium was immediately added to the Eppendorf tube and incubated at 37°C for 1 hour with continuous shaking at 950 rpm. A fraction (~ 150-200 µl) of the transformation mixture was plated out onto selection media plates. Transformed colonies were isolated.

Small scale plasmid isolation from *E. coli*

Small scale plasmid isolation from *E.coli* was performed by alkaline lysis according to Sambrook and Fritsch, 1998, using Plasmid Isolation Mini kit (Qiagen).

Preparation of electrocompetent *A. tumefaciens*

A single colony of *A. tumefaciens* was inoculated into 5 ml of YEB medium and grown o/n at 28°C. The o/n culture was used to inoculate 400 ml of YEB medium and grown to A_{600nm}=0.5. Cells were harvested by centrifugation at 4.9 krpm and successively resuspended in 200 ml, 100 ml and 10 ml of ice-cold 1 mM Hepes (pH=7.5). Finally cells were resuspended in 800 µl of 1 mM Hepes (pH=7.5) and 10% v/v glycerol, aliquoted and frozen at –70°C.

Electroporation of *A. tumefaciens* cells

An aliquot of frozen electrocompetent *A. tumefaciens* was thawed on ice and mixed with 450 µl of 10% glycerol. The electroporator was set to 25 µF, 2.5 kV and 200 Ω. A single electroporation pulse was given and 1 ml of YEB medium immediately added. After incubation at 28°C for 2 hrs, cells are plated on selective YEB medium and incubated for 2 d at 28°C. Transformed colonies were isolated.

Transformation of Arabidopsis plants

Agrobacterium clones carrying respective plasmid were grown in 5 ml of YEB medium with gentamycin (25 mg/l), kanamycin (25 mg/l), carbenicillin (50 mg/l) and rifampicin (50 mg/l) o/n at 28°C. The o/n culture was used to inoculate 400 ml of YEB medium and grown for another 16-20h. Cells were harvested by centrifugation at 4.9 krpm and resuspended in 50 ml of 5% sucrose solution. The resuspended culture was brought to A_{600nm}=0.8 by dilution with 5% sucrose solution. Before transformation benzylaminoprine (10 µl/l) and Silwet L-77 (500 µl/l) were added to the *A. tumefaciens* culture. Arabidopsis *aos* plants were grown under greenhouse conditions at a density of 5 plants/pot (9 cm diameter). The first emerged floral bolts were monitored for male sterility phenotype and cut off to remove the apical dominance, and therefore encourage growth of multiple secondary bolts. Transformation was performed 5-10 days after clipping. The plants were dipped for 30 s into *A. tumefaciens* culture and covered with a plastic dome for 24 hrs to maintain high humidity. After removal of the plastic domes, plants were transferred to a growth-chamber with high humidity conditions for two days and then to the greenhouse until seeds were harvested.

Transient transformation of *N. benthamiana* leaves

2 ml overnight culture of *Agrobacterium* carrying binary construct was grown at 28°C in liquid YEB medium containing antibiotics. Bacteria were spun down and resuspended in 1 ml induction medium. After transferring to new test tubes, 3-5 ml induction medium (with antibiotics) were added. Culture was grown for another 4-6 hrs and bacteria harvested. Subsequently, bacteria were resuspended in infiltration medium to OD 0.4-0.6. Healthy, fresh-looking leaves of young *N. benthamiana* plants were infiltrated with a needleless syringe on the underside. Leaf material was harvested 72 hrs after infiltration to continue subsequent experiments.

Transient transfection assay in Arabidopsis and leek epidermal cells using particle delivery system (for 10 bombardments)

Note: This protocol is compiled by Brigitte Schauf at the MPIZ Cologne, Abt. Molekulare Phytopathologie headed by Paul Schulze-Lefert.

This method works on the principle to use helium pressure introducing gold/tungsten particles (microcarriers) coated DNA into living cells. 30mg of 1µm gold microcarrier was weighed and transferred into a 1.5ml Eppendorf tube. After adding 1ml of 70% ethanol, the suspension was vigorously vortex for 3-5 minutes. Subsequently, 15 minutes break to allow the particles soaking. Micro-particles were centrifuged for 5 seconds to pellet. After the complete removal of supernatant, 1ml sterile dH_2O was added. Subsequently, rigorous vortexing for 1 min and allowing the particles to sediment for 1min. afterwards, gold particles were microfuged briefly to remove the supernatant. These steps were repeated three times to get proper washing of micro-particles.

Finally, micro-particles were suspended in 500µl sterilized 50% glycerol: 50%dH_2O. These washed micro-particles can be used within to 2 weeks with the condition storing at 4°C.

Coating Microcarriers with DNA

Pre-washed micro-carriers in 50% glycerol (60mg/ml) were votexed for 5 minutes to resuspend and disrupt agglomerated particles. For one bombardment, 50µL was aliquoted into a new Eppendorf tube and subsequently added in order 5µl DNA (1µg/µl),

50µl 2.5M $CaCl_2$ by slowly pipetting and 20µl 0.1M spermidine. Meanwhile, the tube was kept carrying on vigorous vortexing. Vortexing was continued 2-3 min. more and then stopped. In further sediment step, microcarriers were stayed in rest for 1 min. Micro-carriers were centrifuged for 2 seconds and after discarding the supernatant, 140µ 70% EtOH was added. The next step was done at low speed to vortex the micro-carriers for 2 seconds and spun for 2 seconds. Supernatant was removed and 140µl 100% EtOH was added. The step was repeated one more time and micro-carriers were resuspended in 48 µl 100% EtOH. DNA coated gold particles were kept on ice until bombardment.

Transient transfection assay

Arabidopsis leaves or leek peelings were cut in appropriate sizes and placed on MS-medium plates. Macrocarriers were positioned in the appropriate seven places the macrocarrier holder (Hepta Adapter™ BioRad) and fix those tightly with a holder. 6 µl of DNA coated microcarriers was taken while vortexing and pasted on pre-placed mrocarriers. Once the ethanol was fully evaporated, this Hepta Adapter™ was placed inside the BioRad particle delivery system (Biolistic-PDS-1000/He) and fixed it tightly with a provided key. A vacuum of 27 mm Hg was applied. Under this constant pressure, rupture discs were burst at a pressure of 900 psi led to the start of bombardment process. The bombarded leaves were placed in a light chamber at 18°C for 24 h to allow the protein expression.

2.2.3. Plant treatments

1-aminocyclopropane-1-carboxylic acid (ACC): ACC was purchased from Sigma Chemical Co. and either infiltrated or supplemented with MS-medium at final concentration of 100µM) while mock solution was Tween 20 of 0.01% concentration.

2,4-dichlorophenoxyacetic acid (2,4 D): 2, 4 D was purchased from Sigma. The tested concentrations were (0.001%, 0.005% and 0.01%)

β-estradiol: 10 µM DEX solution in DMSO was sprayed on Arabidopsis transgenic plants.

Basta: 0.1% on the cotyledon stage and sprayed by atomizer.

Dehydration stress:

0, 100, 200, 400 mM concentrations of NaCl were tested to observe the germination ratio and growth effect on Arabidopsis seedlings.

Dexamethasone (DEX): 10 μM DEX solution in ethanol was sprayed on Arabidopsis transgenic plants.

gamma-aminobutyric acid (GABA): 1mM to 0.5M concentration soluble in dH_2O and sprayed on Arabidopsis transgenic plants.

Heavy metals:

Following heavy metals were dissolved in dH_2O and supplemented with MS-medium in the below stated concentrations.

$CdCl_2$: 0, 200, 250, 300 μM

$CuSO_4$: 0, 10, 100, 125, 150 and 175 μM

H_2O_2: Arabidopsis seeds were either sown on filter paper containing H_2O_2 or MS-medium gown one-week-old seedlings were shifted to MS-medium supplemented with H_2O_2 to observe the root growth phenotypes (0, 10, 20 mM)

MeJA: (Duchefa Biochemie) The working solution spraying concentration was 100μM while the mock solution was 0.2% ethanol.

Paraquat:

Droplet assay: A 5 to 25μM concentration paraquat droplet was used and leaves of 3-4 weeks old plants were placed in darkness for 2h. Necrosis diameter was observed after 2 days.

Germination assay:. Relative germination ratio was determined on MS medium supplemented with 5 μM paraquat.

Root growth/bending assay: 0.5 to 1.00 uM paraquat, MS plates. Mark root tip, turn 90^o. After one week measure additional root growth.

Root gravitropism:

10 days old vertically MS-medium grown Arabidopsis seedlings were reoriented to a 90^o angle relative to gravity vector and root tip responses were monitored.

Root winding assay:

In order to study wavy root growth patterns seedlings were vertically grown on 1.5% MS medium and tilted to 45^{o} for 3 days.

Salicylic acid (SA): 5mM SA solution (pH 7.0 with KOH) in ethanol was sprayed on Arabidopsis seedlings/plants.

Sugar: 0, 100, 175 mM concentrations were tested to observe the any phenotypic effect on Arabidopsis seedlings.

UV (Stratagene UV transillumination Crosslinker): 50-100 mJ/cm^2 energy was used to monitor any phenotypic effect on Arabidopsis seedlings.

Evaluation of *Arabidopsis thaliana* pollen viability by FDA staining

The fluorochromatic reaction (FCR) procedure (Heslop-Harrison et al. 1984; Shivanna and Rangaswamy 1992) was used for determining the viability of pollen. 0.02 g of fluorescein diacetate (FDA) (Sigma-Aldrich GmbH, Munich, Germany), was mixed with 10 ml of acetone. A 20% sucrose solution was made up and 5 ml removed into a separate container. The FDA solution was added drop by drop to this 5 ml of sucrose until persistent turbidity. This solution was used within 30 min of mixing. One 10 µl drop of the mixture was added to a clean microscope slide and subsequently pollen was added. Each slide was incubated at room temperature for 15 min. Then a cover slip was placed over the sample. Viability of pollen grains was examined under a fluorescence microscope (Leica MZ12, excitation filter 450–490nm). Using the fluorochromatic reaction test for the complementation lines pollen, the viable pollen grains fluoresced in a bright green colour, whilst the non-viable grains were of a dull yellow colour. A hundred grains visible on the slide were scored and the percentage viability calculated.

GUS staining assay

Plant tissue were stained for GUS activity using a solution containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) in 0.1 M Na2HPO4, pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide/ferrocyanide, and 0.06% Triton X-100 (Jefferson *et al.*, 1987) at 37°C for 16 hours. The samples were cleared of chlorophyll by sequential washing in 70% ethanol.

2.2.4. Protein analyses

Preparation of the crude-extract

0.25 g and 0.75 g leaf material for *N. benthamiana* and *A. thaliana*, respectively was ground in liquid nitrogen to get a fine powder. The ground tissue was thawed by adding 0.75 ml of *StrepII*- extraction buffer and kept on grinding until uniform slurry was obtained. The slurry was transferred into a 2 ml Eppendorf tube, sonicated for 30 seconds then centrifuged for 10 min at 4°C. The supernatant was transferred into a new Eppendorf and used as crude extract for protein analysis by adding 2xloading buffer.

SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting and protein detection

All denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Mini-blot Protean system (BioRad). 10% resolving gel and 4% stacking gel was prepared (see materials). After placing the gel in electrode assembly, the samples were carefully loaded using Hamilton syringe and migrated. Immunoblotting was done by Trans-Blot System SD Semi-Dry Transfer Gel (Bio-Rad Trans-Blot® semi-Dry) as instructed by Trans-Blot® semi-Dry Quick Reference Guide. Recombinant protein via the *StrepII* tag was detected following the manual instruction of IBA (BioTAGnology, www.iba-go.com) and Witte et al. 2004.

3. Results

3.1. Expression pattern of various *AtWRKY* family members in *A. thaliana* mutants impaired in different signaling pathways

In contrast to six other plant transcription factor families, over 70% of all *At*WRKY family members appear to respond to various abiotic and biotic stresses (Chen et al., 2003; Kalde et al., 2003). As a first step in order to get a general overview of the expression pattern of individual members of the *At*WRKY superfamily, an attempt was made to analyze the entire set of these genes. This expression profiling was studied using different defined signaling Arabidopsis mutant plants specifically affected in different plant defense signaling pathways (Table 2). Macroarray analyses were carried out using a reverse RNA blot approach. The majority of the *AtWRKY* cDNA clones were available in a Gateway Entry vector in our laboratory clone collection. PCR products of each *AtWRKY* cDNA were amplified using Gateway *att*B1 and *att*B2 universal primers (Fig. 5A).

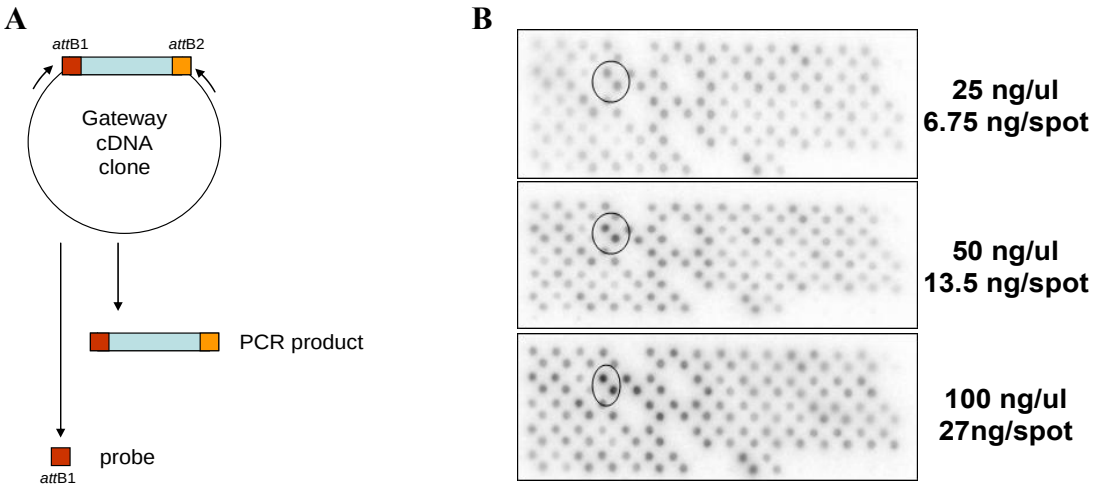


Fig. 5. Optimizing the vector construct used for macroarray analyses. **A.** Schematic representation of a Gateway clone for PCR product amplification. Red, blue and orange boxes indicate *att*B1, cDNA clone cassette and *att*B2, respectively. Arrow bars indicate position of the primers flanking the sites of *att*B1 and *att*B2. **B.** Each duplicate dot

represents a *AtWRKY* cDNA spotted on the nylon membrane and hybridized with the *att*B1 probe. The different amounts spotted are shown on the right side of the diagram. Small round circle highlights the good correlation between the cDNA amount spotted and signal intensity.

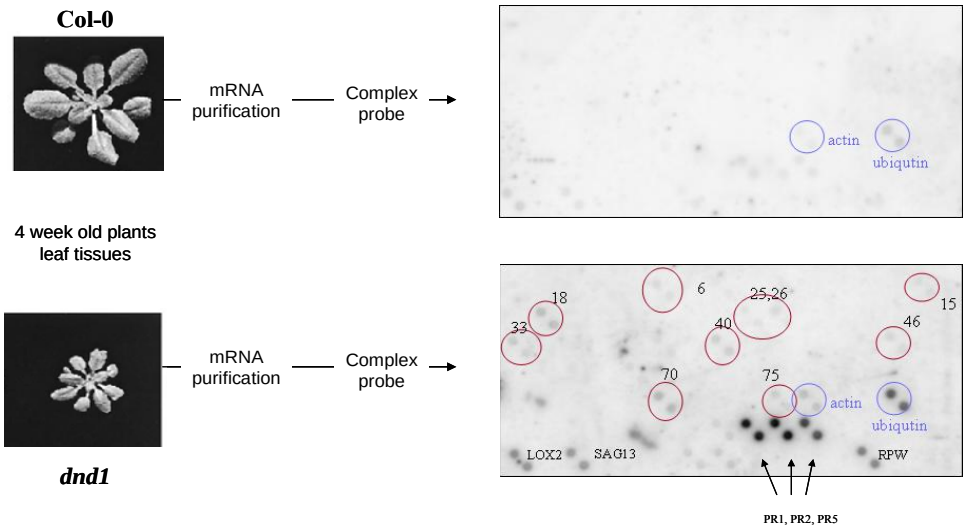


Fig. 6. Example of a expression comparison between wild-type and mutant plants via macroarray analyses. Pictures of the wild-type Col-0 plant and the *dnd1* mutant are shown at the left. The various steps performed include mRNA purification from plants, complex probe preparation and hybridization to duplicate nylon membranes. Blue circles mark the spots of constitutively expressed marker genes. Red circles represent the spots of different *AtWRKY* genes indicated by their numbers. Small arrows points towards the spots of plant defense *pathogenesis-related* (*PR1, PR2* and *PR5*) marker genes. LOX2: lipoxygensae 2, SAG13: senescence associated gene 13, RPW: resistance to powdery mildew.

Various amounts of 72 *AtWRKY* cDNA (6.75, 13.5 and 27 ng/spot) were spotted in duplicate on nylon membranes in order to determine the optimal concentration of cDNA needed per spot. The twin spots served as internal controls for differences in signal

intensity. *att*B1 probe was used for hybridization to the filters. A good correlation was observed between cDNA amount and signal intensity (Fig. 5B).

Table 2. *A. thaliana* mutants impaired in different signaling pathways used in these experiments.

Mutant name	Full name	Annotation/Description
aos	***A***llene ***O***xide ***S***ynthase	Defect in the allene oxide synthase gene (completely blocked in the synthesis of jasmonic acid. Allene oxide synthase, catalyses dehydration of the hydroperoxide to an unstable allene oxide in the jasmonic acid biosynthetic pathway (Laudert et al. 1996; Park et al., 2002).
ctr1-1	***C****onstitutive* ***T****riple* ***R****esponse1*	CTR1 encodes a Raf-like protein kinase which acts as a negative regulator of the ethylene signaling pathway (Kieber et al., 1993).
cpr5	***C***onstitutive expressor of ***P***athogensis ***R***elated genes 5	A transmembrane protein regulating expression of pathogenesis-related (PR) genes. Participates in signal transduction pathways involved in plant defense (systemic acquired resistance - SAR) (Clarke et al. 2001).
dnd1	***D***efense, ***N***o ***D***eath 1	Cyclic nucleotide-gated ion channel, also known as *CNGC2* (Clough et al. 2000).
edr1	***E***nhanced ***D***isease ***R***esistance 1	A mitogen-activated protein kinase kinase kinase (MAPKKK) that confers resistance to powdery mildew disease caused by fungus *Erysiphe cichoracearum* (Frye et al. 2001).
fls1-2	***F****lagellin* ***I****nsensitive***1**	This is also an *fls2* mutant having a stop-codon in the kinase domain. It also shows strong flg22 insensitivity in growth inhibition assays, and cannot bind flg22. *FLS2* encodes a receptor-like protein kinase.(Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Gómez-Gómez and Boller, 2002).
ein2	***E****thylene* ***I****nse****n****sitive 2*	EIN2 is a bifunctional transducer of ethylene and stress response (Guzman and Ecker, 1990; Alonso et al., 1999).
jar1	***Ja***smonate ***R***esponse 1	An auxin-induced gene encoding a cytoplasmic localized phytochrome A signaling component protein similar to the GH3 family of proteins (Staswick et al. 2002).
ndr1	***N***on-Race-Specific ***D***isease ***R***esistance 1	Required for non-race specific resistance to bacterial and fungal pathogens. Many CC-NBS-LRR type proteins require NDR1. NDR1 encodes a 219 AS long protein with putative trans-membrane domain. Mediates systemic acquired resistance (SAR) response (Century et al., 1995; Century et al., 1997; Tornero et al., 2002).
npr1	***N***on-Expressor of ***PR*** genes 1	Adaptor molecule containing ankyrin repeats, controls systemic acquired resistance (SAR), also known as *NIM1* and *SAI1* (Cao et al. 1997).

For all the subsequent experiments, 13.50g/spot (50ng/ul) was selected. Various marker genes, both constitutively expressed and pathogen induced were also spotted on the filter in order to validate the results (Table 3).

Constitutively Expressed marker genes	***Actin, Ubiquitin***
Plant Defense Related Marker genes	***CHS (Chalcone Synthase) , CPR5 (Constitutive Expressor of Pathogensis Related genes 5) , EDS1(Enhanced Disease Susceptibility 1), Eli3 (Mannitol Dehydrogenase, Lox2 (Lipoxygenase 2) , MPK4 (Mitogen-Activated Protein Kinase 4) , NDR1 (Non-Race-Specific Disease Resistance 1) , NPR1 (Non-Expressor of PR1) , PAD4 (Phytoalexin-Deficient 4) , PAL2 (Phenylalanine Ammonia-Lyase 2) , PDF1.2, PDF1.4 (Plant Defensin), PR1, PR2, PR5 (Pathogensis Related) , RPW8 (Resistance to Powdery Mildew 8), SAG13 (Senescence-associated gene 13)***

Table 3. Various marker genes included on the macroarray. Constitutively expressed molecular markers (Actin and Ubiquitin) are shown at the upper panel of the table while plant defense associated molecular markers are given in the lower panel of the table.

mRNA populations were purified from leaves of 3-week old wild-type or from the respective mutant plants to generate complex cDNA probes. These probes were used to hybridize duplicate filters (Fig. 6). The signal intensity or pixel value of each twin spot on a filter, representing a *AtWRKY* cDNA or control gene, was measured After correcting for background of each spot, the signal intensities were spread on an Excel-sheet. Pixel values of all spots were normalized based on the signal intensities of constitutively expressed marker genes. These normalized values were used to create a histogram (Fig. 7). The data were corroborated on the basis of plant defense related marker genes expressed differentially in certain plant defense signaling networks.

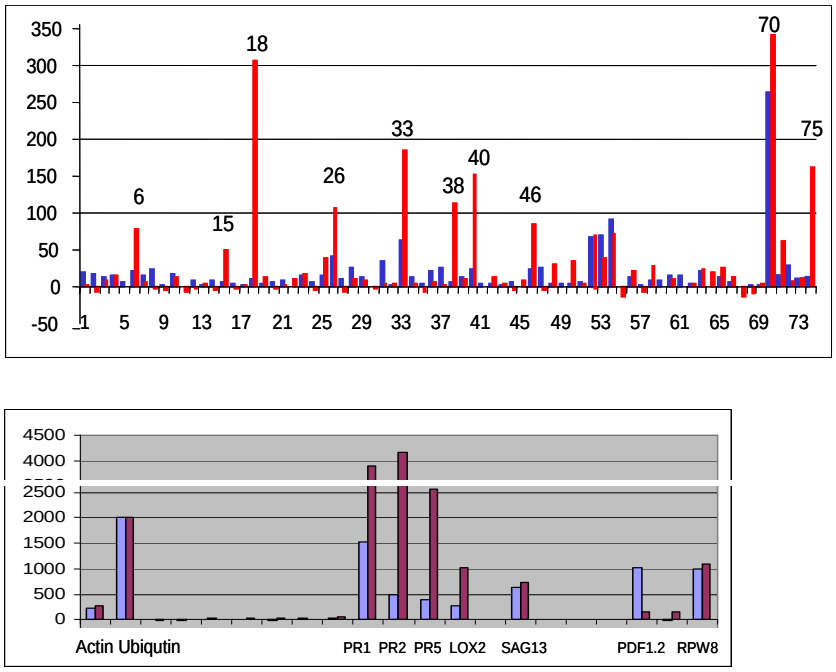


Fig. 7. Diagramtic representation of normalized pixel values of the various obtained signals. **A.** Light blue and light red bars in the histogram represent wild-type and mutant signal intensities of various *AtWRKY* genes, respectively. The differentially regulated *AtWRKY* genes are labeled by an algorithmic number. **B.** Dark blue and dark red bars in the histogram correspond to wild-type and mutant normalized signal intensities of marker genes, respectively.

For instance, *dnd1* (*defense no death 1*) is known to exhibit constitutively elevated levels of salicylic acid and mRNAs for pathogenesis-related (*PR*) genes (Glazebrook, 2001). Overall the levels of transcripts for 12 *AtWRKY* genes were constitutively elevated in various tested mutants compared to wild-type (*AtWRKY6, AtWRKY15, AtWRKY18, AtWRKY25, AtWRKY26, AtWRKY33, AtWRKY40, AtWRKY46, AtWRKY53, AtWRKY54, AtWRKY70* and *AtWRKY75*), while transcript levels of *AtWRKY18* and *AtWRKY40* were induced in wounded plants compared to control plants (Table 4).

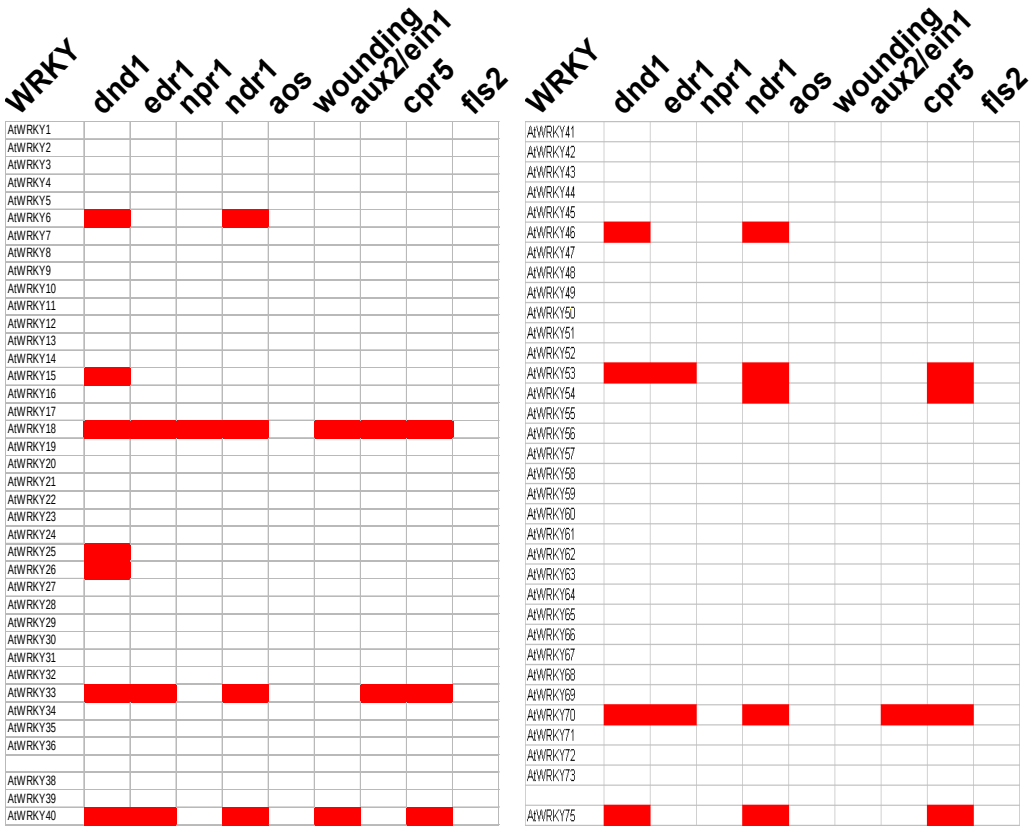


Table 4. Differentaily expressed *AtWRKY* genes with respect to the tested mutants are highlighted in red. The serial number represents the designation of various *AtWRKY* genes listed in the first columns of the Excel spread sheet. Mutants and treatments are given at the top.

Furthermore, comprehensive expression patterns of *AtWRKY18, AtWRKY25, AtWRKY33, AtWRKY53* and *AtWRKY70* were analyzed in different mutants impaired in plant defense networks upon elicitor/stimuli application. 3-week old plants of wild-type, *ndr1*, *edr1*, *cpr5-2* and *npr1* were treated with salicylic acid (SA), *aos* and *jar1* with Methyl jasmonate (MeJa), and *ctr1* and *ein2* with 1-aminocyclopropane-1-carboxylic acid (ACC), a natural precursor of ET. Total RNA was isolated from leaves 3 hours after start of treatment. Full length cDNAs of *AtWRKY18, AtWRKY25, AtWRKY33, AtWRKY53* and *AtWRKY70* were PCR-amplified and used as probes. The transcript levels of *AtWRKY18*, *AtWRKY*33, *AtWRKY53, AtWRKY60* and *AtWRKY70* were induced

by SA in the wild-type, *ndr1*, *edr1*and *cpr5-2* plants but the transcript levels were either unchanged or actually decreased in *npr1* mutant plants (Fig. 8 A and B).

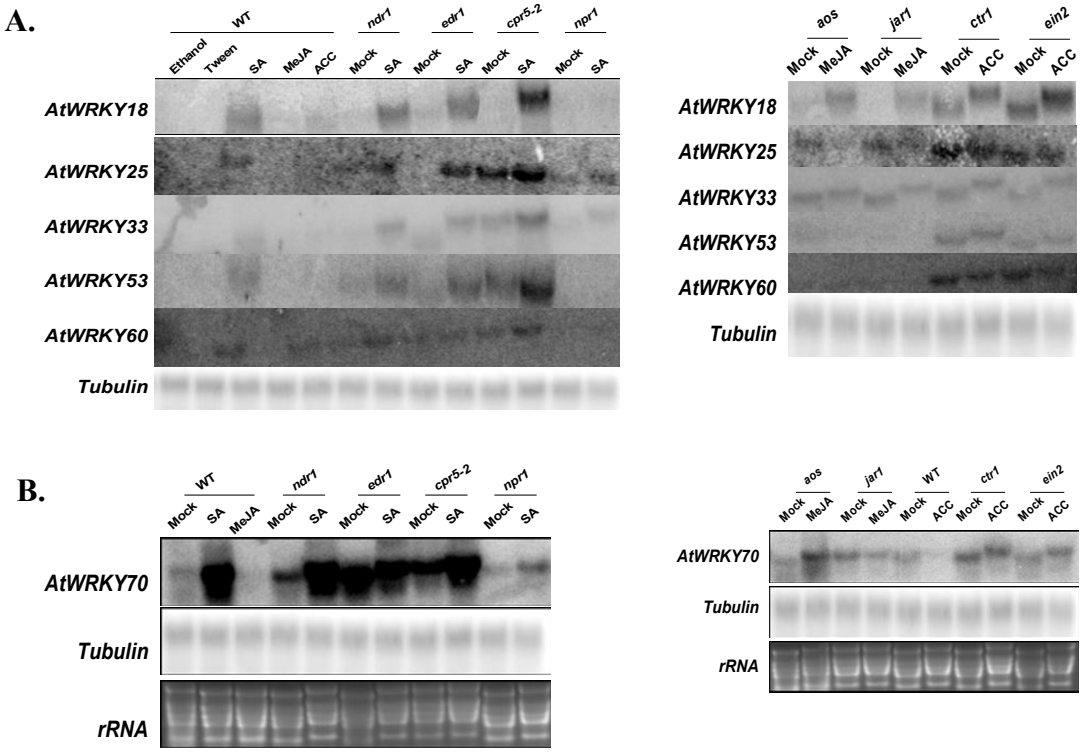


Fig. 8. **A & B.** Northern blot analysis to analyze the expression patterns of specific *AtWRKY* genes. RNA samples were prepared from leaves of 21 days old wild-type (WT) or respective mutant plants. After electrophoretic separation on the gel and blotting onto nylon membranes, the blots was probed with *AtWRKY18, AtWRKY25, AtWRKY33, AtWRKY53* and *AtWRKY70* specific DNA fragments as indicated on the left. *Tubulin* probe or Ethidium Bromide staining of rRNA was used as loading controls. Wild-type and mutant plants are indicated at the top of the blots and underlined, and the respective treatments are given for each lane. SA: salicylic acid, MeJa: methyl jasmonate, ACC: 1-aminocyclopropane-1-carboxylic acid.

AtWRKY25, *AtWRKY53* and *AtWRKY70* showed both basal and SA-induced higher transcript levels in *cpr5* mutant plants compared to wild-type plants. By contrast, *AtWRKY18* and *AtWRKY53* exhibited SA-induced higher transcript levels only in *cpr5* mutant plants compared to wild-type. *AtWRKY18, AtWRKY*25 and *AtWRKY33* exhibited an increased transcript level in JA/ET signaling deficient mutant plants compared to wild-type plants after appropriate treatment. Interestingly, *AtWRKY53* and *AtWRKY60* showed constitutively elevated transcript levels only in ET signaling deficient (*ctr1* and *ein2*) mutant plants.

3.2. Pathogen tests to define *Atwrky* loss of function mutants showing differential phenotypes compared to wild-type plants

Since the complete Arabidopsis genome sequence is available, reverse genetics can be a major tool to study gene function. Although it has been estimated that the Arabidopsis genome is saturated with T-DNA and transposable element insertions, relatively few informative knockouts have been reported that provide a clue to gene function (Bouche and Bouchez, 2001). Structural and functional redundancy, due to the partial duplication of the Arabidopsis genome, may explain the lack of phenotypical alterations in certain cases (Vision et al., 2000; Simillion et al., 2002). The redundancy problem can be especially acute for transcription factors (TFs) because they are, in general, members of large gene families that often include closely related genes (Riechmann et al., 2000). It is also possible that the lack of phenotypes in knockouts is conditional and does not alter plant morphology even in the presence of severe physiological defects or is due to our inability to detect small phenotypic changes.

In Arabidopsis, WRKY proteins were categorized among one of the major families of transcription factors (Eulgem et al., 2000), comprised of 74 members. Usually, single gene mutation at most of individual *At*WRKY loci do not exhibit altered phenotypes under natural growth conditions (Bekir Uelker and Imre E. Somssich, personal communication). A plausible hypothesis would propose that subtle, conditional phenotypical changes can be identified under specific environmental or biological changes. In order to dissect the role of *AtWRKY* factors in the context of plant defense, diverse biotic and abiotic stresses were tested. One of the employed methods was to

subject a set of *Atwrky* knock out (KO) mutants to diverse pathogen infections (host, non-host, virulent/avirulent) and analyze specific altered responses. Among the virulent pathogens selected, *Ralstonia solanacearum* strain GMI1000 was also included in the screening experiments.

3.3. Phenotypic responses of *Atwrky27* knock out mutant to *R. solanacearum* strain GMI1000

R. solanacearum strain GMI1000 is virulent on most of *A. thaliana* ecotypes, including Columbia, causing severe bacterial wilt and leading to death of the entire plant within 10-12 days post inoculation. However, ecotype Nd-1 displays complete resistance to *R. solanacearum*. The molecular phenomenon underlying this difference was elucidated by Deslandes and co-workers (1998, 2003), who showed the presence of two alleles of *AtWRKY52*, named *AtRRS1-S* and *AtRRS1-R*, isolated from Col-5 and Nd-1, respectively. Sequence analysis of the *RRS1* genes present in two homozygous intragenic recombinant lines indicates that several domains of RRS1-R are essential for its resistance function.

Fig. 9. Phenotypic responses of various *Atwrky* knock-out mutant plants to *Ralstonia solanacearum* strain GMI1000 (10^8 bacteria ml^{-1}), 8 days post inoculation. In each row, several plants of 15 different *Atwrky* knock out mutant and wild-type Col-0 plants were root-inoculated. *Atwrky27ETL* knock-out mutant displayed delayed symptom development compared to the other tested *Atwrky* knock-out mutants and wild-type plants (experiment performed by Y. Marco, CNRS/INRA Toulouse, France).

A collection of *Atwrky* KO mutant plants (all in Columbia background) was subjected to *R. solanacearum* strain GMI1000 infection experiments. Due to the general nature of this screen, both enhanced resistance and enhanced susceptible mutants were expected. In Col-0 wild type plants, first wilt symptoms were observed on older leaves 6-8 days post inoculation, which led to wilting of the entire plant 3-5 days later. Interestingly, among all the tested *Atwrky* KO mutants, *Atwrky27* KO mutant plants were found to exhibit a delayed disease symptoms response to *R. solanacearum* strain GMI1000 (Fig. 9) 8 days post inoculation.

Based on this preliminary observation, the foremost hypothesis is that *AtWRKY27* or the component (s) under the control of this TF could correspond to a plant susceptibility factor (s), which is required for the bacteria to provoke wilt symptoms.

3.4. Response of *Atwrky27* KO mutants to various biotic stresses

3.4.1. Host pathogens

***Pseudomonas syringae* pv. *tomato* DC3000**

Pseudomonas syringae is a gram negative, plant pathogenic bacterium divided into more than 40 pathovars on the basis of its host specificity for different plant species (Hirano and Upper 2000). *P. syringae* pv. *tomato* DC3000 causes bacterial speck of tomato and Arabidopsis but elicits the defense-associated hypersensitive response (HR) in bean, tobacco, and many other plants (Whalen et al., 1991; Zhao et al., 2000). A key factor in the pathogenicity of *Pst* DC3000, and many other plant and animal bacteria including *Ralstonia solanacearum,* is the virulence effector proteins delivered via a type III secretion system (TTSS) directly into host cells (Bonas and Lahaye, 2002; Collmer et al., 2000; Cornelis and van Gijsegem, 2000; Galan and Collmer, 1999; He, 1998; Staskawicz et al., 2001). In this study, strain *Pst* DC3000 which is virulent on Col-0 Arabidopsis accession was employed (Whalen et al. 1991). The Arabidopsis gene *Enhanced Disease Susceptibility1* (*AtEDS1)* encodes a lipase-like protein and has been shown to be a key component of the SA-dependent pathway. *eds1* mutants exhibit enhanced susceptibility to *Pst* DC3000 and the virulent oomycete *Peronospora parasitica* Noco2 (Parker et al., 1996; Feys et al., 2001). On the contrary, *constitutive expression of pathogenesis related protein-5* (c*pr5-2*) mutant (Bowling et al., 1994,

1997) shows enhanced resistance to virulent strains of *P. syringae* and/or *P. parasitica* and exhibits spontaneous lesion formation and constitutive expression of *PR* genes. *eds1* and *cpr5-2* mutant plants were both also included into the experiments as controls.

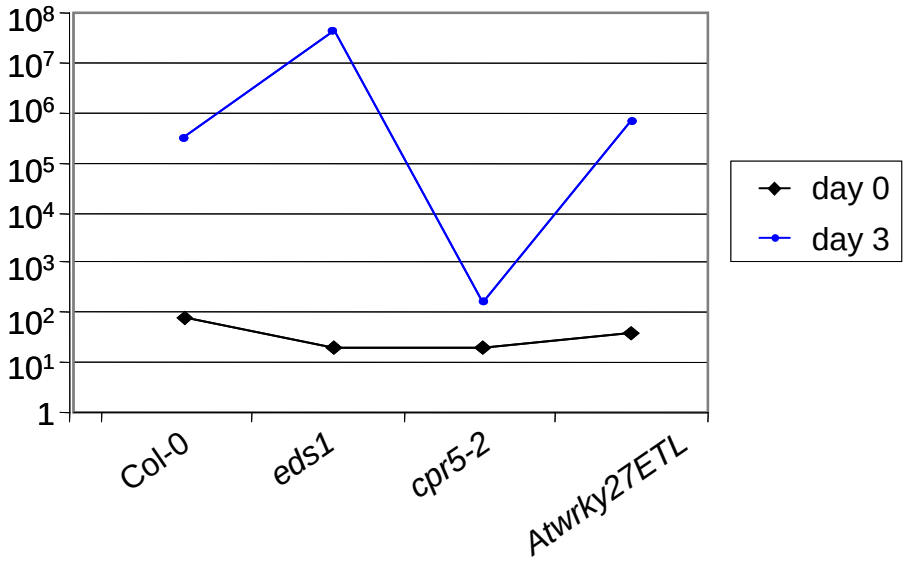


Fig. 10. Bacterial growth kinetics following *P. syringae* pv. *tomato* DC3000 infection. Bacterial density in leaves after inoculation of Col-0, *eds1*, *cpr5-2* and *Atwrky27ETL* plants with *P. syringae* pv tomato DC3000 (initial inoculation set at OD600=0.001). For each time point triplicate assays were performed on 9 plants for each accession/mutant line.

3-week-old plants of Col-*1*, *eds1*, *cpr5-2* and *Atwrky27ETL* were vacuum-infiltrated with the virulent strain *Pst* DC3000. *eds1* clearly showed more extensive chlorosis compared to Col-*0* and Col-*1* at day 3 post inoculation, while *cpr5-2* plants were highly resistant confirming that the infection was properly performed. No visible differences were found between *Atwrky27ETL* and wild-type plants in terms of bacterial chlorotic phenotype. Furthermore, bacterial density was also monitored on 0 and day 3 post inoculation. Both *Atwrky27ETL* and wild-type showed similar levels of bacterial growth (Fig. 10).

***Peronospora parasitica* Noco2**

Peronospora parasitica is an obligate biotrophic oomycete pathogen, which is virulent on the *Arabidopsis* accession Col-0 (Parker et al. 1993). This pathogen usually effects young seedling and leaves causing a very destructive disease known as downy mildew. Two-week old seedlings of Col-*1*, *eds1*, *cpr5-2* and *Atwrky27ETL* were sprayed with a spore suspension of *P. parasitica* isolate Noco2 ($4x10^4$ spores ml^{-1}) and conidiophores formation was quantified 7 days later. *eds1* plants exhibited enhanced susceptibility indicated by vast sporulation of the oomycete, while *cpr5-2* seedlings remained resistant (Fig. 11). The *Atwrky27ETL* seedlings showed a similar magnitude of conidiophore formation compared to wild-type (Fig. 12).

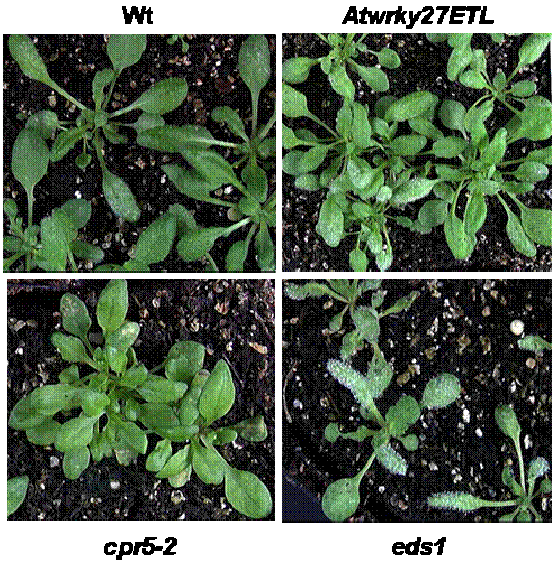


Fig. 11. Symptoms response of *Atwrky27ETL* plants upon infection with *P. parasitica* isolate Noco2. Two-week old seedlings of wild-type Col-0, *eds1*, *cpr5-2* and *Atwrky27ETL* were spray-inoculated with a suspension of *P. parasitica* isolate Noco2 conidia (5x 10^4/ml) and incubated in highly humid chambers. P icture was taken 7 days post-inoculation.

Golovinomyces orontii

Golovinomyces orontii is a biotrophic fungus (Braun, 1999), which grows and penetrates into epidermal cells to establish nutrient-harvesting organs. This pathogen infects Arabidopsis wild-type Col-0 plants and typically does not induce the ET/JA-dependent pathogen responsive marker genes *PDF1.2* and *Thi2.1* (Plotnikova et al., 1998). 4-week-old plants of two independent *Atwrky27* insertion lines (*Atwrky27 ETL* and *Atwrky27-90-1*), two *AtWRKY27* overexpressor lines (see 3.13; *OE-4 strepII* and *OE-7 strepII*) and

wild-type control plants were inoculated with *G. orontii* and macroscopically visualized for fungal sporulation 10 days post inoculation. No difference in the susceptibility was observed for all of the tested plants (data not shown).

S: Suceptible
R: Resistant
HS: HighlySuceptible

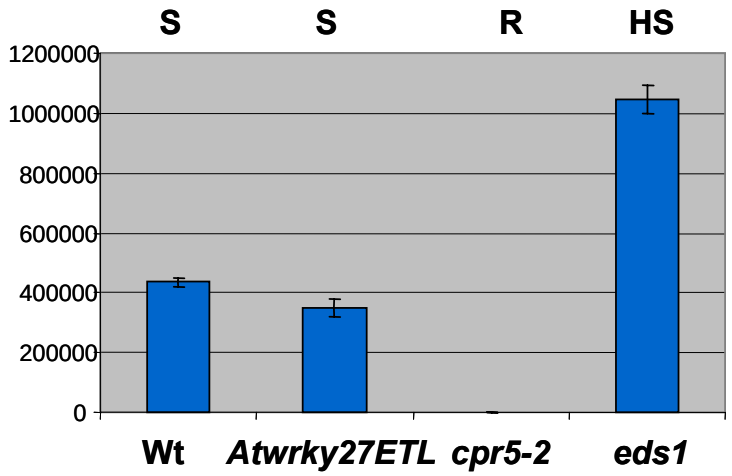


Fig. 12. Quantification of conidiophores formation 7 days post inoculation with *P. parasitica* isolate Noco2. Degree of pathogen infection was determined by harvesting 25 leaves per sample in 1 ml H_2O. After vigorous vortexing, two 10 ml aliquots of the spores from one sample were counted in a hemocytometer and averaged. Two replicate samples per genotype were assayed to obtain means and standard deviations. Error bars represent the standard deviations.

3.4.2. Non-host pathogens

Erysiphe pisi* and *Blumeria graminis* f.sp. *hordei (Bgh)

Erysiphe pisi and *Blumeria graminis* f.sp. *hordei (Bgh)* are referred to as non-host pathogens of Arabidopsis. Four-week-old plants of two independent *Atwrky27* insertion lines, two *AtWRKY27* overexpressor lines (see 3.13; *OE-4 strepII* and *OE-7 strepII*) and wild-type plants were inoculated either with *Bgh* or *E. pisi*. Leaf samples were collected 7 days and 11 days post inoculation, respectively. Samples were stained individually with aniline blue and analyzed under UV-light to determine alteration in the penetration rate and cell death. No differences were observed between the evaluated plants (Fig. 13).

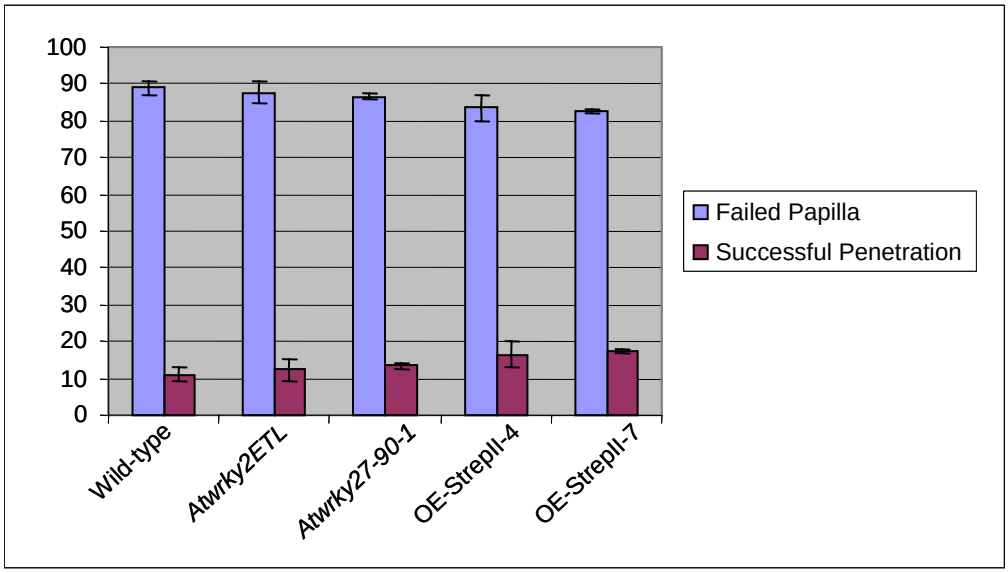


Fig. 13. Quantification of *Bgh* growth on *Atwrky27* mutant plants and *AtWRKY27* overexpressor plants. Rosette leaves of 4-week-old plants of *Atwrky27ETL*, *Atwrky27-90-1*, *OE-4-StrepII*, *OE-7-StrepII* and wild-type were inoculated with *Bgh* conidiospores. Samples were collected 7 days post inoculation. The graphic represents the frequency events of successful penetration (in red) and failed papilla (in blue). The data reported in the graphic represent the average of two independent experiments from 9 plants per replicate.

3.5. *At*WRKY27

The *At*WRKY27 protein is composed of 348 amino acids, with the molecular mass of 38.7 kDa and theoretical pI of 4.72. The aliphatic index was calculated as 58.76 and the GRAVY (Grand average of hydropathicity) index is equal to -0.927, indicating a hydrophobic nature of the protein. The instability index (II) is computed to be 77.07, which classifies the protein as rather unstable. Theoretical post-translational modifications were also surveyed. Two highly probable SUMOylation motifs were detected: ^{257}V**K**EE^{260} and ^{94}L**K**QE ^{97}, with probabilities of 0.93 and 0.91, respectively (http://us.expasy.org/).

As the N-terminal glycine is lacking, the *At*WRKY27 protein is unlikely to be a target for myristylation. Neither O-, nor N-glycosylation sites could be found while scanning the protein in the various databases available. However, a number of likely

phosphorylation sites are predicted for *At*WRKY27. In particular, serines 87, 118, 239 and 342 (highest scores 0.986, 0.986, 0.983 and 0.987, respectively), and threonine 226 (highest score 0.983) are prominent as targets for phosphorylation (Fig. 14).

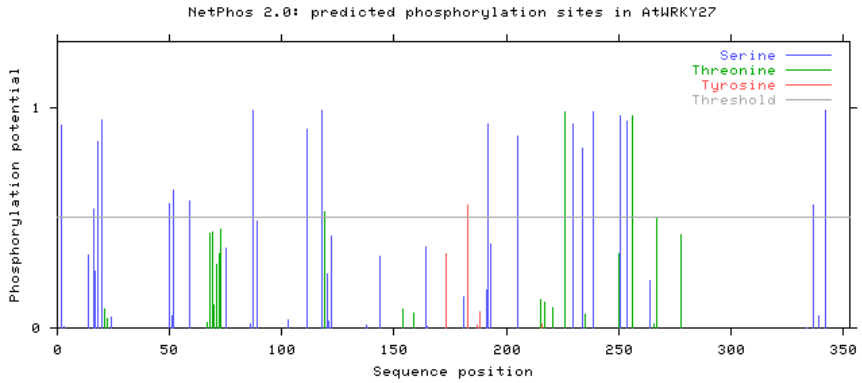


Fig. 14. Numerous phosphorylation sites were predicted in *At*WRKY27 using NetPhos2.0 (http://us.expasy.org/). Amino acid sequence position is given along the x-axis, while phosphorylation potential is marked from 0-1 on the y-axis. The cut-off value is set at 0.5 for putative phosphorylation sites.

*At*WRKY proteins are divided into 3 subgroups on the basis of number of WRKY domains and features of Zn finger like motif (Eulgem et al., 2000). *At*WRKY27, with a single WRKY domain within the C-terminus, belongs to group-IIe in the phylogenetic tree. Structurally closest members are *At*WRKY22 and *At*WRKY29 (Fig. 15) to which *At*WRKY27 shares 54% and 62% identity and 64% and 73% similarity, respectively (http://www.sdsc.edu/MEME).

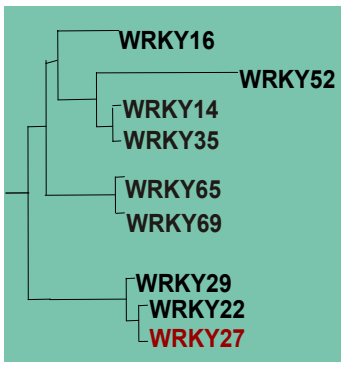


Fig. 15. Phylogenetic analysis of *At*WRKY group-IIe. The amino acid sequences of 9 members of this sub-group were aligned using CLUSTALW (Thompson et al 1994) and hierarchy analysis was done using TREEVIEW program (www.sdsc.edu/MEME). *At*WRKY27 was found to be closely related to *At*WRKY22 and *At*WRKY29.

3.5.1. Subcellular localization of *At*WRKY27

One of the main features of transcription factors (TFs) and a pre-requisite for their functionality is nuclear localization. Previously, it was shown that Arabidopsis WRKY proteins localized to nucleus (Robatzek and Somssich, 2001; Ingo Ciolkowski, Dierk Wanke and Bekir Uelker, personal communication). Computer based subcellular localization prediction program "PSORT" (http://psort.nibb.ac.jp/) deduced at least 2 putative nucleus localization signals (NLS): ^{144}RKRK^{147} and ^{140}PLRSRKR^{146}. In contrast, the certainty value for nuclear localization of *At*WRKY27 is not significant (table.) instead suggests rather mitochondrial and chloroplastic subcellular localization.

Subcellular localization of *At*WRKY27 was determined experimentally by means of transient expression of fluorescently tagged proteins. For this, the coding sequence of the green fluorescent protein (GFP) was fused in frame to the 3' end of the full-length *AtWRKY27* cDNA while the control construct consisted of the coding sequence of red fluorescent protein (DsRed). Both these constructs were driven by the constitutively expressed double cauliflower mosaic virus *CaMV 35S* promoter (*2x CaMV 35S*) (Odell et al., 1985).

Table 5. Predicted subcellular localization probabilities in various cellular compartments using PSORT (Prediction of protein subcellular localization) software (http://psort.nibb.ac.jp/). Cut-off value for probability is 0.5.

Cellular compartment	Probability
Mitochondrial matrix space	0.627
Chloroplast stroma	0.467
Nucleus	0.420
Mitochondrial inner membrane	0.326

The fusion construct *2x35S::AtWRKY27-GFP* and the control construct *2x35S::DsRED* were coated onto gold particles and co-bombarded into leek epidermal cells. Confocal laser scanning microscopy observations illustrated the detection of red fluorescence in

the entire transformed cells 24 h post transfection (Fig. 16). By contrast, the *At*WRKY27-GFP was visualized solely in the nucleus. In the overlay, *At*WRKY27-GFP and RFP can be observed in the same transformed cell. These data clearly indicate that *At*WRKY27-GFP is targeted to nucleus.

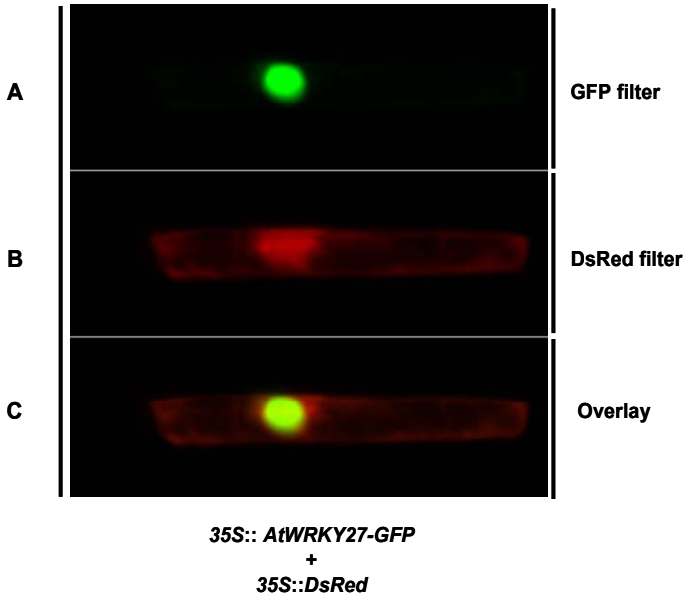


Fig. 16. Transient, heterologous expression of *At*WRKY27-GFP and DsRed in leek epidermal cells. Subcellular localization was studied by confocal laser scanning microscopy. A) *At*WRKY27-GFP was targeted to the nucleus. B) DsRed expression is observed in the entire transformed cell. C) Overlay for GFP and DsRed channels.

3.5.2. Monitoring the endogenous expression of *AtWRKY27* in *Atwrky27ETL* and using *AtWRKY27* promoter GUS reporter constructs

To gain clues concerning the function of a particular gene, it's important to characterize its temporal and spatial expression pattern. A number of reporter genes have been used as convenient markers to visualize gene expression and protein localization in a wide spectrum of prokaryotes and eukaryotes (Jefferson et al., 1987; Davis and Vierstra, 1998). The *Escherichia coli uidA* gene (also referred to as GUS reporter), which encodes ß-glucuronidase (GUS), has been extensively used in plants (Jefferson et al., 1987). The ß-glucuronidase assay is very sensitive, and it is possible to obtain both qualitative (histochemical) and quantitative (fluorometric) data (Jefferson et al., 1987).

The *Atwrky27ETL* loss-of-function mutant is an exon trap line, in which the GUS reporter gene has inserted into intron-2. The bonus of the gene trap T-DNA based mutagenesis is that besides disrupting a particular gene by insertional mutation, it allows detection and monitoring of the endogenous expression pattern of the targeted gene (Martienssen and Springer, 1998). Constructs used to generate exon trap lines don't have a promoter sequence, so that the expression of the reporter gene can occur only if inserted within transcribed chromosomal regions and are under the influence of endogenous regulatory sequences creating a transcriptional/translational fusion.

Genomic and transcript sequence analyses revealed that *AtWRKY27* introns as well as left and right triple splice acceptors (T-DNA borders) are spliced out after the transcription events resulting in an in-frame translational fusion between *AtWRKY27* exon-2 and the GUS reporter gene as well as leading to the disruption of the endogenous gene (Fig. 17).

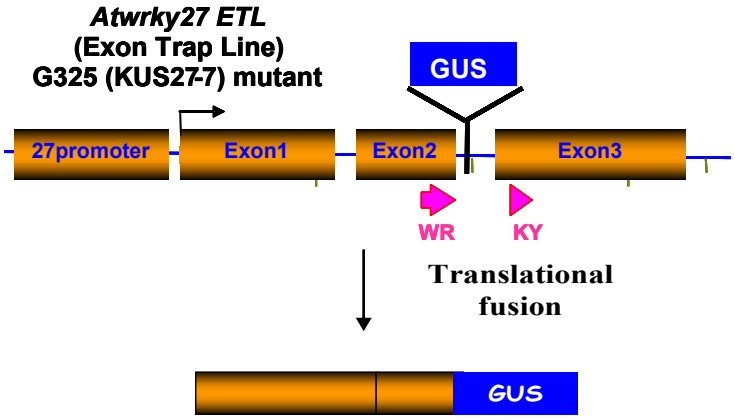


Fig. 17. Schematic representation of the *Atwrky27ETL* (Exon Trap Line) knock-out mutant. Position of the GUS reporter gene within *AtWRKY27* is shown. Sequence region encoding the WRKY domain is marked by the red arrows. Disruption of the endogenous gene leading to the generation of a translational fusion encoded by the exon1, exon2 and the GUS reporter gene. *Atwrky27 ETL* was kindly provided by S. Kushnir, Ghent University, Belgium).

Therefore, the detectable GUS activity staining in *Atwrky27ETL* represents expression of the entire native 5' flanking regulatory region of *AtWRKY27*, and was systematically analyzed to determine the endogenous expression of this gene in various tissues during different developmental stages of the plant.

The full-length 5' upstream flanking intergenic region of *AtWRKY27* is 6792 bp. A construct comprising 2 kb of upstream DNA sequence relative to the translational start site was fused to the GUS reporter gene and the strepII sequence(*AtWRKY27p::GUS::StrepII-terminator*) (Fig. 18). 10 independent *AtWRKY27p::GUS::StrepII-terminator* stable transgenics lines were analyzed and compared *AtWRKY27* expression in *Atwrky27ETL*.

Fig. 18. Diagrammatic representation of the *AtWRKY27promoter::GUS::StrepII-terminator* construct. The size of the *AtWRKY27* promoter is 2kb (relative to the translational start) which was fused upstream of a chimeric sequences encoding the GUS reporter, StrepII sequence and terminator sequence. StrepII sequence allows detection of the protein via use of a commercially available StrepII antibody. This construct was transformed into Columbia-0 wild-type plants.

GUS activity staining was examined at various stages of plant development to monitor endogenous *AtWRKY27* expression. Overall, similar, consistent but weak GUS expression was detected for both the *Atwrky27ETL* and the different *AtWRKY27p::GUS* lines. In 2 days old seedlings, detectable GUS expression was confined to areas of roots: the elongation zone, and to vascular tissue. At early stages of plant development (7 days and 14 days old plants), both *Atwrky27ETL* and *AtWRKY27p::GUS* lines display consistent GUS activity patterns (Fig. 19) within the root elongation zone and the vasculature (Fig. 19). In stem, 7 and 14 days old plants, GUS expression is highly restricted to vascular tissue (Fig. 19). Similar expression patterns can be detected in 21, 28 and 35 days old plants (data not shown).

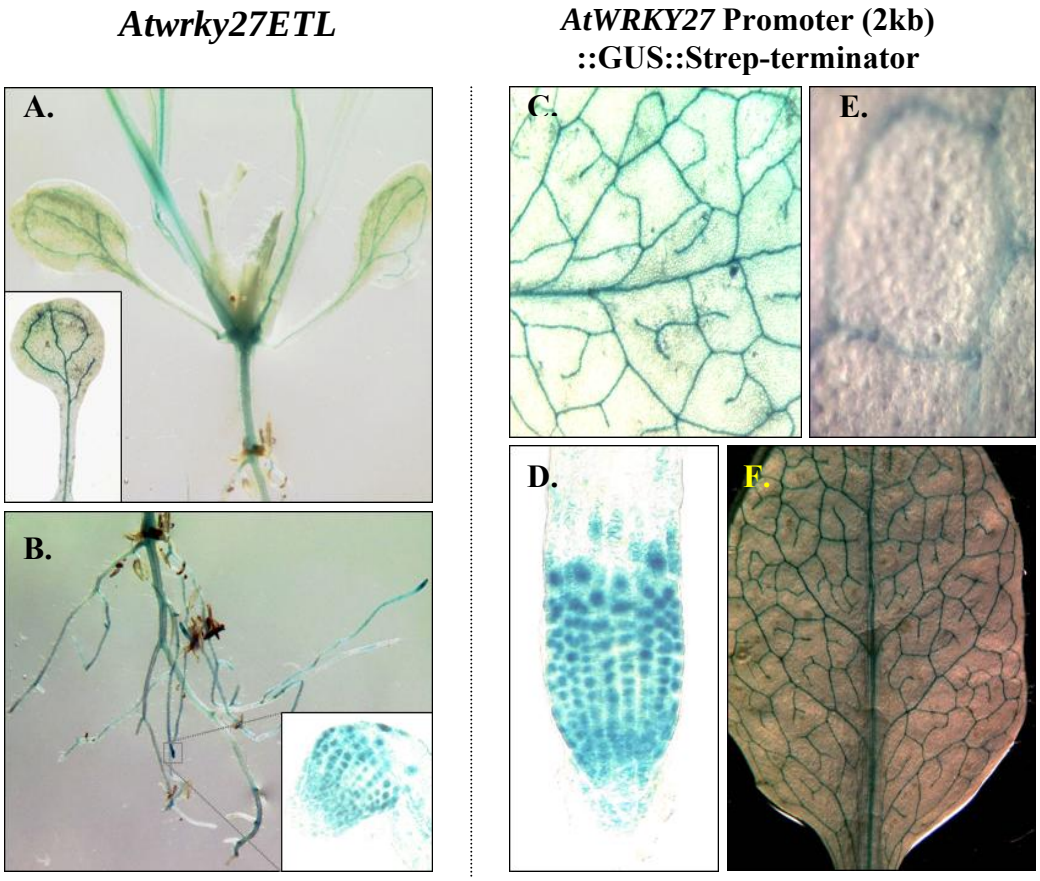


Fig. 19. Comparative GUS expression analysis between the *Atwrky27ETL* line and a representative *AtWRKY27* promoter (2kb)::*GUS::StrepII-terminator* transgenic plant. A comparable GUS activity was detected between *Atwrky27ETL* and *AtWRKY27p::GUS* lines in 7 and 14 days old plants. **A, C, E & F:** GUS activity was monitored specifically in stem and leaf vasculature. The close up (inset in A) shows the restriction of the activity to the vasculature. **B:** GUS activity is also seen in the vasculature of primary and secondary roots. GUS activity is highly restricted to the root elongation zone (inset in B). **D:** GUS activity is highly restricted to root elongation zone.

During early plant maturation, at the bud stage, GUS activity staining was transiently observed in various floral tissues such as stigmatic papillae, anthers, pollen grains, and the flower abscission zone. However, at later stage of flower development, GUS activity

staining was no longer detected in mature flowers and siliques, whereas it remained persistent in the abscission zone (Fig. 20). Moreover, GUS activity staining was not observed in pollen grains at later stages of flowers nor in pollen tubes that were germinated on artificial medium (Fig. 21).

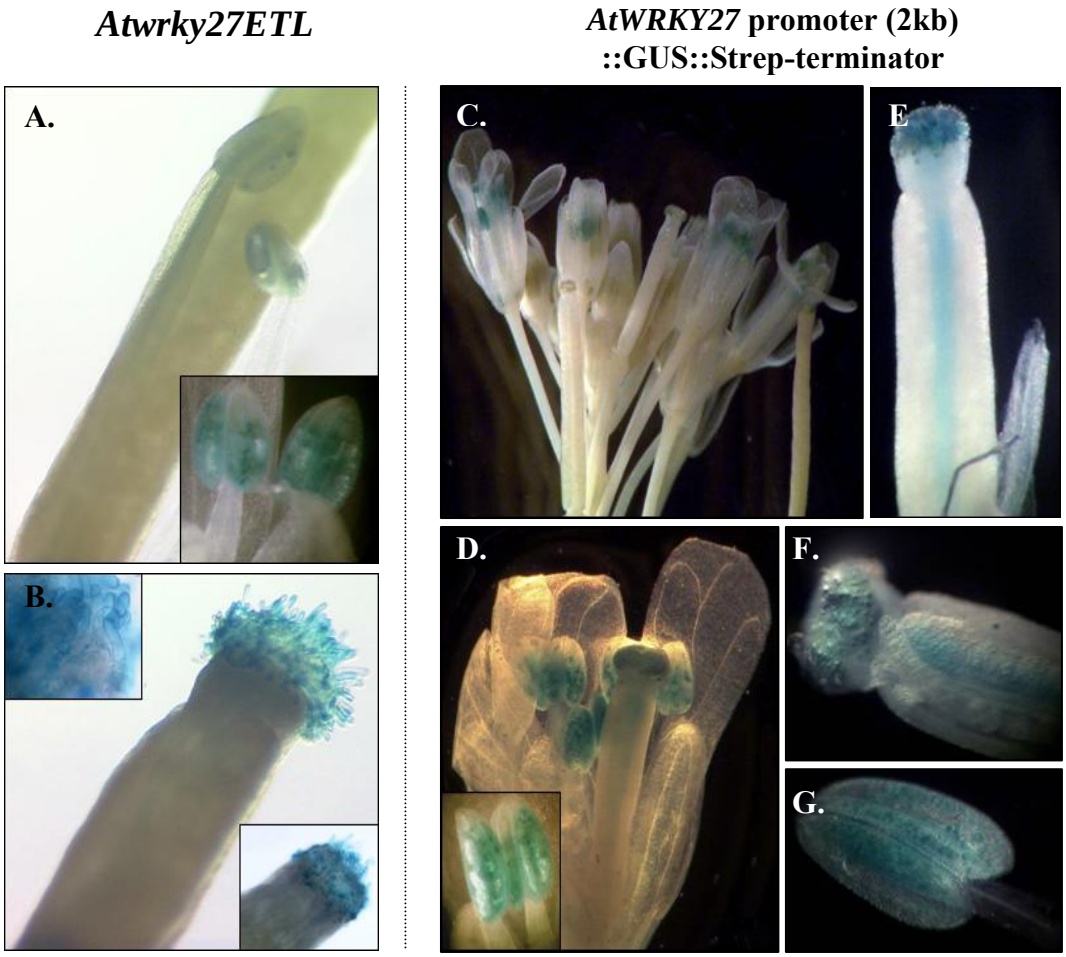


Fig. 20. *AtWRKY27* promoter driven GUS expression in floral organs. **A, D & G:** GUS activity was detected in anthers of older flower. **C & D:** GUS activity was detected in total inflorescences. **B, E & F:** GUS activity can also be seen in the residual stigmatic papillae. **E & F:** GUS activity was detected in the transmission tract of the septum.

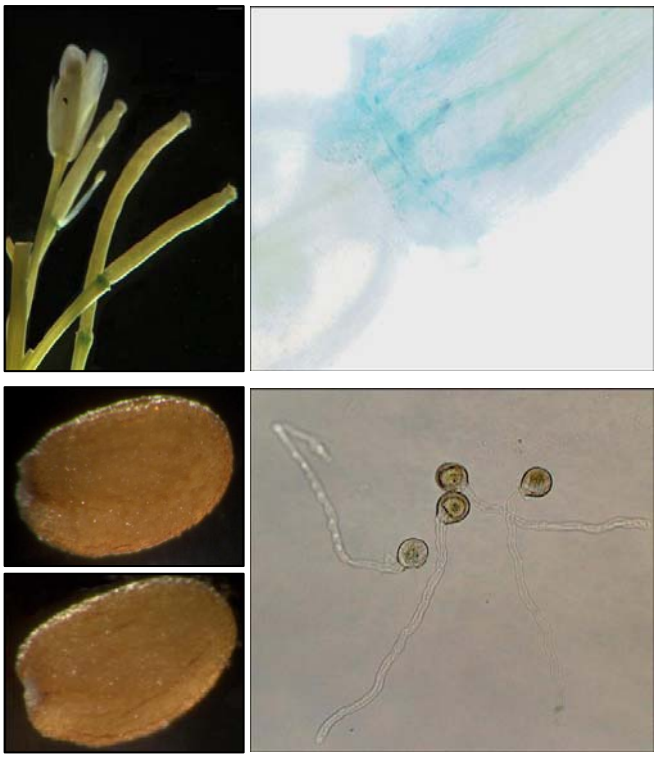


Fig.21. *AtWRKY27* promoter driven GUS expression analysis in anther and flower abscission zones. **A & C:** GUS activity in the flower abscission zones was persistent at early and later stages of flowering, while GUS activity is absent in stigmatic papillae at later stages of flower/silique development (A). **B:** GUS activity is undetectable in pollen grains and **D:** in pollen tubes germinated on artificial media.

To more precisely localize GUS activity staining in the vasculature, cross and longitudinal sections of *Atwrky27ETL* stems were made. GUS staining was only observed in phloem tissue (Fig. 22). For more detailed studies, transgenic lines containing a *AtWRKY27p::GFP* construct were generated. GFP has several advantages as an *in vivo* reporter for monitoring dynamic processes in living cells or organisms, as its fluorescence can be measured directly without additional proteins, substrates, or cofactors (Chalfie at el., 1994; Fey et al., 1995; Gubin et al., 1997; Kain et al., 1997).

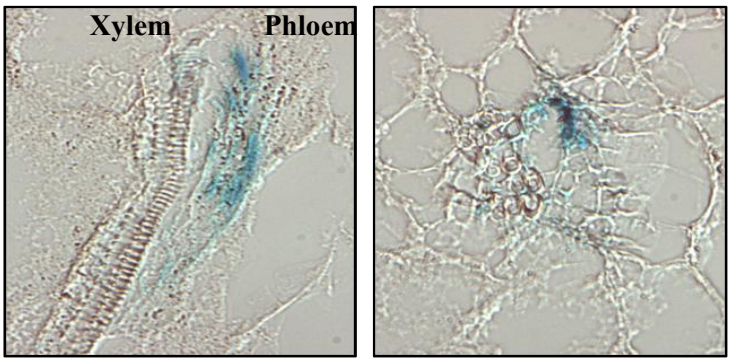


Fig. 22. Longitudinal section (left) and cross section (right) of *Atwrky27ETL* stem tissue. GUS activity staining (regions of blue staining) appears to be localized to phloem tissue.

For this, two new constructs were generated using the 2 kb promoter of *AtWRKY27* either driving the expression of the *AtWRKY27* cDNA fused to the coding sequence of GFP and a terminator (Fig. 23: A*tWRKY27p::AtWRKY27::GFP-terminator)* or driving the expression of a soluble modified-green fluorescent protein reporter gene (smGFP) and respective terminator sequence (Fig. 23: A*tWRKY27p::smGFP::terminator*).
Confocal laser scanning microscopy was used to detect GFP in these transgenic lines. Unfortunately, no GFP was detected in several analyses of independent lines (data not shown) expressing either the A*tWRKY27p::AtWRKY27::GFP-terminator* or A*tWRKY27p::sm-GFP::terminator* constructs. A possible explanation could be that the expression of *AtWRKY27* was below the sensitivity level despite the use of a brighter variant of GFP (smGFP).

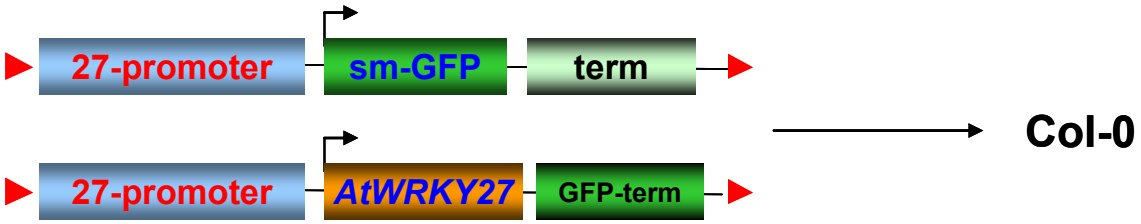


Fig. 23. Schematic representation of *AtWRKY27promoter::smGFP-cDNA::I*-Terminator (top) and *AtWRKY27promoter::AtWRKY27::GFP-cDNA*-Terminator (bottom) constructs used for transformation of Arabidopsis Col-0 wild-type plants.

3.5.3. Analysis of *AtWRKY27* expression upon treatments with various elicitors/stimuli

For a better insight into the possible function of *AtWRKY27*, a stimuli-dependent expression analysis was equally important. Taking advantage of the *cis*-regulatory element software PlantCare (Higo et al. 1999), 2 kb 5´ upstream DNA regulatory region of *AtWRKY27* was evaluated for the presence of various known regulatory motifs (Fig. 24). Computational analysis showed the presence of at least 4 W box (TTGACC/T) - like sequences. W box is a *cis*-regulatory DNA element which is shown to be the binding site of *At*WRKY proteins (Uelker and Somssich, 2004). Interestingly, two W boxes are

present in the direct physical proximity (between -300 bp and -200 bp relative to the translational start site), while the other two W boxes are also located together (between -2000 bp and -1800 bp relative to the translational start site).

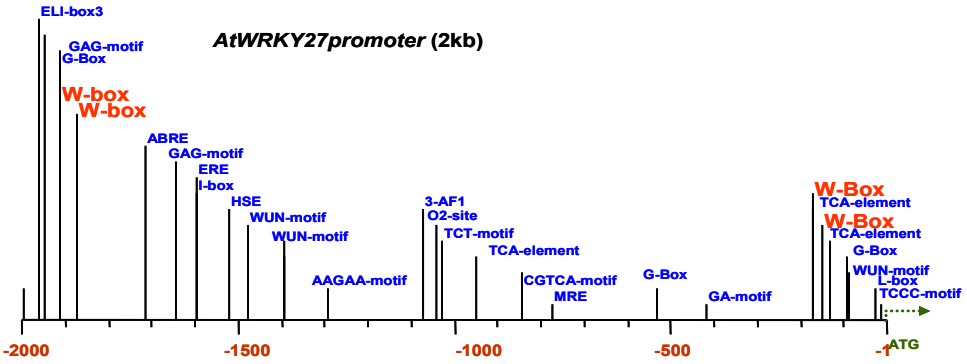


Fig. 24. Analysis of the 2kb *AtWRKY27* promoter (relative to translational start) using the PlantCare program to detect known *cis*-regulatory DNA elements (Higo et al. 1999). Position of the classified motifs within this promoter region is shown. Further information see text.

A large number of other known *cis*-regulatory sequences were also predicted that have been shown to be involved in both activation and repression of gene expression. These include a wound responsive element (WUN-motif: tCATTacct, Elliott and Shirsat, 1998), numerous light responsive elements like GT1-consensus sequences (GRWAAW, Zhou 1999), ethylene responsive element (ERE: AWTTCAAA, Itzhaki et al., 1994), abscisic acid responsive element (ABRE: cgtACGTaac Hagen et al., 2002), auxin responsive element (AuxRR-core: GGTCcct, Ulmasov et al., 1999, Hagen et al., 2002), MeJA responsive element (CGTCA-motif, Kubigsteltig et al., 1999), SA responsive element (TCA-element: aAGAAcaaga, Zhou, 1999), and endosperm specific elements (AACA_motif: aAACAtactata, Ulmasov et al., 2001).

Based on these *in silico* predictions, various stimuli/elicitors (table) which are known to mediate the expression of such *cis*-elements were analyzed to check for changes in basal GUS expression levels in the *Atwrky27ETL* at various phases of plant development, using appropriate controls, from 2 days old seedlings up to 28 days old plants.

Somewhat unexpectedly, all the tested treatments did not noticeably influence *AtWRKY27*expression levels (Table 6).

Stage / Treatments	2 days	7 days	14 days	21 days
Flg22 (1μM)	-	-	-	-
MeJa (100μM)	-	-	-	-
SA (2mM)	-	-	-	-
ACC (100μM)	-	-	-	-
Wounding	ND	ND	-	ND
UV (50 mj/cm²)	ND	ND	-	ND
Cold (2 hours)	ND	ND	-	ND
Heat (2 hours)	ND	ND	-	ND

- = No response
ND = not determined

Table 6. Expression analysis of *AtWRKY27* by means of promoter::GUS studies using the *Atwrky27ETL* line with various indicated stimuli or elicitors. The selected treatments are listed in the left column, while the various stages of plant development are indicated on the top row. 2- and 7-day-old-seedlings were grown on MS-medium while 14 and 21 days old plants were grown on soil in the greenhouse. flg22: 22-amino acid motif of the bacterial flagellin (Felix et al., 1999), SA: salicylic acid, MeJa: methyl jasmonate, ACC: 1-aminocyclopropane-1-carboxylic acid, UV: ultra violet light.

A parallel, complementary approach to gather more information on the responsiveness of the *AtWRKY27* promoter was to make use of expression data available in the NASC and Genevestigator databases (http://arabidopsis.info/, https://www.genevestigator.ethz.ch/). Both cover a vast part (currently available data from 2292 Affymetrix Arabidopsis Gene Chip [ATH1], dated September 08, 2005) of the *Arabidopsis thaliana* transcriptome (~25.000 genes) and allow monitoring of gene expression under various conditions,

developmental stages, treatments, in wild type plants and numerous mutants, etc. However, despite screening of a number of experiments currently available in the databases, no significant alterations in the level of *AtWRKY27* expression have been documented (Fig. 25).

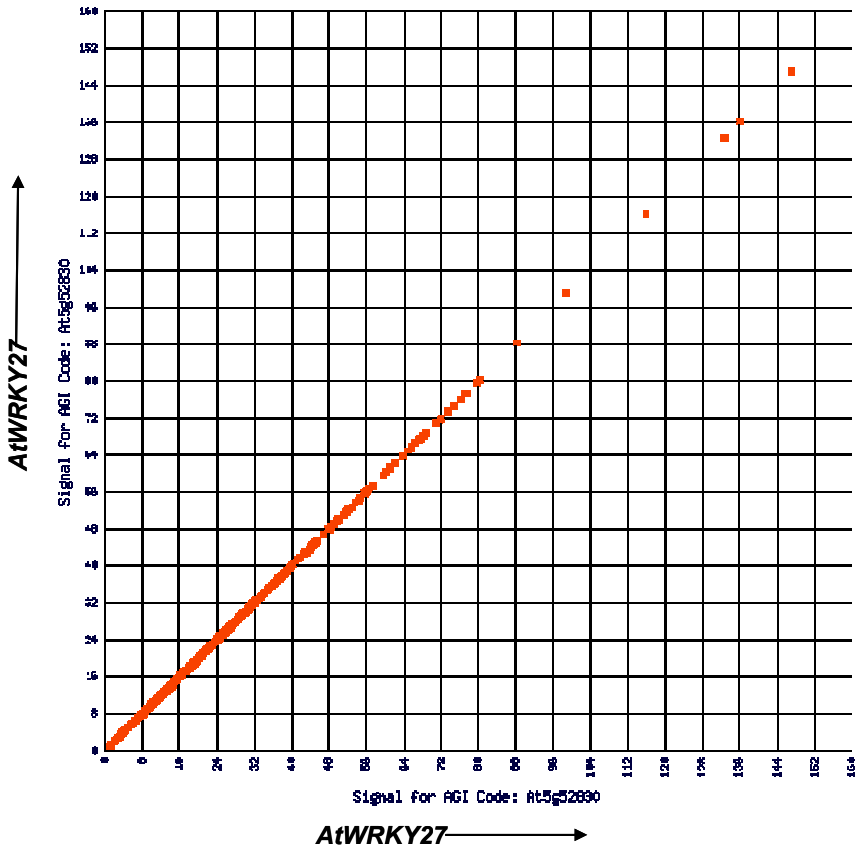


Fig. 25. *AtWRKY27* expression pattern over all slides available in Nottingham Arabidopsis Stock Centre (NASC) database (September 08, 2005). Spot history and Gene scatter plot tools were used to plot the signal intensities of *AtWRKY27*. These data indicate that overall, *At*WRKY27 expression levels are very low and are not influenced under various condition tested.

3.5.4. Study of *AtWRKY27* transcript levels in various plant organs

In order to corroborate the *AtWRKY27::GUS* basal expression data, an extensive expression analysis of *AtWRKY27* was pursued as an additional experimental approach. A series of semi-quantitative RT-PCRs were performed in wild-type Col-0 plants. mRNA from root, shoot, leaf, flower and silique tissue was isolated, and subsequently first strand cDNA was synthesized. This material was further used as PCR-template in the experiment. Firstly, *At*tubulin β-subunit transcript was amplified to adjust for equal loading of the samples, while genomic DNA template was also included in the experiment to rule out possible DNA contaminations. The same procedure was repeated for all subsequent RT-PCR analyses. Relatively weak but differential transcript levels of *AtWRKY27* were observed (Fig. 26), which were highly consistent with previous results using GUS reporter lines.

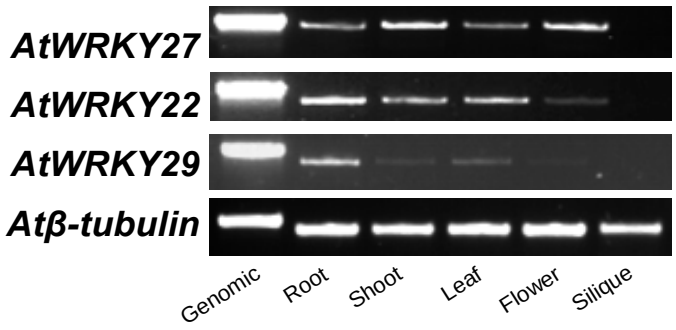


Fig. 26. *At*WRKY27 transcript levels tested in various organs. Steady state expression levels of *AtWRKY27*, *AtWRKY22* and *AtWRKY29* in root, shoot, leaf, flower and silique derived tissue detected by RT-PCR analysis.

Additionally, semi-quantitative RT-PCR was also performed, in order to compare the basal expression patterns of two closely related gene family members namely *AtWRKY22* and *AtWRKY29*, using specific primer combinations. Interestingly, all three members showed similar levels of expression in root, while none of them was expressed in siliques. *AtWRKY29* displayed relatively weak expression in shoot, leaf and flower tissue which contrasts to the expression of *AtWRKY22* and *AtWRKY27*. Importantly, *AtWRKY27* exhibited a comparatively higher expression level in floral tissue compared to the other tested *AtWRKY* sub-group members (Fig. 27).

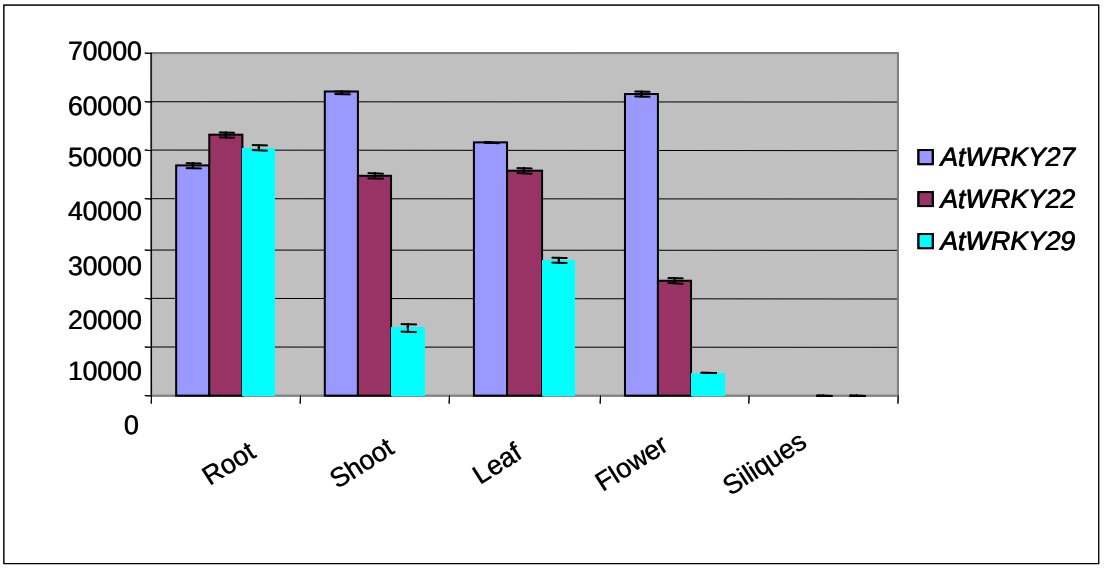


Fig. 27. Semi-quantitative measurements of *AtWRKY27* expression levels. Transcript levels of *AtWRKY27* (in blue), *AtWRKY22* (in red) and *AtWRKY29* (in green) in root, shoot, leaf, flower and silique tissue analyzed by RT-PCR.

3.6. Importance of known host defense signaling pathways for *AtWRKY27* function

An important aspect is to find out the resistance mechanism operating in *Atwrky27* loss-of-function mutants and furthermore to evaluate whether or not known host defense pathways play any role in this type of resistance. If *AtWRKY27* or a component(s) under the control of this transcription factor is a susceptibility factor, then *Atwrky27* resistance hould be largely independent of these defense signaling networks.

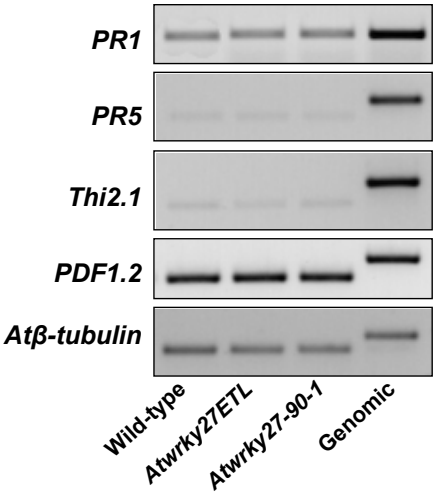


Fig. 28. Basal expression levels of pathogenesis responsive marker genes in *Atwrky27* mutant plants. Transcript levels of *PR1*, *PR5*, *Thi2.1* and *PDF1.2* in Col-0 wild-type, *Atwrky27ETL* and *Atwrky27-90-1* plants were detected by RT-PCR. PCR on Genomic DNA showed no DNA contamination. *PR1*: *pathogen related 1, PR5*: *pathogen related 5 PDF1.2*: *plant defensin1.2, Thi2.1*:*Thionin2.1.*

In Arabidopsis, at least three genetically distinguishable pathways: the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) mediated resistance, through which defense genes can be activated, are well characterized (Glazebrook, 2001 & 2005).
Pathogen related proteins, *e.g.* PR1 and PR5 act downstream to the SA dependent signaling pathway, while JA and ET act together to activate the expression of plant defensin1.2 (*PDF1.2*) and Thionin2.1 (*Thi2.1*). To determine the activation state of the SA, JA and ET pathways in the *Atwrky27* mutant, the steady-state transcript levels of *PR1*, *PR5*, *Thi2.1* and *PDF1.2* were evaluated in both wild-type and *Atwrky27* mutant plants. As shown in Fig. 28, no difference in mRNA levels was detected for the respective SA and JA/ET-responsive defense marker genes when comparing wild-type and *Atwrky27* plants. Thus, *Atwrky27* mediated resistance is not associated with altered regulation of these defenses response genes.

3.7. Basal expression pattern of *AtWRKY27* in different defense signaling mutants

A range of mutants or transgenic lines are genetically well defined at distinct positions within known host defense signaling pathways, which either block or constitutively enhance the activation of downstream defense responsive genes. To further analyze the association of defense signaling pathways to *Atwrky27* resistance and possibly integrate *AtWRKY27* into the network, basal expression of *AtWRKY27* was examined in these mutants or transgenic lines. The transcript level of *AtWRKY27* was not significantly altered in any of the mutants or transgenic lines under study (Fig. 29). Thus, *Atwrky27*-mediated resistance does not appear to signal through SA and JA/ET pathways leaving open the possibility that *AtWRKY27* or a component(s) under the control of this transcription factor are either required for bacterial growth or that the *Atwrky27* mutation leads to the activation of an undetermined defense pathway.

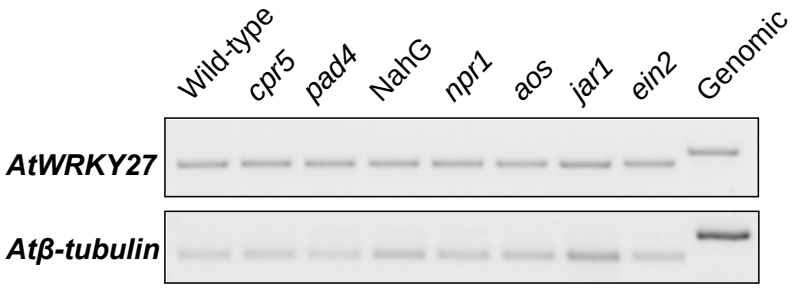


Fig. 29. Basal *AtWRKY27* transcript analysis in various signaling mutants. Comparative basal *AtWRKY27* transcript levels among a number of selected Arabidopsis mutants impaired in different defense signaling pathways. *cpr5*, *pad4*, *NahG* and *npr1* are defined in the SA dependent pathway, *aos* and *jar1* are in the JA-dependent and *ctr1* and *ein2* are in the ET-dependent pathway.

3.8. Analysis of independent *Atwrky27* T-DNA insertion lines

Taking advantage of various publicly available T-DNA insertion sources, three independent T-DNA insertion lines were selected from the SIGnAL database (accession numbers: SALK_048949, SALK_048952 and SALK_109290; http://signal.salk.edu/cgi-bin/tdnaexpress?GENE=&FUNCTION=&TDNA=salk_109290). All these lines were predicted to contain T-DNA insertions in the exon-2 of the *AtWRKY27* locus. PCR was performed using SALK left border T-DNA specific primer sets which verified the presence of the T-DNA in two insertion lines, designated SALK_048952 and SALK_109290. However, no T-DNA was detected in the SALK_048949 insertion line. Further on, sequence analysis was done to specify the precise site of T-DNA insertion in the SALK_048952 and SALK_109290 lines. The position of the T-DNA insertion in the SALK_048952 insertion line was detected in intron-1 rather than of the previously predicted exon-2, while in the case of the SALK_109290 line, the presence of the T-DNA was confirmed to be in exon-2 as shown in Fig. 30A.

In order to select for homozygous T-DNA insertion lines (insertions in both homologous chromosomes), PCR analysis was performed using designed gene specific primers flanking the sequence of the T-DNA. Heterozygous plants were sorted out from homozygous ones by the presence or absence of a specific band, respectively (Fig. 30B).

RT-PCR analysis confirmed the absence of *AtWRKY27* transcript in the selected homozygous progeny for the T-DNA insertion lines (Fig. 30C). SALK_109290 (*Atwrky27-90-1*) line was selected for subsequent experiments, as the T-DNA insertion was located in an exon.

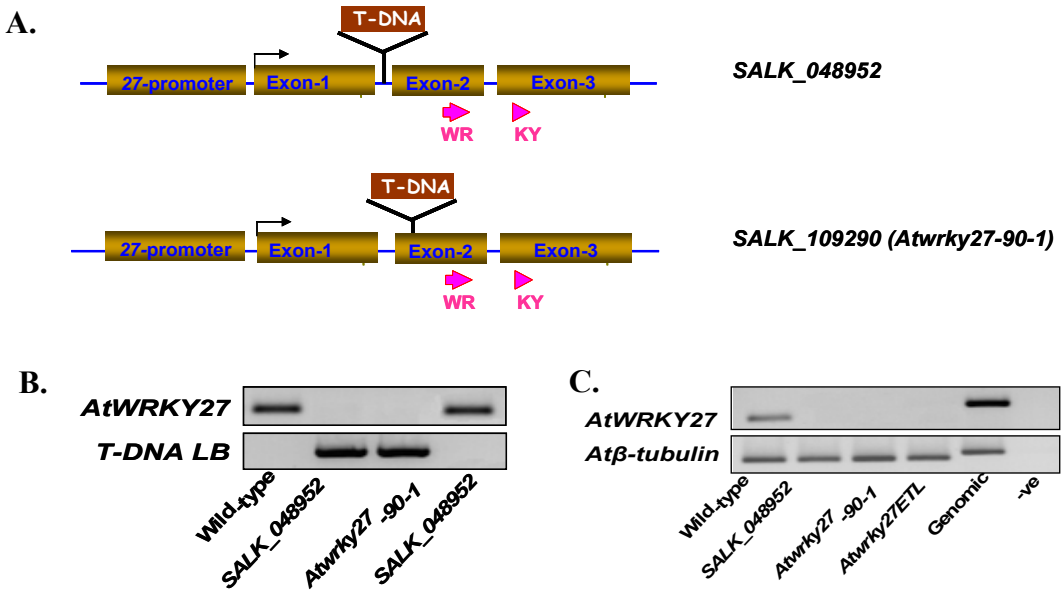


Fig. 30. Molecular identification of additional *Atwrky27* T-DNA insertion lines.

A. Schematic picture of the T-DNA insertions in the *AtWRKY27* locus. Exons are highlighted. Coding sequence for the WRKY domain is indicated in pink. Positions of T-DNA insertions are marked.

B. Homozygous lines were selected by PCR analysis. Equal amount of DNA template from wild-type plants and the T-DNA tagged lines (SALK_048949, SALK_048952 and SALK_109290=*Atwrky27-90-1*) were used to confirm the presence of the respective insert using the *AtWRKY27* primers (W27-exon1-FRW and W27Exon3_684Rev; details available in materials and methods) and T-DNA left border (LB) specific primer pairs (SALK_LBb1_Rev). No T-DNA was found in the case of the SALK_048949 line.

C. RT-PCR analysis of *AtWRKY27* expression levels in the selected T-DNA lines. No endogenous *AtWRKY27* transcript was detected in the homozygous insertion lines. *Atwrky27ETL* line was included in this analysis. Genomic DNA was used as control for contamination. –ve: no DNA template was added to the reaction.

3.8.1. Analysis of two the independent *Atwrky27* insertion lines in response to *R. solanacearum* GMI1000 strain infection

To further solidify the involvement of *Atwrky27* in the establishment of a delayed disease symptoms response to *Ralstonia solanacearum* strain GMI1000, two independent insertion lines namely *Atwrky27ETL* and *Atwrky27-90-1* were analyzed. Both of the independent insertion lines and appropriate wild-type control plants (*Atwrky27ETL* line is derived from Col-1 plants, while *Atwrky27-90-1* is in Col-0 background) were root inoculated with *Ralstonia solanacearum* strain GMI1000. Infected plants were monitored for symptom appearance and development (Disease Index) over a period of 13 days.

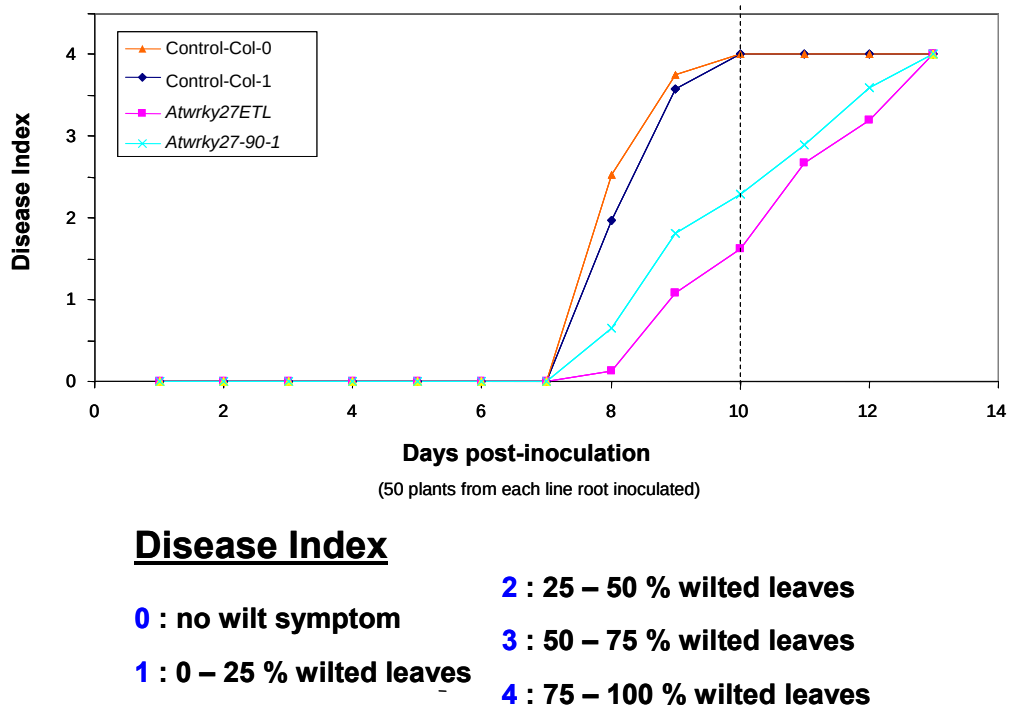


Fig. 31. Disease Index curve over a course of time of infection. A clear-cut difference in the development of wilt symptoms starting 8 days post inoculation can be observed between wild-type plants and *Atwrky27* mutant plants. By day 10 100% of the control Col-0/Col-1 plants wilted, while the Disease Index for both *Atwrky27* knock-out mutants

stayed in the range of 25-50%. (Experiment performed by Dr. Y. Marco, CNRS/INRA, Toulouse France).

The Disease Index curve in the course of time showed a clear-cut difference between wild-type and mutant plants starting 8 days post inoculation. Further on, at 10 days post inoculation, 100% of the control wild-type plants wilted completely, while the Disease Index for both of the independent *Atwrky27* insertion lines remained in the range of 25-50% (Fig. 31). The infection experiment was performed in replications (50 plants per replication) and confirmed the consistent differential response of the two independent *AtWRKY27* KO lines.

3.9. Test for abiotic stresses

3.9.1. Dark-induced and natural leaf senescence studies

Leaf senescence is a continuous and complex process regulated by numerous exogenous and endogenous factors with light playing a major role. Several lines of evidence have demonstrated the involvement of *AtWRKY* factors in both the induced and natural senescence process (Robatzek and Somssich, 2002; Miao et al., 2004; Bekir Uelker and Imre Somssich, personal communication). Thus, a possible role of *AtWRKY27* in leaf senescence was also investigated by employing both a dark-induced and natural senescence regime.

2-week-old soil grown plants of the two independent *Atwrky27* insertion lines and wild-type plants were placed in darkness or individual detached leaves were covered with a black cloth in a box (data not shown). Under the above tested conditions, no visible differences in terms of yellowing time were found. Further on, both *Atwrky27* insertion lines and wild-type plants were grown in standard conditions to monitor natural senescence. *Atwrky27* knock out mutant plants did not exhibit any differences compared to wild-type plants in the rate or efficiency of seed germination, elongation of the root system, seedling development, bolting, flowering time and senescence (data not shown). These data indicate that mutations within *Atwrky27* do not influence gross plant development and in particular leaf senescence.

3.9.2. Root gravitropism

Arabidopsis seedlings immediately respond to a gravistimulated state by developing a downward root tip curvature. Gravity is perceived mainly by the columella cells of the root cap, whereas the differential growth response associated with gravistimulation occurs in the root elongation zone (Blancaflor and Masson, 2003). Since *AtWRKY27* is specifically expressed in the root elongation zone (see 3.5.2), I tested whether *AtWRKY27* plays any role in root gravitropism. 10 days old vertically grown seedlings of the two independent *Atwrky27* insertion lines and wild-type plants, were reoriented by 90^{o} angle relative to the gravity vector and root tip responses were monitored. No visible differences were found in root tip curvature of *Atwrky27* insertion lines compared to wild-type seedlings (Fig. 32).

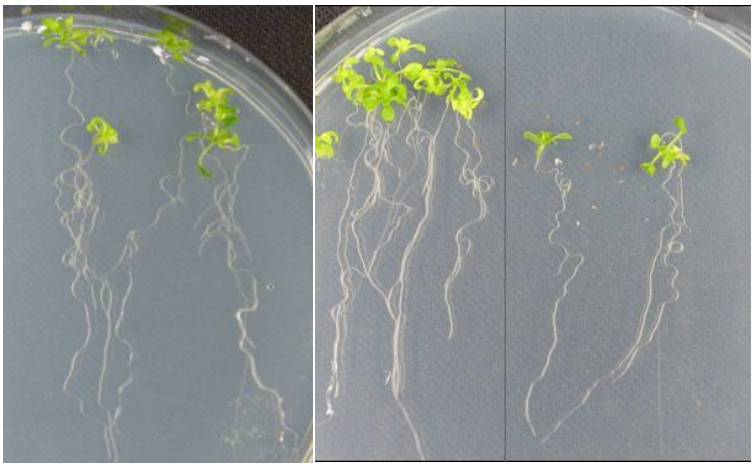
2xCaMV35S::AtWRKY27 ***Atwrky27ETL***

Fig. 32. The *AtWRKY27* overexpressor and *Atwrky27ETL* seedlings were vertically grown on MS media for 10 days and then reoriented by 90^{o} angle relative to the gravity vector. The root tip responses were monitored. Pictures were taken 2 days later. No visible differences were found in root tip curvature of *AtWRKY27* overexpressor lines compared to *Atwrky27ETL* seedlings.

3.9.3. Root winding assay

Furthermore, wavy root growth patterns were also studied to check for a possible role of *AtWRKY27* in root gravitropism. The two independent *Atwrky27* insertion lines and wild-type seedlings were vertically grown on 1.5% MS medium and tilted by 45^{o} for 3

days. *Atwrky27* insertion lines and wild-type all exhibited similar patterns of root growth (Fig. 33).



Atwrky27-90-1 **Wild-type** ***Atwrky27ETL***

Fig. 33. Independent *Atwrky27* insertion lines and wild-type seedlings were vertically grown on 1.5% agar-agar MS medium for 10 days and tilted by 45°. Pictures were taken after 3 days after tilting the plates.

3.9.4. Other abiotic stresses

Atwrky27 insertion lines and wild-type seedlings/plants were also subjected to various other abiotic stresses including cold, heat shock, paraquat, H_2O_2 and KNO_3. Soil grown plants were either placed at 4° for 2 days to observe cold stress or at 37° for one hour to examine a heat shock effect. Seedlings were grown on MS media containing 5 µM paraquat for germination tests, while various concentrations (0, 10, 20 mM) of H_2O_2 were used to study root growth. Both *Atwrky27* insertion lines showed similar responses to all above mentioned stresses compared to the wild-type plants. (Fig. 34).

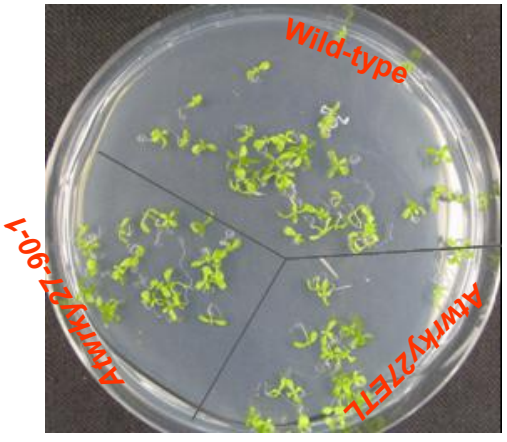


Fig. 34. The two independent *Atwrky27* knock-out mutants and wild-type seedlings were grown on MS media supplemented with KNO_3. No visible differences were found.

Pictures were taken 13 days after the germination.

3.10. *AtWRKY27* complementation lines

Based on the GUS studies (see 3.5.2), it appears that 2 kb of *AtWKY27* 5´ regulatory region is sufficient to mediate all observed expression patterns of the gene. Thus, a transcriptional fusion construct was generated using the *AtWRKY27* 2kb promoter sequence, a full length *AtWRKY27* cDNA and the *StrepII*-terminator sequence as shown in Fig. 35A. This construct was transformed into *Atwrky27ETL* plants using Agrobacterium. After Basta selection, RT-PCR was performed to detect *AtWRKY27* transcript in several T_1 and T_2 lines (Fig. 35B and C). Differential levels of *AtWRKY27* transcript were detected in various complementation lines, most likely due to position events of T-DNA insertion into different genomic loci.

Two T_2 lines (*Comp5-4*, *Comp5-8*) and one T_1 line (*Comp-8*) were further subjected to subsequent *Ralstonia solanacearum* strain GMI1000 root inoculation experiments. The Disease Index curve of *Comp5-4*, a line showing *AtWRKY27* expression levels similar to wild-type plants, fully overlapped with that of wild-type plants during the entire period of symptoms development. Similarly, *Comp5-8* and *Comp-8* both showed a positive correlation between expression levels and the magnitude of susceptibility (Fig. 36).

3.11. Bacterial growth kinetics

To better understand the molecular mechanisms and type of resistance displayed by the *Atwrky27* KO mutant, bacterial growth was monitored in the aerial parts of the plants. A relatively low bacterial titer was root-inoculated and the magnitude of pathogen multiplication was determined over time. Surprisingly, the bacterial density in both *Atwrky27* KO lines versus wild-type plants increased at a similar rate, and reached high densities of 10^{10} CFU per gram fresh weight (Fig. 37).

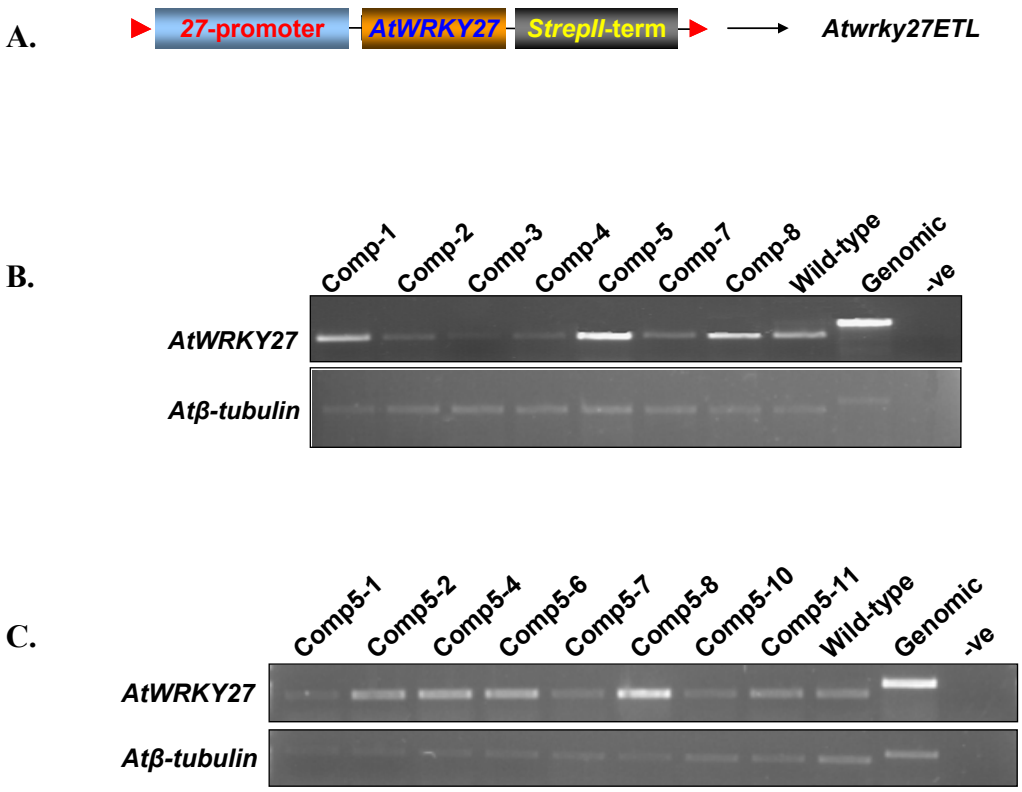


Fig. 35. *AtWRKY27* transcript levels in various complementation lines.

A. Schematic representation of the construct used for complementation of the *Atwrky27ETL* knock-out line. The 2kb fragment of *AtWRKY27* upstream regulatory sequence was fused to the full length cDNA of *AtWRKY27* and Strep-II epitope terminator sequence.

B. A number of individual T_1 complementation lines were screened by RT-PCR for the presence of *AtWRKY27* transcript.

C. A number of T_2 siblings originating from selfing of the line *Comp-5* were screened by RT-PCR and showed the presence of *AtWRKY27* transcript compared to wild type plants. Genomic DNA was used as control for contamination. –ve: no DNA template added to the reaction.

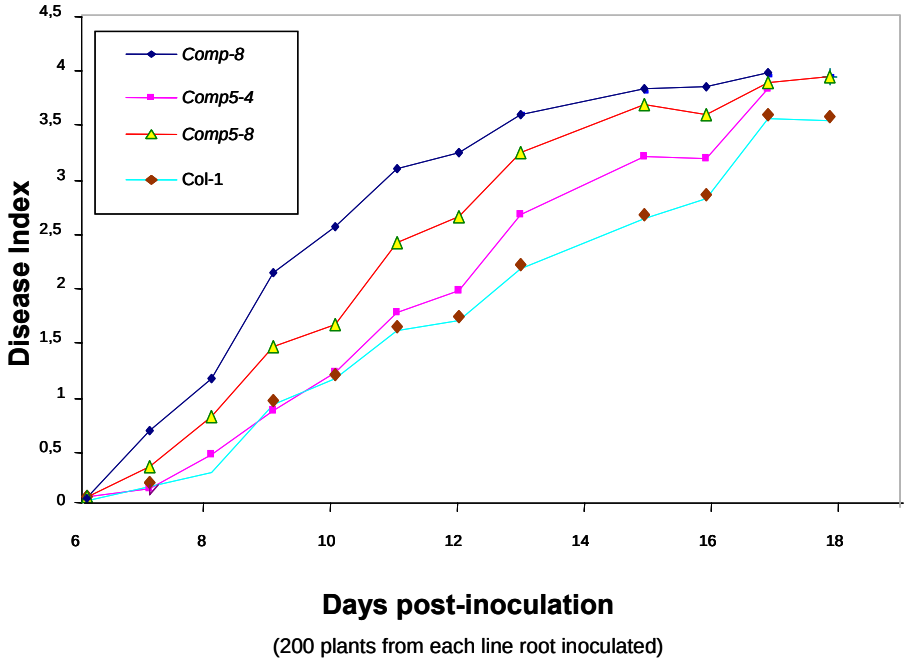


Fig. 36. Infection of *R. solanacearum* strain GMI1000 on wild type Col-1 and *AtWRKY27* complementation lines. Disease Index curve during the course of time. Symptom development is indistinguishable between wild-type and *Comp5-4* lines, while *Comp8* and *Comp5-8* lines show enhanced susceptibility. (Experiment performed by Dr. Y. Marco, CNRS/INRA, Toulouse, France).

3.12. Tissue-specific expression of the *AtWRKY27* transgene

In order to track down where in the *AtWRKY27* KO mutant plants the normal *R. solanacearum* strain GMI1000 infection process is affected, a series of constructs were generated, expressing *AtWRKY27* under three tissue specific promoters.

The *Arabidopsis sucrose transporter2* (*AtSUC2*) is expressed in the plasma membrane of companion cells within the phloem, a specialized tissue of the plant vasculature (Ward et al., 1998). 2067 bp of 5´ untranslated promoter fragment of *AtSUC2* was previously shown to specify the expression pattern restricted only to companion cells (Truernit and Sauer, 1995).

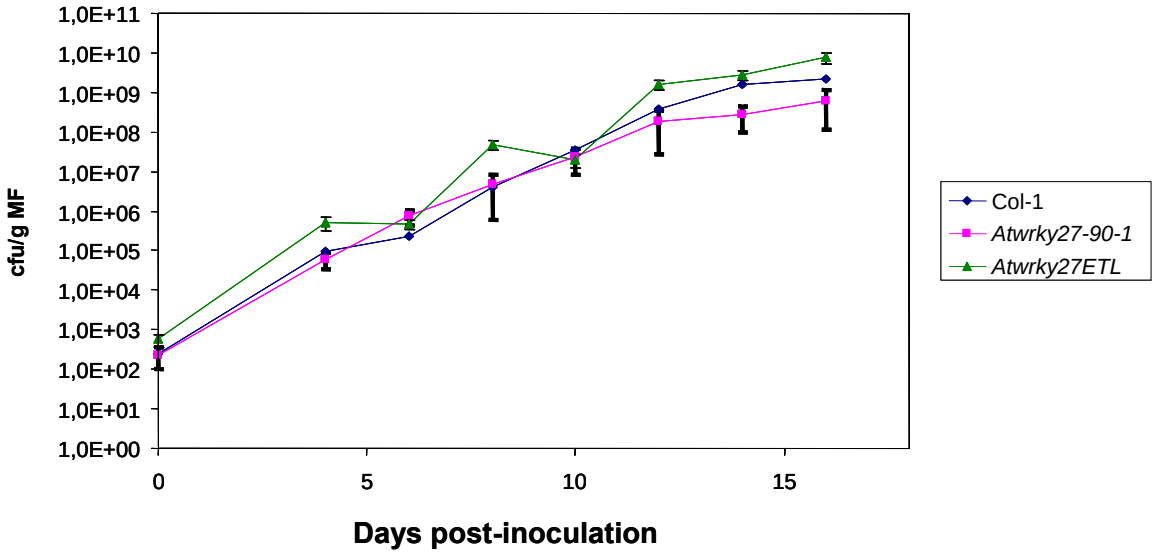


Fig. 37. Bacterial growth kinetics upon infection with *R. solanacearum* strain GMI1000. Bacterial multiplication in leaves of Col-1, *Atwrky27-90-1* and *Atwrky27ETL* upon inoculation with *R. solanacearum* strain GMI1000 (10^8 bacteria ml^{-1}). For each time point triplicate assays were performed on 3 plants for each accession/mutant line. (Experiment performed by Dr. Y. Marco, CNRS/INRA, Toulouse, France).

In situ localization of GUS activity in Arabidopsis plants showed that *4-coumarate:coenzyme A ligase (4CL)* promoters (with the exception of *4CL-3*) are specifically expressed in xylem, root sub-apical cells and pigmented portions of petals (Douglas et al., 1991; Hauffe et al., 1991). 1550 bps of *4CL-2* upstream regulatory DNA sequence is sufficient to ensure the expression in the appropriate tissues (Stuible and Kombrink, personal communication).

Transgenic *Arabidopsis* carrying a construct of 1648 bps of the *endo-1,4-β-glucanase (Cel1)* promoter fused to the GUS reporter gene, demonstrated its expression specifically within both shoot and root elongation zones (Shani et al ., 1997; Tsabary, 2001).

The above described promoters (*SUC2*, *4CL-2* and *Cel1*) were individually fused to generate a series of transcriptional fusion constructs, either with the *AtWRKY27*-cDNA or with the GUS reporter gene, respectively (Fig. 38A). The former constructs were transformed into *Atwrky27ETL* KO mutant plants to assay for tissue-specific

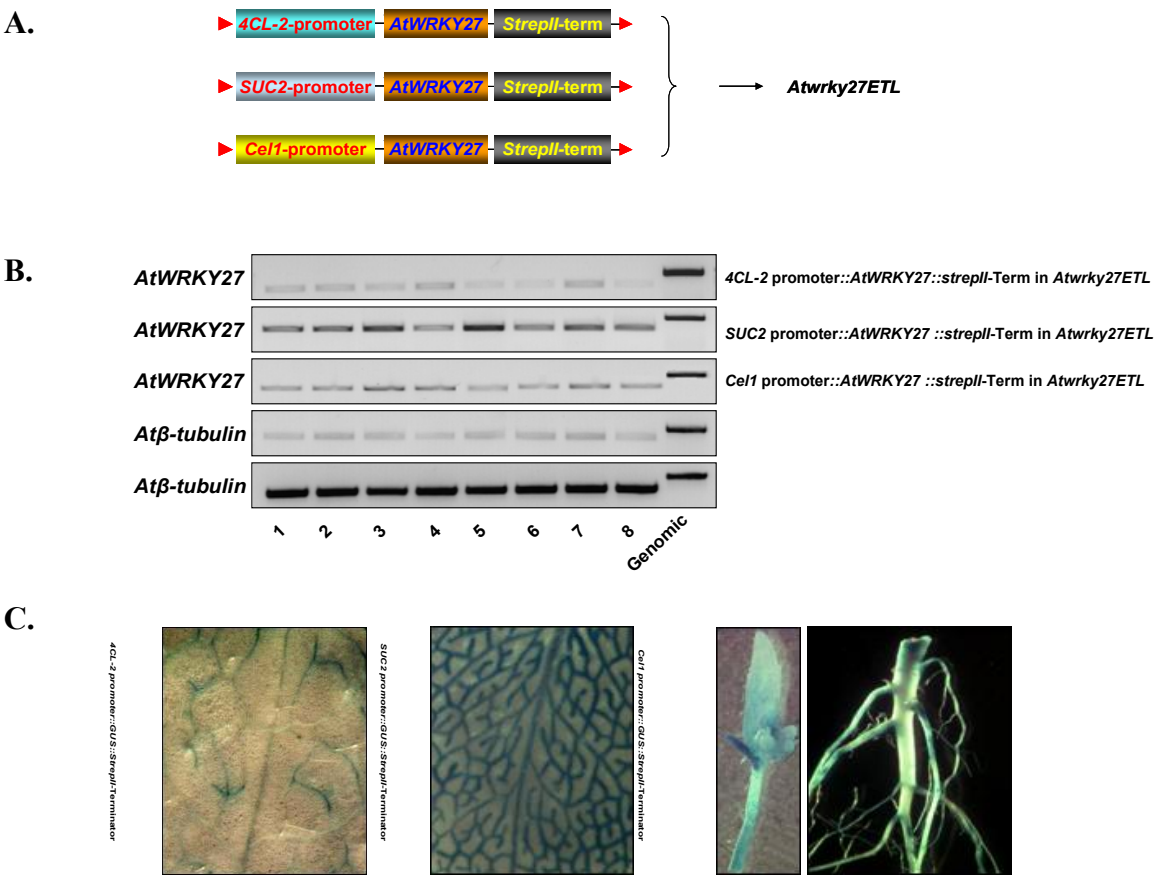


Fig. 38. Tissue-specific expression of *AtWRKY27*

A. Schematic representation of the constructs used for tissue-specific complementation of *Atwrky27ETL* with the cDNA of *AtWRKY27*. Three different promoters: (*At4CL-2*, *AtSUC2* and *AtCel1)* were used and each construct contained a StrepII sequence that allows for the detection of the protein using a StrepII antibody.

B. RT-PCR analysis of selected *AtWRKY27* complementation lines. *AtWRKY27* transcript was detected in the lines with differential intensities. Genomic DNA was used as control for contamination.

C. GUS activity was detected in leaf vasculature of *4CL-2 promoter::GUS::StrepII*-Terminator (left) and *SUC-2 promoter::GUS::StrepII*-Terminator (middle) transgenic plants. GUS activity was detected in the elongation zones of plant tissue harboring the *Cel1 promoter::GUS::StrepII*-Terminator (right) transgene.

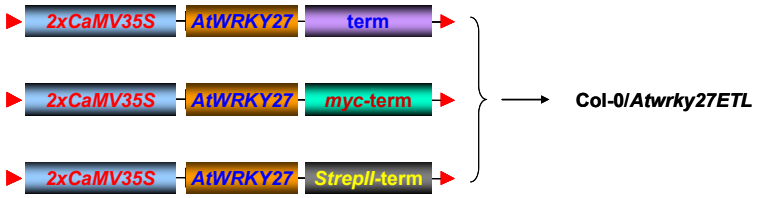
complementation, whereas reporter gene constructs were transformed into wild-type plants. This served the purpose of a positive control, to note accurate transcriptional fusion as well as to monitor GUS expression within proper compartments (Fig. 38C). RT-PCR analysis confirmed the presence of *AtWRKY27* transcript expressed under the various tissue specific promoters (Fig. 38B). These transgenic lines will be analyzed to document their responses to *Ralstonia solanacearum* strain GMI1000.

3.13. Ectopic overexpression of *AtWRKY27*

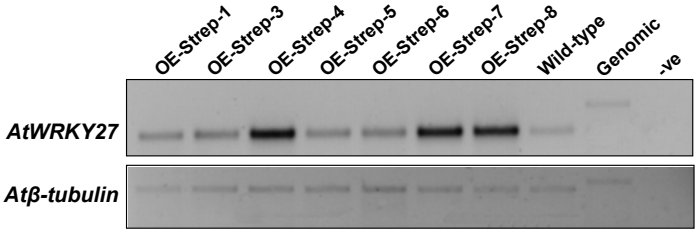
Besides using diverse biotic and abiotic stresses to identify altered effects related to *Atwrky27* loss-of-function mutants, overexpression advocates an alternative and complementary approach to define *AtWRKY27* function that is less affected by functional redundancy. A set of overexpressor constructs, driven by the *2xCaMV35S* promoter, were generated using the full-length cDNA of *AtWRKY27* fused with the 3′ terminator element or in frame to a *myc/StrepII* sequence allowing epitope tagging of the respective protein (Fig. 39A). The constructs: *2x35S::AtWRKY27*-terminator, *2x35S::AtWRKY27-myc*-terminator and *2x35S::AtWRKY27-StrepII*-terminator, were transformed into Arabidopsis wild-type and *Atwrky27ETL* KO mutant plants. RT-PCR and Northern blot analyses clearly showed elevated but varying levels of *AtWRKY27* transcript in three T_1 *2x35S::AtWRKY27-StrepII*-terminator overexpressor lines (*OE-Strep-4*, *OE-Strep-7* and *OE-Strep-8*) compared to wild-type plants (Fig. 39B and C). Similarly, high *AtWRKY27* transcript levels were also detected by RT-PCR in several other transgenic lines, expressing either the *2x35S::AtWRKY27*-terminator or the *2x35S::AtWRKY27-myc*-terminator constructs. *OE-Strep-4* and *OE-Strep-7* were further used in the subsequent experiments (Fig. 39D).

Moreover, stable *OE-Strep-4* and *OE-Strep-7* overexpressor lines were confirmed by detection of epitope tagged *At*WRKY27 protein on Western blots (Fig. 39E). Equal volumes of crude extracts from leaves of the transgenic Arabidopsis plants were dissolved in loading buffer and separated by SDS-PAGE. As controls, crude extracts derived from *Nicotiana benthamiana* leaves transiently expressing the *2x35S::AtWRKY27-StrepII*-terminator and *2x35S::AtEDS1-StrepII*-terminator constructs,

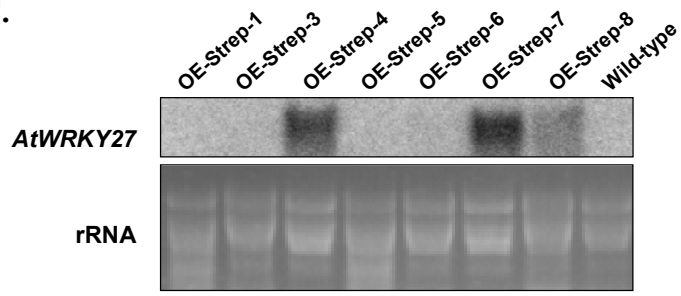
A.


2xCaMV35S
AtWRKY27
term
2xCaMV35S
AtWRKY27
myc-term
2xCaMV35S
AtWRKY27
StrepII-term
Col-0/Atwrky27ETL

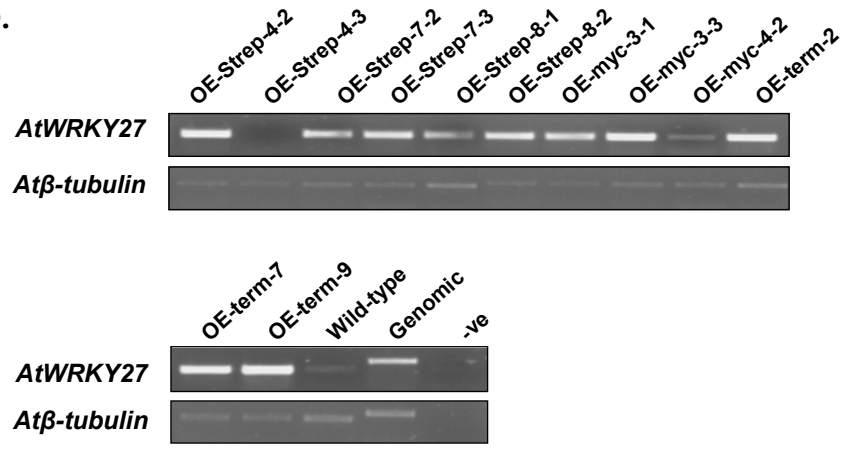
B.


OE-Strep-1
OE-Strep-3
OE-Strep-4
OE-Strep-5
OE-Strep-6
OE-Strep-7
OE-Strep-8
Wild-type
Genomic
-ve
AtWRKY27
Atβ-tubulin

C.


OE-Strep-1
OE-Strep-3
OE-Strep-4
OE-Strep-5
OE-Strep-6
OE-Strep-7
OE-Strep-8
Wild-type
AtWRKY27
rRNA

D.


OE-Strep-4-2
OE-Strep-4-3
OE-Strep-7-2
OE-Strep-7-3
OE-Strep-8-1
OE-Strep-8-2
OE-myc-3-1
OE-myc-3-3
OE-myc-4-2
OE-term-2
AtWRKY27
Atβ-tubulin
OE-term-7
OE-term-9
Wild-type
Genomic
-ve
AtWRKY27
Atβ-tubulin

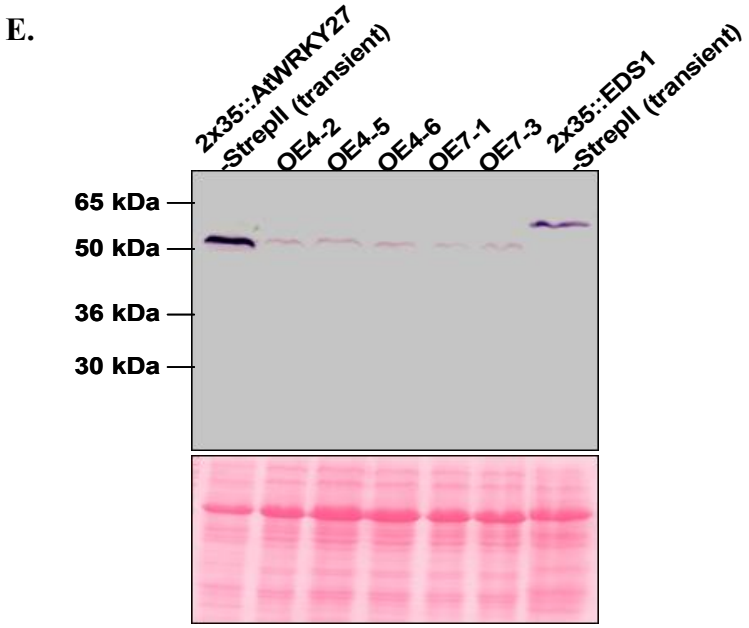


Fig. 39. Ectopic overexpression of *AtWRKY27*. **A.** Schematic representation of the constructs used for ectopic expression of *AtWRKY27* in Col-0/*Atwrky27ETL* plants. For the *AtWRKY27* overexpressor constructs, the *2x CaMV35S* constitutive promoter was fused with *AtWRKY27* cDNA and with terminator sequences (in violet box), *myc* and terminator sequences (in green box) and *StrepII* and terminator sequences (indicated in grey box).

B. RT-PCR analysis on 8 T_1 *AtWRKY27* overexpressor *StrepII*-terminator lines. Transcript level was compared with the wild-type plants. Genomic DNA was used as control for contamination. –ve: no DNA template.

C. Northern blot analysis on 8 T_1 *AtWRKY27* overexpressor *StrepII*-terminator lines. *AtWRKY27* transcript abundance was examined in 3 lines (*OE-Strep-4*, *OE-Strep-7* and *OE-Strep-8*). Ethidium bromide stain of rRNA was used to monitor for equal loading of the samples.

D. RT-PCR analysis of *AtWRKY27* expression level in T_2 selected transgenic lines carrying 3 differnet overexpressor constructs.

E. Western blot to detect StrepII-tagged *At*WRKY27 protein. Immunoblot of crude extracts derived from overexpressor transgenic lines. For controls, crude extracts were from *Nicotiana benthamiana* leaves transiently expressing *2x35S::AtWRKY27-StrepII-*

terminator or the *2x35S::AtEDS1-StrepII*-terminator, respectively. Ponceau S staining of gel was used for equal loading of the samples.

respectively were included. *AtEDS1* encodes a lipase-like protein (Parker et al. 1996) unrelated to WRKY factors. The protein is larger than *At*WRKY27 and was used as a control. After immunoblotting with the *Strep*-Tactin alkaline phosphate conjugate (ST-AP), *At*WRKY27-StrepII and *At*EDS1-StrepII proteins were detected at the expected molecular weights in all samples (Fig. 39E).

3.13.1 Pleiotropic phenotypes of ectopic *AtWRKY27* overexpressor plants

During the entire vegetative growth, *2x35S::AtWRKY27* (regardless of which transgene construct was used) plants exhibited visible phenotype associated with stunted growth and irregular leaf shape (Fig. 40). The most striking observation, however, was partial sterility and a delay in senescence in perianth organs. The young siliques of the overexpressor transgenic plants were undeveloped and empty, while at very late stages of flowering, the siliques started to elongate. Nevertheless, the normal size was never attained and the seed content was clearly reduced, compared to those of wild-type plants (Fig. 40).

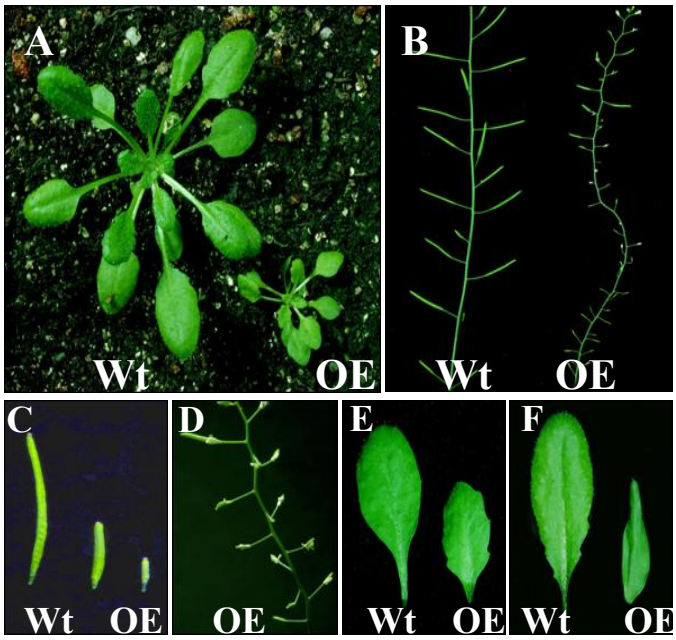


Fig. 40. Pleiotropic phenotypes of ectopic *AtWRKY27* overexpressor plants. Wt – wild type plant, OE – *AtWRKY27* overexpressor plant. **A.** *AtWRKY27* overexpressor transgenic lines exhibit dwarf phenotypes compared to wild-type plants. **B & C.** partial sterility was found in plants expressing increased levels of *AtWRKY27* compared to wild-

type plants. **D.** Delayed senescence in perianth organs of the *AtWRKY27* overexpressor line. **E & F.** Altered leaf shape of *AtWRKY27* overexpressor transgenic plants (to the right in E & F) compared to wild-type leaves (to the left in E & F).

The sterility phenotype was neither reversible by exogenous application of jasmonic acid (JA), gibberelic acid (GA) and gamma-amino butyric acid (GABA), nor was growth under various temperature conditions (22^0C, 26^0C and 28^0C) affected. Using scanning electron microscopy, at higher magnification, anther dehiscence defect was observed in the developing flowers of ectopic *AtWRKY27* overexpressor plants (Fig. 41). Furthermore, viability of the pollen produced by the *AtWRKY27* overexpressor lines was also investigated. Pollen grains from five independent *2xCaMV35S::AtWRKY27* overexpressor lines were stained with FDA (fluoresceine diacetate), which is a substrate processed by an esterase in the living cell. Released fluoresceine results in bright fluorescence of a viable grain indicative of the intactness of the plasma membrane. The viability of stained grains was inspected under a fluorescence microscope. The count of viable grains revealed that a very low percentage of the pollen derived from the overexpressor lines has restored fertility (Fig. 41). In addition, *AtWRKY27* overexpressor flowers were artificially pollinated with wild-type pollen at an early flowering stage. This resulted in the restoration of fertility. Thus, overexpression of *AtWRKY27* results in male sterility.

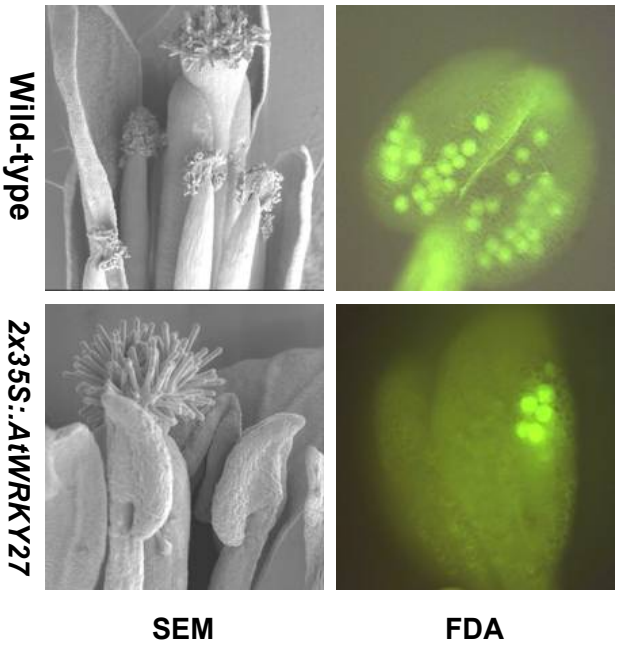


Fig. 41. *AtWRKY27* overexpressor lines show reduced fertility. **A.** Scanning electron microscopy (SEM) analysis of *2x35S::AtWRKY27* flower development. Unopened anthers of *AtWRKY27* overexpressor transgenic plants compared to those of wild-type plants. **B.** Fluoresceine diacetate (FDA) assay on unopened anthers from *2x35S::AtWRKY27* overexpressor lines showing reduced pollen viability compared to wild-type plants.

3.13.2. Response of *AtWRKY27-StrepII* overexpressor lines to *R. solanacearum* strain GMI1000 infection

Based on phenotypic data from the complementation experiments, which showed a tight association between expression levels and the degree of susceptibility, the response of strong ectopic *AtWRKY27* overexpressor lines to *R. solanacearum* strain GMI1000 infections was studied. *OE-Strep-4*, *OE-Strep-7* and wild-type plants were root-inoculated and their disease symptoms were monitored and evaluated during the course of time. Disease Index curve of both the *OE-Strep-4* and *OE-Strep-7* lines indicated a substantial enhanced susceptibility phenotype compared with that of the wild-type plants (Fig. 42).

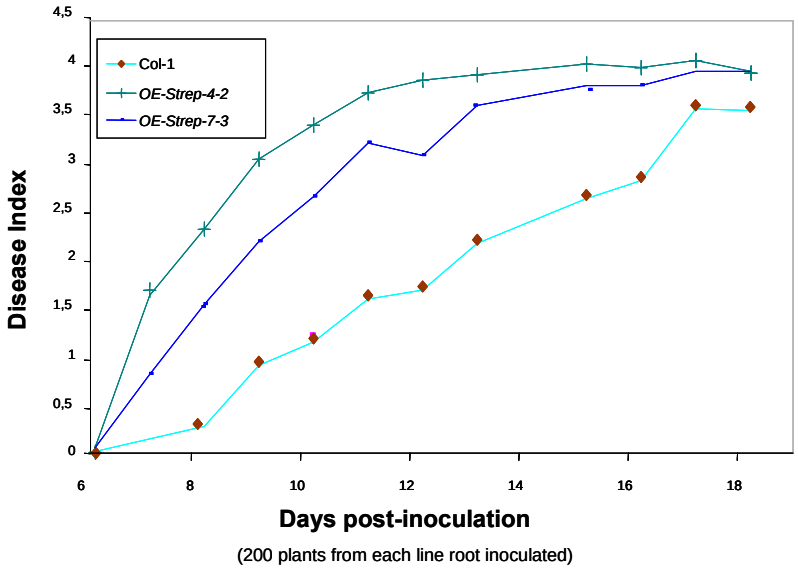


Fig. 42. Response of *2x35S::AtWRKY27* transgenic plants to *R. solanacearum* strain GMI1000 infection. Disease index curve of two ectopic *AtWRKY27* overexpressor lines showed significant enhanced susceptibility to this strain compared to wild-type. 200 plants for each line were used for root inoculation with the pathogen. (Experiment performed by Dr. Y. Marco, CNRS/INRA, Toulouse, France).

3.14. Search for target genes of *At*WRKY27

3.14.1. *At*WRKY27 does not autoregulate its own promoter

2 kb of upstream DNA sequence of *AtWRKY27* (relative to the translational start site) was analyzed to examine various DNA binding motifs particularly the consensus sequences of the W box. The presence of at least 3 putative W box like elements hinted towards possible regulation by *At*WRKY factors. *At*WRKY22 and *At*WRKY29, the structurally closest members of *At*WRKY27, have been shown to autoregulate their own promoters by a positive feedback loop (Asai *et al*., 2002). In order to test the possibility of *At*WRKY27 autoregulation of its own promoter, the 2 kb *AtWRKY27* promoter-driven GUS reporter gene construct (*AtWRKY27p::GUS*) was co-bombarded using the particle gun together with a *2xCaMV*35S-driven *AtWRKY27* cDNA construct (*2xCaMV*35S::*AtWRKY27*) into detached green leaves of wild-type plants. *AtWRKY22p::GUS* and *AtWRKY6p::GUS* were also co-bombarded in combination with a *2xCaMV*35S::*AtWRKY22* and *2xCaMV*35S::*AtWRKY6*, as positive and negative controls, respectively (Asai *et al*., 2002; Robatzek and Somssich, 2002). As expected, strong GUS activity staining was detected in co-bombardments of *AtWRKY22p::GUS* with *2xCaMV*35S::*AtWRKY22* while, no GUS activity was detected in the combinations of *AtWRKY6p::GUS or AtWRKY27p::GUS* with *2xCaMV*35S::*AtWRKY6* and *2xCaMV*35S::*AtWRKY27*, respectively (Fig. 43).

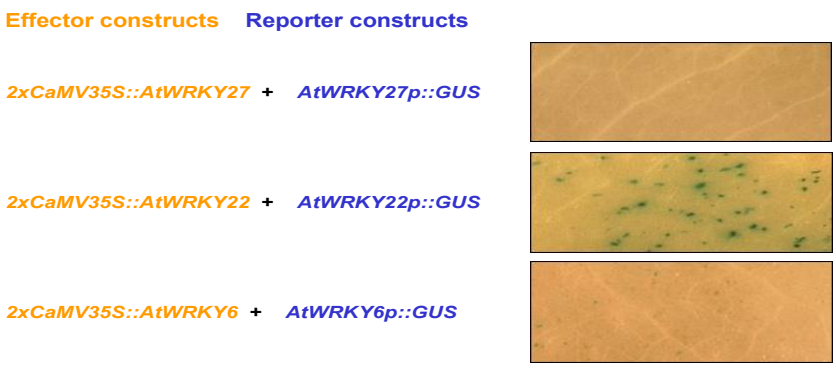


Fig. 43. *At*WRKY27 does not activate its promoter. Representative leaves of three independent biolistic-mediated transient transfection assays are shown. The effectors and

reporter constructs used for co-bombardment are indicated to the left. *At*WRKY22 and *At*WRKY6 were used as positive and negative controls in the experiment.
These results suggest that *At*WRKY27 does not function as an activator of a reporter gene driven by the *AtWRKY27* promoter.

3.14.2. *At*WRKY27 is able to activate *AtSIRK* promoter mediated expression

Senescence Induced Receptor Kinase (*AtSIRK*)/Flagellin Receptor Kinase 1 (*AtFRKI*) is one of the putative targets of several *At*WRKY family members including *At*WRKY6, *At*WRKY22, *At*WRKY29 and *At*WRKY42 (Robatzek and Somssich, 2002, Asai *et al*., 2002; Zhou and Somssich unpublished). Transient transfection assays of detached leaves were used to monitor possible *At*WRKY27-dependent activation of the *AtSIRK* promoter. *2xCaMV*35S::*AtWRKY6*, *2xCaMV*35S::*AtWRKY11* or *2xCaMV*35S::*AtWRKY27* clones were co-bombarded in combination with a *AtSIRKp::GUS* reporter construct. Strong GUS activity staining was detected with the *2xCaMV*35S::*AtWRKY6* or *2xCaMV*35S::*AtWRKY27* effector constructs suggesting that *At*WRKY27 is able to regulate *At*SIRK expression (Fig. 44). As expected, GUS activity staining was not detected using the *2xCaMV*35S::*AtWRKY11* effector construct (Robatzek and Somssich, 2002; Ciolkowski and Somssich unpublished). *AtWRKY11* has recently been shown to have a repressor activity on the *AtSIRK* promoter (Ciolkowski and Somssich unpublished).

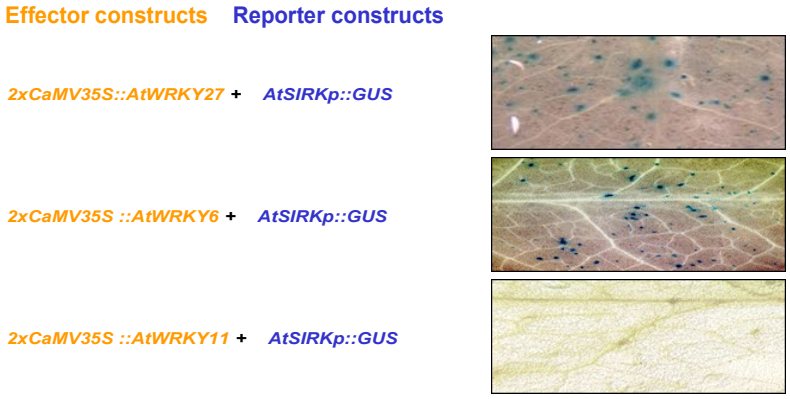


Fig. 44. *At*WRKY27 can activate the *AtSIRK* promoter driven GUS reporter gene. Representative leaves of three independent biolistic-mediated transient transfection assays using an *AtSIRK* promoter-driven GUS reporter gene. Bombardments of detached

wild-type leaves were performed in combination with the effector and reporter constructs indicated to the left.

Arabidopsis plants harboring a *AtSIRKp::GUS* reporter cassette were used as starting material to generate new transgenic lines containing $P_{G10\text{-}90}$*:: AtWRKY27-GR* and $P_{G10\text{-}90}$*::GFP-GR* constructs (Fig. 45). $P_{G10\text{-}90}$ is a synthetic promoter allowing strong constitutive expression (Ishige et al., 1999). The *GR* sequence encodes the glucocorticoide receptor. In these transgenic plants, $P_{G10\text{-}90}$ allows strong expression of *At*WRKY27-GR or GFP-GR but the nuclear translocation of *At*WRKY27-GR or GFP-GR solely requires dexamethasone (Dex).

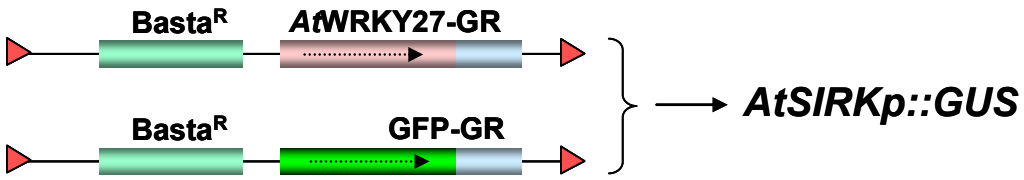


Fig. 45. Schematic representation of the $P_{G10\text{-}90}$*:: AtWRKY27-GR* (top) and $P_{G10\text{-}90}$*::GFP-GR* (bottom) constructs. These constructs were transformed into transgenic plants carrying *AtSIRK* promoter::GUS reporter gene cassettes. The construct carries the *pat* gene for Basta selection. $P_{G10\text{-}90}$ is a synthetic promoter allowing strong constitutive expression (Ishige et al., 1999). *GR* codes for the glucocorticoid receptor.

$P_{G10\text{-}90}$*:: AtWRKY27-GR* and $P_{G10\text{-}90}$*::GFP-GR* T_2 plants were treated with Tween-20, Dex or flg22. flg22 was shown to also activate *AtSIRK* expression (Robatzek and Somssich, 2002:; Navarro et al., 2004). GUS activity staining was detected in both $P_{G10\text{-}90}$*:: AtWRKY27-GR* and $P_{G10\text{-}90}$*::GFP-GR* transgenic plants after flg22 treatment. GUS activity staining was not detected neither by the application of Dex or Tween-20 in $P_{G10\text{-}90}$*::GFP-GR* plants. A strong GUS activity was only observed in $P_{G10\text{-}90}$*:: AtWRKY27-GR* plants after Dex application but not by Tween-20 treatment demonstrating positive regulation of *AtSIRK* by *At*WRKY27 *in planta* (Fig. 46).

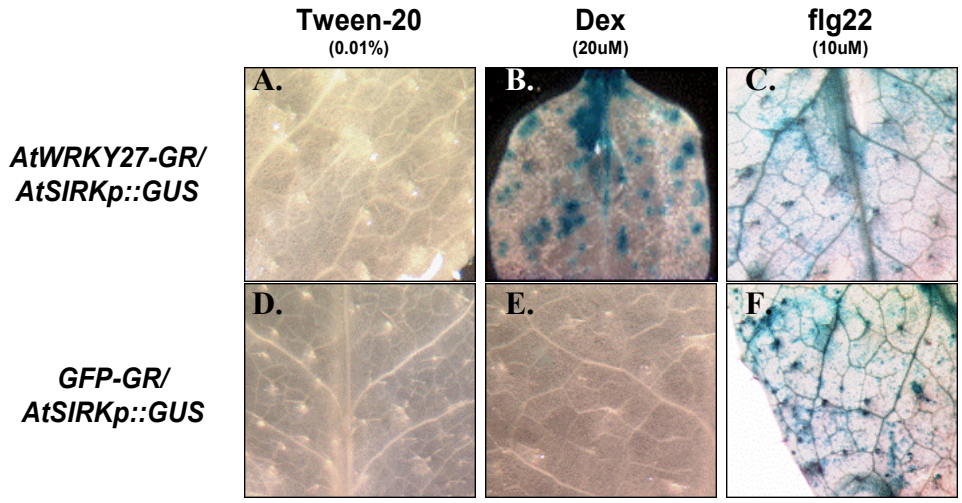


Fig. 46. *At*WRKY27 regulates *AtSIRK* promoter activity based on the detection of induced GUS staining. **A, B & C.** GUS assay on detached leaves of transgenic plants harboring the $P_{G10\text{-}90}$*:: AtWRKY27-GR* and the *AtSIRKp::GUS* constructs following Tween-20, Dex and flg22 application, respectively. **A.** GUS activity staining was not detected after Tween-20 application. **B & C.** Strong GUS activity staining was detected either after Dex or flg22 application. **D, E & F.** GUS assay on detached leaves of transgenic plants harboring the $P_{G10\text{-}90}$*::GFP-GR* and the *AtSIRKp::GUS* constructs, after Tween-20, Dex and flg22 application, respectively. **D & E.** GUS activity staining was not detected either after Tween-20 or Dex application. **F.** GUS activity was detected after flg22 application for one hour.

3.14.3. Potential genes regulated by *At*WRKY27

Additional expression constructs were designed to allow for simultaneous β-estradiol inducible expression of *At*WRKY27 and the GUS reporter gene (Fig.). For this, the pre-existing XVE (pER8) based inducible system (Zuo et al., 2000) was modified by inserting the coding sequences for Gateway-StrepII and the GUS reporter gene each behind a LexA operator sequence. $P_{G10\text{-}90}$ promoter driven XVE results in production of the chimeric transcription factor that can bind to LexA sites but requires β-estradiol for transactivation function. This new vector was named *pMD* (Fig. 47A).

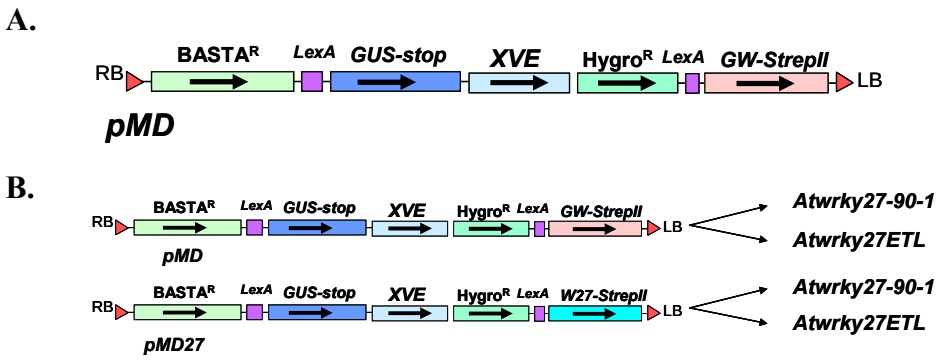


Fig. 47. Schematic representation of the β-estradiol inducible expression constructs designed for use in the *Atwrky27* mutant lines. **(A)** Important features of the *pMD* vector: Only the region between the right and left borders is shown (not to scale)., XVE expression is under the control of the $P_{G10\text{–}90}$ promoter; XVE encodes a chimeric transcription factor containing the LexA DNA-binding domain, the transcription activation domain of VP16 and the regulatory region of the human estrogen receptor; HPT, hygromycin phosphotransferase II coding sequence; Basta, coding sequences for the *pat* gene, eight copies of the LexA operator sequence; –46, the –46 35S minimal promoter; GUS-stop, coding sequences of the GUS reporter gene with terminal stop codon, GW: Gateway cloning site for target genes allowing fusions to coding sequences of StrepII;. Arrows indicate the direction of transcription**. (B)** *pMD* vector (upper) and *pMD27*construct (bottom) containing the *AtWRKY27* cDNA. Both of these plasmids are transformed into *Atwrky27-90-1* and *Atwrky27ETL* mutant plants.

The *AtWRKY27* cDNA was cloned into the Gateway cassette resulting in *pMD27*. This construct and an appropriate vector control were used to transform into *Atwrky27-90-1* and *Atwrky27ETL* mutant plants (Fig. 47B). Inclusion of the GUS reporter cassette enabled rapid selection of appropriate transformation lines (preferentially with no background activity) based solely on GUS activity staining (Fig. 48).

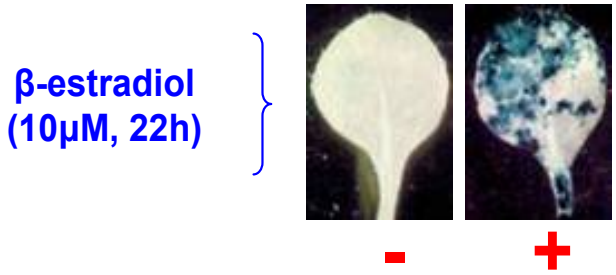


Fig. 48. High inducibility and tight control of the *XVE*-expression system in transgenic *Atwrky27* mutant plants. Strong GUS activity staining was detected in transgenic plants expressing either *pMD* or *pMD27* constructs after β-estradiol application. Conditions for β-estradiol based induction are indicated at the left. +: β-estradiol treated, -: β-estradiol untreated.

Additionally, stable *pMD27* construct-expressing transgenic lines in two independent *Atwrky27* mutants were confirmed by detection of *At*WRKY27-StrepII protein on Western blots (Fig. 49). Equal volumes of crude extracts from leaves of transgenic Arabidopsis plants, pre-treated with β-estradiol for 22 hours prior to sample collection, were separated by SDS-PAGE electrophoresis. As control, crude extracts derived from *Nicotiana benthamiana* leaves transiently expressing a *2xCaMV35S::AtEDS1-StrepII*-terminator construct was also included. After immunoblotting with the *Strep*-Tactin alkaline phosphate conjugate (ST-AP), *At*WRKY27-StrepII and *At*EDS1-StrepII proteins were detected at the expected molecular weights in the appropriate samples (Fig. 49). Furthermore, optimal induction conditions for β-estradiol-induced expression of the *pMD27*construct were identified. *AtWRKY27* transcript accumulation was detected as early as 4 hours after β-estradiol application in a series of time course experiments ranging from 4 to 22 hours (Fig. and data not shown). *AtWRKY27* transcript was not detected in transgenic mutant plants expressing the *pMD* construct after β-estradiol

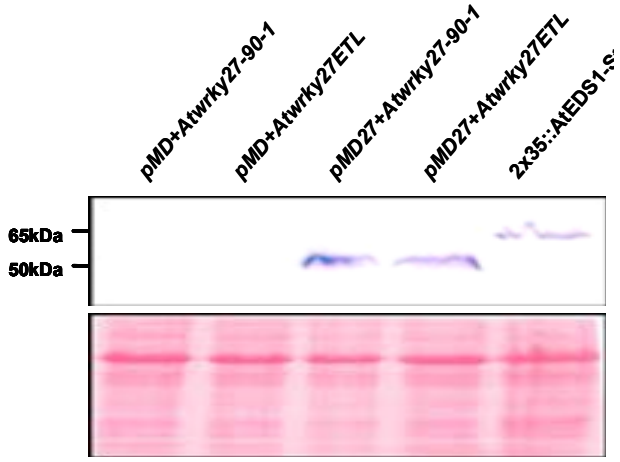


Fig. 49. Detection of StrepII-tagged *At*WRKY27 protein. Immunoblot of crude extracts derived from indicated transgenic plants either expressing *pMD* or *pMD27* after β-estradiol application. Extracts from *Nicotiana benthamiana* leaves transiently expressing *2xCaMV35S::AtEDS1-StrepII*-terminator was used to monitor the specificity of the antibody. Ponceau S staining of the gel was performed for protein loading.

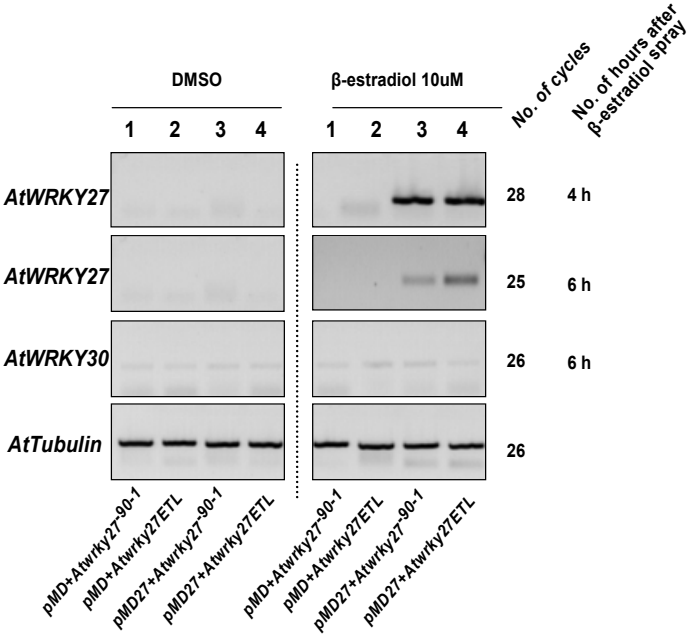


Fig. 50. Optimal induction conditions for β-estradiol-induced expression of the *pMD27*construct. Induction of *AtWRKY27* expression 4 and 6 hours after DMSO (left RT-PCR panel) and β-estradiol (right RT-PCR panel) (10 μM) treatment. The number of PCR cycles and the number of hours of β-estradiol treatment are indicated on the right side of the diagram. *AtWRKY30* (lowly expressed) and *AtTubulin* (moderately expressed) were used as loading controls in the experiment. RNA samples were derived from the transgenic plants as indicated below for each lane.

application nor in transgenic lines expressing either *pMD* or *pMD27* following DMSO treatment (Fig. 50). *AtWRKY30* and *AtTubulin* were included as loading controls in this experiment. For the identification of putative *At*WRKY27 target genes, transgenic plants expressing *pMD* or *pMD27* in *Atwrky27-90-1* and *Atwrky27ETL* mutants background were treated with β-estradiol for 6 hours and total RNA was isolated from these four samples. Using the Affymetrix Arabidopsis Gene Chip (ATH1) representing ~25000 genes, (performed by the service unit of Nottingham Arabidopsis Stock Centre) normalized transcriptional profiles of *pMD* or *pMD27* in the *Atwrky27-90-1* and *Atwrky27ETL* mutant backgrounds were analyzed. Initially, the data were sorted out in excel spread sheets by dividing the normalized *pMD27*signal intensity by *pMD* with respect to their corresponding mutants. Genes that showed a consistent difference (>1.5fold) in their expression pattern (induction or repression) in both mutants (background and high signal intensity >100) are listed in Table 7 and were considered for further analysis.

Up-regulated candidates

Putative candidate targets	*pMD27/pMD in Atwrky27-90-1*	*pMD27/pMD in Atwrky27ETL*
Nitrate Reductase1 (NR1)	4.19	2.21
Asparagine Synthetase 2 (ASN2)	3.28	1.76
Nitrate Reductase 2 (NR2)	2.75	1.48
Expressed Protein (At3g10930)	1.56	1.61
Sterile Alpha Motif (SAM) domain-containing protein	1.54	1.54
DSS1/SEM1 family protein (At1g64750)	1.77	1.74

Down-regulated candidates

Putative candidate targets	*pMD27/pMD in Atwrky27-90-1*	*pMD27/pMD in Atwrky27ETL*
Dof-type zinc finger domain-containing protein	0.41	0.72
Protease Inhibitor/seed storage/Lipid Transfer protein (LTP) family protein	0.49	0.7
Auxin-Responsive GH3 protein, putative (DFL-1)	0.52	0.74
Acireductone Dioxygenase (ARD/ARD') family protein	0.55	0.67
AtWRKY 38	0.55	0.71

Table 7. A partial list of putative target genes of *At*WRKY27 identified in the microarray experiments. Data obtained using the Arabidopsis ATH1 chip, were sorted out in an Excel spread sheet by dividing the normalized *pMD27*signal intensity by *pMD* with respect to their corresponding mutants. Up-regulated and down-regulated genes are listed at the top and bottom panel of the table. Cut-off signal intensity was set at 100. Genes exhibiting more than 1.5 fold differential regulation were selected.

Putative candidate targets	W box(TTGAC[C/T])	W-like box (TTGACA)
Nitrate Reductase1 (NR1)	2	1
Asparagine Synthetase 2 (ASN2)	1	3
Nitrate Reductase 2 (NR2)	1	3
Acireductone Dioxygenase (ARD/ARD') family protein	0	1
AtWRKY 38	2	1

Table 8. List of putative differentially regulated genes by *At*WRKY27 confirmed by independent RT-PCR experiments. W box or W-like box distribution is within 2kb promoter sequences

Independent RT-PCR analyses were performed to validate the differential expression of these genes. In the up-regulated class of genes, transcript levels of *Nitrate Reductase1 (NR1)*, *Nitrate Reductase1 (NR1)* and *Asparagine Synthetase 2(ASN2)* were higher in *pMD27* expressing plants compared to *pMD* (Fig. 51A) plants, while transcript levels of *Expressed Protein (At3g10930)*, *Sterile Alpha Motif (SAM) domain-containing protein* and *DSS1/SEM1 family protein (At1g64750)* were found to be similar in both *pMD27* and *pMD* expressing plants. Within the down-regulated class of genes, *AtWRKY38* and *Acireductone Dioxygenase (ARD/ARD') family protein* exhibited rather low transcript levels in the *pMD27* expressing plants compared to *pMD* plants while, *Protease Inhibitor/seed storage/Lipid Transfer protein (LTP) family protein*, *Dof-type zinc finger domain-containing protein* and *Auxin-Responsive GH3 protein, putative (DFL-1)* showed similar levels of transcript in both *pMD27* and *pMD* expressing plants (Fig. 51B). *AtSIRK*, *AtGlutathionine* and *AtTubulin* were included as loading control in these experiments (Fig. 51C). Thus, RT-PCR was able to confirm some putative target genes. The confirmed candidate genes contain W box/W box like sequences found in their 2 kb promoter regions (Table 8). However, the differences observed were not always that obvious.

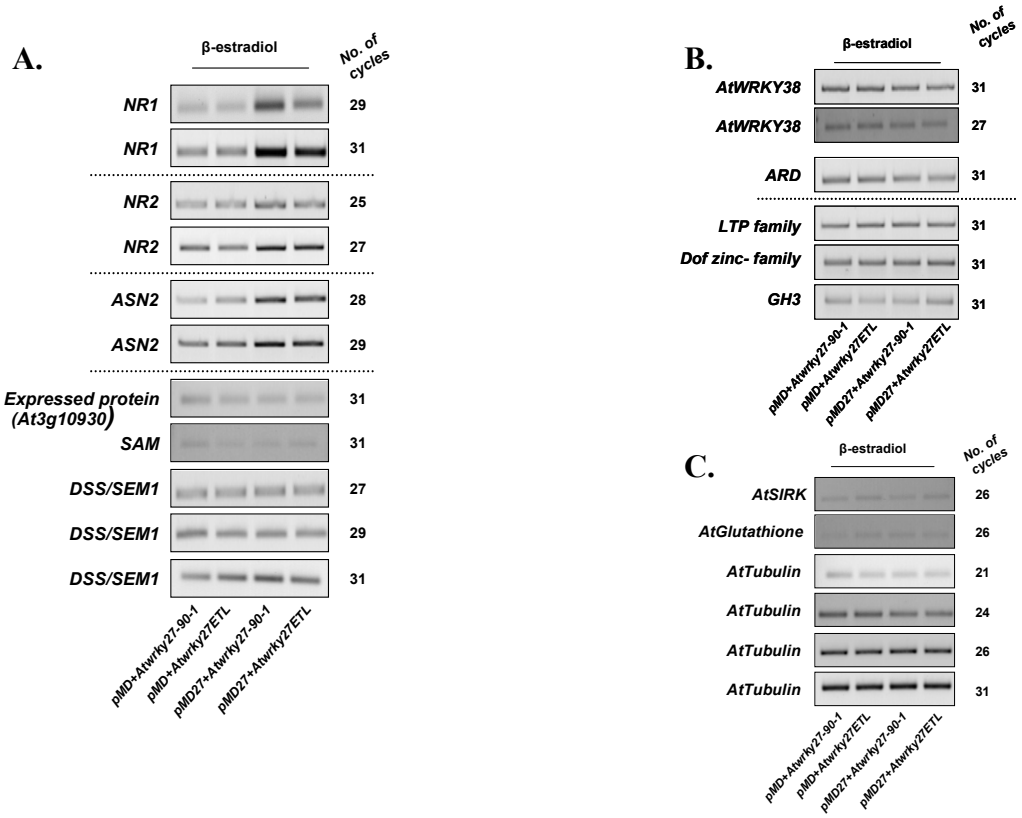


Fig. 51. RT-PCR evaluation of the putative *At*WRKY27 target genes identified by microarray analysis. A: Analysis of putative up-regulated candidate genes from table No. 7. B: Analysis of putative down-regulated candidate genes from table. Transgenic lines from which the RNA was isolated are given below each lane. Number of PCR cycles is indicated to the right in all experiments. The abbreviations for each gene are listed in the table. C: *AtSIRK* (lowly expressed), *AtGlutathionine* (lowly expressed) and *AtTubulin* (moderately expressed) were used as loading control.

4. Discussion

For understanding the molecular basis of plant development it is necessary to acquire detailed knowledge on the functions of transcription factors and thus on the plant gene regulation processes. The biological functions of some representatives of plant transcription factors families are already elucidated. Completely different roles were found to be played by them, in processes as diverse as organ identity determination, development and stress defense mediation. So far, the *At*WRKY factors were predominantly described to act in plant defense responses against abiotic and biotic stresses (Eulgem et al., 2000). In the scope of this work, the accomplished functional analysis of an *AtWRKY* genes superfamily representative offered new insights into plant – microbes interactions (*Arabidopsis thaliana-Ralstonia solanacearum*), namely the enhanced pathogen tolerance phenomenon.

4.1. The *At*WRKY family expression signatures: shared and specific expression patterns

In the present study, unique basal and induced expression patterns of some *AtWRKY* genes were identified. Yet, genetic analyses of Arabidopsis have yielded experimental proof of function for only 3,500 genes (Berardini et al., 2004). One powerful approach to get clues for assigning the function of a particular gene is high throughput gene expression analysis (microarray-based transcript analysis) (D'Haeseleer et al., 2000; Hughes et al., 2000; Maki et al., 2001; Lee et al., 2002). Expression profiling analyses have revealed that plants express similar sets of defense genes in response to different pathogens. An array of about 8000 genes representing nearly one-third of the total number of protein-encoding genes in *Arabidopsis*, was used to study the gene-for-gene type resistance response to the bacterial pathogen *Pseudomonas syringae* (Zhu and Wang, 2000; Glazebrook et al., 2003). More than 2000 genes showed altered expression levels within nine hours of inoculation with the pathogen (Tao et al., 2003). Accordingly, a similar ratio could be expected for more than 27,000 genes in Arabidopsis.

Thus, a large array of the genes shows altered expression in response to a given pathogen. In order to narrow down the candidate genes number, (Katagiri, 2004) the subsequent confirmation needs to be performed under defined biological conditions, to validate the specificity of gene expression related to a particular pathogen infection. One way to gain meaningful information about the specific expression regulation is to perform expression profiling on loss-of-function mutants and transgenics, impaired in defense signaling (Katagiri, 2004; Uelker and Somssich, 2004).

A comparative expression profile of approximately 8,000 Arabidopsis genes was analyzed using wild-type and mutant plants disrupted in three different *R*-gene dependent defense pathways upon pathogen infection either with compatible or incompatible isolates of oomycete *Peronospora parasitica*. These genetically separable *R*-gene dependent defense pathways are: the *RPP4* (*Resistance to Peronospora parasitica4*) that is dependent on PAD4 (Phytoalexin Deficient4), SA accumulation, and SGT1b (suppressor of G2 allele of suppressor of kinetochore protein1 (skp1)); the *RPP7* (*Resistance to Peronospora parasitica7*) pathway that is dependent on SGT1b but independent of PAD4 or SA accumulation; and the unique *RPP8* (*Resistance to Peronospora parasitica8*) pathway that is independent of PAD4, SA accumulation, or SGT1b. The analysis revealed the convergence of these three pathways leading to up-regulation of a common set of target genes (Eulgem et al., 2004).

Similarly, the expression profiles of the *Arabidopsis* WRKY gene family revealed that almost two-third (49 out of 72) of the *AtWRKY* genes were differentially regulated in plants infected by an avirulent strain of the bacterial pathogen *Pseudomonas syringae* or treatment with SA (Dong et al., 2003). However, in the *npr1-3* mutant plants (impaired in systemic acquired resistance) altered expression patterns of *AtWRKY* were documented. For instance, the pathogen induced expression of *AtWRKY51*, *AtWRKY55* and *AtWRKY62* appeared to be substantially reduced in the *npr1-3* mutant. In the transgenic *nahG* plants, *AtWRKY38*, *AtWRKY50*, *AtWRKY51*, *AtWRKY55*, *AtWRKY59*, *AtWRKY60*, *AtWRKY62*, and *AtWRKY66* had substantially reduced levels of pathogen-induced expression (Dong et al., 2003). A similar study only on group-III *At*WRKY factors showed strong transcript accumulation for 11 out of the 13 *AtWRKY* gene members upon SA treatment or pathogen infection (Kalde et al., 2003). The transgenic

nahG plants express the bacterial *nahG* gene encoding a salicylate hydroxylase that degrades SA to catechol, thereby making *nahG* transgenic plants SA deficient (Gaffney, 1993; Delaney, 1994). Transcript levels of *AtWRKY30*, *AtWRKY41*, *AtWRKY53*, and *AtWRKY70* were not changed in *nahG* transgenic compared with wild-type plants infected with *P. parasitica* Cala2 among the 13 members tested of *At*WRKY sub-group III (Kalde et al., 2003). Transcript accumulation of *At*WRKY members of group III was tested in *pad4-1* and wild-type plants after SA treatment. Expression patterns of all tested *AtWRKY* genes were unchanged with the notable exception of *AtWRKY64* whose expression was dampened in *pad4-1* plants (Kalde et al., 2003). PAD4, is a lipase like protein, affecting the synthesis of the phytoalexin camalexin in response to infection with *Pseudomonas syringae* pv. *maculicola* and is also an important component of SA mediating signaling cascade leading to systemic acquired resistance. The *pad4* knock-out fails to accumulate SA in response to pathogen infection which makes it a valuable tool for analysis of SA-dependent plant defense.

A large set of microarray experiments has been performed and can be found in the Nottingham Arabidopsis Stock Centre microarray database (http://arabidopsis.info). However, the Affymetrix ATH 8,000 and 22,000 gene arrays did not include a significant proportion of the 74 *AtWRKY* genes. In addition, the whole genome gene expression analyses were performed in only a few Arabidopsis defense signaling mutants.

A so far unique wide-range expression study of all the *AtWRKY* family members in *nahG* transgenic and *npr1-3* mutant plants was performed by Dong and colleagues (2003). However, information is still lacking about the *AtWRKY* expression patterns in other defense signaling mutants, especially in the SA-, JA- and ET-dependent pathways.

In the present study, the basal expression patterns of 12 *AtWRKY* genes were found to be affected in SA-dependent (*dnd1*, *ndr1*, *npr1* and c*pr5*), JA-dependent (*aos*) and ET-dependent (*ein2*) mutant plants. These distinct expression patterns demonstrated that regulation of different subsets of the *At*WRKY factor family requires different signaling pathways. The SA-induced expression of some of these defense-related *AtWRKY* genes was greatly inhibited in the *npr1* mutant. Thus, there appears to be NPR1-dependent and NPR1-independent pathways for regulated expression of the *AtWRKY* genes.

Interestingly, *AtWRKY18, AtWRKY*25 and *AtWRKY33* exhibited increased basal transcript levels in all JA/ET signaling mutants tested (*aos*, *jar1*, *ctr1* and *ein2*). Moreover, transcript levels of these genes are shown to be up-regulated by pathogen infection and SA treatment. *AtWRKY18*, *AtWRKY25* and *AtWRKY33* showed altered expression after ozone treatment and pathogen infection (Nottingham Arabidopsis Stock Centre microarray, http://arabidopsis.info). Present study revealed that JA/ET pathway directly/indirectly repressed *AtWRKY18, AtWRKY*25 and *AtWRKY33*. In contrast, *AtWRKY53* and *AtWRKY60* only showed constitutively elevated transcript levels in the mutants affected in ET pathway (*ctr1* and *ein2*). Moreover, *AtWRKY53* showed altered expression patterns upon pathogen infection, cold treatment, wounding and SA treatment (Nottingham Arabidopsis Stock Centre microarray, http://arabidopsis.info; Uelker and Somssich, 2004). *AtWRKY53* and *AtWRKY60* are also shown to be involved in senescence (Uelker and Somssich, 2004). The observations made in the present study also indicate the existence of separate ET-signaling pathway independent of JA.

In another study, the expression level of *AtWRKY70* was shown to be strongly up-regulated in the plants treated with SA, but not in ACC (1-aminocyclopropane-1-carboxylic acid a natural precursor of ET) treated plants. MeJA treatment, on the other hand, appeared to repress *AtWRKY70* expression at rather later stages of treatment (Li et al., 2004). Basal and SA-induced expression levels of *AtWRKY70* are completely blocked in plants expressing the *nahG* transgene and are significantly reduced in *npr1* mutant plants. Enhanced accumulation of *AtWRKY70* transcripts was also absent in *coi1* (*coronatine insensitive1*), a mutant impaired in JA-signaling pathway, plants. These and other lines of evidence suggest that *AtWRKY70* acts as a node of convergence for integrating SA- and JA-signaling events during plant defense (Li et al., 2004). These results of Li et al stand partly in contrast to this study that showed basal and MeJa-induced transcript levels of *AtWRKY70* are not affected in *aos* and *jar1* mutant plants (mutants impaired in JA-signaling pathway) compared to wilt-type plants. They also are in conflict with a report showing that *AtWRKY70* expression was not significantly altered in the mid-flowering stage of *coi1* (*coronatine insensitive1*), mutant plants (V. Buchanan-Wollaston, Nottingham Arabidopsis Stock Centre microarray, http://arabidopsis.info). These discrepancies may be due to differences in the

experimental set-up used or the age of the tested plants. However, an extensive transcription profiling and detailed genetic evidences are still needed in order to state that *AtWRKY70* functions as a point for cross talk in SA-, JA/ET-signaling network.

4.2. The enhanced pathogen tolerance mechanism in *Atwrky27* knock-out mutant

In a screen, a set of *Atwrky* knock-out mutants were subjected to diverse pathogen infections (host, non-host, virulent/avirulent). *Atwrky27* knock-out mutant plants showed an altered phenotypic response specifically to *R. solanacearum* strain GMI1000. Loss-of-function mutants of *AtWRKY* genes have rarely resulted in obvious phenotypes most likely due to functional redundancy in the transcription factor (TF's) familes (Riechmann et al., 2000; Vision et al., 2000; Simillion et al., 2002). Nevertheless, generation of multiple knock-out lines based on the sequence redundancy or overlapping expression patterns together with phenotypic profiling under altered environmental conditions can be alternative approach (Cutler and McCourt, 2005). Individual knock-out for the *TGA* TF's don't exhibit sensitivity to SA. However, SA-induced *PR1* expression in *tga6-1 tga2-1 tga5-1*(a triple *tga* mutant) plants was completely blocked as shown in *npr1* mutant plants (Zhang et al., 2003). The elegant example in the *At*WRKY superfamily which shows noticeable phenotypes is *AtWRKY44*, also known as *Transparent Testa Glabra2* (*TTG2*). *Atwrky44/ttg2* knock out mutant plants have unbranched trichomes that are reduced in number, in addition to reductions in mucilage production and tannin synthesis in the seed coat (Johnson et al., 2002). RNAi and knock-out lines of *AtWRKY53* exhibited delayed senescence phenotype under normal growth conditions (Miao et al., 2004). Next to other subtle physiological changes, antisense *AtWRKY70* lines were shown to be more sensitive to *E. c. carotovora* strain SSC1 (Li et al., 2004). In the case described here, *Atwrky27* loss-of-function mutant plants exhibit clearly delayed wilting symptoms in response to *R. solanacearum* GMI1000 although the plants do ultimately die.

Currently, two genetic components have been studied in detail in Arabidopsis that mediate resistance to *R. solanacearum* GMI1000, namely *RRS1* and *NWS1* (Deslandes et al., 2002, Feng et al., 2004). In the case of *RRS1*, resistance is mediated by the

interaction of a major *R*-gene product RRS1 with the bacterial *avr* gene product PopP2 in the plant cell nucleus (Deslandes et al., 2003). This specific gene for gene interaction is a particular strong form of plant defense leading to full resistance by inhibiting bacterial growth. Thus, the type of resistance conferred by *RRS1-R* in Nd-1 plants is different from the delayed wilt symptoms phenotypes observed in *Atwrky27* mutant plants as the latter will wilt eventually. Moreover, *RRS1-R*-mediated resistance is partially salicylic acid- and NDR1 (*Non-Race-Specific Disease Resistance 1*)-dependent, as Nd-1 plants containing salicylate hydroxylase or *RRS1-R*/*RRS1-R ndr1*/*ndr1* plants exhibit wilting symptoms, suggesting the existence of similar signaling pathways to those controlled by resistance genes in specific resistance (Deslandes et al., 2002).

In a large genetic screen (12000 fast-neutron mutagenized Col-0 plants) to find additional components operating in the wilt symptoms response to *R. solanacearum* strain GMI1000 led to the identification of a novel recessive *Arabidopsis thaliana* mutant, *nws1* (no wilt symptoms) (Feng et al., 2004). The *nws1* mutant plants completely failed to develop wilt symptom in response to virulent strains of the phytopathogenic bacterium (Feng et al., 2004). Thus, the type of phenotypic response displayed by *nws1* knock-out mutant plants is also different from the delayed wilt symptom phenotypes observed in *Atwrky27* mutant plants as the latter will wilt eventually. The identity of the *AtNWS1* locus is not known so far.

In order to understand the role of other known signaling pathways contributing to resistance in response to *R. solanacearum* strain GMI1000 infection, several mutants or transgenic lines impaired in SA-, JA-, and ET-dependent signaling pathways, all in a susceptible Col-0 background, were tested in another study (Hirsch et al., 2002). These included *nahG* transgenics, affected in SA accumulation, *cpr1* and *cpr5* plants, overexpressing a number of *PR*-related genes, *jar1* plants, JA-insensitive mutant, and various mutants affected in the ethylene signaling pathway such as *etr1-3*, *ein2-1*, *ein3-1*, *ein4-1* (ethylene insensitive mutants), and *eto3* (ethylene-overproducing mutant). This study revealed that development of wilt symptoms was strongly delayed in *ein2-1* and *eto3* mutant plants compared with Col-0 wild-type plants (Hirsch et al., 2002).

Thus, it appears that Arabidopsis plant responses to *R. solanacearum* strain GMI1000 are composite of multiple signaling pathways. However, the nature of the host

components required for disease development, including targets of virulence factors, negative regulators of plant defense responses, and susceptibility factors required for both pathogen growth or fitness, remains largely elusive (Glazebrook, 2001; Vogel at al., 2002; Nishimura et al., 2003; Belkhadir et al., 2004;Vogel at al., 2004: Kim et al., 2005). The steady-state transcript levels of *AtWRKY27* are not significantly altered in any of the mutants or transgenic lines under study which block signaling through the SA or JA/ET pathways. Additionally, *Atwrky27* mediated delayed symptoms phenotype is not associated with constitutive expression of defense response marker genes like *PR1*, *PR5*, *Thi2.1* or *PDF1.2*. Most of the mutants in these signaling pathways, such as *acd11* (*accelerated cell death11*), *cpr* (*constitutive expressor of PR*), *dnd* (*defense no death*), *edr1* (*enhanced disease resistance*) and *cev1* (*constitutive expression of VSP1*) confer enhanced resistance to virulent strains of different pathogens either by constitutively expressing elevated levels of defense genes or influencing steps leading to accelerated cell death (Glazebrook, 2001). These mutants are affected in genes that appear to act as negative regulators of signaling molecules such as SA and JA. However, the cost for this type of resistance is extremely high and these mutants have pleiotropic phenotypes including stunted growth, nectrotic lesions of leaves and stress alterations (Bowling et al., 1994; Glazebrook, 2001; Katagiri, 2004).

This is in contrast to Arabidopsis *pmr* (*powdery mildew resistance*) mutants or rice *ebr* (*enhanced blast resistance*) mutants, required for compatible plant microbe interactions. *pmr5* and *pmr6* mutants exhibit enhanced resistance to a virulent pathogen *E. cichoracearum* but not to the unrelated pathogens *Pseudomonas syringae* or *Peronospora parasitica* (Vogel et al., 2002; Vogel at al., 2004). Similarly, *ebr1* and *ebr2* display enhanced resistance to *Magnaporthe grisea* (Campbell and Ronald, 2005). The resistance mechanisms operating in these mutants display no constitutively elevated expression of the defense genes and do not require the activation of either the SA or JA/ethylene defense pathways. Therefore, it is very likely that pathogens target these genes or their respective products for successful proliferation within the plant (susceptibility/compatible factors) (Vogel at al., 2004; Campbell and Ronald, 2005). *NWS1* may also constitute such as susceptibility factor. The *nws1* mutation is recessive and appears to be highly specific to *R. solanacearum*. Similarly, the basal transcript

levels of defense responsive molecular markers don't accumulate to higher levels compared to wild-type plants. However, *nws1* plants showed no wilt symptoms and inhibit bacterial growth (Hirsch et al., 2002). In case of *Atwrky27*, the delayed symptom phenotypes are not due to the inhibition of bacterial growth, which revealed the fact that *AtWRKY27* is not a compatibility factor. However, it is not excluded that the subtle difference in bacterial growth could not be detected at these time points using the described assays. An alternative possibility is that the *Atwrky27* loss-of-function mutant plants may be affected in a specific defense mechanism known as enhanced pathogen tolerance (Bent et al., 1992). Pathogen tolerance is considered to be a defense mechanism which doesn't limit infection but reduces or offsets pathogen fitness. For example, *rTGA2.1* (rice TGA transcription factor 2.1) mutant plants and *rTGA2.1*–silenced transgenic plants exhibited reduced lesion development after infection with the rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), but bacterial growth in infected leaves did not differ when compared with wild-type plants (Fitzgerald et al., 2005). *Xoo*, like *R. solanacearum*, is a vascular pathogen whose excessive production of extracellular polysaccharides eventually impedes nutrient movement through xylem tissues (Ou, 1972). In another study, the effect of silicon accumulation on the resistance level of tomato (a silicon-non-accumulator plant) in response to *R. solanacearum* was elucidated (Dannon and Wydra, 2004). Both tomato genotypes L390 (susceptible) and King Kong2 (moderately resistant) showed reduced bacterial wilt incidence following silicon treatment but bacterial numbers in roots, midstems and leaves of the genotype L390 was not affected, while significantly bacterial growth was reduced in all organs of genotype King Kong2 (Dannon and Wydra, 2004). This observation also points at a mechanism of an induced tolerance in genotype L390, where silicon treated plants showed less symptoms with similar bacterial numbers compared to non-treated plants and increased tolerance in King Kong2 after silicon treatment, when disease severity was further reduced, (Dannon and Wydra, 2004). Tolerance and resistance traits can be genetically linked (Stowe, 1998), or they can be pleiotropic, with a single host trait altering both the incidence of disease and host tolerance (Vanderplank, 1984; Roy and Kirchner, 2000). For instance, *ethylene-insensitive* (*ein2*) mutant plants of different species such as Arabidopsis, tobacco, soybean, and tomato, exhibit both enhanced

resistance and enhanced tolerance to various pathogens which ultimately contribute to plant defense (Bent et al., 1992; Knoester et al., 1998; Hoffmann et al., 1999; Thomma et al., 1999; Wubben et al., 2001; Hirsch et al., 2002). Arabidopsis *ein2* mutant plants also display a delayed symptoms response to *R. solanacearum* by inhibiting bacterial growth (Hirsch et al., 2002) but show minimal disease development to *P. syringae* pv. *maculicola*, or *X. campestris* pv. c*ampestris* without effecting bacterial growth (Bent et al., 1992). Additionally, the tomato dominant ethylene-insensitive mutant, *Nr* (*Never ripe*; impaired in ethylene perception), exhibits a significant reduction in disease symptoms which is not due to the reduction in bacterial titer or decreased ethylene synthesis (Lund et al., 1998).

Tolerance strategies and resistance defenses can both contribute to improve host fitness in response to phytopathogens (Roy and Kirchner, 2000). However, resistance traits exert direct pressure on pathogen fitness leading to alterations of the pathogens to offset such host defenses. Thus, host resistance defenses create a co-evolutionary combat with pathogens. By contrast, host tolerance strategies avoid such combat because they don't threaten the pathogen survival. As a consequent, enhanced tolerance may be co-evolutionarily stable (Roy and Kirchner, 2000).

4.3. *In silico* predictions versus *in vivo* functional analysis of *AtWRKY27*

GFP-tagged version of *At*WRKY27 revealed its nuclear localization. This could be expected for transcription factor and have also been demonstrated by other *At*WRKY factors (Robatzek and Somssich, 2001; Ingo Ciolkowski, Dierk Wanke and Bekir Uelker, personal communication). However, several computer predictions did not indicate nuclear localization but rather chloroplastic/mitochondrial targeting. Therefore, the computer-assisted forecast of the subcellular localization should be treated with caution and always must be experimentally validated.

The *At*WRKY27 is also predicted to undergo various post-translational modifications. Recent reports suggest that *At*WRKY factors can be targets of modifications particularly, involving mitogen-activated protein kinase (MAPK) (Asai et al., 2002; Liu et al., 2004; Wan et al., 2004; Ülker and Somssisch, 2004). An exciting possibility would be that *At*WRKY27, like its closest neighbors in the phylogenetic *At*WRKY tree, *At*WRKY22

and *At*WRKY29, would be a target to phosphorylation (Asai et al., 2002), activating the early defense signaling via a MAP kinase cascade. Whether or not these sites are biologically functional is an interesting question to be addressed in future experiments.

The entire *Arabidopsis thaliana WRKY27* 5` intergenic region (with respect to the translational start) is 6,800 bp in length. *Atwrky27ETL*, where the GUS reporter transgene is inserted in-frame with the second exon of the gene, and thus remians under the control of the endogenous *AtWRKY27* regulatory region was compared to the transgenic line containing a 2 kb *At*WRKY27 promoter GUS reporter construct. The distribution and intensity of GUS expression overlapped consistently between the two transgenic lines tested, offering evidence that 2 kb promoter fragment is sufficient to driving the *AtWRKY27* expression under the conditions tested.

A large number of known *cis*-regulatory sequences are also predicted within the 2 kb *AtWRKY27* promoter that have been shown to be involved in both activation and repression of gene expression. The GCC box (AGCCGCC) has been shown to mediate ethylene-responsiveness and is often found in activators of defense genes (Ohme-Tagaki and Shinshi, 1995; Rushton et al. 2002). A similar element (JERE, AGACCGCC, Menke et al., 1999) directs jasmonic acid and elicitor-responsive expression. Some of the *cis* elements also respond to both wounding and pathogen attack; in addition, a functional separation from elements in both signal pathways is conceivable. Although no more than one single element is sometimes sufficient for pathogen inducibility, usually promoters contain different elements, and frequently several elements contribute to the better activation of gene expression (Rushton and Somssich, 1998; Rushton et al. 2002). Numerous motifs responsive to a number of abiotic (wounding) and biotic (phytohormons, light) treatments were identified *in silico*. However, none of these elements were shown to be functional under the experimental conditions tested. Moreover, *Atwrky27ETL* plants don't show altered phenotypes in response to various abiotic stresses analyzed on the basis of different predicted elements in the *AtWRKY27* promoter. It cannot however be excluded that the *AtWRKY27* promoter may respond to certain treatments at certain defined developmental stages or in a highly specific tissue of the plant. In addition, the induced *AtWRKY27* expression levels might have been below the detection threshold of the reporter used. On the other hand, the *in silico*

prediction of promoter DNA elements were not accurate. According to the literature reports, the online tools available currently can predict a certain element with 80% confidence, *i.e.* 20 false positives in a hundred will be detected (Shahmuradov et al. 2005).

4.4. The phenotypical effects of *AtWRKY27* endogenous expression and ectopic overexpression in complementation analyses

The complementation approach, employing native regulatory sequences of a gene, requires the determination of promoter size for the successful complementation effect. The majority of literature reports state that the usage of ~2 kb of native upstream intergenic regulatory sequences is sufficient for the complementation analyses. However, it cannot be excluded that certain regulatory elements (e.g. enhancers) are present within the entire intergenic region or in some other position on the chromosome. Based on the GUS studies it appears that 2 kb of *AtWKY27* 5′ regulatory region is sufficient to mediate all observed expression patterns of the gene and capable of restoring the wild-type disease susceptibility.

The overexpressor *AtWRKY27* plants showed early wilting symptoms in response to *R. solanacearum* strain GMI1000. Moreover, a positive correlation between wilting symptoms and *AtWRKY27* transcript levels were observed. This provides an additional line of evidence that *At*WRKY27 or the component (s) under this TF could contribute to wilt disease development.

The use of Cauliflower Mosaic Virus (*CaMV*) *35S* promoter induces a strong expression throughout the plant in various developmental stages. This promoter allows expression in virtually all tissues and is largely independent of environmental and developmental factors. This can well serve a purpose of defining basic gene functionality, but is not suitable for subtle investigations of gene regulation or protein activity (Meyer et al. 1996, Stacey et al. 1999). While elevated levels of the transcript are the consequence of strong constitutive overexpression, decreased amounts of *AtWRKY27* mRNA in some of the overexpressor transgenic plants are likely due to the phenomenon called “co-suppression” (Napoli et al. 1990).

The lines with highest levels of *AtWRKY27* mRNA displayed additionally numerous alterations in the morphological phenotype, related to stunted growth, irregular leaf shape, and drastically reduced male fertility. Endogenous expression patterns of *AtWRKY27* coincide with these aberrant phenotypes. Similar aberrations were previously observed by Delessert et al. (2005), who overexpressed *AtAF2*, a member of the plant-specific NAC-domain transcription factor family. Transgenic plants displayed an increased biomass and yellowing of the leaves, while no obvious phenotype could be observed in two independent *AtAF2* T-DNA insertion lines. Interestingly, *AtAF2* overexpressing plants showed a higher susceptibility to the soil-borne fungal pathogen *Fusarium oxysporum*, similarly to the results obtained in this study with *AtWRKY27* overexpression toward *R. solanacearum*.

Evidence accumulates also for other members of the *At*WRKY family (Chen 2005). For example, overexpression lines for *AtWRKY18*, *AtWRKY40* and *AtWRKY48* have significantly stunted plants and more serrated leaves. These plants also displayed delayed flowering. On the other hand, overexpression lines for other *AtWRKY* genes such as *AtWRKY7*, *AtWRKY25* and *AtWRKY26* flowered earlier than wild type plants. Moreover, overexpression of multiple *WRKY* genes was found to play a role in defense responses to bacterial and fungal pathogens. Overexpressor lines of ten *AtWRKY* family members displayed so far pathogen-related phenotypes (Chen 2005; Bekir Uelker and Imre Somssich, personal communication; this study).

It is unclear whether the *AtWRKY* genes mentioned above affect plant growth and development directly or indirectly through affecting expression of genes involved in plant stress responses. One of the possibilities would be that some of the *At*WRKY family members are capable of occupying the W boxes present in the promoters of developmentally crucial genes, and in this way repressing their expression, leading to disturbed life cycle (Turck et al. 2004). Taken together, these data validate the usage of 2 kb *AtWRKY27* promoter for the complementation analysis, as the native regulatory sequence, mimicking the wild type situation, contrasting to the *CaMV 35S* promoter, which resulted in the early wilt symptom development and morphological disorders of ectopic overexpressor lines. The results mentioned above indicate a significant role for *AtWRKY* transcription factors in regulation of plant development and defense responses,

and suggest that the ectopic overexpression may offer a valuable tool to track down the biological role of genes clustered in families, and therefore functionally redundant.

4.5. Choice of selective promoters for tissue-specific expression of *AtWRKY27* transgene

In this present study, the main emphasis was to elucidate the molecular mechanism, through which *At*WRKY27 is involved in the wilt disease establishment. An attempt to determine where exactly within the plant *At*WRKY27 is needed was made initially employing the *Atwrky27ETL*. The GUS expression pattern was detected within the vascular bundles of stem and leaves, as well as within the cells of the root elongation zone, whereas the bacterial propagation sites are xylem vessels. As the xylem cells are dead and empty of cell contents at maturity, parenchymatic cells are often present in xylem tissue, where they help to maintain water balance and carry out metabolism within the tissue. The parenchymatic tissue would be, therefore, a likely target for the *AtWRKY27* expression site. Alternatively, the phloem tissue constitutes another possible location for the *AtWRKY27* transcription. Unlike xylem, phloem is alive at maturity, but usually with a much reduced cell contents and no nucleus. The only transcriptionally active phloem components are parenchymatous companion cells. Despite multiple attempts and applications of various enhanced versions of fluorescent proteins, neither the longitudinal and cross sections through the stem of *Atwrky27-ETL* nor *AtWRKY27* promoter driven GFP transgenic lines didn't provide any ultimate answer where in plant's vasculature *AtWRKY27* is expressed. In order to overcome the problems with microscopy-based *AtWRKY27* detection, a series of constructs were generated, expressing *AtWRKY27* under three Arabidopsis tissue specific promoters (phloem-specific *SUC2,* xylem-specific *4CL-2* and elongation zone-specific *Cel1*). These lines were aimed at providing genetic data, indicating whether a site-specific expression of *AtWRKY27* would restore the wild-type wilt susceptibility in response to *Ralstonia solanacearum* strain GMI1000. The analysis of these transgenic lines will give an insight into the spatial regulation of the gene expression. However, it is not known whether the temporal and circadian activity of the tissue-specific promoters would be supportive for

or interfering with the complementation effect. This possibilities need to be resolved experimentally.

Currently, the most active research for tissue-specific promoters focuses on cancer-targeted systems (Fukazawa et al. 2004); however, numerous reports of plant tissue-specific promoters appear in the literature (Funk et al. 2002, Ito et al. 2003, Koiwai et al. 2004). Unlike constitutive expression of genes, tissue-specific expression is the result of several interacting levels of gene regulation. As such, it is then preferable to use promoters from homologous or closely related plant species to achieve efficient and reliable expression of transgenes in particular tissues. This is one of the main reasons for the large amount of tissue-specific promoters isolated from particular plants and tissues found in scientific literature. Some of agronomically important tissue-specific promoters are covered by patents, such as beta-amylase gene or barley hordein gene promoters (for seed gene expression), tomato *pz7* and *pz130* gene promoters (for ovary gene expression), tobacco *RD2* gene promoter (for root gene expression), banana *TRX* promoter and melon actin promoter (for fruit gene expression), etc. (Roa-Rodríguez 2003).

4.6. Potential genes regulated by *At*WRKY27

In a candidate gene approach to find the potential targets of *At*WRKY27, it was found that *At*WRKY27 does not autoregulate its own promoter. However, it is still unclear whether *At*WRKY27 can repress its own promoter activity. Moreover, loss-of-function or overexpression of *AtWRKY27* gene was not found to exhibit differential expression of *pathogenesis-related* defense marker genes. WRKY proteins show high DNA binding affinity to a *cis*-regulatory DNA element known as W box (TTGACT/C) (Rushton *et al*., 1996; Eulgem. *et al*., 1999; Yang *et al*., 1999; Uelker and Somssich, 2004). W boxes are present in clusters within short promoter stretches of many *AtWRKY* genes as well as the genes representing other transcription factor families (Eulgem *et al*., 2000; Maleck *et al*., 2000) suggesting that WRKY proteins may act synergistically with other family members or other classes of transcription factors. In addition, W boxes are also present in the promoters of many plant defense genes including the well studied *PR* genes (Rushton et al., 1996; Eulgem. et al., 1999; Dong et al., 2003; Kalde et al., 2003; Eckey

et al., 2004; Kim and Zhang, 2004; Uelker and Somssich, 2004). WRKY proteins dependent *PR1* regulation appears to be complex and may involve both activation and repression functions (Turck et al., 2004; Rocher et al., 2005).

Transient silencing of *HvWRKY1* gene showed reduced fungal penetration of the cells, suggesting that this *HvWRKY1* gene functions as a negative regulator of powdery mildew resistance (Eckey et al., 2004). Mutation analyses revealed that a cluster of three W box sequences in the promoter of the *AtWRKY18* gene reduced its own promoter activity (Chen and Chen, 2002). *At*WRKY6 was shown to act as a negative regulator on its own and on *AtWRKY42* expression by unknown mechanism (Robatzek and Somssich, 2002). Similarly, *At*WRKY53 was shown to influence its own expression in a negative feed back loop (Miao et al., 2004). *In vitro* binding assay showed that recombinant *At*WRKY53 protein can bind to the promoter region of *AtWRKY42* in a W box dependent manner and *At*WRKY53 can negatively regulate *AtWRKY6* and *AtWRKY42* expression patterns. Additional evidences showed the involvement of *At*WRKY proteins in plant defense by acting as positive regulators. Interestingly, *At*WRKY22 and *At*WRKY29, structurally closest members of *At*WRKY27, have been shown to autoregulate their own promoters by a positive feedback loop (Asai *et al*., 2002). *At*WRKY6 positively influences *PR*-promoter activity. Moreover, *AtWRKY6* overexpressor plants showed increased *PR1* expression upon pathogen infection leading to a significant enhancement of resistance or increased cell death (Robatzek and Somssich, 2002). Similarly, overexpression of *AtWRKY18* and *AtWRKY70* led to constitutive or enhanced expression of defense-related genes, including SA-induced *PR1*, and increased resistance to virulent pathogens (Chen and Chen, 2002; Li et al., 2004).

In this study, it was also shown that *At*WRKY27 is able to activate *AtSIRK* expression. Increasing sets of data showed the induced expressions of many *AtWRKY* genes during the onset of senescence (Hinderhofer and Zentgraf, 2001; Lin and Wu, 2004). Senescence process is at least regulated via a key component known as senescence induced receptor kinase (*AtSIRK*). *At*WRKY6, *At*WRKY42, *At*WRKY53 positively regulate *AtSIRK* expression in a W box selectivity dependent manner (Robatzek and Somssich, 2002; Miao et al., 2004; Zhou and Somssich unpublished). Moreover,

*At*WRKY22 and *At*WRKY29, structurally closest members of *At*WRKY27, were also shown to activate flagellin induced kinase1 (*AtFRK1*) expression. *AtFRK1* was found to be identical to *AtSIRK* (Navarro et al., 2004). These lines of evidence suggest that the regulation of WRKY factors is rather complex. *At*WRKY factors can also act in a regulatory network by influencing the transcription of each other, rather than in a linear signal transduction pathway. Besides the annotation of specific function of an individual *At*WRKY factor, regulation of common sets of genes by *At*WRKY proteins reveals a high functional redundancy within family.

Microarray analysis using Affymetrix genome arrays was employed to identify downstream potential regulon genes of *At*WRKY27. Gene expression profiling using membrane-spotted macroarray, cDNA-AFLP (amplified fragment length polymorphism), SAGE (serial analysis of gene expression) and oligonucleotide-based array provides a general overview without distinguishing direct versus indirect putative target genes (Aharoni and Vorst, 2002; Donson et al., 2002; Wyrick and Young, 2002; Chen and Zhu, 2005). Target gene analysis of *AtWRKY6* using cDNA-AFLP differential display indicated that the single *Atwrky6* knockout does result in altered gene expression profiles including *AtSIRK* (senescence induced receptor kinase). Further studies showed that the regulation of *AtSIRK* by *At*WRKY6 in a W box selectivity dependent manner (Robatzek and Somssich, 2002). A similar study showed the identification of few indirect putative target genes regulated by *At*WRKY70 in a microarray analysis using Affymetrix genome arrays (approx. 8300 genes) (Li et al., 2004).

Target gene analysis of *AtWRKY27* revealed the identification of up-regulated gene expressions of *Asparagine Synthetase 2 (ASN2)*, *Nitrate Reductase1 (NR1)* and *Nitrate Reductase2 (NR2)*. In Arabidopsis, asparagine synthetase (Asn) is encoded by a small gene family (*ASN1, ASN2*, and *ASN3*). Phylogenetic analysis revealed that although *ASN1* clustered with all dicot Asn genes, *ASN2* and *ASN3* are more closely related to monocot Asn genes (Lam et al., 1998). *ASN1* and *ASN2* were shown to be reciprocally regulated by light and metabolites in Arabidopsis (Lam et al., 1998). *ASN1* was proposed to play a role related to primary nitrogen assimilation and transport (Lam et al., 1998). However, ammonium and stress treatments-dependent *ASN2* induction revealed that the physiological role of *ASN2* may be related, directly or indirectly, to the recapturing of

lost nitrogen resources under stress conditions (Wong et al., 2004). Ammonium accumulation may also occur when plants are under abiotic and biotic stresses. For instance, an increase of ammonium level was observed in tomato (*Lyocopersicon esculentum*) plants subjected to water and salinity stresses (Feng and Barker, 1993). On the other hand, when tomato was infected with root-knot nematode, foliar accumulation of ammonium was observed (Barker, 1999).

In Arabidopsis, nitrate reducatse (NR) is encoded by at least two genes; *NR1* (*NIA1*) and *NR2* (*NIA2*). In a chlorate-resistant mutant screen, *nia2* null mutant was identified which still retained 10% of wild-type nitrate reductase activity (Wilkinson and Crawford, 1991). However, a NR-null mutant (*nia1/nia2*) exhibits no nitrate reductase activity both in roots and shoots (Wang et al., 2004). At least, Arabidopsis *NIA2* was shown to express in the living cells of the vascular tissue, based on GUS staining activity (Sherameti et al., 2005). Endogenous expression patterns of *AtWRKY27* was also detected on the basis of GUS staining activity at the root elongation zone and root-, stem- and leaf-vasculature which coincides with the sites of bacterial colonization and propagation.

NR is the key enzyme of nitrate assimilation in plants (Neill et al., 2003). In plants, NO is produced nonenzymatically through light-mediated conversion of NO_2 by carotenoids or enzymatically from NO_2 by nitrate reductase (NR) (Millar and Day 1997; Neill *et al*., 2003). A recent study revealed that the NO production site is mainly in both phloem fibres and xylem cells regardless of the cell differentiation status, but spatial and temporal NO gradient is inversely related to the degree of xylem differentiation (Gabaldon et al., 2005). Xylem vessels are also shown to be the colonization and propagation sites of *Ralstonia solanacearum* (Saile et al., 1994). NO is involved in many physiological processes in plants, where it serves as a synchronizing chemical messenger involved in cytotoxicity and programmed cell death (PCD) (van Camp *et al*., 1998; Durner and Klessig, 1999; de Pinto *et al*., 2002; Neill *et al*., 2003). PCD is one of the most distinctive characteristics exhibited by the differentiating xylem in plants (Fukuda, 1996; Robert and McCann, 2000) and is invariably coordinated with processes of secondary cell wall formation and lignification (Groover & Jones, 1999).

NO works with O_2^-/H_2O_2 to trigger PCD through a finely balanced $NO/O_2^-/H_2O_2$ cooperation (Delledonne *et al*., 2001). Therefore, the species which characterize xylem differentiation also work together during the PCD. Lignifying plant tissues are capable of sustaining both NO and O_2^- production (Ogawa et al., 1997; Ros Barceló, 1998; Gabaldon et al., 2005). Therefore it is likely that in the differentiating xylem both NO and O_2^- react to produce the peroxynitrite anion ($ONOO^-$). Peroxynitrite is postulated to play a major role in cytotoxicity (Delledonne *et al*., 2001; Wendehenne *et al*., 2001), although it's real role in PCD is uncertain (Fukuto & Ignarro, 1997). Regardless of this uncertainty, NO does not affect O_2^-/H_2O_2 production by the lignifying xylem (Ferrer & Ros Barceló, 1999) or H_2O_2 production by isolated plant mitochondria (one of the organelles involved in H_2O_2 production during PCD) (Yamasaki *et al*., 2001). These lines of evidence indicate that the synthesis of O_2^-/H_2O_2 and NO in plant cells is simultaneously coordinated in response to environmental/hormonal stimuli with no negative crosstalk between them (Delledonne *et al*., 1998).

Nitric oxide selectively regulates Ca^{2+}- sensitive ion channels in plant cells by promoting Ca^{2+} release from intracellular stores to raise cytosolic-free Ca^{2+} (García-Mata *et al*., 2003; Gould *et al*., 2003). Execution of cell death in xylem elements involves a Ca^{2+} influx into the cell, and it is manifested by a rapid collapse of the vacuole leading to the release of hydrolytic enzymes, and the cessation of cytoplasmic streaming (Groover and Jones, 1999). Moreover, differentiating (noncollapsed) thin-walled xylem cells showed a burst of NO production just before the late processes of secondary cell wall formation and cell autolysis suggesting a certain role for NO in xylem differentiation/cell death (Gabaldon et al., 2005). Thus, cell differentiation/ vacuolar collapse and cell death via at least NO and Ca^{2+} is one of the possible metabolic cascades that may be present in the differentiating xylem (Gabaldon et al., 2005).

NO appears to involved in controlling plant growth and development, fruit ripening, senescence, stomatal movement and pathogen defense (Neill et al., 2003). High levels accumulation of NO (40–80 pphm) exhibit reduced growth in tomato and tobacco, whereas low levels (0–20 pphm) enhanced growth in lettuce and pea (Hufton *et al*., 1996; Leshem and Haramaty, 1996; Morot-Gaudry *et al*., 2002). NO also increased chlorophyll content in pea leaves, particularly in guard cells (Leshem *et al*., 1997). A

potential role for NO in delaying flower senescence is indicated by the increased longevity of several varieties of cut flowers induced by application of NO donors (Leshem, 2001). Expression patterns of *NR* genes were shown to be induced upon inoculation with an incompatible race of *P. infestans* or treated with a crude elicitor (Yamamoto et al., 2003). During the PCD events, kinetics of accumulation of NO was studied by employing 4-amino-5-methylamino-2′, 7′-difluorofluorescein (DAF-FM) diacetate, upon infection with *Pseudomonas syringae* pv. tomato DC3000 carrying either *avrB* or *avrRpt2* (Zhang et al., 2003). Moreover, the experiments with an NO scavenger and an nitric oxide synthase (NOS) inhibitor demonstrated that NO accumulation can slow down hypersensitive response (HR) progression and can function in cell-to-cell spread of the HR (Zhang et al., 2003; Mur et al., 2005). In another elegant experiment, co-delivery of *R. solanacearum* with NOS inhibitor exhibited delay of HR development without inhibitory growth effect on bacterium (Huang and Knopp, 1998).

On the basis of these data, it is hypothesized that *At*WRKY27 acts upstream of *NR1 and NR2* in the process of nitric oxide accumulation. Additionally, enhanced pathogen tolerance observed in *Atwrky27* mutant plants (in terms of delayed symptoms response without the inhibition of bacterial growth) is regulated by the suppression/blockage of NO accumulation via NR in the vasculature. Moreover, pleiotropic phenotypes (stunted growth, delayed in perianth organs and partial sterility) observed in *AtWRKY27* overexpressor plants can be due to the enhanced accumulation of NO. However, still comprehensive genetic and biochemical analyses needed to demonstrate the regulation of *NR1* and *NR2* by *At*WRKY27.

5. Summary

5.1. Summary (English)

In the present study, it was shown that Arabidopsis plants lacking a functional gene, *AtWRKY27*, coding for a WRKY-type transcription factor, displayed an altered disease response towards the soil-borne pathogen *Ralstonia solanacearum* strain GMI 1000. Two independent *Atwrky27* knockout (KO) lines consistently exhibited clearly delayed wilting symptoms in response to the bacterium. The steady-state transcript levels of *AtWRKY27* were not significantly affected in any of the SA or JA/ET signaling pathway mutants under study. Additionally, *Atwrky27*-mediated delayed symptoms phenotype was not associated with constitutive expression of defense response marker genes such as *PR1*, *PR5*, *Thi2.1* or *PDF1.2*. Loss of *AtWRKY27* function did not affect the response of the plants towards other tested pathogens nor towards diverse abiotic stresses. Complementation of the KO lines with *AtWRKY27* under the control of its own promoter restored wild type susceptibility to the GMI1000 strain, whereas ectopic overexpression of *AtWRKY27* led to an even earlier wilting symptom response than wild type plants. Surprisingly, the bacterial density in aerial parts of both KO lines versus wild type plants increased at similar levels throughout the period assayed. These observations point to a role of *At*WRKY27 in a specific defense mechanism known as enhanced pathogen tolerance. *AtWRKY27* expressions appear mainly restricted to specific root parts and in vascular tissue that is highly consistent with sites of bacterial colonization and propagation. Interestingly however, *AtWRKY27* also appears to be expressed in defined floral organs and the ectopic overexpressor lines showed significant partial male sterility. Our data suggest that *At*WRKY27 or a component(s) under the control of this transcription factor can contribute to enhanced pathogen tolerance. There also reveal however that *At*WRKY27 has additional functions within certain stages of anther and pollen development.

5.2. Zusammenfassung (Deutsch)

In der vorliegenden Arbeit wurde gezeigt, dass *Arabidopsis*-Pflanzen mit einem defekten *AtWRKY27*-Gen, dass einen WRKY-Transkriptionsfaktor kodiert, ein verändertes Resistenzverhalten gegenüber dem bodenbürtigem, pathogenen Bakterium *Ralstonia solanacearum* (Stamm GMI 1000) zeigen. Zwei unabhängige *Atwrky27*-Nullmutanten zeigten übereinstimmend deutlich reduzierte Welkesymptome nach Infektion mit dem Bakterium. Die Expression des *AtWRKY27*-Gens, gemessen als "steady-state" mRNA-Menge, war in allen untersuchten Mutanten mit Defekten in den durch Salicylsäure (SA) oder Jasmonsäure (JA) bzw. Ethylen (ET) vermittelten Signaltransduktionswegen unverändert. Auch die Expression von Markergenen der pflanzlichen Pathogenabwehr, wie z.B. der Gene *PR1*, *PR5*, *Thi2.1*, oder *PDF1.2*, war in den Nullmutanten im Vergleich zum Wildtyp unverändert und damit nicht mit dem verzögerten Welkephänotyp korreliert. Der Verlust der *At*WRKY27-Funktion führte nicht zu einer veränderten Reaktion der Pflanzen gegenüber anderen Pathogen oder unterschiedlichen Arten von abiotischem Stress. Expression des *AtWRKY27*-Gens unter der Kontrolle seines eigenen Promotors in der *Atwrky27*-Nullmutate führte zur Komplementation des Phänotyps und damit zur Restauration der Suszeptibilität gegenüber *R. solanacearum* (Stamm GMI 1000) wie in Wildtyp-Pflanzen. Im Gegensatz dazu führte ektopische Expression des *AtWRKY27*-Gens zu verstärkten und früher einsetzenden Welkesymptomen. Die Bakterienanzahl nahm in den überirdischen Pflanzenteilen sowohl im Wildtyp als auch in beiden Nullmutanten im gesamten Untersuchungszeitraum in gleichem Masse zu. Dies Befund war unerwarte und deutet darauf hin, dass *At*WRKY27 eine spezifische Funktion bei der Vermittlung von erhöhter Toleranz gegenüber Pathogenen hat. Die Expression des *AtWRKY27*-Gens ist beschränkt auf spezifische Regionen in Wurzeln und auf Leitbündel, was den Geweben entspricht, die von Bakterien befallen werden. Darüber hinaus wird das *AtWRKY27*-Gen in begrenztem Maß in Blütenorganen exprimiert und ektopische Expression führt zu eindeutiger, partieller männlicher Sterilität. Insgesamt zeigen die vorgelegten Daten, dass *At*WRKY27 selbst, oder eine Komponente, die von diesem Transkriptionsfaktor kontrolliert wird, zur erhöhten Toleranz von Pflanzen gegenüber Pathogen beitragen kann. Darüber hinaus hat *At*WRKY27 vermutliche zusätzliche Funktionen in bestimmten Entwicklungstadien von Antheren und Pollen.

6. Outlook

The results available so far require extended and detailed experimental continuation, which is already initiated to a large extent. The ongoing characterization of the tissue-specific *AtWRKY27* complementation lines will provide an insight into the spatial requirements for the gene expression during the *R. solanacearum* infection process. The kinetics of bacterial growth in *Atwrky27* mutant plants in various organs including root-, shoot- and leaf-vasculature will provide evidence whether the delayed symptom responses are due to enhanced pathogen tolerance strategies or enhanced disease resistance mechanisms. On the other hand, the wilt resistance phenotype of the *Atwrky27 ein2* double knock-out needs to be analyzed. This would show whether *At*WRKY27 functions via the ethylene-mediated signaling pathway and displays an additive effect together with *At*EIN2, or acts independently.

The preliminary results of the ATH1 microarray analysis indicated *AtNIA1*, *AtNIA2* and *AtASN1* as putative early targets of the *At*WRKY27. Further analyses of *nia1nia2* double knock-out plants will provide a genetic proof of the involvement of genes coding nitrate reductase in wilting disease by *R. solanacearum* strain GMI1000. Usage of a NO scavenger/inhibitor on wilt-type plants and a NO donor on *Atwrky27* knock-out plants would further strengthen the hypothesis of *At*WRKY27 involvement in the wilting symptom response via the nitric oxide accumulation. Phenotypic response of *AtWRKY27* overexpressor plants grown on MS-media supplemented with ammonium succinate would allow supporting the above mentioned hypothesis. Furthermore, chlorophyll contents measurement and nitrate reductase activity analysis in *AtWRKY27* overexpressor plants would confirm the involvement of *At*WRKY27 in NO accumulation. Chromatin-immunoprecipitation of *At*WRKY27 with *NR1* and *NR2* promoters would provide a final line of evidence that these two genes are indeed *in vivo* regulated by *At*WRKY27 in the process of NO accumulation upon pathogen infection.

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9. Eidesstatliche Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen - die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Paul Schulze-Lefert betreut worden.

Köln, im Dezember 2005

(Muhammad Shahid Mukhtar)

10. Publications

Ein Teil dieser Arbeit wird zur Veröffentlichung vorbereitet:

Mukhtar MS, Deslandes L, Marco Y and Somssich IE (*Manuscript in preparation*). *AtWRKY27*, a gene affecting *Ralstonia solanacearum* disease development in Arabidopsis.

11. Lebenslauf

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