Verticillium longisporum induced gene expression in *Arabidopsis thaliana*

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Abbreviations

A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
ABA	abscisic acid
aba1-1	ABA deficient mutant
AG	research group
amp	ampicillin
AOC3	allene oxide cyclase 3
AP2-TF	AP2 domain-containing transcription factor
APRR5	pseudo-response regulator 5
At, at	marks a protein from *Arabidopsis thaliana*
AtFBX7	F-box family protein
ATP	adenosine triphosphate
atrbohD/F	mutant of *Arabidopsis* impaired in ABA-induced closing of the stomata
AXR1	AUXIN RESISTANT PROTEIN 1
B. cinerea	*Botrytis cinerea*
BGL2	PATHOGENESIS-RELATED PROTEIN 2
bp	base pairs
BSA	bovine serum albumine
BT4	BTB AND TAZ DOMAIN PROTEIN 4
Bur	*Arabidopsis thaliana* ecotype Burren
C24	*Arabidopsis thaliana* ecotype C24
Cal	*Arabidopsis thaliana* ecotype Calver
CCD	charge coupled device
cDNA	copy DNA
CHIB	CHITINASE B
Co	*Arabidopsis thaliana* ecotype Coimbra
COI1	CORONATINE INSENSITIVE 1
Col-0	*Arabidopsis thaliana* ecotype Columbia
CORI3	coronatine induced 2
CPD	Czapek Dox

C_T	threshold cycle
C-terminal	carboxy-terminal
CTR1	constitutive triple response
Cvi	*Arabidopsis thaliana* ecotype Cape Verde Islands
dde2-2	delayed-dehiscence 2-2
ddNTPs	didesoxy nucleotides
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleotides
dpi	days post infection
dT	desoxyribonucleotide thymine
DTT	dithiotreitol
E. coli	*Escherichia coli*
EB	elusion buffer
EDTA	ethylenediaminetetraacetic acid
EF-Tu	elongation factor Tu
e.g.	exempli gratia; for example
ein2-1	ET insensitive
EIN3	ET INSENSITIVE 3
elo	elongation
ERF	ET responsive factor
ERF1	ETHYLENE RESPONSE FACTOR 1
etc	et cetera
ET	ethylene
et al.	et alii (and others)
EtBr	ethidium bromide
EtOH	ethanol
etr1-1	defect in ethylene perception
EXLA, EXPR	EXPANSIN-LIKE PROTEINS
FDR	false discovery rate
FW	fresh weight
g	gram
gm	gentamycin
GNAT	GCN5-related N-acetyltransferase
GO	gene ontology

GRP7	glycine rich protein 7
h	hours
H_2O_2	hydrogen peroxide
HEL	HEVEIN-LIKE PROTEIN
HR	hypersensitive response
HSB	high salt buffer
HSP	heat shock protein
IAA	indole-3-acetic acid
i.e.	id est; that is
INA	isonicotinic acid
JA	jasmonic acid
l	litre
lacZ	gene coding for ß-galactosidase
LB	Luria-Bertani Broth
LB	lipid binding
Ler	*Arabidopsis thaliana* ecotype Landsberg *erecta*
LOX1	lipoxygenase 1
LOX2	lipoxygenase 2
LRR	leucine-rich repeat
LTP	lipid transfer protein
M	molarity [mol l^{-1}]
MAP	mitogen activated protein
MAPKKK	MAP kinase kinase kinase
MBF1c	multiprotein bridging factor 1c
MeSA	methyl salicylate
min	minutes
M-JA	methyl jasmonate
MKK	MAP kinase kinase
MPK	MAP kinase
mRNA	messenger RNA
MS	Murahige and Skoog
MT1A	METALLOTHIONEIN 1A
MT1C	METALLOTHIONEIN 1C
MUB5	MEMBRANE-ANCHORED UBIQUITIN-FOLD PROTEIN 5 PRECURSOR

NPR1	NON-EXPRESSOR of *PR*-GENES 1
N-terminal	amino-terminal
OD	optical density
PAEs	phosphate acquisition efficiencies
PCD	programmed cell death
PCL1	phytoclock 1
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PDF1.2	plant defensin 1.2
PEG	polyethylenglycol
PER	peroxidase
pH	negative log10 of proton concentration
PIP	plasma membrane intrinsic protein
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PR	Pathogenesis related
Psm	pseudomonades
qRT-PCR	quantitative reverse transcription PCR
RB	right border
RD22	responsive to dehydration 22
RD29a	responsive to dehydration 29a
R-genes	resistance genes
rif	rifampizin
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	rotations per minute
RPP5	RECOGNITION OF PERONOSPORA PARASITICA 5
RR	ready reaction
rRNA	ribosomale RNA
RT	room temperature
sec	second
SA	salicylic acid
SABP2	SA-binding protein 2
SAR	systemic acquired resistance

SDS	sodium dodecylsulfate
SEN1	senescence 1
SFP	subtilase family protein
sid2-2	SA induce deficient
spp.	species
SSU-rRNA	small-subunit rRNA
SXM	simulated xylem medium
TAE	Tris-acetate-EDTA
TAIR	The *Arabidopsis* Information Resource
TE	tris-EDTA buffer
temp	temperature
THI2.1	THIONIN2.1
TIP	tonoplast intrinsic protein
Tris	tris-hydroxymethylamino methane
U	unit (quantity for enzyme activity)
UBQ5	ubiquitin 5
UV	ultra violet
V. dahliae	*Verticillium dahliae*
V. longisporum	*Verticillium longisporum*
v/v	volume per volume
Vd830	*V. dahliae* isolate 830
VdBOB.70	*V. longisporum* isolate used by Veronese et al., 2003
VET1	*V. dahliae* tolerance 1
Vl18	*V. longisporum* isolate 18
Vl43	*V. longisporum* isolate 43
VliG(s)	*V. longisporum*-induced gene(s) at 18 dpi
VliG5(s)	*V. longisporum*-induced gene(s) at 5 dpi
VSP1	vegetative storage protein 1
VSP2	vegetative storage protein 2
w/v	weight per volume
Ws-2	*Arabidopsis thaliana* ecotype Wassilewskija
Wt, WT	wildtype plants

1 Introduction

As primary producers of organic material, plants provide an attractive habitat for microorganisms. However, plants are not merely a substrate for microorganism growth. They are able to defend themselves against most pathogenic microorganisms by either constitutive barriers or by the activation of multicomponent defense responses. Different types of interactions are described including killing of infected plant cells by necrotrophic microorganisms (e.g. *Botrytis cinerea;* Colmenares et al., 2002), exploitation of resources from living host cells by biotrophic pathogens (e.g. *Cladosporium fulvum;* Perfect et al., 2001), and symbiotic interactions (e.g. *Laccaria bicolor;* Lammers et al., 2004), which benefit both sides. If pathogenic microorganisms overcome the defense barriers of their hosts, they often cause devastating effects, irrespective of whether they derive nutrients from living host tissues or from damaged or dying cells.

1.1 Plant – fungus interactions

Plants developed different strategies to defend themselves against pathogens. If all members of a given plant species are able to prevent the propagation of any member of a given pathogen species, this interaction is called resistance one or non-host resistance (Halim et al., 2006). According to the general models, pathogenic species acquired virulence factors to overcome the plant defense through evolutionary processes. In turn, plant resistance genes (*R*-genes) evolved as a response of the plant to new virulence factors. Flor, (1971) reviewed the details of this gene-for-gene hypothesis.

The gene-for-gene resistance plays an important role in plant resistance against biotrophic pathogens (Glazebrook, 2005). The *R*-gene mediated defense response is usually accompanied by an oxidative burst which is required for a hypersensitive response (HR), a type of programmed cell death. The HR can induce a systemic acquired resistance (SAR) (Durrant and Dong, 2004; Glazebrook, 2005) which is dependent on salicylic acid (SA) production. In tobacco plants methyl salicylate (MeSA) is converted into SA by the methyl salicylate (MeSA) esterase activity of SA-binding protein2 (SABP2) (Park et al., 2007). Downstream of SA the expression of several *PATHOGENESIS RELATED* (*PR*) genes is triggered by the NON-EXPRESSOR OF PATHOGENESIS RELATED PROTEIN 1 (NPR1) in *Arabidopsis* (Glazebrook,

2005). The exact function for most *PR*-genes is unclear, but roles in the defense responses have been suggested (Kitajima and Sato, 1999; van Loon et al., 2006).

To cope with necrotrophic pathogens, plants have developed two strategies. One is strictly jasmonic acid (JA) dependent, the other needs both JA and ethylene (ET). For instance, resistance to *B. cinerea* is JA/ET dependent, whereas resistance to *Alternaria brassicicola* is rigorously JA-dependent (Glazebrook, 2005). Increased JA synthesis in response to pathogen attack, like *B. cinerea,* leads to an induction of defense effector genes such as *PDF1.2*. Both phytohormones, JA and ET, are necessary for the induction of *PDF1.2*. In the case of an infection of *A. brassicicola* the JA-inducible gene *VSP1* is induced. ET is not required for the expression of *VSP1* (Glazebrook, 2005).

JA, SA and ET defense pathways do not function independently (Kunkel and Brooks, 2002). As mentioned above, JA and ET signaling are required for the expression of the defense-related gene *PDF1*.2. SA can inhibit the JA pathway. Kunkel and Brooks (2002) reviewed the cross talk between the signaling pathways in pathogen defense and published a working model of the interaction of SA, JA and ET in *A. thaliana* (Figure 1).

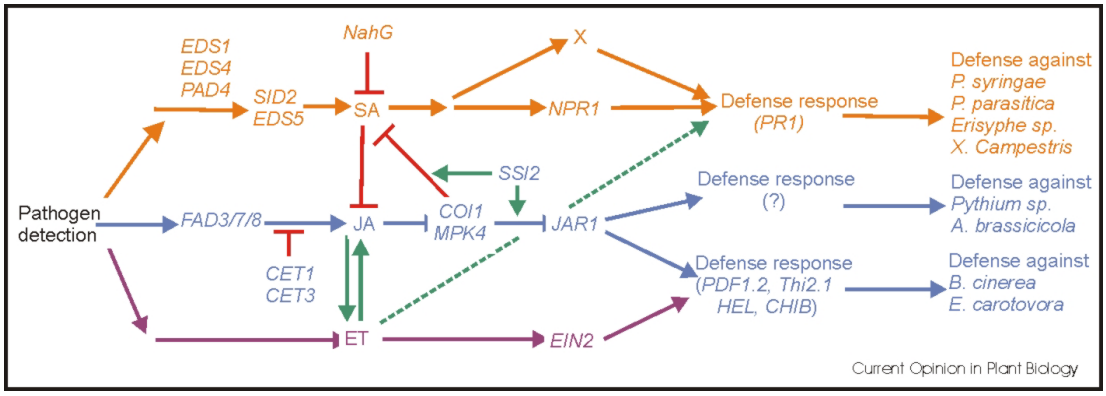


Figure 1: Working model of the SA, JA, and ET pathogen defense pathways in *A. thaliana*
Source: Kunkel and Brooks, 2002; Biotrophic pathogens can trigger a defense response dependent on SA and NPR1 or only dependent on SA which leads to the expression of *PR*-genes. Necrotrophic pathogens trigger a JA or JA/ET dependent defense pathway. The SA defense signaling inhibits the production and signaling of JA. The synthesis of both ET and JA triggers the expression of several defense related genes like *PDF1.2, THI2.1, HEL*, and *CHIB*. Positive regulatory interactions between these signaling pathways are indicated by green arrows, antagonistic interactions by red lines. For more detailed information see (Kunkel and Brooks, 2002).

1.2 *Verticillium*-induced diseases

Verticillium spp. is a soil-borne phytopathogenic fungus responsible for *Verticillium* wilt disease in temperate and sub-tropical regions. *Verticillium longisporum* occurs in northern Europe, where it predominantly infects *Brassica* oil crops (Zeise, 1990; Fahleson et al., 2003; Johansson et al., 2006). In Sweden,

Verticillium wilt has been known as the cause of economic losses of oilseed rape since 1960s and has become a significant problem since the 1970s (Dixelius, 2005). In Germany, the occurrence of *V. longisporum* has increased rapidly since mid-1980s (Daebeler et al., 1988; Günzelmann and Paul, 1990; Zeise and Seidel, 1990). A detailed review of physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum* has been recently published by Fradin and Thomma, 2006.

1.2.1 Symptom development and life cycle of *Verticillium spp.*

In vascular diseases, fungi colonize the xylem elements that supply water and mineral salts to the aerial plant tissues. Many vascular fungi, such as *Fusarium ssp*. (Beckman, 1987), *Verticillium dahliae, V. albo-atrum* (Fradin and Thomma, 2006), and *Ophiostoma ulmi*, the cause of the Dutch Elm Disease (Hubbes, 1999), induce wilting of the host plant as a result of vessel clogging or destruction. In contrast, *V. longisporum* colonizes only a few xylem vessels, which might explain the absence of the wilting symptoms (Zeise and von Tiedemann, 2002; Eynck et al., 2007). Partial xylem colonization by *V. longisporum* correlates with stunted growth, leaf chlorosis (Figure 2), and early flowering of its host.

Figure 2: Typical stunting of *Brassica napus* after inoculation with *V. longisporum*
Mock-treated plants on the left, *V. longisporum*-infected plants on the right. Source: www.angenetik.fu-berlin.de/diederichsen.html

The infection cycles of *V. longisporum* and *V. dahliae* are very similar (Johansson et al., 2006) and consist of three stages: dormant, parasitic, and a limited saprophytic stage (Figure 3). During the dormant stage, the excretion of carbon and nitrogen from root exudates elicits the germination of the microsclerotia, which are persistent in the soil (Bishop and Cooper, 1983) (Figure 3A). The length of hyphae, which are growing out of a microsclerotium, does not exceed 300 µm (Huisman, 1982). However, every single cell of the microsclerotium can germinate, which increases the efficiency of an infection. At the start of the parasitic stage, hyphae penetrate the roots. Entry sites are

typically the root tips (Figure 3B) or the sites of the lateral root formation (Bishop and Cooper, 1983). After colonizing the root cortex (Figure 3C), *Verticillium* reaches the vascular tissues (Figure 3D). This is only possible if the endodermis is physically damaged or not yet developed, as in the root tips (Pegg and Brady, 2002; Schnathorst, 1982; Bowers et al., 1996).

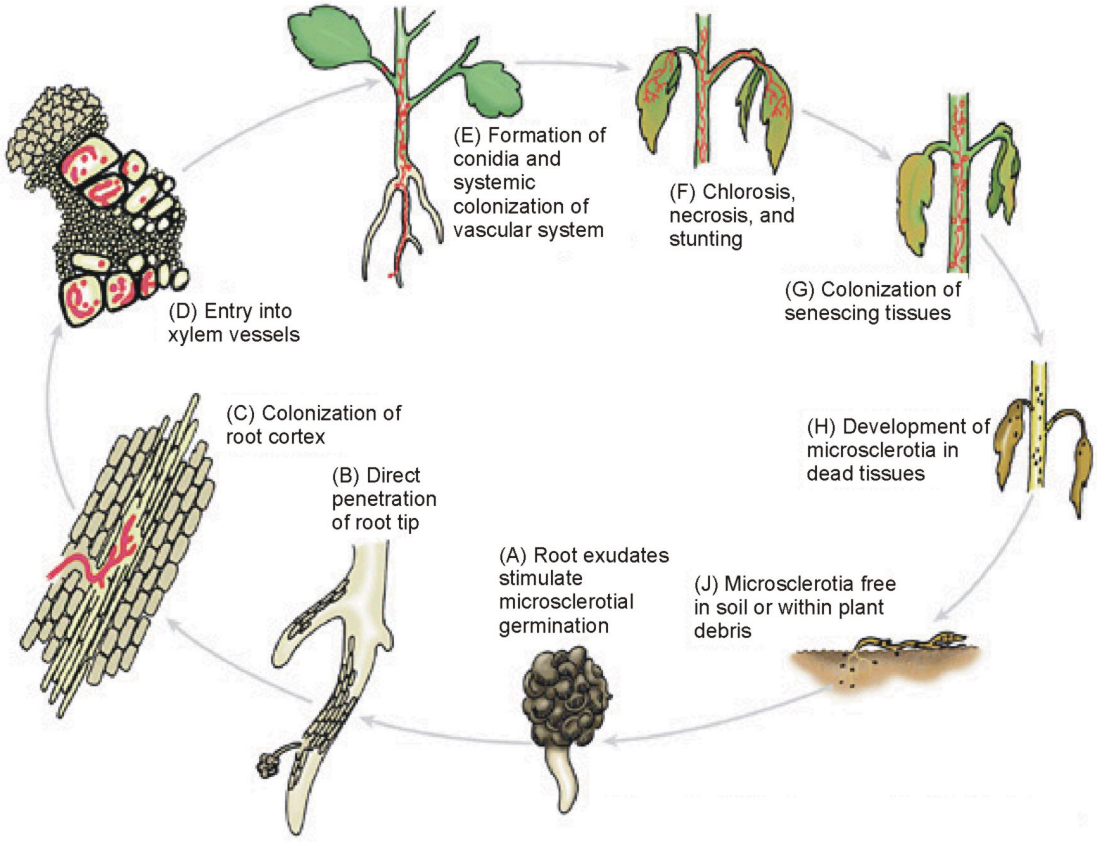


Figure 3: Life cycle of *Verticillium spp*.
Adapted from a drawing of Vickie Brewster, colored by Jesse Ewing (Source: http://www.apsnet.org/Education/lessonsPlantPath/Verticillium/discycle.htm). Life cycle of *Verticillium spp.* consists of three stages: I: parasitic stage A-E; II: limited saprophytic stage F-H; III: dormant stage J;

Verticillium is predominantly constricted to the vascular system, where it exhibits both hyphal and "yeast-like" growth. During its parasitic life stage, new conidia bud directly from the hyphae or are formed on short phialides. Conidia are carried upwards through the vascular elements with the transpiration stream, until they are trapped in bordered pits or at vessel ends. Germ tubes subsequently penetrate into the upstream vessel elements, where new conidia are produced (Figure 3E), so that the plant becomes increasingly colonized and develops typical symptoms (Figure 3F). The later stages of the disease cycle are characterized by the beginning of senescence of the foliage (Figure 3G) and the fungus enters a limited saprophytic growth phase in which microsclerotia are formed in the dead tissues (Figure 3H). The microsclerotia persist as dormant

structures in the soil (Figure 3J) (Olsson and Nordbringhertz, 1985). *Verticillium* can stay in this dormant phase for 10 -15 years and starts to germinate again when it senses a host plant (Pegg and Brady, 2002).

1.2.2 *Verticillium longisporum* and *V. dahliae*

The genus *Verticillium* comprises seven species: *V. albo-atrum, V. dahliae, V. longisporum, V. nigrescens, V. nubilum, V. tricorpus* and *V. theobromae* (Pegg and Brady, 2002). *V. longisporum* was first described as a variant to *V. dahliae* by Stark (1961). Karapapa et al. (1997) suggested *longisporum* to be a heterozygous diploid between *V. dahliae* and *V. albo-atrum* and hence, a separate species, *V. longisoproum* based on molecular and morphological differences. Four years later, Karapapa and Typas (2001) further supported the proposed phylogeny by detecting an intron of 839 bp in the SSU-rRNA gene in *V. longisporum* that was absent in *V. dahliae* and *V. albo-atrum*. The morphological and physiological parameters that distinguish between *V. longisporum* and *V. dahliae* are listed in Table 1 (Zeise and von Tiedemann, 2001).

Table 1: Morphological and physiological characteristics of selected *Verticillium* isolates
Classification cited: (Zeise and von Tiedemann, 2001); Morphological and physiological parameters for discrimination of *V. longisporum* and *V. dahliae*

	***V. longisporum* 43 and 18**	***V. dahliae* 830**
Conidial length [µm]	7.0 – 7.9	3.2 – 5.5
Colony color (solid medium)	black	white
Dark mycelium (liquid medium)	present	absent
Microsclerotia	long	rounded
Polyphenol oxidase activity	none	none
Pigment secretion (liquid medium)	none	weak
Sporulation rate (shake cultures)	low	high

In addition, *V. longisporum* differs from its close relative *V. dahliae* with respect to host plant species. Whereas *V. dahliae* can be isolated from a broad range of hosts (e.g. tomato, cotton, sunflower etc; Bhat and Subbarao, 1990), *V. longisporum* predominantly infects *Brassicaceae* (Zeise and von Tiedemann, 2002).

1.2.3 Disease control

Verticillium-induced diseases are difficult to control. The disease usually spreads through use of contaminated equipment on crop fields and transfer of infected plant material, such as rootstocks, bulbs, and tubers. Because of the very long viability of the

microsclerotia and the broad host range of *Verticillium spp*., the fungus can persist in the soil for many years (Pegg and Brady, 2002). Particularly when Verticillium enters the xylem, fungicides are ineffective as they do not reach the pathogen. The microsclerotia in the soil are resistant against many chemicals (Pegg and Brady, 2002). Furthermore, applying chemicals in an attempt to control fungal persistence leads to the development of resistance by the fungus.

To optimize the productivity of the crop fields, tolerant or resistant plants need to be used. Polygenic resistance to *Verticillium spp*. has been found in some plant species, including alfalfa, cotton, potato, and strawberry (Bolek et al., 2005; Simko et al., 2004; Hunter et al., 1968). Several specific loci have been identified that are involved in resistance against *Verticillium* wilt. For instance, locus *Ve* provides resistance against *Verticillium* wilt in tomato (Schaible et al., 1951). It has been fully characterized by Kawchuk et al. (2001). *Ve1* and *Ve2* genes encode leucine-rich repeat (LRR) proteins that belong to the class of so-called receptor-like proteins (Kruijt et al., 2005). Veronese et al. (2003) found a locus (*VET1*: *V. dahliae* tolerance) controlling *V. dahliae*-induced disease in *Arabidopsis* and mapped it to the top of chromosome IV. Interestingly, genes like *FRI*, *FCA*, and *VRN2* involved in the vernalization/autonomous pathway controlling flowering time (Hepworth et al., 2002) and the *RPP5* supergene family controlling resistance to the oomycete pathogen *Hyaloperonospora prasasitica* (Parker et al., 1997; Knoth and Eulgem, 2008) are also mapped to this region.

1.3 *Arabidopsis thaliana* ecotypes

A. thaliana was chosen as a model plant to study the plant-*Verticillium* interaction in the present study. In contrast to other vascular diseases, *V. longisporum* does not induce wilt in its host plants. Thus, the interaction between a xylem-localized microorganism and its host plant can be studied in the absence of pleiotropic effects. Together with the natural host, oilseed rape, *A. thaliana* belongs to the family of *Brassicaceae*. Many mutants in signal transduction processes or defense reactions are available for the *A. thaliana* ecotype Col-0. For example, *sid2-2* (SA-induced deficient; Nawrath and Metraux, 1999; Wildermuth et al., 2001), *dde2-2* (delayed-dehiscence2-2; von Malek et al., 2002) and *ein2-1* (ethylene insensitive 2; Guzman and Ecker, 1990) are mutants of *A. thaliana* Col-0. With the use of theses mutants it is possible to investigate the role of the different plant hormones (respectively SA, JA, and ET) in the *Arabidopsis*-*Verticillium* interaction.

An ecotype-specific genetic variability exists in *Arabidopsis* (Narang et al., 2000; Sauer et al., 2004; Schmelz et al., 2004; Chen et al., 2005; Kalbina and Strid, 2006). Chen et al. (2005) reported that genes with presumed functions in signal transduction, transcription and stress response show substantial genetic variations among different accessions. Van Leeuwen et al. (2007) studied the natural variation among different *Arabidopsis thaliana* accessions for transcriptome response to exogenous salicylic acid. Narang et al., 2000 investigated 36 *Arabidopsis* accessions for phosphate acquisition efficiencies (PAEs) and showed that C24, Co, and Cal accessions have a high PAE, while Col-0 and Te exhibit a low PAE. After analyzing these accessions in detail, significant differences were found in root morphology, phosphate uptake kinetics, organic acid release, rhizosphere acidification, and the ability of roots to penetrate substrates (Narang et al., 2000).

In addition, the symptom development of *A. thaliana* accessions after infection with *Verticillium* was described by Veronese et al. (2003) and Steventon et al. (2001). Veronese et al. (2003) showed that C24 is a more tolerant ecotype compared to Col-0. This correlates with the presence of *VET1* locus in C24. Steventon et al. (2001) inoculated two other ecotypes, Ler and Ws-2, with different *Verticillium* isolates. They observed ecotype-specific differences in the plant response towards the same fungal isolates.

1.4 Aims of the study

The *Verticillium* research unit (FOR546), which consists of nine partners of the Georg-August-University Göttingen, has chosen *V. longisporum* as a common experimental system to address the questions as to how plants detect and react to foreign organisms in the xylem and how microorganisms adapt to the growth conditions inside the plant. In the framework of this collaborative project, the present study focuses on the analyses of gene expression by *A. thaliana* after infection with *V. longisporum*. To investigate which genes are induced after the development of the first symptoms, a whole genome profiling was done. Genes induced after infection with *V. longisporum* (VliGs) were used as candidate genes for analyzing the role of defense related phytohormones. To understand the effect of different fungal isolates on the host, expression of VliGs was monitored. Transgenic plants containing a construct of a candidate gene promoter fused to the luciferase reporter gene were used in a bioassay to provide more information about the putative elicitor for induced plant responses. In addition, to further understand which genes are induced in the early stages of infection,

a second whole genome profiling was done. Different *Arabidopsis* ecotypes were compared and the course of symptom development was analyzed.

2 Materials and Methods

2.1 Materials

2.1.1 Organisms

2.1.1.1 Bacteria

Bacterial strain	Description	Reference
Agrobacterium tumefaciens GV3101	PMP90RK *rif*^{r}, *gm*^{r}	Deak et al., 1986
Escherichia coli DH5α	F-, *gyr*A96 (Nalr), *rec*A1, *end*A1, *thi*-1, *hsd*R17 (rkmk+), *gln*V44, *deo*R, D (*lac*ZYA-*arg*F) U169 [p80dD(*lac*Z)M15]	Hanahan, 1983

2.1.1.2 Fungi

Fungal isolate	Description	Reference
Verticillium longisporum 43	isolated in Mecklenburg/Germany	Zeise and von Tiedemann, 2002
Verticillium longisporum 18	isolated in Mecklenburg/Germany	Zeise and von Tiedemann, 2002
Verticillium dahliae 830	isolated in Ohio/USA	Zeise and von Tiedemann, 2002

2.1.1.3 Plants – *Arabidopsis thaliana*

Plant	Description	Reference*
Burren, Bur	Wildtyp	N1029
Columbia, Col-0	Wildtyp	N1092
Cape Verde Islands, Cvi	Wildtyp	N8580
C24	Wildtyp	N906
dde2-2	mutation in the *ALLENE OXIDE SYNTHASE*	Park et al., 2002
ein2-1	ethylene insensitive	Guzman and Ecker, 1990
Landsberg *erecta*, Ler	Wildtyp	N1686

P_{PER21}:*luciferase*	Promotor of *PER21* gene cloned upstream of the *FIREFLY LUCIFERASE* reporter gene (Col-0)	this thesis
$P_{LTP(2)}$:*luciferase*	Promotor of $LTP_{(2)}$ gene cloned upstream of the *FIREFLY LUCIFERASE* reporter gene (Col-0)	this thesis
$P_{TIP2.2}$:*luciferase*	Promotor of *TIP2.2* gene cloned upstream of the *FIREFLY LUCIFERASE* reporter gene (Col-0)	this thesis
sid2-2	SA-induced deficient	Nawrath and Metraux, 1999; Wildermuth et al., 2001
Wassilewskija,Ws-2	Wildtyp	N1601

* The number beginning with "N" refers to NASC (the European *Arabidopsis* stock center)

2.1.2 Media and additives

All media and heat-stable solutions were sterilized by autoclaving for 20 min at 121°C. The heat sensitive solutions were sterilized by filtering through a 0.2 µm membrane filter (Heinemann Labortechnik GmbH, Germany). To solidify the media 15g l^{-1} select agar were added.

2.1.2.1 Media

Media	**Components and concentrations / Reference**
CPD	Czapek Dox (SIGMA, Steinheim, Germany)
LB	10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, pH 7.0 (NaOH)
2 MS	2.2 g l^{-1} MS salt, 2.5 % sucrose, 4 g l^{-1} gelrite (Carl Roth GmbH, Karlsruhe, Germany), pH adjusted to 5.7 with KOH
PDB	Potato Dextrose Broth (SIGMA, Steinheim, Germany)
SOC	20 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 0.5 g l^{-1} NaCl, 186.38 mg l^{-1} KCl, 2.033 g l^{-1} $MgCl_2$, 3.6 g l^{-1} glucose, pH 7.0 (NaOH)

2.1.2.2 Additives

Additives	Working Concentration	Stock solution, Solvent
Cefotaxim	500 mg l^{-1}	250 mg ml^{-1}, H_2O
Kanamycin	50 mg l^{-1}	50 mg ml^{-1}, H_2O

2.1.3 Nucleic acids

2.1.3.1 Plasmids

Plasmids	Description	Reference
pSK-T	Cloning and sequenicing vector; pBluescriptII SK (Stratagene, Cedar Cree, Texas) was restricted with *Eco*RV and treated with terminal transferase in presence of ddTTP; *lacZ*α, *amp*^{r}	Guido Kriete, unpublished
pSK-T_At2g37130	pSK-T vector with At2g37130 cDNA sequence as insert; *amp*^{r}	this thesis
pSK-T_At3g18280	pSK-T vector with At3g18280 cDNA sequence as insert; *amp*^{r}	this thesis
pSK-T_At3g53980	pSK-T vector with At3g53980 cDNA sequence as insert; *amp*^{r}	this thesis
pSK-T_actin8	pSK-T vector with *actin8* insert (genomic DNA); *amp*^{r}	Katja Rindermann, unpublished
pSK-T_AtPR1	pSK-T vector with ArPR1 cDNA sequence as insert; *amp*^{r}	Brenner, 2002
pSK-T_RD22	pSK-T vector with *RD22* coding sequence insert; *amp*^{r}	Heupel, 2006
pSK-T_RD29a	pSK-T vector with *RD29a* cDNA sequence as insert; *amp*^{r}	Heupel, 2006
pGEM-T	Cloning and sequenicing vector; pGEM-5Zf(+) (Promega) was restricted with *Eco*RV and treated with terminal transferase in presence of ddTTP; *amp*^{r}	Promega, Mannheim, Germany

pAt2g37170	pGEM-T-Easy vector with PIP2.2 coding sequence insert; *amp*^{r}	Baiges et al., 2002
pAt3g54820	pGEM-T-Easy vector with PIP2.5 coding sequence insert; *amp*^{r}	Baiges et al., 2002
pAt4g17340	pGEM-T-Easy vector with TIP2.2 coding sequence insert; *amp*^{r}	Baiges et al., 2002
pGEM-PDF1.2	pGEM-T vector with PDF1.2 coding sequence insert; *amp*^{r}	Herde, 2006
pDONR223	Gateway-vector ® *kan*^{r}	Shevchenko et al., 2002
pGWB235	Gateway-vector ® *kan*^{r}	Nakamura et al., 2006 accession number AB289803

2.1.3.2 Oligonucleotides

Primer	**Sequence 5' – 3'; *= catalog number of Qiagen for QuantiTect®**
Sp980	CCG TCC CGT CGG TTA CAA GTG T
AS980	CGA AAT TTG CAC CGT TTA GGG
SP130	GCC AAT GCG AAG CCC TTT TG
AS130	TGT TGT CCA CAA CCA TCG GA
SP280	GGT GAT CAT CAA GAC CAC AAT G
AS280	ACC TTG GGA TGG GGA GTT TG
uni24	ACG ACG TTG TAA AAC GAC GGC CAG
ref23	TTC ACA CAG GAA ACA GCT ATG ACC
Olg70	CAG CGA AAC GCG ATA TGT AG
Olg71	GGC TTG TAG GGG GTT TAG A
act8fow	GGT TTT CCC CAG TGT TGT TG
act8rev	CTC CAT GTC ATC CCA GTT GC
UBQ5forw	GAC GCT TCA TCT CGT CC
UBQ5rev	GTA AAC GTA GGT GAG TCC A
PER21forw	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG GGA CTT TAC ATG GCT ATA AAT GAC
PER21rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT GGC TCT ATA TGT GTT CCA ATG TAG G
PIP2.2forw	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGG TGT AAG CTG AGT GTT AAA TCT G

PIP2.2rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AAG ACT GAA GAG ACA ATG AAA GTT G
LTP2forw	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC TTG GCA AGC AAA TGC AAA AAA TGA G
LTP2rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TGT TGT GGT TTT TGA CAT CTC CTC C
RD22 fwd	GGT GGC TAA GAA GAA CGC AC
RD22 rev	CAC ACA ACA TGA GTC TCC GG
RD29a fwd	TTA CAC CAA ACC CAC TGA ATC
RD29a fwd	TTA CAC CAA ACC CAC TGA ATC
P1	QT00862078*
P3	QT00820764*
P5	QT00816893*
PLTP(2)	QT00774263*
PPER21	QT00718277*
PTIP2.2	QT00806862*
VSP2	QT00837879*

2.1.3.3 Probes for northern blot analysis

Probe	**Properties**	**Reference**
PIP2.2	495 bp amplificatd with uni24 and rev23; plasmid: pAt2g37170	Baiges et al., 2002
PIP2.5	495 bp amplificatd with uni24 and rev23; plasmid: pAt3g54820	Baiges et al., 2002
TIP2.2	495 bp amplificatd with uni24 and rev23; plasmid: pAt4g17340	Baiges et al., 2002
PER21	721 bp amplificated with SP130 and AP130; plasmid: pSK-T_At2g37130	this thesis
$LTP_{(2)}$	300 bp amplificated with SP280 and AP280; plasmid: pSK-T_At3g18280	this thesis
PDF1.2	731 bp amplificated with uni24 and rev23; plasmid: pGEM-PDF2.1	Heupel, 2006
PR-1	500 bp *EcoRI*-fragment; plasmid: pSK-AtPR1	Brenner, 2002

RD22	307 bp with RD22fwd and RD22rev; plasmid: pSK-RD22	Heupel, 2006
RD29A	1031 bp with RD29afwd and RD29arev; plasmid: pSK-RD29a	Heupel, 2006

2.1.4 Buffers and solutions

Buffer/Solution	**Component and concentration**
Buffer I for alkaline lysis	50 mM Tris-HCl, pH 8.0 10 mM EDTA 100 µg/µl RNase A
Buffer II for alkaline lysis	0.2 M NaOH 1 % (w/v) SDS
Buffer III for alkaline lysis	29.4 g potassium acetate 5 ml formic acid water up to 100 ml
DNA loading buffer	67 % (w/v) sucrose 50 mM EDTA, pH 8.0 0.42 % (w/v) bromophenolblue 0.42 % (w/v) xylenecyanol 0.42 % (w/v) orange G
SSC (20 x)	2 M NaCl, 0.3 M sodiumcitrat, pH 7.0 with HCl
TAE (20 x)	0.8 M Tris, 2.3 % (v / v) acetic acid, 20 mM EDTA
TE	10 mM Tris, 1 mM EDTA, pH 7.5
MEN (10x)	200 mM MOPS 50 mM NaOAc 10 mM EDTA, pH 7.0 with 1 M NaOH
RNA loading buffer (3x)	100 µl bromphenolblue/xylenecyanol 80 µl 0.5 M EDTA, pH 8.0 3333µl 10x MEN 1200 µl glycerol (100 %) 4286 µl formamide 1001 µl fomaldehyde add 6 µl EtBr per ml loading buffer direct before useing
Church buffer	500 mM $NaPO_4$, pH 7.2 7 % (w/v) SDS 1 mM Na_2EDTA, pH 8.0

Trizol buffer	380 ml l^{-1} phenol with 0.1M citric buffer, pH 4.3 saturated 0.8 M guanidinium thiocyanat 0.4 M ammonium thiocyanat 33.4 ml Na-Acetate, 3 M, pH 5.2 5% glycerol
HSB	1.2 M NaCl 0.8 M tri-sodium-citrate

2.1.5 Kits

Kit name	**Manufacturer**
BigDye™ Terminator Cycle Sequencing, Ready Reaction Kit	Perkin-Elmer Corporation (Massachusetts, USA)
DNeasy Kit	Quiagen (Hilden, Germany)
Nucleo Spin® Extrakt II	Macherey-Nagel (Düren, Germany)
Nucleo Spin® Plasmid	Macherey-Nagel (Düren, Germany)
Qiagen Plasmid Preparation Kits (Midi, Maxi)	Qiagen (Hilden, Germany)

2.1.6 Consumables

Product	**Manufacturer**
3 mm paper	Whatman (Dassel, Germany)
Glass material	Brand Shott (Zwiesel, Germany)
Nylon Membrane Hypond N+	Amersham Pharmacia (Munich, Germany)
Plastic ware	Sarstedt (Nümbrecht, Germany), Greiner (Frickenhausen, Germany), Eppendorf (Hamburg, Germany)
Fluted filter	Macherey-Nagel (Düren, Germany)
Sand	Vitakraft, Nr. 12262 (Bremen, Germany)
Seramis	Masterfoods GmbH (Verden/Aller, Germany)

2.1.7 Software

Program	**Manufacturer**
Acrobat Reader 8.1.2	Adobe (http://www.adobe.com/de)
Chromas 1.55	Technelysium Pty Ltd (Shannon Co. Clare, Ireland)
Clone v7	Scientific and Educational Software (Groningen, Netherlands)

Office XP	Microsoft (Unterschleißheim, Germany)
Oligo 4.0	MedProbe (Olso, Norway)
Bildanalyseprogramm 1.0.4.6	Datinf GmbH (Tübingen, Germany)
Bio-Rad iQ5	BioRad (Munich, Germany)
Wasabi 1.5	Hamamatsu Photonics (Herrsching am Ammersee, Germany)

2.1.8 Equipment

Equipment	**Model**	**Manufacturer**
Autoclave	3870 ELV	Tuttnauer (Breda, Netherlands)
Automatic pipette		Gilson (Middleton, USA)
Balance	SP052, SAC62, 1207 MP2	Scaltec (Göttingen, Germany) , Sartorius (Göttingen, Germany)
Cooling centrifuge	Sorvall RC 5B Plus	DuPont (Bad Homburg, Germany)
Digital camera	Canon Powershot A510	Canon (Krefeld, Germany)
DNA/RNA calculator	GeneQuantII	Pharmacia (Munich, Germany)
Gel-documentation station		MWG Biotech (Ebersberg, Germany)
Heating block		Unitek Boekel Scientific (Delray Beach, USA)
Heating stirrer	RCT Basic	IKA Labortechnik (Staufen, Germany)
Ice machine	Af 20	Scotsman (South Yorkshire, Great Britain)
Incubation chamber		WTC Binder (Tuttlingen, Germany), Memmert (Schwabach, Germany)
PCR termocycler	MiniCycler PTC 150, PTC-200	MJ Research (Miami, USA)
pH-meter	HI 9321	Hanna Instruments (Kehl am Reihn, Germany)
Shaker	ST5 M	Zipperer GmbH (Staufen, Germany)
Sequencer	ABI Prism 310	Perkin-Elmer (Massachusetts, USA)

Sterile bench	Microflow	Nunc (Wiesbaden, Germany)
Table-top centrifuge	Biofuge Pico	Heraeus Christ (Osterrode, Germany)
Table-top centrifuge with cooling	5403	Eppendorf (Hamburg, Germany)
Freezer (-80°C)	C54285	New Brunswick Scientific (Nürtingen, Germany)
Vortex	L46	Labinco BV (Breda, Germany)
Water deionization dystem	Option 4, Maxima	ELGA (Celle, Germany)
Water Bath	1086	GFL (Burgwedel, Germany)

2.2 Methods

2.2.1 Culture and storage of microorganisms

2.2.1.1 Growth conditions for microorganisms

	E. coli	***A. tumefaciens***	***Verticillium spp.***
Temperature	37°C	28°C	21°C
Medium	LB	YEB	CPD or PDB
Light conditions	dark	dark	dark
O_2 conditions	aerobe	aerobe	aerobe

For fungal propagation, droplets of glycerol-spore suspensions were plated onto potato dextrose agar (PDA, SIGMA, Steinheim, Germany) and incubated for 14 days at 21°C in the dark. Spores were obtained by transferring blocks of agar containing mycelium to 120 ml PDB (SIGMA, Steinheim, Germany) supplemented with 0.5 mg l^{-1} cefotaxim. The cultures were subsequently incubated for 2 to 4 weeks on a rotary shaker at 21°C in the dark. To start sporulation, PDB was replaced by CPD broth (SIGMA, Steinheim, Germany). After 4 to 7 days, spores were harvested by filtering through a fluted filter (Macherey-Nagel, Düren, Germany). The conidia were washed two times with sterile tap water. Spore concentration was determined with a haemocytometer and diluted to 1 x 10^6 spores ml^{-1} with sterile tap water.

2.2.1.2 Preparation of chemical-competent *Escherichia coli*

E. coli DH5α was used for the preparation of competent cells. The procedure was followed as per Inoue et al. (1990). An 8 h liquid culture was used as an inoculum for an overnight 5 ml SOC-culture. Number of cells in a liquid culture was determined by measuring the optical density at 600 nm (OD_{600}). The overnight culture was diluted with 300 ml SOC medium and cultivated in a 2 l flask at 37°C to OD_{600} 0.2 – 0.25. The cultivation conditions were changed to 18°C as long as needed for OD_{600} 0.4 – 0.5. The cells were centrifuged (4000 rpm, 4°C) and suspended in 90 ml cold transformation buffer (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7 with $MnCl_2$). After an incubation of 15 min on ice the centrifugation step was repeated. The cells were suspended in 30 ml transformation buffer. 1050 µl DMSO were added and incubated on ice for 5 min. This step was repeated twice. Aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C.

2.2.1.3 Preparation of electro-competent *Agrobacterium tumefaciens*

An overnight culture (YEB medium) was used as an inoculum for a 250 ml YEB culture. It was incubated at 28°C to an OD_{600} of 0.5 and then centrifuged (5000 rpm, 5 min). The cells were washed three times in 4°C sterile water. They were suspended in 1 ml 15 % glycerol and aliquots of 50 µl were frozen in liquid nitrogen and stored at -80°C.

2.2.1.4 Transformation of bacteria

The heat shock method was used only to transform *E. coli* chemical competent cells. The transformation procedure was followed as per Hanahan (1983). In brief, 200 µl competent *E. coli* cells were thawed on ice for 20 min, 50 ng of plasmid DNA were added to the cells and mixed gently. The mixture was incubated on ice for 30 min. After a heat shock for 90 sec at 42°C the cells were placed immediately on ice for at least 3 min. Eight hundred µl of LB medium were added to the tube and the suspension was mixed on a roller for 45-60 min at 37°C depending on selectable antibiotic resistance marker. Different volumes of the culture were plated on plates containing LB medium supplemented with antibiotics. The plates were incubated overnight at 37°C. Genetransfer in *Agrobacterium tumefaciens* was done by electroporation with a Gene Pulser II. Bacterial competent cells were thawed on ice slowly before adding 2 µl of plasmid DNA. The mixture was transferred into an ice-cooled electroporation cuvette (2 mm electrode distance). The cuvette was subjected to electroporation at 25 µF, 2.5 kV, 400 Watt. The cells were suspended immediately in 1 ml SOC medium and

incubated for 60 min at 28°C. The culture was plated on selective media supplemented with antibiotic and incubated 2 – 3 days at 28°C.

2.2.1.5 Cryoconservation of microorganisms

2.2.1.5.1 *Escherichia coli*

A single colony of *E. coli* cells was cultured at 37°C in LB liquid or solid media in the presence of selective antibiotic. The liquid culture was grown under continuous shaking at 250 rpm in a 37°C shaker, while solid culture was grown in a 37°C incubator. Number of cells was determined. A liquid culture with OD_{600} equal to 0.1 corresponds to 2 x 10^7 cells ml^{1-}. For long-term storage, the overnight culture was supplemented with 20 % glycerol and stored at -70°C.

2.2.1.5.2 *Verticillium* isolates

The *V. longisporum* 43, *V. longisporum* 18 and *V. dahliae* 830 were obtained from A. von Tiedemann, Georg-August-University Göttingen (Göttingen, Germany). Long term storage was performed as conidial suspensions in a concentration of 1-3 x 10^9 conidia ml^{-1} in CPD broth (SIGMA, Steinheim, Germany) supplemented with 25 % glycerol at -80°C.

2.2.2 Analyses of nucleic acids

2.2.2.1 Separation of DNA on agarose gel

The electrophoretic separation of DNA for analytical and preparative purpose was done in a horizontal agarose gel (10 cm x 7 cm x 0.3 cm, 16 lanes) with 1x TAE as running buffer. DNA fragments ranging between 500 bp and 14 kb were run on 1 % agarose gel, as DNA fragments with lower size were run on 2 % (w/v) agarose gel. DNA samples were mixed with 1/10 volume of 10 x DNA loading buffer, loaded in separate lanes and run at 120 V for 40-45 min. Ethidium bromide solution (0.1% w/v) was used to stain the DNA fragments. The detection of DNA was done under UV light (260 nm). Preparative gels were examinated at larger wavelengths UV light (320 nm). Before exposure to the UV light, the gel was rinsed briefly in H_2O to reduce background staining. In a gel-documentation station, gels were visualized on a UV-transilluminator and documented. The sizes and amount of the DNA fragments were determined using a DNA standard, MassRuler™ DNA Ladder Mix (MBI Fermentas, St Leon Rot, Germany).

Fragment lengths [bp]:

10000 / 8000 / 6000 / 5000 / 4000 / 3500 / 3000 / 2500 / 2000 / 1500 / 1200 / 1031 / 900
800 / 700 / 600 / 500 / 400 / 300 / 200 / 100

2.2.2.2 Isolation of DNA fragment from agarose gel

The elution of DNA fragments from agarose gel was done using the Nucleospin Extract II Gel Extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The eluted fragments were verified by electrophoresis as described above.

2.2.3 DNA isolation methods

2.2.3.1 Alkaline lysis

Small amounts of plasmid DNA for analytical purposes were isolated from *E. coli* using a modification of the alkaline lysis method (Le Gouill et al., 1994). 1.5 ml of an overnight culture of *E. coli* (stationary phase) was collected by centrifugation at 13000 rpm for 1 min. The supernatant was descanted and the cells were resuspended in 100 µl of buffer I for plasmid DNA. The cell suspension was lysed for 5 min on ice using 200 µl of buffer II. The suspension was neutralized with 150 µl of buffer III. The solution was mixed well by inverting 8 times and the suspension was centrifuged for 10 min at 13000 rpm at room temperature. The aqueous solution (~ 400 µl) was transferred into a new reaction tube containing 1 ml of 96 % (v/v) ethanol. The DNA was left to precipitate for 20 min at -20°C. Plasmid DNA was collected by centrifugation for 10 min at 13000 rpm and 4°C. The pellet was washed with 70 % (v/v) ethanol and air-dried for 10 min at 37°C. The DNA was dissolved in 20 µl of TE buffer.

2.2.3.2 High-purity plasmid DNA isolation

2.2.3.2.1 *Spin Miniprep*

For sequencing and transformation purposes, high-purity plasmid DNA was isolated using Nucleospin Mini kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Optional steps were always followed according to the manufacturer's recommendation. A 4 ml overnight culture was used to isolate plasmid and the isolated DNA was eluted with 50 µl (high copy) or 30 µl (low copy) EB buffer.

2.2.3.2.2 *Preparative quantity (Midi and Maxiprep)*

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

2.2.3.3 Isolation of plant DNA for quantification of *Verticillium* DNA

Fungal biomass was quantified by determination of fungal DNA in infected plant extracts with real-time PCR. DNA extraction from infected plant leaf material was conducted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Manufacturer's protocol including the optional recommendations was followed and two times eluted with 50 µl of EB.

2.2.3.4 Estimation of nucleic acids concentration and purity

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

2.2.4 Enzymatic and cloning techniques

2.2.4.1 Restriction analysis of DNA molecules

Type II endonucleases were used to digest a double stranded DNA molecule for analytical and cloning purposes. The enzymes cut the DNA either as 5′ or 3′ "sticky" overhangs or as blunt ends. The digestion reactions were incubated in a buffer system optimized for the used enzyme. In the case of double digestion a universal buffer system was used. The activity of the restriction enzymes was estimated in "units" (U), where 1 U was defined as the amount of enzyme cutting completely 1 µg of λ DNA in 60 min at optimal conditions. The minimal amount of enzyme necessary for each restriction was determined according to the following formula: U min = bp [λ] * No. of restriction sites in target DNA * incubation period (hour) * (restriction sites in [λ])$^{-1}$ * (bp of target

DNA)$^{-1}$ Where, λ = 48500 bp. The incubation temperature was 37°C unless otherwise mentioned for particular restriction enzyme. Due to the adverse effect of high glycerin concentration, the total volume of restriction enzymes was kept less than 10 % in reaction mixture.

2.2.4.2 Ligation of DNA fragments

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

2.2.4.3 Generation of FIREFLY LUCIFERASE reporter lines

To generate binary vectors for the expression of promoter:LUCIFERASE constructs, the Gateway technology (Invitrogen GmbH, Karlsruhe, Germany) was used by Dr. Meik Dilcher and Dr. Corinna Thurow (both AG Gatz). The promoter regions were amplified from genomic DNA with the following primer combinations:

Name	AGI -Code	Primer	Fragment[a]
PER21	At2g37130	PER21forw; PER21 rev	-1973 to +19
PIP2.2	At2g37170	PIP2.2forw and PIP2.2rev	-2007 to +3
LTP$_{(2)}$	At3g18280	LTP2forw and LTP2rev	-2007 to -1

[a] The numbers indicate positions in base pairs relative to the transcriptional start site (+1) as announced by The *Arabidopsis* Information Resource (At-TAIR v7)

The promoter fragments were cloned into the pDONR223 vector (Shevchenko et al., 2002) and subsequently recombined into the binary destination vector pGWB235 (Nakamura et al., 2006; accession number AB289803). Binary plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting agrobacteria were used to transform Col-0 plants using a floral dipping method (Clough and Bent, 1998). In the present thesis the primary transformants were selected on kanamycin containing medium. Resistant plants were allowed to self and the resulting progeny was tested for reporter gene expression after *V. longisporum* infection.

2.2.5 Polymerase chain reaction (PCR)

Amplification of target DNA fragments was performed by polymerase chain reaction in thermal cycler according to the protocol from Mullis et al., 1986 with required modification. The reaction started with the denaturation of two strands of a DNA template. The 5′ complementary strands of the denatured DNA were recognized and hybridized with specific primers (annealing). A high fidelity polymerase (iProof or Phusion) was used which catalyzed elongation of a newly synthesized chain and the complementary polymerization of nucleotides to the free 3′-OH group of the primer. Repeating the denaturation, annealing and elongation for x cycles (usually from 25 to 35) exponentially enriched the reaction with the primer-flanked DNA sequence.

2.2.5.1 Standard PCR reaction

The PCR reaction was carried out in a 25 µl reaction volume with the following constituents: 10-50 ng template DNA, 10 pmol sense primer, 10 pmol antisense primer, 0.2 mM dNTPs, 2 µl of 10x buffer, 2 U polymerase and H_2O filled to a total volume of 25 µl. The amplification reaction was done in a PCR thermocycler. The used program is mentioned in Table 2.

2.2.5.2 Determination of fungal DNA

The iCycler System (BioRad, Hercules, CA, USA) was used for the amplification and quantification of *Verticillium* DNA using primers OLG70 and OLG71. The amplification mix consisted of NH_4-reaction buffer (Bioline, Luckenwalde, Germany), 3 mM $MgCl_2$, 200 µM of each dNTP, 0.3 µM of primer OLG70 and OLG71, 0.25 U BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM Fluorescein (BioRad, Hercules, CA, USA), 100,000 x diluted SYBR Green I solution (Cambrex Bio Science Rockland Inc., Maine**,** USA) and 20-30 ng of template DNA and double distilled water filled to a total volume of 25 µl. PCR program is shown in Table 2. The amount of *V. longisporum* DNA was estimated from a calibration curve constructed with purified fungal DNA dissolved in plant DNA. To normalize for different DNA preparations, the *Arabidopsis actin8* gene (At1g49240) was amplified with the primers act8fow and act8rew. The amount of *actin8* DNA in the samples was calculated with a reference plasmid pSK-T_actin8 encoding *actin8* sequence. Copy number of the product was calculated from the threshold cycles of duplicate real-time PCR assays using the standard curve.

2.2.5.3 Quantitative PCR after cDNA synthesis (qRT-PCR)

The iCycler System (Bio Rad, Hercules, CA, USA) was used for the amplification and quantification of cDNA using QuantiTect®-primers (Qiagen, Hilden, Germany) for *LTP(2)* (At3g18280), *PER21* (At2g37130), *TIP2.2* (At4g17340) and *UBQ5* (At3g62250) as a reference. The amplification mix consisted of 1x NH_4-reaction buffer (Bioline, Luckenwalde, Germany); 2 mM MgCl2; 100 µM of dNTPs; 0.4 µM of primers, 0.25 U BIOTaq DNA polymerase (Bioline Luckenwalde, Germany); 10 nM Fluorescein (BioRad, Hercules, CA, USA); 100,000 times diluted SYBR Green I solution (Cambrex, Rockland, ME, USA); 1 µl of a 1:10 dilution of cDNA as template and double distilled water filled to a total volume of 25 µl. The amplification reaction was done in an iCycler System (BioRad, Hercules, CA, USA) using program mentioned in Table 2. Quantitation of Results was done by the comparative C_t method. This involves comparing the C_t values of the samples of *Verticillium*-treated with the mock-treated control RNA. The C_t values of both the mock- and *Verticillium*-treated samples are normalized to an appropriate endogenous housekeeping gene (*UBQ5*). The comparative C_t method is also known as the $2^{-[delta][delta]Ct}$ method, where

$$[delta][delta]C_t = [delta]C_{t,\ sample} - [delta]C_{t,\ reference}$$

Here, $[delta]C_{t,\ sample}$ is the C_t value for any *Verticillium*-treated sample normalized to the endogenous housekeeping gene and $[delta]C_{t,\ reference}$ is the C_t value for the *UBQ5* calibrator also normalized to the endogenous housekeeping gene.

Table 2: PCR-Programs

	Standard PCR		***Verticillium* PCR**		**RT-qPCR**	
	Temp.	**Time**	**Temp.**	**Time**	**Temp.**	**Time**
1) First denaturation	95°C	1 min	94°C	2 min	95°C	3 min
2) Denaturation	95°C	15 sec	94°C	20 sec	95°C	20 sec
3) Annealing	58°C	30 sec	59°C	30 sec	55°C	20 sec
4) Extension	72°C	2 min	72°C	40 sec	72°C	40 sec
5) x time to 2	30		36		40	
6) Final extension	72°C	10 min	72°C	5 min	72°C	4 min

2.2.5.4 DNA sequencing

The DNA sequencing was done using the BigDye Terminator RR Mix Cycle Sequencing kit (Perkin-Elmer Corporation, Massachusetts, USA). The principle of DNA sequencing is based on the chain-termination method described by (Sanger et al.,

1977). In the chain-termination method, didesoxynucleotides (terminators) are incorporated into a newly synthesized complementary chain that will lead to stop its elongation in a PCR reaction. Each of didesoxynucleotides is labeled with a specific fluorescent dye and the terminated chains can be specifically detected using an ABI Prism 310 Capillary Sequencer (Applied Biosystems). The PCR sequencing reaction was performed using 500-1000 ng plasmid DNA, 5 pmol primer, 2 µl RR mix (ready reaction) and H_2O up to a total volume of 10 µl. The samples were subjected to 25 cycles of: 10 sec at 95°C, 5 sec at 50°C, 4 min at 60°C in a thermocycler. The DNA product was precipitated using 9.5 µl water and 30.5 µl of absolute ethanol and left for 1 hour. The DNA was collected by centrifugation for 20 min at 13000 rpm. The pellet was washed using 125 µl 70 % ethanol and then centrifuged for 10 minutes at 13000 rpm. The pellet was dried at 95°C for 1 min and resuspended in 15 µl of template-suppression reagent (TSR, Perkin-Elmer). After the final denaturing step at 95°C for 2 min tubes were directly put on ice. The reaction was loaded on an ABI-Prism 310 capillary electrophoresis sequencing station for analysis.

2.2.6 RNA and cDNA specific analysis

2.2.6.1 RNA isolation

150 mg plant material was ground in liquid nitrogen and 1.3 ml trizol buffer were added. After shaking the samples for 20 min, 260 µl chloroform were added and another 20 min shaking was performed. Centrifugation with 13000 rpm at 4°C for 30-60 min was performed. 900 µl supernatant were transferred to a new reaction tube. 325 µl HSB were added and mixed well by inverting. 325 µl of isoproanol were added and the tube was inverted again. After 10 minutes the samples were centrifuged at room temperature. The supernatant was discarded and the dried pellet was dissolved in 40 µl water.

2.2.6.2 Northern blot analysis

Total RNA was extracted from 100 mg plant tissue using the trizol method and analyzed by Northern blot analysis (Heinekamp et al., 2002). A 284 bp *LTP*$_{(2)}$ (At3g18280) specific fragment was amplified by PCR using the primers SP280 and AS280. A 766 bp peroxidase 21 (*PER21,* At2g37130) specific fragment was amplified by PCR using the primers SP130 and AP130. Both fragments were cloned into the *Eco*RV restriction site of pBluescript II SK (Stratagene, Cedar Creek, Texas) which was treated with terminal transferase in the presence of ddTTP. pGEM-T Easy vectors

(Promega, Madison, USA) containing the 3'-UTR sequences of *TIP2.2, PIP2.2* and *PIP2.5* were provided by Anton Schäffner, GSF Munich, Germany (Glombitza et al., 2004). Sequences were amplified using the standard universal primers (uni24 and rev23) flanking the multiple cloning site. Probes were radioactively labeled using the random-priming method with the Megaprime DNA labeling system (Amersham, Munich, Germany). Aliquots of total RNA were fractionated on denaturing agarose gels and transferred to nylon membranes (Hybond N^+; Amersham, Munich, Germany). Hybridization was performed over night. Membranes were washed with 2x SSC / 0.1 % SDS at 65°C for 1 h and with 1x SSC / 0.1 % SDS at 65°C for 1 h. Quantification of RNA levels was performed using Bio-imager analysis (BAS-1000, Fuji, Tokyo).

2.2.6.3 cDNA synthesis

RNA extraction of plant leaf material was performed as described above. The RNA samples get a DNase restriction to prevent any DNA contamination in the samples. 1 µg of RNA, 1 µl of 10x reaction buffer with $MgCl_2$ (Fermentas, St. Leon-Roth, Germany), 1 µl deoxyribonuclease I (DNase I), and RNase-free was added with water to a total volume of 10 µl. The mixture was incubated at 37°C for 30 min. To denatured the DNaseI 1µl 25 mM EDTA was added and incubated at 65°C for 10 min.

cDNA synthesis was performed with 1 µg total RNA (DNA-free), 20 pmol of oligo-dT primer and 200 pmol of random nonamer oligonucleotides. Water was added to a total volume of 12.5 µl. The mixture was heated to 70°C for 10 min, 20 nmol dNTPs, 4 µl 5x reaction buffer (Fermentas, St. Leon-Roth, Germany) and 30 U ribonuclease inhibitor (Eppendorf, Hamburg, Germany) were added and the mixture was heated to 37°C for 10 min. 100 U of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas, St. Leon-Roth, Germany) was added (final volume 20 µl) and the mixture was incubated at 42°C for 70 min, then heated to 70°C for 10 min.

2.2.6.4 Whole genome array

Mock-treated plants were compared with *V. longisporum*-inoculated plants using three biological replicates in a DRLL (diagonal right left loop)-design with nine microarrays (Landgrebe et al., 2004) for leaf material from plants harvested at 18 dpi. Mock-treated plants were compared also at 5 dpi with *V. longisporum* infected plants. Petioles from 36 plants were accumulated together in a dye swap design with 4 microarrays (Landgrebe et al., 2004). The material was frozen and ground in liquid nitrogen. Total RNA was extracted according to the trizol method and purified using the

RNeasyMini Kit (Qiagen, Hilden, Germany). Microarrays spotted with the *Arabidopsis* Genome Oligo Set version 3.0 (Qiagen, Hilden, Germany) were obtained from D. Galbraith (University of Arizona, Tucson Arizona, USA). Slides were rehydrated at 60^{o}C and UV-cross linked according to the supplier's web page (http://www.ag.arizona.edu/microarray/). The Amino Allyl MessageAmp^{TM} II aRNA Amplification Kit (Ambion, Darmstadt, Germany) was used for cDNA synthesis, *in vitro* transcription and Cy3/Cy5-labeling of the 5-(3-aminoallyl)-UTP-containing aRNAs with the following modifications: Purification and concentration of double-stranded cDNA was done using the DNAclear^{TM} Kit (Ambion, Darmstadt, Germany), and the large scale transcription reaction was purified with the MEGAclaer^{TM} Kit (Ambion, Darmstadt, Germany). Hybridization and washing was done as recommended on the supplier's web page (http://www.ag.arizona.edu/microarray/). The slides were scanned with a G2505B Microarray Scanner (Agilent Technologies, Böblingen, Germany). Image processing, including spot finding and quantification of signal intensity, was done using the software "Automatic Imageprocessing for Microarrays" (Katzer, 2004). Normalization of the local background corrected raw intensity data was done with nonlinear lowess regression. To increase the comparison of all slides, each normalized dataset was scaled by division with its standard deviation. The preprocessing of the data was done by using "R" (http://can.r-project.org, http://www.bioconductor.org). Differentially expressed genes were identified by an ANOVA mixed effects model using SAS PROC MIXED, where false discovery rate (FDR)-adjusted p-values were obtained by the Benjamini-Hochberg method (Bretz et al., 2005). Normalization and statistical computation was done independently for a high and a low gain dataset, allowing the recovery of lost data from saturated spots.

2.2.7 Infection procedure

2.2.7.1 Plant inoculation

Two different methods for plant inoculation with comparable results were used in this study.

	Method one	**Method two**
substrate (before infection)	sand/seramis	MS-plates
age of plants at infection	four weeks	four weeks

growth chamber conditions (before and after infection)	22°C, ~ 140 µmol^{-1} m^2 sec^{-1} PAR	22°C, ~ 140 µmol^{-1} m^2 sec^{-1} PAR
day length	8-h-light/16-h-dark	8-h-light/16-h-dark
Incubation time	45 min	permanent
spore concentration	10^6 conidia ml^{-1}	10^6 conidia ml^{-1}
substrate (after infection)	steam-sterilized soil	soil
high humidity	2 dpi	2 dpi
infection procedure	- gently uprooting - rinse with water - root incubation with condial suspension - re-potting after 45 min	- gently uprooting - roots cut 1 cm above tip - roots transferred to small soil cavity filled with 10 ml conidial suspension

2.2.7.2 Plant sampling

For the experiment shown in Fig. 1 and 2, 60 plants were either mock-infected or infected with *V. longisporum* (Vl43). After 10, 14, 21, 28, and 35 dpi, 10 plants were harvested and the material was used for measurements of the parameters displayed in Figure 1 and 2. Due to the amount of material needed for the analysis, material from several plants had to be combined at the earlier time points. The following pools of plants were formed: 10 dpi: 1 pool of mock-infected plants and 1 pool of Vl43-infected plants; 14 dpi: 2 pool of mock-infected plants and 2 pools of Vl43-infected plants; 21 dpi: 4 pools of mock-infected plants and 6 pools of Vl43-infected plants; 28 dpi: 5 pools of mock-infected plants and 7 pools of Vl43-infected plants; 35 dpi: 10 individual mock-infected plants and 8 pools of Vl43-infected plants. The experiment was repeated four times with similar results.

2.2.7.3 Leaf surface area measurement

Pictures were taken with a digital camera and custom-made software (Bildanalyseprogramm, Datinf GmbH Tübingen, Germany) was used to quantify the projected leaf area.

2.2.8 Biochemical methods

2.2.8.1 Fragmentation of fungal culture supernatant

V. longisporum was grown in SXM (simulated xylem medium, Neumann et al., 2003) for secondary metabolite extraction by the AG Karlovsky. Spores (10^4 – 10^5 spores per flask) were inoculated into 600 ml medium in Fernbach flasks, the cultures were grown for 4 weeks at 20°C and mycelium was separated from the culture medium by filtration. Medium was extracted three times with 1 vol of ethyl-acetate, extracts were combined and re-extracted with 1 vol n-butanol (Figure 4). Extracts were dried in vacuum and resuspended in tab-water. Petioles of reporter-plants were fed by these extracts over night.

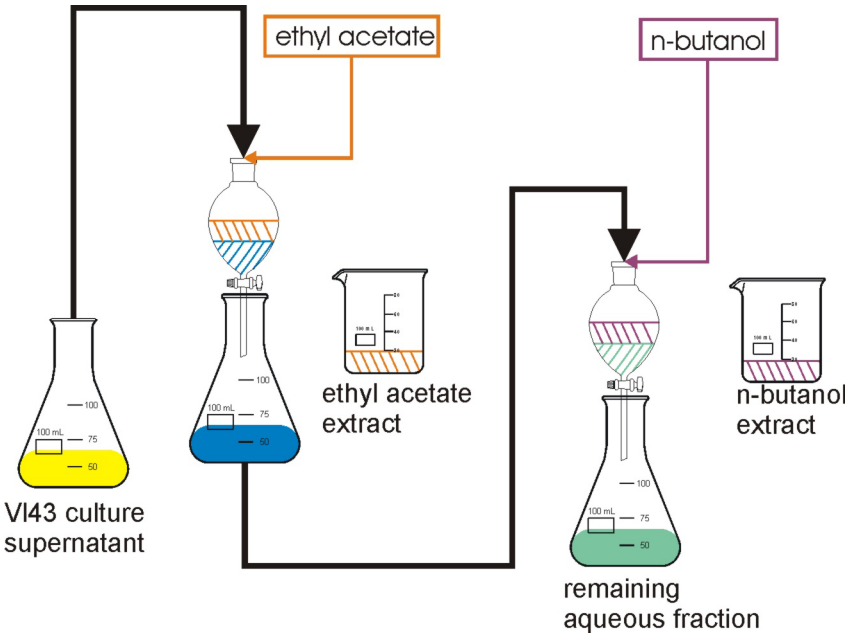


Figure 4: Extraction procedure for *Verticillium* culture supernatant
Vl43 culture supernatant was extracted three times with 1 vol of ethyl-acetate, extracts were combined and re-extracted with 1 vol n-butanol.

2.2.8.2 Luciferase reporter assay

Plants were sprayed with 1 mM luciferin in 0.01% Triton X-100. Subsequently incubated for 10 min in the dark, and then imaged with a Hamamatsu C-4742-98 cooled CCD camera (Hamamatsu Photonics, Herrsching am Ammersee, Germany). The Wasabi 1.5 software (Hamamatsu Photonics, Herrsching am Ammersee, Germany) was used for automating image exposure and processing.

3 Results

3.1 *Verticillium longisporum*-induced progression of disease symptoms in *Arabidopsis thaliana*

After infection of *Arabidopsis thaliana* Col-0 plants with *Verticillium longisporum* isolate 43 (Vl43) by root-dip inoculation, stunted growth, decreased fresh weight, and leaf chlorosis were observed. A time course of the progression of symptoms is displayed in Figure 5.

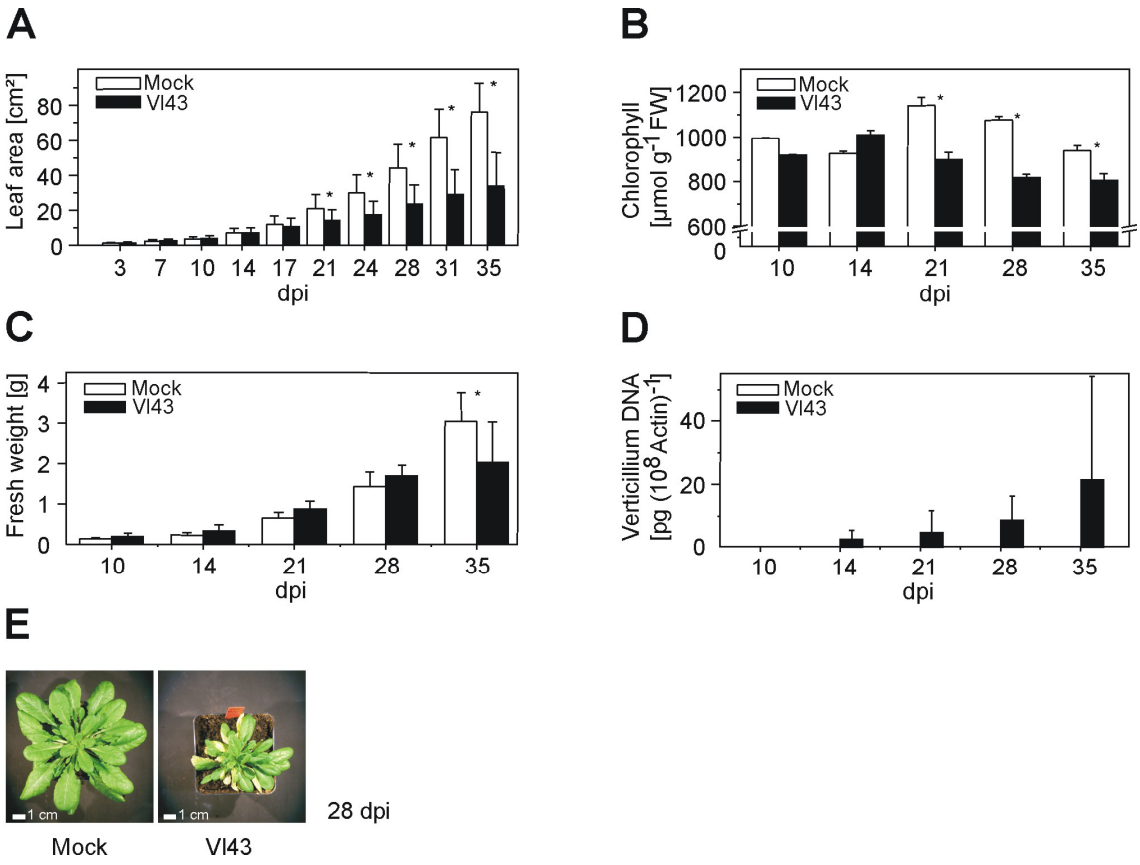


Figure 5: Disease phenotype of *V. longisporum*-infected *Arabidopsis* plants
Four-week-old plants grown on sterile substrate were inoculated with *V. longisporum* isolate 43 following a root-dip procedure. Disease parameters were determined at the indicated time points (dpi, days post infection). Plotted are mean values of 20 (A) or 10 (B, C, D) individual plants, some of which were combined to one sample as described in Materials and Methods. Error bars indicate the standard deviation. Asterisks indicate significant differences at $P \leq 0.05$ between mock- and *V. longisporum* (Vl43)-infected plants. A, Projected leaf area of mock- and *V. longisporum*-infected *A. thaliana* plants at different dpi. B, Chlorophyll content of mock- and *V. longisporum*-infected *A. thaliana* plants at different dpi. Chlorophyll-analysis was done by Saskia Flörl (AG Polle) C, Fresh weight of mock- and *V. longisporum*-infected *A. thaliana* plants at different dpi. D, Fungal DNA of mock- and *V. longisporum*-infected *A. thaliana* plants (same material as for (B and C)) at different dpi. Fungal DNA was determined by real-time PCR using *Verticillium*-specific primers and normalized for the amount of plant DNA using the *actin8* gene (pg fungal DNA per 10^8 copies *actin8*). E, Phenotype of a typical diseased plant at 28 dpi.

The "stunted phenotype" as quantified by measurements of the projected leaf area (Figure 5A) was observed at 21 days post infection (dpi). At this time point, differences in chlorophyll content also became evident (Figure 5B). This measurement was done by Saskia Flörl (AG Polle). However, as visible chlorosis was primarily affecting older leaves (Figure 5E), the overall effect on chlorophyll content was rather low. Reduction of fresh weight was observed at 35 dpi (Figure 5C) in this experiment, but occurred concomitantly with the reduction in leaf area in other experiments (e.g. Figure 12 and Figure 13). Similar symptoms have been observed in oilseed rape (Zeise and von Tiedemann, 2002) and in *A. thaliana* infected with the *V. longisporum* isolate VdBob.70 (Veronese et al., 2003). Electrolyte leakage was not detected up to 35 dpi (Saskia Flörl (AG Polle), personal communication). Fungal DNA could not be amplified in the shoot at 10 dpi but since 14 dpi (Figure 5D). As frequently observed, stunting affected one half of the rosette more vigorously than the other half (Figure 5E).

In general, infection of *Arabidopsis* plants with biotrophic pathogens leads to the activation of salicylic acid (SA)-mediated defense responses, whereas infection with necrotrophic pathogens elicits jasmonic acid/ethylene (JA/ET)-dependent responses (Glazebrook, 2005).

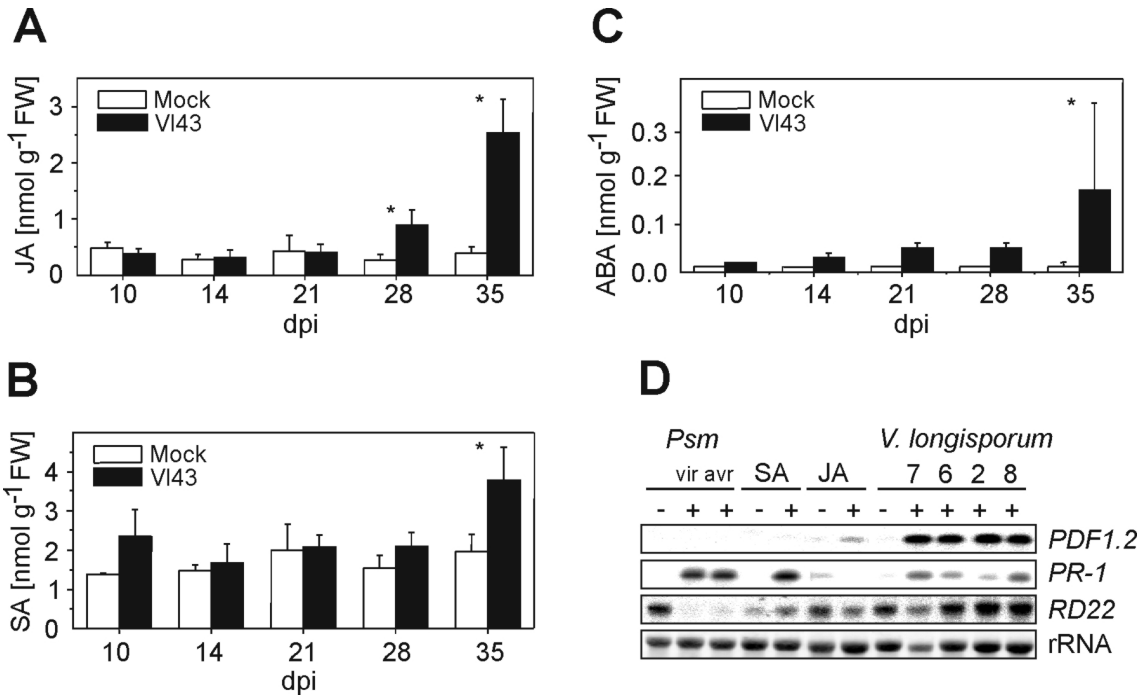


Figure 6: Phytohormone levels and corresponding marker transcripts in *V. longisporum*-infected *A. thaliana* leaves
Levels of phytohormones of leaves of mock- and *V. longisporum* 43-infected *Arabidopsis* plants were determined (same material as used in Fig. 1 B, C, D) by Jan-Gerrit Carsjens (AG Feußner). Error bars indicate the standard deviation of the mean values; asterisks indicate significant differences ($P \leq 0.05$) between mock- and *V. longisporum* (Vl43)-infected samples. Values are given in nmol g^{-1} fresh weight

(FW). A, jasmonic acid (JA); B, salicylic acid (SA); C, abscisic acid (ABA); D, expression of *PDF1.2*, *PR-1* and *RD22* of *A. thaliana* leaves at 35 dpi. Numbers above the line indicate different individual plants. For comparison, RNA from plants infected with avirulent or virulent strains of *Pseudomonas syringae pv maculicola* (*Psm*) (30 hours) and from plants treated with 1 mM SA (12 hours) or 50 µM JA (48 hours), respectively, was loaded on the gel. RNA was isolated and subjected to Northern blot analysis. Hybridization was carried out with radioactively labeled probes for the indicated genes. rRNA is displayed as a loading control.

The measurement of phytohormones levels was done by Jan-Gerrit Carsjens (AG Feußner). At least in shoots, JA levels did not start to increase until 28 dpi, and a further increase was observed at 35 dpi (Figure 6A). Significantly increased SA levels were observed only at 35 dpi (Figure 6B). The amounts of abscisic acid (ABA), which is another plant hormone that influences plant-pathogen interactions (Anderson et al., 2004; Ton et al., 2005), also increased towards the end of the experiment (Figure 6C). Expression of the SA marker gene *PATHOGENESIS-RELATED-1* (*PR-1*; Lebel et al., 1998) and the JA/ET marker gene *PDF1.2* (Penninckx et al., 1998) corresponded to the increase of the inducing phytohormones. No detectable pathogen-induced activation of either gene was observed before 28 dpi. At 28 dpi, *PR-1* and *PDF1.2* transcript levels started to increase (Figure 6D). Even at 35 dpi, induction of *PR-1* after *V. longisporum* infection was not as efficient as after treatment of plants with either virulent or avirulent strains of *Pseudomonas syringae pv maculicola (Psm)* for 30 h (Figure 6D). In contrast, *PDF1.2* induction, which requires JA and ET (Penninckx et al., 1998), was quite pronounced when compared to mRNA levels induced by treatment with JA alone. The ABA-responsive gene *RD22* (Yamaguchishinozaki and Shinozaki, 1993) was not induced at 35 dpi, although higher levels of ABA were detected (Figure 6C and D).

3.2 Identification of *Verticillium longisporum*-induced genes (VliGs) at 18 dpi

To investigate whether *V. longisporum* elicits plant responses before obvious disease symptoms become visible, the transcriptome was monitored by using a whole genome oligonucleotide array covering 26,173 protein coding genes. Leaf material from three mock- and three *V. longisporum*-infected plants was harvested at 18 dpi for RNA extraction. At this time point, the levels of the phytohormones JA, SA and ABA were not increased compared to the controls (Figure 6), so that secondary effects due to the activation of the corresponding signaling cascades were minimized. As plants were kept under short day conditions, the early flowering phenotype observed under long day conditions was not yet evident (Veronese et al., 2003). Thus, it was possible to compare RNA from vegetative grown plants rather than comparing RNA from flowering and

non-flowering plants. RNAs were first transcribed into cDNAs and then into biotinylated complementary RNAs that were hybridized to *Arabidopsis* oligonucleotide microarrays fabricated by the University of Arizona. Table 2 lists genes with more than 2.5-fold (log_2) altered expression levels. The estimated false discovery rate (FDR) of this candidate set is 10 %.

Table 3: List of genes which expression is affected (FDR = 0.1, Fold Expr ≥ |2.5|) upon infection of *A. thaliana* with *V. longisporum* isolate 43 at 18 dpi (analyzed material: rosettes)

	AGI-Code	Description	Expr [a]
1	**Cell wall / defence**		
1.1	**Polycarbohydrate modifying enzymes**		
	At1g23460	putative polygalacturonase	3.7
	At4g19420	pectinacetylesterase	3.0
	At3g61490	putative polygalacturonase (EC 3.2.1.15)	3.0
	At5g03260	putative laccase	2.8
	At5g09760	pectinesterase	2.8
	At1g14890	invertase/pectin methylesterase inhibitor	2.6
	At4g23500	putative polygalacturonase (EC 3.2.1.15)	2.5
1.2	**Lipid transfer proteins**		
	At3g53980	$LTP_{(1)}$	9.9
	At3g18280	$LTP_{(2)}$	7.2
	At4g33550	$LTP_{(3)}$	5.4
	At5g05960	$LTP_{(4)}$	4.5
1.3	**Peroxidase**		
	At2g37130	peroxidase 21 (EC 1.11.1.7)	8.7
	At4g37520	peroxidase 50 (EC 1.11.1.7)	4.3
	At5g64120	peroxidase	4.1
	At4g33420	class III peroxidase ATP32	4.1
1.4	**Glycine-rich proteins**		
	At2g05540	glycine-rich protein	4.9
	At4g01985	similar to glycine-rich protein	3.9
	At4g30460	glycine-rich protein	3.5
2	**Aquaporins**		
	At2g37170	PIP2.2	6.9
	At3g54820	PIP2.5	6.6
	At4g17340	TIP2.2	6.4
	At2g36830	TIP1.1	3.2

At1g01620	PIP1.3	3.0

3 Oxidative stress

3.1 Peroxidases (see 1.2)

3.2 Heavy metal detoxification

At1g07610	MT1C	6.8
At1g07600	MT1A	3.9

4 Biotic stress

At4g35770	SEN 1	4.5
At4g23690	disease resistance-responsive family protein	2.8

5 Proteins related to degradation

At1g20160	subtilase family protein	5.0
At2g45170	autophagy 8e (APG8e)	2.6
At1g10110	AtFBX7	3.8

6 Signal transduction

6.1 Proteins related to Ca^{2+} signalling

At3g50770	calmodulin-related protein	4.4
At2g41410	putative calmodulin-like protein	3.7
At5g55530	C2 domain-containing protein low similarity to cold-regulated gene SRC2 *Glycine max* GI:2055230; contains Pfam profile PF00168: C2 domain	2.9
At2g46600	calcium-binding protein, putative similar to EF-hand Ca^{2+}-binding protein CCD1 *Triticum aestivum* GI:9255753	2.5

6.2 Others

At4g35750	Rho-GTPase-activating protein-related	3.1
At1g21000	zinc-binding family protein	2.5

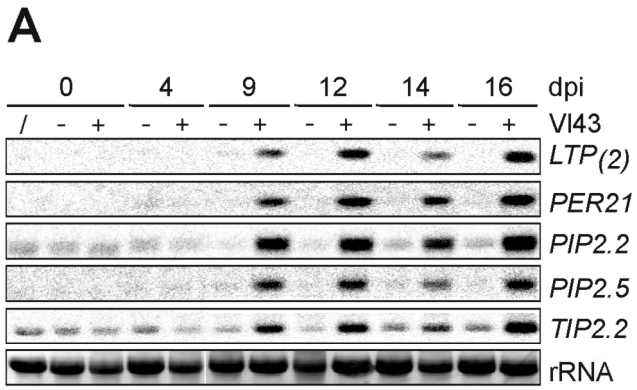
7 Others

At1g20070	expressed protein	8.3
At4g27450	similar to ARG10	4.5
At1g07590	pentatricopeptide	4.3
At1g65500	expressed protein	4.3
At1g64360	expressed protein	3.8
At2g20670	expressed protein	3.3
At4g14020	rapid alkalinization factor (RALF) family protein	2.9

At3g13310	DNAJ heat shock N-terminal domain-containing protein similar to J11	2.9
At5g20250	raffinose synthase family protein	2.9
At4g36040	DNAJ heat shock N-terminal domain-containing protein; identical to DNAJ heat shock protein J11	2.7
8	**Down regulated genes**	
At3g16530	legume lectin family protein	-3.4
At3g23810	putative adenosylhomocysteinase (EC 3.3.1.1)	-2.6

[a] Numbers show the magnitude of change (log_2) between mock and fungal treatments; positive values represent up-regulation, negative values represent down-regulation in *V. longisporum*-infected plants (Fold Expr ≥ |2.5|). The estimated FDR of this candidate set is 10 %.

To verify the data obtained from the microarray experiment, the expression profiles of five selected genes (*lipid transfer protein LTP(2)* (At3g18280), *peroxidase21 PER21* (At2g37130), and three aquaporins *PIP2.2* (At2g37170); *PIP2.5*, (At3g54820); *TIP2.2* (At4g17340)) were assessed by Northern blot analysis of plant material harvested from an independent time course experiment (Figure 7A). Re-hybridization of the blot shown in Figure 6 documented exemplarily for *PER21* that *Verticillium*-induced genes (VliGs) were only slightly induced after infection with *Psm*. SA or JA treatment had a rather negative than a positive effect on *PER21* expression (Figure 7B), supporting the idea that VliG expression is not connected to the classical defense pathways.



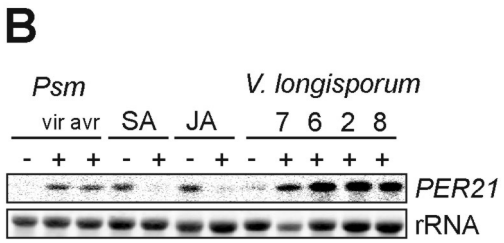


Figure 7: Expression analysis of five selected *V. longisporum*-induced genes in mock- and *V. longisporum*-infected *Arabidopsis* plants
A, Four-week-old plants grown on a layer of sand/seramis were inoculated with *V. longisporum* isolate 43 following a root-dip procedure. At each of the indicated time points (dpi), RNA from a whole rosette of one individual plant was extracted and subjected to the Northern blot analysis. Hybridization was carried out with radioactively labeled probes for the indicated genes. B, Rehybridization of the blot shown in Figure 6 with probe *PER21*. rRNA is displayed as a loading control.

3.3 Functional classification of genes differentially expressed after infection with *Verticillium longisporum* at 18 dpi

Of the 50 *V. longisporum*-dependent genes, that were more than 2.5-fold (log_2) up- or down-regulated, 45 were annotated by The *Arabidopsis* Information Resource (TAIR; Rhee et al., 2003), whereas five were "expressed" genes. The *V. longisporum*-dependent genes were classified into 8 groups (Table 3). Forty-eight genes were up-regulated (*V. longisporum*-induced genes: VliGs; groups 1 to 7) and two genes were down-regulated (group 8). Eighteen of the 48 up-regulated genes are potentially related to modification of pectin in the cell wall (group 1). Group 2 contains four aquaporins. Group 3 and 4 encompass genes involved in oxidative or biotic stress. Group 5 classifies genes involved in degradation processes. In group 6, which contains genes involved in signal transduction, four genes related to Ca^{2+}-signaling were found. Genes with other or unknown functions were classified in group 7.

3.4 Contribution of known stress signaling pathways to the expression of VliGs

Arabidopsis mutants deficient in SA- and JA synthesis *sid2-2* (Nawrath and Metraux, 1999; Wildermuth et al., 2001), *dde2-2* (von Malek et al., 2002) and ET signaling/perception (*ein2-1;* Guzman and Ecker, 1990) were infected with *V. longisporum 43.*

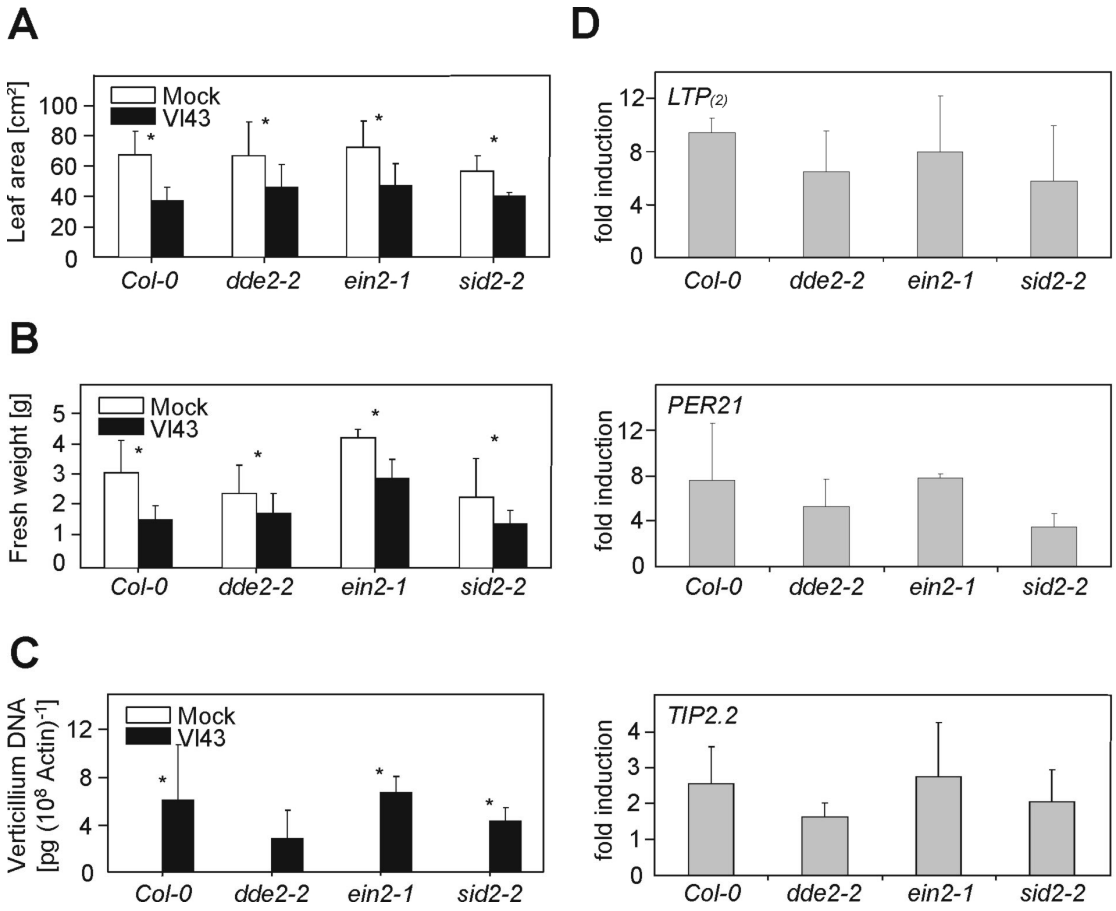


Figure 8: Pathophenotype, fungal propagation and expression of selected VliGs in known stress signaling pathway mutants
Four-week-old mutant (*dde2-2, sid2-2, ein2-1*) or wildtype plants (Col-0) were inoculated with *V. longisporum* isolate 43 following the root-dip procedure. A, Leaf area, B, fresh weight, C, fungal DNA and D, gene expression were determined at 35 dpi. The amount of fungal DNA (C) was determined by real-time PCR using *Verticillium*-specific primers and normalized for the amount of plant DNA using the *actin8* gene (pg fungal DNA per 10^8 copies *actin8*). Data indicate mean values of the means of three independent experiments, each including 15-20 uninfected and infected plants. Error bars indicate the standard deviation. Asterisks indicate significant differences at $P \leq 0.05$ between mock- and *V. longisporum* (Vl43)-infected plants. D, Expression analysis was done by qRT-PCR with *UBQ5* gene as a reference. The y-axis indicates mean values of two experiments and the error bars show the standard deviation.

As documented in Figure 8A and B, all of these mutants revealed the typical *V. longisporum*-induced symptoms (reduced leaf area, reduced fresh weight). Fungal propagation was also similar to wildtype plants (Figure 8C). As expected from the late increase of the stress-related phytohormones, qRT-PCR analysis revealed that none of the corresponding hormones is required for expression of the genes *LTP(2), PER21*, and *TIP2.2* (Figure 8D).

To get an impression how all VliGs are affected by the phytohormones an analysis with the Genevestigator V3 (Zimmermann et al., 2005; https:\\www.genevestigator.ethz.ch) was done.

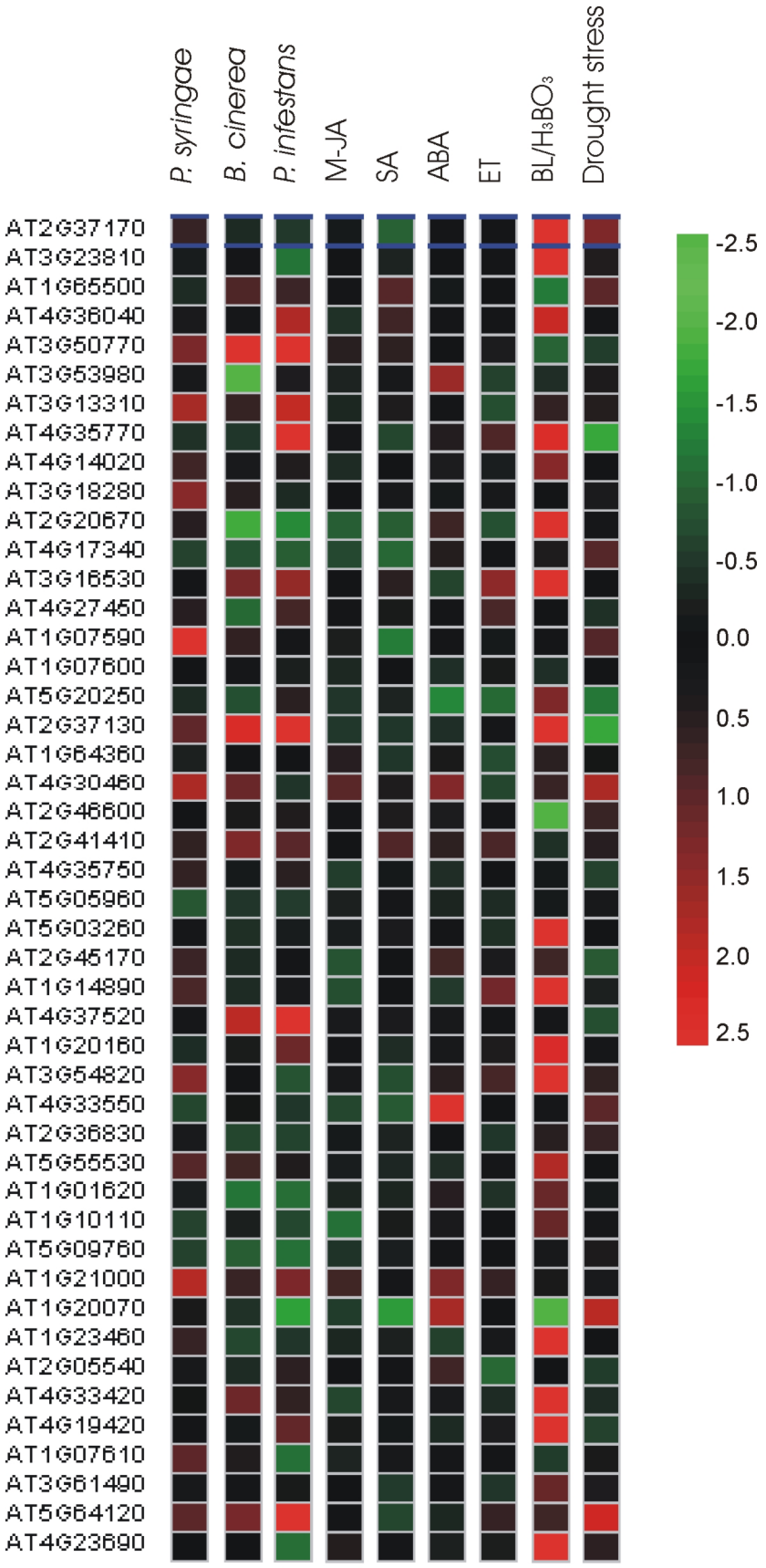


Figure 9: Analysis with Genevestigator V3 of VliGs
Adapted from Genevestigator V3. Genes whose expression is affected 18 dpi upon infection of *Arabidopsis* with *V. longisporum* isolate 43 were analyzed by the Genevestigator V3 (https://www.genevestigator.ethz.ch). Presented are treatments related to the classical defense pathways (jasmonic acid (JA), salicylic acid (SA), ethylene (ET)) and infections with pathogens. Descriptions of the

genes are listed in Table 3, page 45. Color code: lower expression levels are light-green; higher expression levels are light-red (log_2 scale).

The Genevestigator V3 software suite (https://www.genevestigator.ethz.ch) provides categorized quantitative information about elements (genes or annotations) contained in large microarray databases. It is possible to analyse the expression of the VliGs in respond to different stimuli (Zimmermann et al., 2005). The expression pattern differs for individual VliGs within a treatment (Figure 9), so that some of the VliGs are down-regulated, whereas others are up-regulated.

Induction of five aquaporin encoding genes upon infection with *V. longisporum* (Table 3) as well as increase in ABA levels over time (Figure 6C) suggested that the infected plants try to counteract inefficient water transport. However, classical drought- and ABA-responsive genes such as *RD29A* (Nakashima et al., 2006) and *RD22* (Yamaguchishinozaki and Shinozaki, 1993) were not identified by microarray analysis (Table 3). This result was reproduced in an independent experiment (Figure 10).

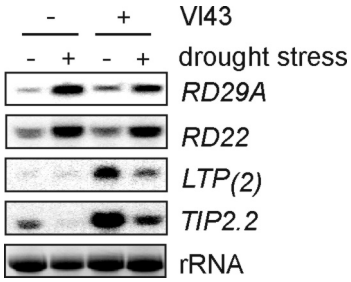


Figure 10: Expression of drought-responsive genes after infection of *Arabidopsis* with Vl43
Four-week-old plants grown on a layer of sand/seramis were inoculated with *V. longisporum* 43 (Vl43) following the root-dip procedure. Plants were grown for further 21 days either with (-) or without (+) regular watering. RNA was isolated and subjected to Northern blot analysis. Hybridization was carried out with radioactively labeled probes for the indicated genes. rRNA is displayed as a loading control.

After 21 days without watering, *RD29A* and *RD22* were induced in both mock and *V. longisporum*-infected plants. However, no induction was observed in *V. longisporum*-infected plants at 21 dpi following regular watering. In contrast, the *V. longisporum*-responsive genes *TIP2.2* and $LTP_{(2)}$ were induced by *V. longisporum* infection but not by drought stress, indicating that at least several of the VliGs shown in Table 3 are not induced by some sort of drought stress potentially inflicted upon the plant by the fungus. Moreover, the *atrbohD/F* mutant, which is impaired in ABA-induced closing of the stomata (Kwak et al., 2003) did not show a more severe disease phenotype than the wild-type plants (Figure 11).

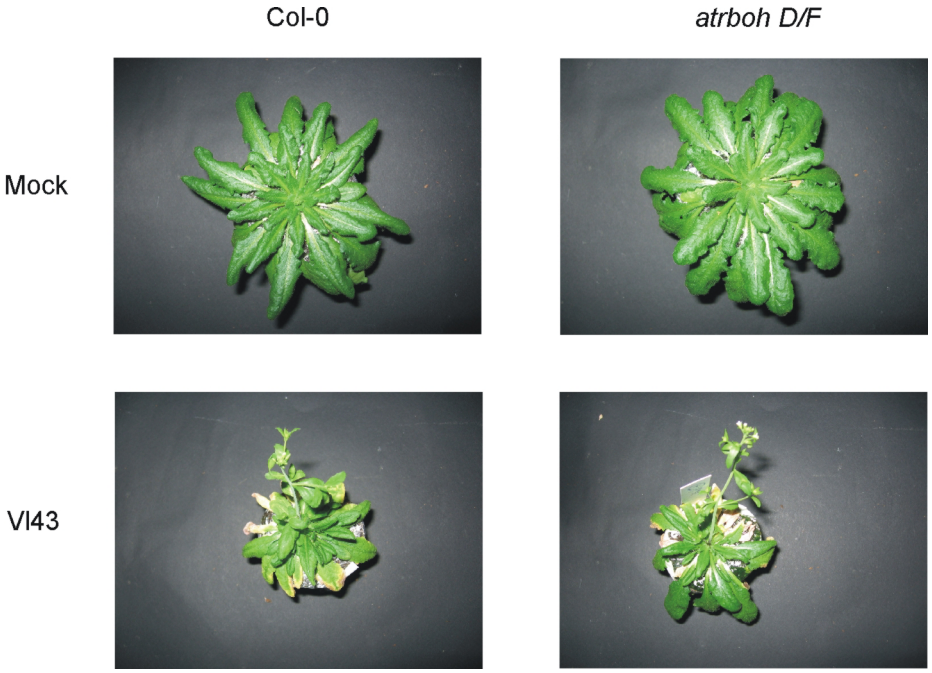


Figure 11: Comparison of Col-0 and *atrbohD/F*
Four-week-old plants grown on a layer of sand/seramis were inoculated with *V. longisporum* 43 (Vl43) following a root-dip procedure. Plants were grown for further 33 days. The *atrbohD/F* mutant is impaired in ABA-induced stomata closing.

3.5 Symptom patterns of *Arabidopsis* after infection with different *Verticillium* isolates

In 2001, Zeise and Tiedemann (Zeise and von Tiedemann, 2001) categorized 24 isolates of *Verticillium dahliae* from various geographical regions and host species into groups. In a dendrogram based on morphological and physiological characteristics of the isolates, *V. longisporum* 43 (Vl43) and *V. longisporum* 18 (Vl18) clustered next to each other. Both isolates showed no differences in the tested parameters (Table 1), such as sporulation rate or conidial length. Vl43 and Vl18 were isolated in one geographical area, Mecklenburg/Germany, from *Brassica napus* as a host plant (Zeise and von Tiedemann, 2001), in 1990 and 1989, respectively. *V. dahliae* 830 (Vd830) clustered as a different species separately from *V. longisporum* isolates (Table 1). Vd830 was isolated in Ohio/USA from *Solanum tuberosum,* as a host plant. Vl43, Vl18 and Vd830 were used for infection of *A. thaliana* Col-0 to investigate whether different isolates elicit differences in the *Verticillium*-induced disease pattern.

Infection with these different *Verticillium* isolates led to an isolate-dependent symptom development (Figure 12). The infection with Vl43 showed the typical fresh weight loss and reduction of the projected leaf area by nearly 50 % compared to the mock-treated plants (Figure 12A and B). Interestingly, the infection with Vl18 did not

induce symptom development and infection with Vd830 led to an intermediate pathophenotype. The reduction of fresh weight and projected leaf area was approximately 75 % compared to the mock-treated plants (Figure 12A and B). Fungal DNA was detected after each infection (Figure 12C), but showed no significant differences among the different strains. The *V. longisporum*-responsive gene $LTP_{(2)}$ was induced by the infection with Vl43 but not by Vl18, whereas Vd830 induced only a very weak expression (Figure 12D).

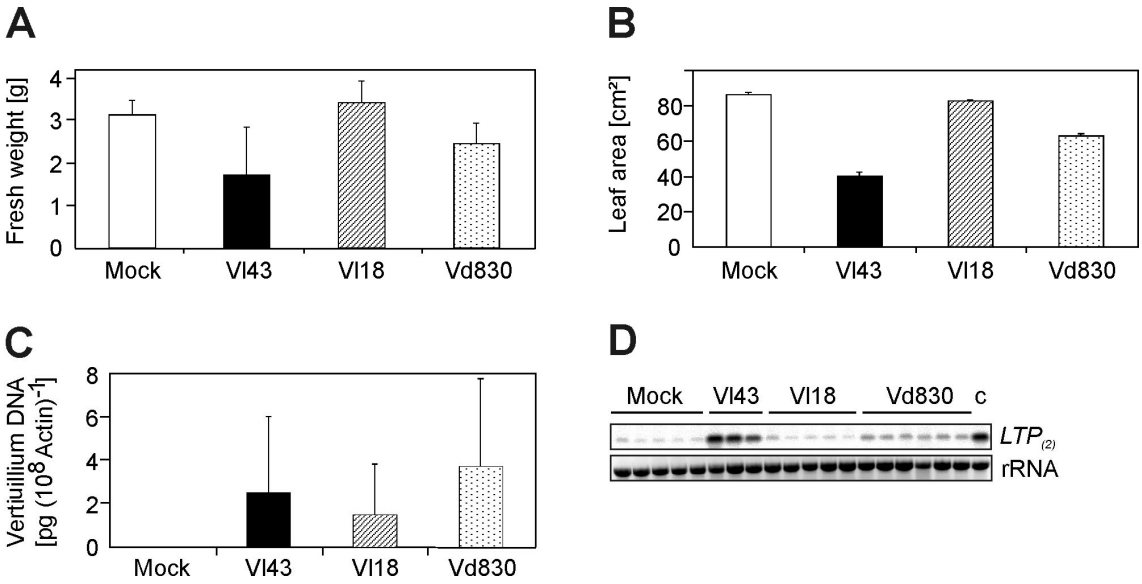


Figure 12: Pathophenotype, fungal propagation and expression of $LTP_{(2)}$ in *A. thaliana* Col-0 infected with different fungal isolates
Four-week-old plants were inoculated with *V. longisporum* 43 (Vl43), *V. longisporum* 18 (Vl18) and *V. dahliae* 830 (Vd830) following a root-dip procedure. A, Projected leaf area, B, fresh weight, C, fungal DNA and D, gene expression were determined at 25 dpi. Fungal DNA (C) was determined by real-time PCR using *Verticillium*-specific primers and normalized for the amount of plant DNA using the *actin8* gene (pg fungal DNA per 10^8 copies *actin8*). Bars indicate mean values of 18 individual plants with error bars depicting the standard deviation (A, B, C). D, Northern blot analysis of mock- and *Verticillium*-infected plants at 25 dpi using $LTP_{(2)}$ as a probe. The results for single individual plants per treatment are shown, exemplarily for the 18 individual plants per treatment that showed the same expression levels. rRNA is displayed as a loading control.

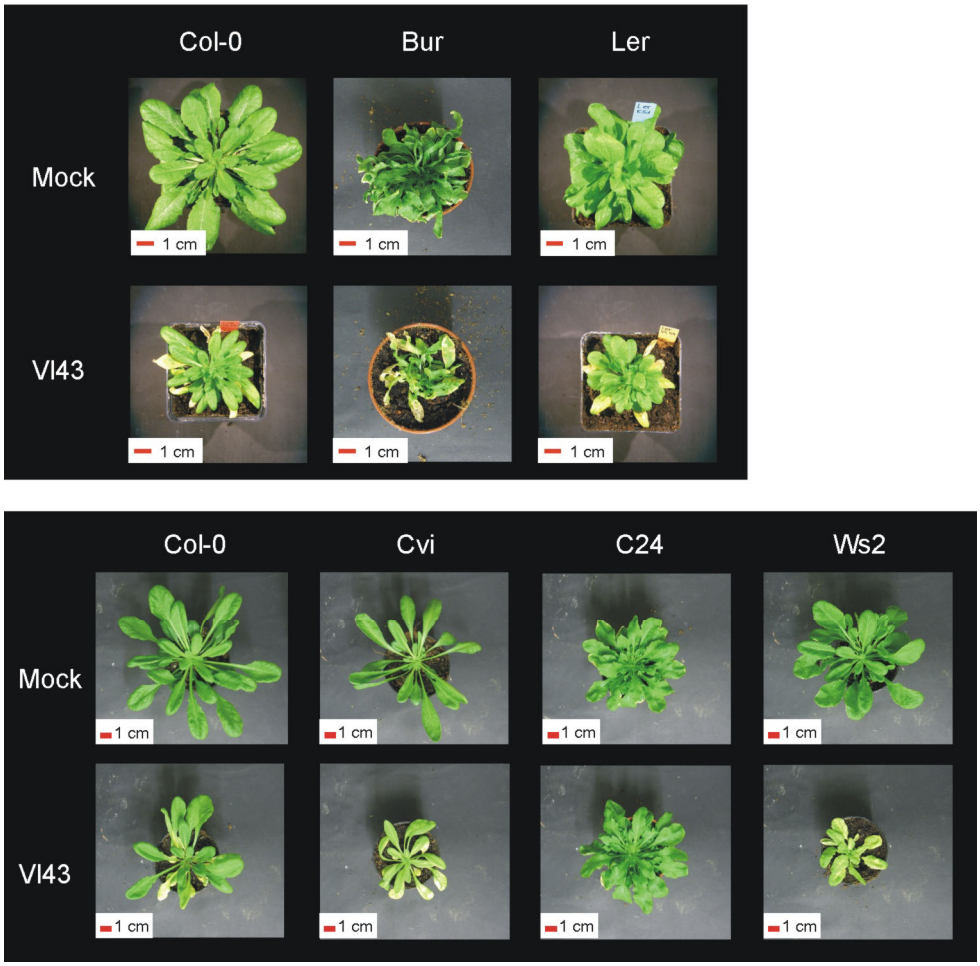
3.6 Symptom patterns after infection of different *Arabidopsis thaliana* accessions with *V. longisporum* 43

To answer the question whether the *V. longisporum*-induced gene expression is dependent on the infected ecotype, different *Arabidopsis* accessions were analyzed after infection with Vl43.

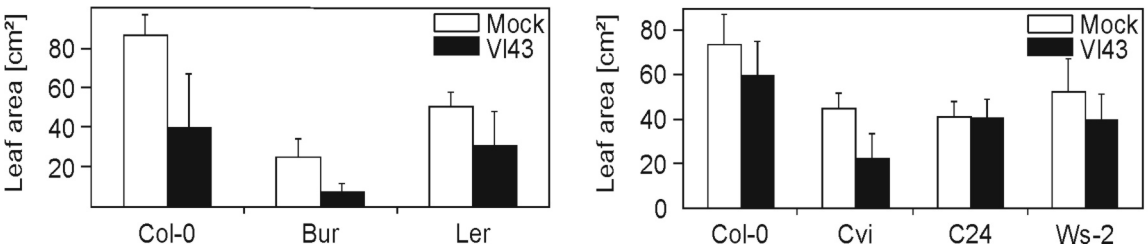
The symptom development of Col-0, Bur, Ler, Cvi, and Ws-2 was comparable with each other (Figure 13A). The projected leaf area and fresh weight levels showed the same decrease compared to the respective mock-treated plants. Interestingly, *A. thaliana* ecotype C24 showed no visible symptom development at 21 dpi (Figure 13B

and C). No *LTP(2)* induction was detectable in the ecotype C24 by Northern blot analysis, while Col-0, Bur, Ler, Cvi, and Ws-2 revealed a clear *LTP(2)* induction (Figure 13D). Figure 13 displays two independent infections. The first was done with Col-0, Bur, and Ler; the second was done with Col-0, Cvi, C24, and Ws-2. Comparisons of the two Col-0 infections demonstrate that the second infection leads in general to less symptom development.

A



B



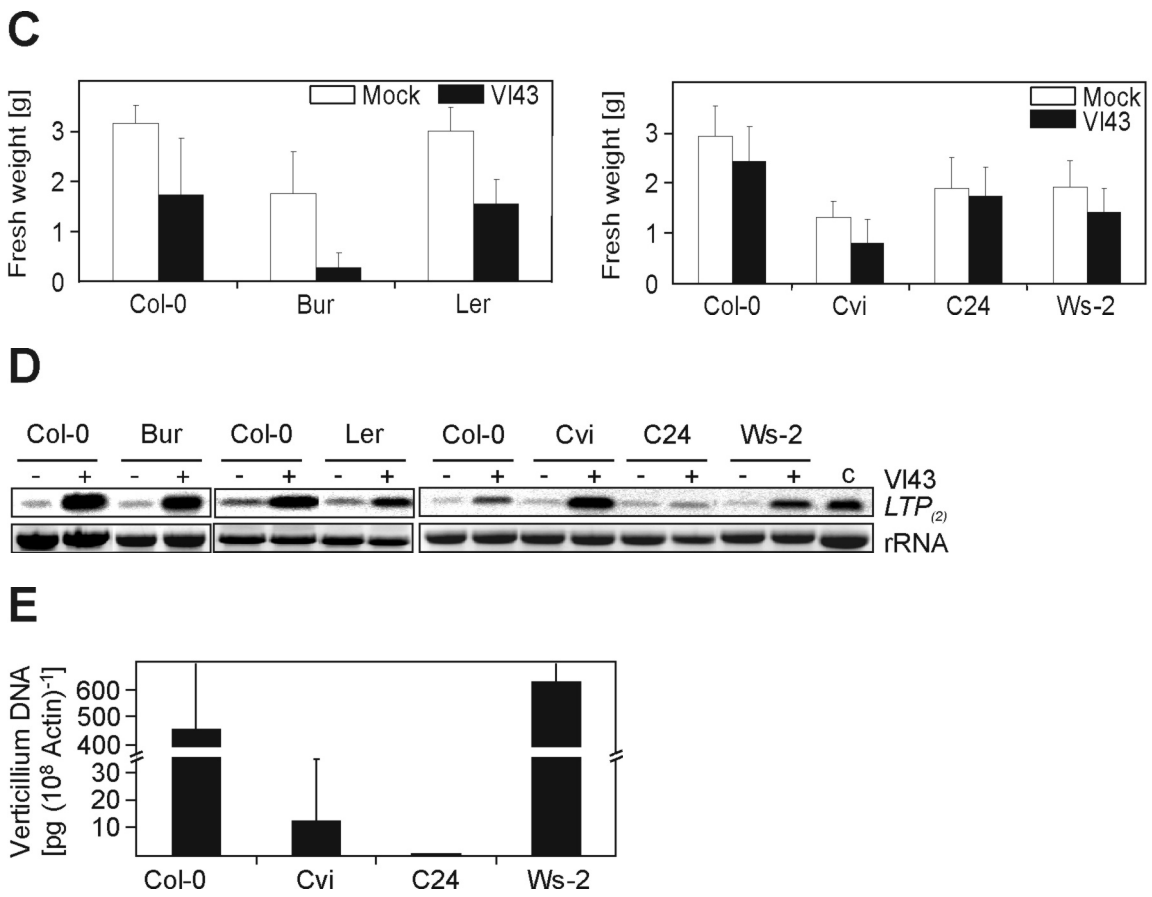


Figure 13: Pathophenotype, fungal propagation and expression of $LTP_{(2)}$ in different *Arabidopsis* ecotypes

Four-week-old Col-0, Bur, Ler, Cvi, C24 and Ws-2 plants were inoculated with *V. longisporum* 43 (Vl43) following the root-dip procedure. A, Phenotype of a typical diseased plant B, leaf area, C, fresh weight, D, gene expression, and E, fungal DNA was determined at 21 dpi. D, Leaf material of 18 individual plants was collected and RNA was isolated and subjected to Northern blot analysis. Hybridization was carried out with radioactively labeled probes for the indicated gene. rRNA is displayed for loading control. E, Fungal DNA was determined by real-time PCR using *Verticillium*-specific primers and normalized for the amount of plant DNA using the *actin8* gene (pg fungal DNA per 10^8 copies *actin8*). Mean values of 18 individual plants are displayed for B, C, D, and E, respectively. Error bars indicate the standard deviation.

Fungal propagation in different ecotypes was analyzed at two different time points. At 14 dpi C24 contained an amount of fungal DNA (Figure 14) comparable to that of other ecotypes (only 5 individual plants were tested). At 21 dpi the concentration of fungal DNA did not change in the ecotype C24 (Figure 13E), whereas in the other tested accession, the fungus spread and reached higher concentrations (18 individual plants were tested).

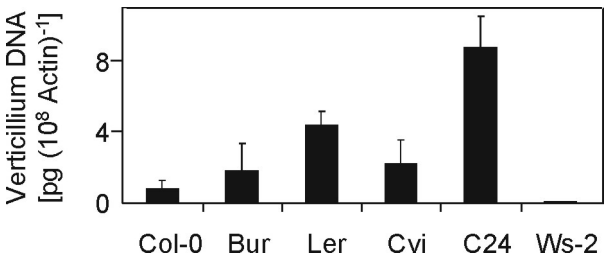


Figure 14: Fungal propagation in different *Arabidopsis* ecotypes at 14 dpi
Four-week-old Col-0, Bur, Ler, Cvi, C24 and Ws-2 plants were inoculated with *V. longisporum* 43 (Vl43) following the root-dip procedure. At 14 dpi, fungal DNA was determined by real-time PCR using *Verticillium*-specific primers and normalized for the amount of plant DNA using the *actin8* gene (pg fungal DNA per 10^8 copies *actin8*). Data indicate mean values of 5 individual plants. Error bars indicate the standard deviation.

3.7 Transcript levels of VliGs in aerial parts of *A. thaliana* at different time points

The aerial parts of *A. thaliana* Col-0 were investigated to determine the earliest time point and the localization of VliGs expression induced by *Verticillium longisporum* 43. Expression of VliGs started as early as 6 dpi and continued until the end of the experiment at 32 dpi (Figure 15A). Higher levels of transcription were induced in petioles than in leaf lamina (Figure 15A). To obtain a more detailed spatial and temporal image of the expression pattern of VliGs, the promoters of $LTP_{(2)}$, *PER21* and *PIP2.2* were cloned upstream of the *FIREFLY LUCIFERASE* reporter gene and the constructs were stably transformed into *Arabidopsis* plants (M. Dilcher, C. Thurow, unpublished). Four transgenic lines per reporter-construct were tested. As documented in Figure 15B, the promoters are still silent at 2 dpi, but are activated in the vascular tissues at 5 dpi. Interestingly, no fungal DNA was observed in the aerial parts of the plants at 10 days past infection (Figure 5D). This result was independently confirmed by placing surface sterilized petioles of 12 plants per treatment on potato dextrose agar. In this assay, fungal growth was only observed at later time points of infection (Figure 15C). Therefore, it is plausible to assume that a xylem-mobile signal elicits expression of VliGs.

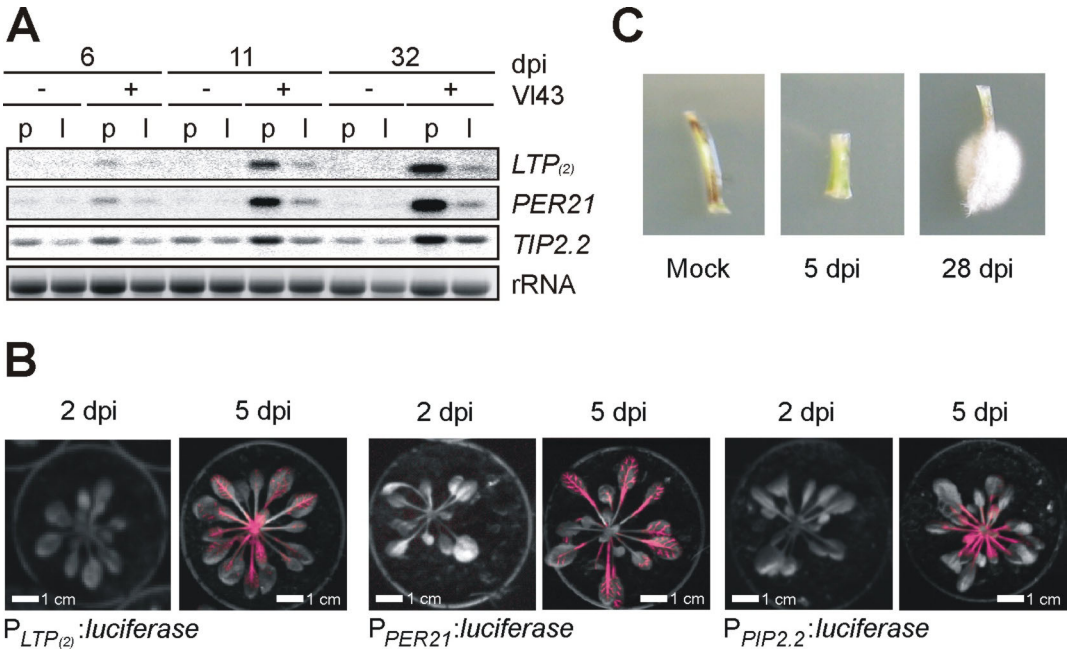


Figure 15: VliGs and fungal propagation at early time points after infection
Four-week-old plants were inoculated with *V. longisporum* 43 (Vl43) following a root-dip procedure. A, Transcript levels of *LTP$_{(2)}$, PER21,* and *TIP2.2* in petioles (p) and leaf lamina (l) at 6, 11 and 32 dpi. B, bioluminescence of transgenic plants encoding the *FIREFLY LUCIFERASE* reporter gene driven by the promoters of *LTP$_{(2)}$*, *PER21*, and *PIP2.2*. Images were taken at 2 and 5 dpi, using the low light imaging system from Hamamatsu, Japan. C, Petioles with out-growing fungus. At 5 and 28 dpi petioles of 12 individual plants were surface sterilized and incubated further 5 days on PDA plates. The mock petioles were harvested at 28 dpi and also incubated for additional 5 days on PDA plates.

To further investigate the characteristics of this putative elicitor an experiment was performed in collaboration with the group of Prof. Karlovsky. *V. longisporum* (Vl43) was grown in a simulated xylem medium for 4 weeks. The culture supernatant was extracted with ethyl acetate and the aqueous phase was partitioned with n-butanol (see 2.2.8.1). The n-butanol and the remaining aqueous fraction were fed through petioles of four-week-old reporter-plants and gene expression was monitored after 3 and 7 days, respectively (Figure 16; #3 butanol extraction of a Vl43 culture; #4 aqueous fraction of a Vl43 culture seven days after treatment; #5 aqueous fraction of a Vl43 culture three days after treatment). As controls, simulated xylem medium without any fungus was subjected to the same extractions (Figure 16; #1 butanol control; #2 aqueous fraction control). *P$_{PER21}$:luciferase* expression was observed at 7 dpi accompanied by a yellowing of the leaves (Figure 16, #4). The putative elicitor remained in the aqueous fraction, indicating that it must be highly polar.

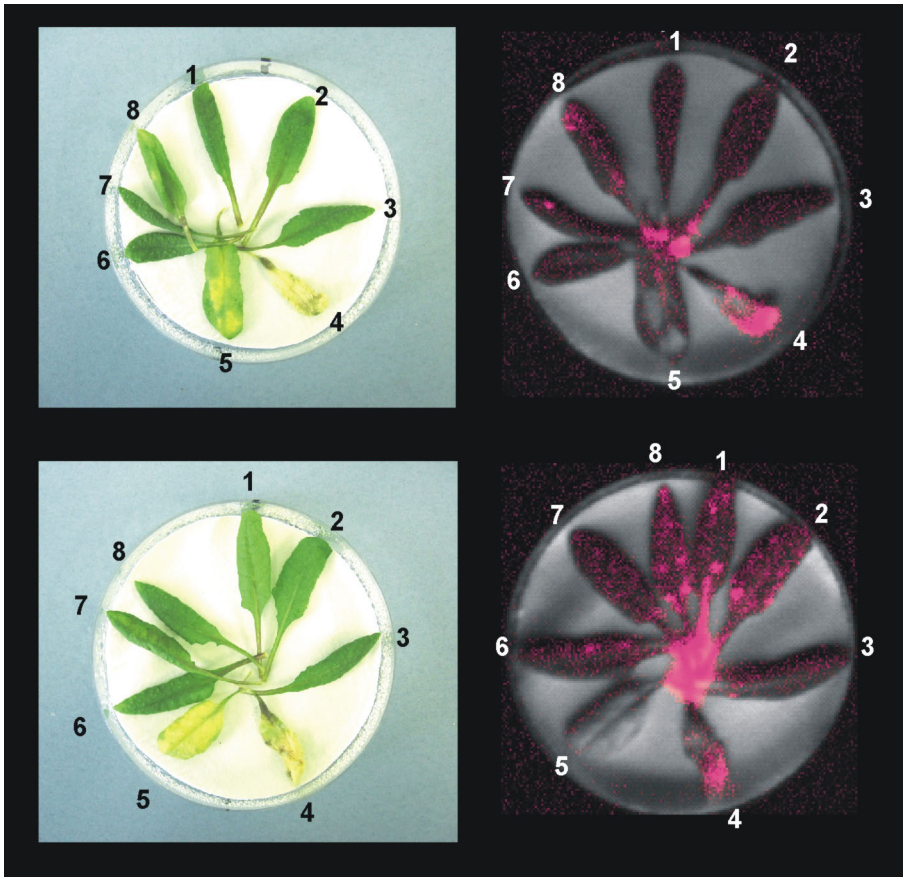


Figure 16: P_{PER21}*:luciferase* plants as biosensors for a putative elicitor
Leaves of four-week-old transgenic plants encoding the *FIREFLY LUCIFERASE* reporter gene driven by the promoter of *PER21* were cut and the petioles were fed with the following solutions: 1, butanol control; 2, aqueous fraction control; 3, butanol Vl43; 4, aqueous fraction Vl43 (7 days); 5, aqueous fraction Vl43 (3 days); 6, tap water; 7, *Brassica napus* xylem sap control (7 days); 8, *Brassica napus* xylem sap Vl43 (7 days). Two biological replicates are shown. On the left: images were taken by a photo camera. On the right: Images were taken with the low light imaging system (Hamamatsu, Japan) to detect the bioluminescence of the transgenic line.

In addition to the fractionated *Verticillium*-culture supernatant, xylem-sap of non-infected and infected *Brassica napus* plants, kindly provided by the group of Prof. Tiedemann, was fed to the leaves (Figure 16; #7 and #8). It was possible to initiate the yellowing of the leaf in one of the two tested plants (Figure 16, #8 upper panel). Also, very weak P_{PER21}*:luciferase* expression was detected as compared to the control leaf (Figure 16, #7 upper panel).

3.8 Identification of *Verticillium longisporum*-induced genes at 5 dpi

As documented in Figure 15B, VliGs induction occurs in the vascular tissues at 5 dpi. At this time point no fungal DNA was detectable in the aerial parts of the plant (Figure 5D and Figure 15C). To investigate which genes were induced after the early colonizing of the roots by Vl43, the transcriptome of the infected plants was monitored

using a whole genome oligonucleotide array covering 26,173 protein coding genes. Thirty six plants per treatment were combined at 5 dpi and the RNA was isolated. Northern blot analysis confirmed the induced expression of $LTP_{(2)}$ (Figure 17) as already evident in P_{PER21}:*luciferase* plant (Figure 16).

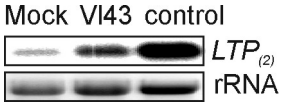


Figure 17: *V. longisporum*-induced gene expression in petioles at 5 days after infection
Northern blot analysis of mock- and *Verticillium*-infected petioles was done with the indicated probe. RNA was harvested from a pool of 36 plants (petioles) at 5 dpi. Control is a sample of a *V. longisporum* 43 (Vl43)-infected plant at 28 dpi. rRNA is displayed as a loading control.

RNAs were first transcribed into cDNAs and then into biotinylated complementary RNAs. Hybridization was done with *Arabidopsis* oligonucleotide microarrays fabricated by the University of Arizona. Genes with more than 2.0-fold (log_2) altered expression levels are listed in Table 4. The estimated FDR of this candidate set is 10 %.

Table 4: List of genes which expression is affected (FDR = 0.1; Fold Expr ≥ |2|) upon infection of *A. thaliana* with *V. longisporum* at 5 dpi (analyzed material: petioles)

	AGI-Code	Description	Expr[a]
1	**Cell wall**		
1.1	**Cellulose and pectin-containing cell wall loosening**		
	At3g45970	EXLA1	2.68
	At3g45960	EXLA3	2.24
	At2g18660	EXPR3	3.93
	At4g16563	aspartyl protease family protein	2.08
	At5g57560	TCH4; hydrolase, acting on glycosyl bonds	2.86
1.2	**Lipid transfer proteins**		
	At3g53980	$LTP_{(1)}$	3.69
	At4g33550	$LTP_{(3)}$	2.82
1.3	**Peroxidase**		
	At4g33420	class III peroxidase ATP32	3.86
1.4	**Glycine-rich proteins**		
	At2g05510	glycine-rich protein	2.19
	At2g15780	glycine-rich protein	4.01
	At2g21660	GRP7	2.71
2	**Phytohormone related**		
2.1	**Response to JA and wounding**		
	At3g45140	LOX2	2.01
	At4g23600	CORI3	2.03
	At1g72520	lipoxygenase, putative	2.15
	At5g24770	VSP2	2.78
2.2	**JA biosynthetic process**		
	At3g25780	AOC3	2.09
2.3	**Response to ABA**		
	At3g02480	ABA-responsive protein-related	2.24
2.4	**Auxin**		
	At2g33830	dormancy/auxin associated family protein	2.01

Signal transduction

3.1	**Transcription factors**		
	At1g21910	AP2 domain-containing transcription factor family protein (AP2, AtERF#012)	2.05
	At4g28140	AP2 domain-containing transcription factor, putative (AP2-TF, AtERF#054)	4.16
	At4g37260	MYB73	2.07
	At5g61600	ethylene-responsive element-binding family protein	2.18
	At5g22570	WRKY38	2.70
	At3g46640	PCL1	2.78
3.2	**Transkription regulators**		
	At5g24470	APRR5	2.87
3.3	**Proteins related to Ca^{2+} signaling**		
	At5g67480	BT4	2.02
	At5g39670	calcium-binding EF hand family protein	2.11
4	**Response to biotic or abiotic stress**		
	At2g34930	disease resistance family protein	2.52
	At2g39030	GNAT family protein (N-acetyltransferase activity)	2.60
	At4g17470	palmitoyl protein thioesterase family protein	2.95
	At4g01700	chitinase, putative	3.38
5	**Proteins related to degradation**		
	At1g20160	subtilase family protein	3.00
6	**Others**		
	At1g35140	PHI-1 (PHOSPHATE INDUCED 1)	2.63
	At1g09932	phosphoglycerate/bisphophoglycerate mutase-related	2.17
	At5g23660	MTN3 (homolog of the *Medicago* nodulin MTN3)	2.11
	At3g28290	AT14A (similar to protein of unknown function of *Medicago)*	2.06
	At1g52690	late embryogenesis abundant protein	2.43
	At1g76790	o-methyltransferase family 2 protein	2.07
	At3g50930	AAA-type ATPase family protein	2.37

At3g10280	fatty acid elongase 3-ketoacyl-Co A synthase	2.31
At2g34600	similar to unknown protein *Medicago truncatula*	3.31
At2g28570	unknown protein	2.91
At1g11210	similar to fiber expressed protein *Gossypium hirsutum*	2.46
At2g38465	unknown protein	2.17
At1g19180	similar to PNFL-2 *Ipomoea nil*	2.10
At5g42900	similar to hypothetical protein DRAFT *Medicago truncatula*	2.79
At5g13220	similar to P0482D040 *Oryza sativa*	2.33
7	**Down-regulated genes**	
At5g57760	contains interpro domain helix-loop-helix DNA-binding	-2.20
At2g08986	similar to TTN-1 *Caenorhabditis elegans*	-3.20
At3g14395	unknown protein	-2.40
At5g35480	unknown protein	-2.50
At2g07981	similar to hypothetical protein G09238 *Caenorhabditis briggsae*	-3.00
At3g55240	similar to unknown protein *Oryza sativa*	-2.01
At1g77870	MUB5	-2.15
At5g58770	dehydrodolichyl diphosphate synthase, putative	-2.27

[a] Numbers show the magnitude of change (log_2) between mock and fungal treatments; positive values represent up-regulation, negative values represent down-regulation in *V. longisporum*-infected petioles at 5 dpi (Fold Expr ≥ |2|). The estimated FDR of this candidate set is 10 %.

To verify the data obtained from the microarray experiment, the expression profiles of four selected genes (*AP2 domain-containing transcription factor, putative* (*AP2-TF,* At4g28140), *subtilase family protein* (*SFP,* At1g20160), *lipid transfer protein (3)* (*$LTP_{(3)}$,* At4g33550) and *vegetative storage protein2* (*VSP2,* At5g24770)) were assessed by qRT-PCR with plant material harvested from an independent experiment (Figure 18). Even at 6 dpi transcript levels of all tested genes were increased following the infection with *V. longisporum* 43. *AP2-TF* showed a strong induction in the petioles and a weak in the leaf lamina material of the same plants at 6 and 11 dpi (Figure 18A). *SFP* was induced in both petioles and leaf lamina to the same level and the induction increased further from 6 to 11 dpi (Figure 18B). The *$LTP_{(3)}$* gene was strongly induced at 11 dpi (Figure 18C). The induction was stronger in the petioles than in the leaf

lamina. *VSP2* showed the strongest induction in the leaf lamina material after 11 dpi (Figure 18D).

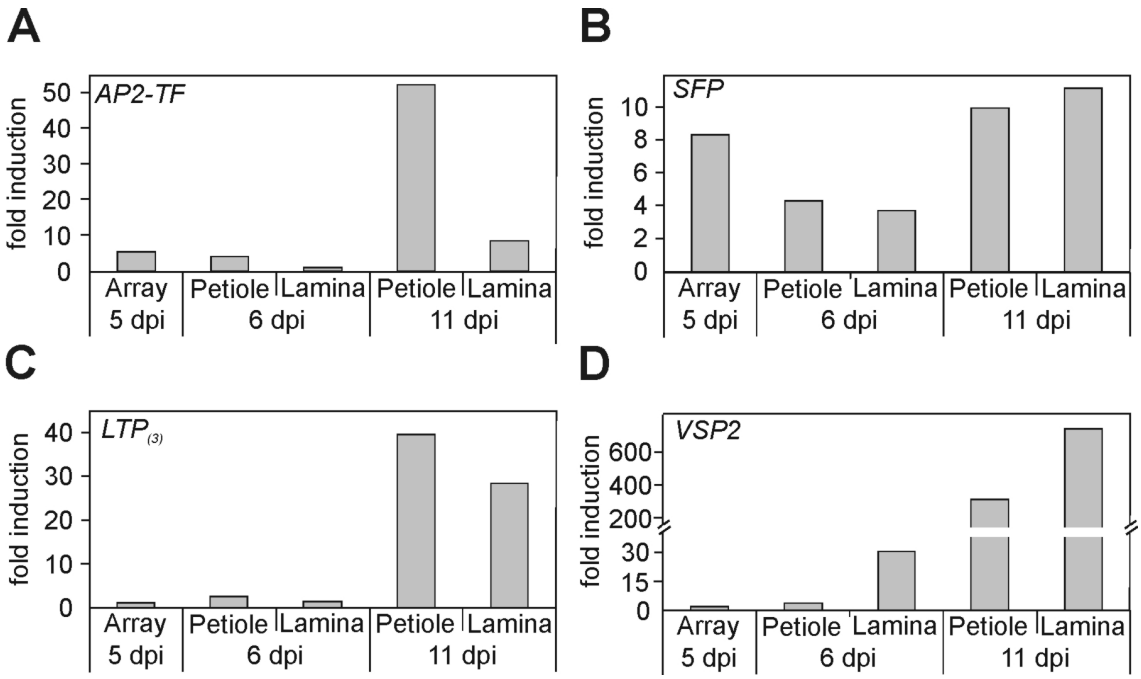


Figure 18: Expression analysis of petioles and leaf lamina at early time points after infection
Expression analysis was done by qRT-PCR with *UBQ5* gene as a reference. Pools of 18 mock- and *V. longisporum*-infected Col-0 plants were harvested at indicated time points. A, Expression of *AP2 domain-containing transcription factor* (*AP2-TF*, At4g28140); B, Expression of *subtilase family protein* (*SFP*, At1g20160*)*; C, Expression of *lipid transfer protein* (*LTP(3)*, At4g33550); D, Expression of *vegetative storage protein 2* (*VSP2*, At5g24770).

3.9 Functional classification of genes differentially expressed after infection with *Verticillium longisporum* at 5 dpi

Fifty-five *V. longisporum*-dependent genes were up- or down-regulated by more than 2-fold (log2) in the petioles at 5 dpi. Fifty-one were annotated by The *Arabidopsis* Information Resource (TAIR, Rhee et al., 2003; http://www.arabidopsis.org/tools/bulk/index.jsp), whereas four were "unknown proteins" genes. The *V. longisporum*-dependent genes were classified into 7 groups using the annotations of TAIR (Table 4). Forty-seven genes were up-regulated (*V. longisporum*-induced genes at 5 dpi: VliG5s; groups 1 to 6) and eight genes were down-regulated (group 8). Of the 47 up-regulated genes 11 (23 %) are potentially related to cell wall modifications (group 1). Group 2 contains genes that are involved in phytohormones synthesis as well as phytohormones signaling by jasmonic acid (JA), salicylic acid (SA), ethylene, abscisic acid (ABA) and auxin. Group 3 to 5 encompass genes involved in signal transduction, response to biotic or abiotic stress, or proteins related to degradation. Genes with other functions were classified in group 6.

3.10 Expression analysis of VliG5s in *A. thaliana* ecotypes Col-0 and C24

As described before, the *Arabidopsis* accession C24 showed no symptom development and no $LPT_{(2)}$ induction after infection with *V. longisporum* 43 at 21 dpi (Figure 14A, B, C and D). To investigate whether the detection of the fungus in early stages of infection leads to ecotype-specific gene expression in *Arabidopsis*, petioles and leaf laminas of 18 Col-0 and C24 plants were analyzed at 5 dpi. Four *V. longisporum* 43-induced genes were examined (Figure 19). No ecotype-specific gene expression was detected in the lamina for *AP2-TF*, $LTP_{(3)}$, and *VSP2*. Interestingly, the C24 petiole tissue showed a much stronger induction of *AP2-TF* and *VSP2* compared to Col-0.

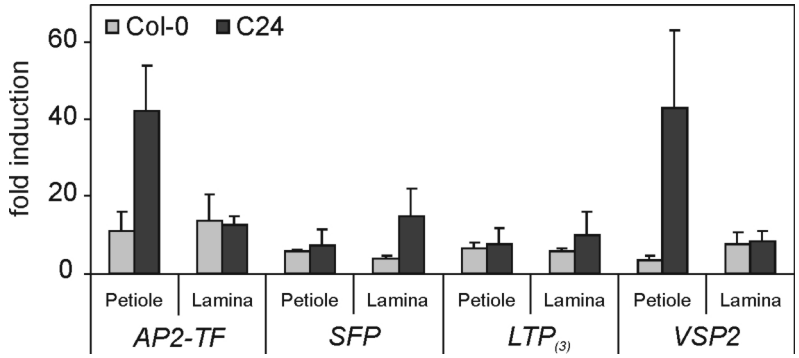


Figure 19: Expression analysis of VliG5s in Col-0 and C24
Expression analysis was done by qRT-PCR with *UBQ5* gene as a reference. Eighteen mock- and *V. longisporum*-infected plants per ecotype were harvested at 5 dpi and combined for the analysis. The y-axis indicates mean values of two technical replicates and the error bars stand for the standard deviation.

4 Discussion

Verticillium dahliae and *V. longisporum* are important soil-borne pathogens causing diseases in a large variety of economically important agronomic and horticultural crops. *Verticillium ssp.* are vascular pathogens that parasitizing the xylem of infected host plants before they macerate the tissue to form resting structures. *V. longisporum* is strictly host specific for *Brassicaceae*. *Arabidopsis thaliana* was chosen as a model plant to investigate the relationship between host and pathogen. The present study focused on gene expression of *Arabidopsis* after infection with *V. longisporum*.

4.1 Disease phenotype of *Verticillium*-infected plants

Consistent with results of earlier experiments (Veronese et al., 2003; Johansson et al., 2006) and observations in oilseed rape (Eynck et al., 2007), *Verticillium*-infected *Arabidopsis* plants showed stunted growth, decreased fresh weight and chlorosis (Figure 5). The described symptom "early flowering time point" (Veronese et al., 2003) was not observed as *A. thaliana* was cultivated under short-day-conditions.

4.2 *V. longisporum*-induced genes may play a role in modifications of the cell wall

Once a vascular fungus has established itself in the xylem, plants can prevent further spread by releasing antimicrobial components or by forming barriers against fungal penetration of cell walls or pits. These barriers can be established by depositing suberin and other coating materials on vascular cell walls (Fradin and Thomma, 2006). In addition, new vessels might be formed to compensate for those being less functional due to clogging by the mycelium. It seems plausible that genes responsible for these processes are induced as soon as the plant detects the presence of a fungus in the xylem. In this thesis, several genes induced by *V. longisporum* (VliGs) were identified at 18 dpi. Their presumed functions are discussed below with respect to their potential defense responses.

Classification of VliGs according to their postulated function revealed a relatively high number of genes (18 out of 48; 37.5%) encoding proteins that are most likely acting in the apoplast. The up-regulation of genes with homologies to pectin acetyltransferases, pectinases, laccases, pectin esterases, plant invertase inhibitors, and

polygalacturonidases (group 1.1, Table 3) suggests that cell wall modifications are initiated after infection with *V. longisporum.* A treatment with brassinolid and borate induces all 18 genes of this group (Figure 9), except for the putative polygalacturonase (At4g23500), which is not analyzed in this experiment (Genevestigator; (Zimmermann et al., 2004)). This treatment evokes differentiation of *Arabidopsis* protoplasts into xylem vessels (Kubo et al., 2005). Since the same set of genes is activated after *V. longisporum* infection, it is likely that *V. longisporum* induces xylem modifications in *Arabidopsis*. Supporting this hypothesis, 27 of the 48 VliGs were also found in the global transcriptome analysis that was undertaken to identify genes regulating secondary xylem development (Ko et al., 2006).

The second subgroup (group 1.2, Table 3) contains four up-regulated genes coding for lipid transfer proteins (LTPs). LTPs are small cysteine-rich lipid-binding proteins that are also being referred to as non-specific LTPs (nsLTPs), since they transfer membrane lipids with no specificity *in vitro*. In plants, LTPs are probably involved in somatic embryogenesis (Toonen et al., 1997), in defense against pathogens (Chassot et al., 2007), in tracheary element differentiation (Garcia-Olmedo et al., 1995), in the formation and reinforcement of waxy cuticle layers in plant surfaces (Orford and Timmis, 2000; Blein et al., 2002), and in long distance signaling (Maldonado et al., 2002). The *V. longisporum*-induced *LTP* genes may play a role in compensating the parasitized xylem vessels. Indeed, two reports relate specific *V. longisporum*-induced LTPs with tracheary element differentiation: (1) $LTP_{(2)}$ (At3g18280) shows similarity to TED4 from *Zinnia elegans* that is induced in immature xylem (Endo et al., 2001). It has been proposed that TED4 functions as a protease inhibitor to protect neighboring cells from proteases. These proteases are released by cells undergoing programmed cell death to generate the vessel elements. (2) Four of the five induced *LTP* genes are expressed during lignification promoting treatments (Rogers et al., 2005) and secondary cell wall modifications of either the hypocotyl or the stem (Brown et al., 2005). Also, the vessel coating, described to occur during the *Verticillium*–host interaction (Fradin and Thomma, 2006), might be supported by the induced LTPs. It is possible that LTPs function in a systemic signaling pathway, which is described for the putative LTP DIR1. It is needed for the transport of an unknown systemic signal necessary for the establishment of systemic acquired resistance after local pathogen attack (Maldonado et al., 2002).

A third group of VliGs includes four peroxidases. Peroxidases have been reported to play a role in tracheary element autolysis and differentiation (Sato et al., 2006) as well as in defense responses (Bindschedler et al., 2006). The peroxidase encoding genes induced by infection with *V. longisporum* are also induced by infection with other fungal pathogens like *Botrytis cinereae* and *Phytophthora infestans* (Figure 1) (Genevestigator; (Zimmermann et al., 2004)). Increased transcription levels of the *Verticillium*-induced peroxidases At2g37130 and At5g64120 have been observed in transgenic *Arabidopsis* plants expressing a fungal cutinase as part of an immune response that is independent of SA, JA and ET (Chassot et al., 2007). Expression of At2g37130 and At5g64120 conveyed partial resistance against the necrotrophic fungus *Botrytis cinereae*, demonstrating the function of these proteins in plant defense.

Another group of genes which is expressed in plants infected with *V. longisporum* are the aquaporins, which facilitate transport of water and other small neutral solutes (e.g. CO_2, NH_3) through membranes (Chaumont et al., 2005). The expression of these genes suggests that some sort of water stress might be experienced by the plant. However, marker genes for drought stress (*RD29A, RD22*) are not co-induced with the aquaporin encoding genes (Figure 10). Indeed, the Genevestigator analysis (Figure 9; Zimmermann et al., 2004) indicates no differential regulation of VliGs by drought, except for At3g54820. However, At3g54820 is also highly expressed by the xylem-inducing brassinolid/borate treatment (Kubo et al., 2005), which activates 27 of the 48 VliGs with more than 1.5-fold induction (Figure 9). Interestingly, 10 aquaporins are down-regulated in roots and hypocotyls of *Fusarium*-infected cotton seedlings (Dowd et al., 2004). This establishes a correlation between, wilting and no expression of aquaporins in *Fusarium*-infected plants, and no wilting but expression of aquaporins in *V. longisporum*-infected plants.

Signaling transduction cascades that may be induced by molecules originating from *V. longisporum* seem to involve Ca^{2+}-dependent processes as displayed in Table 3. Although, it remains to be shown, which of the *V. longisporum*-induced processes are activated by these regulatory proteins.

Interestingly, no transcription factors were identified by this microarray analysis. However, as the RNA for this expression profiling was isolated from the whole rosettes at a distinct time point (18 dpi), it might well be that transient or local alterations of the amount of mRNAs of regulatory genes remained undetected (see in addition 4.7).

In conclusion, the nature of the majority of the 48 differentially expressed genes supports the idea that xylem-modifications are initiated following *V. longisporum* infection. No evidence for the synthesis of anti-microbial substances was obtained. It is tempting to speculate that the "stunted" phenotype elicited by Vl43 is due to the cell wall modifications that might alter their extensibility.

4.3 Relationship between known stress signaling pathways and VliGs

In *Arabidopsis*, SA, JA and ET are the major phytohormones which are required for induction of efficient defense responses against biotrophic (SA) or necrotrophic (JA/ET) pathogens (Glazebrook, 2005). Conflicting results have been reported with respect to the induction of SA- and JA-responsive marker genes in *V. longisporum*-infected *Arabidopsis* plants (Veronese et al., 2003; Johansson et al., 2006). Veronese et al. (2003) did not observe induction of *PR-1* or *PDF1.2* at 4, 6, or 9 dpi. At this time points, no symptoms were yet detectable. In contrast, Johansson et al. (2006) reported induction of the SA marker genes *BGL2* and *PR-1* as early as 5 dpi. As two-week-old seedlings grown on sucrose-containing medium were analyzed in both studies, the reason for the discrepancy is unclear. In the present study SA levels increased primarily in plants showing strong diseased phenotype at 35 dpi, irrespective of the substrate (sand or soil) used for growing plants. Consistently, *PR1* gene expression is induced at 35 dpi but less pronounced when compared to *PR1* gene expression induced by *Psm* (Figure 7B). Mutant analyses showed that SA is not involved in the induction of the VliGs identified at 18 dpi (Figure 6B).

JA levels increased in all infected plants starting at 28 dpi, reaching even higher levels at 35 dpi (Figure 6A). The late increase of JA synthesis corresponds to the findings that VliGs are expressed independently of JA (Figure 8). Increased synthesis of JA at later stages of infection might result from the damaging effects caused by the fungus.

To analyze the impact of ethylene on the expression of several VliGs, the *ein2-1* mutant (Guzman and Ecker, 1990) was chosen which displays an ethylene-insensitive phenotype. No effect on the induction of three tested VliGs was observed in this mutant (Figure 8D). The role of ethylene in the *V. longisporum – Arabidopsis* interaction has been analyzed before: the *etr1-1* mutant, which carries a dominant negative mutation in one of the ethylene receptor genes, did not show reduced chlorophyll content (Veronese

et al., 2003). Johansson et al. (2006) found a more severe reduction of fresh weight in the *ein2-1* and other ethylene-insensitive mutants as compared to wildtype plants post infection. In the current thesis, stunted growth and chlorosis was as pronounced in the *ein2-1* mutants as in wildtype plants (Figure 8). This result suggests the manifestation of the symptoms in different mutants depends on either the growth conditions or the use of specific fungal isolates.

4.4 Symptom development in response to different *Verticillium* isolates

To test the effect of the specific fungal isolates *A. thaliana* Col-0 was infected with three isolates of *Verticillium spp.* Infection with *V. longisporum* 43 (Vl43), *V. longisporum* 18 (Vl18) and *V. dahliae* 830 (Vd830) leads to different symptoms.

Both *V. longisporum* strains cluster next to each other in a dendrogram which rated morphological and physiological characteristics of the isolates (Zeise and von Tiedemann, 2001). Both fungal species were isolated in one geographical area, Mecklenburg/Germany from *Brassica napus*, the original host plant. Vl18 was collected in 1989 and Vl43 in 1990 by Zeise (Zeise and von Tiedemann, 2001). In the interaction with *A. thaliana* Col-0, Vl43 induced showed a symptom development, whereas Vl18 induced no visible symptoms (Figure 12A and B). Infection with Vd830 led to an intermediate phenotype, with fewer symptoms than after infection with Vl43 and with more symptoms than after infection with Vl18 (Figure 12A and B). Isolate-dependent gene induction was also detected. Vl43 induced $LTP_{(2)}$ gene expression but Vl18 did not, whereas Vd830 showed an intermediate induction for the $LTP_{(2)}$ expression (Figure 12D).

Eynck et al. (2007) demonstrated that *V. dahliae* is non-pathogenic to *Brassica napus*. Although *V. dahliae* is able to penetrate the roots, it does not spread systemically into the shoots. Interestingly, *V. dahliae* 830 is able to infect *A. thaliana* Col-0 (Figure 12C) but can not elicit strong symptoms or $LTP_{(2)}$ expression compared to an infection with Vl43. (Figure 12A, B and D). The lack of a complete $LTP_{(2)}$-induction and weak symptoms in *A. thaliana* might be reflecting the *B. napus–V. dahliae* non-host situation.

Zeise and von Tiedemann (2001) showed for both isolates the same morphological and physiological parameters (Table 1). Obviously, both isolates interact with *A. thaliana* Col-0 in a different manner (Figure 12). This observation leads to two different

hypotheses. One scenario could be that Vl43 synthesizes an elicitor and Vl18 does not. The other idea is that Vl18 excretes a factor that suppresses the plant response (symptom development and induction of VliGs expression).

4.5 Symptom development and VliGs expression after infection of *A. thaliana* ecotypes with *V. longisporum* Vl43

It is well known that an ecotype-specific genetic variability exists in *Arabidopsis* (Kalbina and Strid, 2006; Chen et al., 2005; Narang et al., 2000; Meyer et al., 2004; Sauer et al., 2004). Infection of Col-0, Bur, Ler, Cvi, and Ws-2 resulted in similar pattern of symptom development (Figure 13). C24 showed no visible symptoms compared to a Col-0 infection that resulted in weak symptoms at 21 dpi. These findings confirmed the results of Veronese et al. (2003). They tested the susceptibility of 10 ecotypes to the isolate VdBob.70 of *V. dahliae*, which is confirmed to be a *V. longisporum* isolate (von Tiedeman, personal communication). Veronese et al. (2003) showed that C24 is a more tolerant ecotype compared to Col-0. This trait was linked to a single dominant locus, *Verticillium dahliae* tolerance (*VET1*) (Veronese et al., 2003), which was mapped to the top of chromosome IV. Other genes at this locus encode for flowering time control (Hepworth et al., 2002) and resistance to *Hyaloperonospora prasasitica* (Parker et al., 1997; Knoth and Eulgem, 2008).

The measurement of the fungal propagation by Veronese et al. (2003) showed that while the concentration of fungal DNA is equal in both ecotypes at the early stages of infection, it increases in Col-0 but not in C24 at later time points. Consistent with this result the analysis of fungal DNA at 14 (Figure 14) and 21 dpi (Figure 13E) showed that *Verticillium* infected both ecotypes but did not spread any further in C24 as no further increase in the fungal DNA could be observed at 21 dpi. This suggests that ecotype C24 effectively controls the fungal spreading, whereas Col-0 has no means to inhibit the fungal growth.

An infection of the accession C24 with Vl43 showed that *AP2-TF* (At4g28140) and *VSP2* (At5g24770) were more than two-fold stronger induced in the petioles of this ecotype than in the petioles of Col-0 at 5 dpi (Figure 19; Figure 18A and D). At later time point of the infection, no induction of $LTP_{(2)}$ gene and no symptom development was observed in C24 (Figure 13). Therefore, it can be hypothesized that a stronger induction of the defense responsive genes at 5 dpi effectively controls fungal proliferation in the plant. It results in only weak symptom development and reduced

VliGs expression at later time points of the plant-fungus interaction. In this respect, it is interesting to consider the interaction of Vl18 and Col-0. In contrast to Vl43, Vl18 does not elicit symptom development and causes no $LTP_{(2)}$ induction at 25 dpi (Figure 12) in Col-0. Further research is needed to test whether Vl18 produces a strong induction of VliGs in Col-0 at early stages of the infection, similar to the infection response observed in C24. If this is the case, the weak symptoms observed at 25 dpi could be the result of a strong defense response at early stages of infection that minimizes opposite effects.

4.6 Elicitors are likely to be transported from the root to the shoot

Time course experiments demonstrated that expression of VliGs is induced in the shoot before fungal DNA can be detected in this organ (Figure 15C). Presumably, one or several elicitors are transported with the transpiration stream to the shoot where they promote gene expression. Bioluminescence imaging of plants expressing the *FIREFLY LUCIFERASE* gene under the control of selected VliG promoters revealed promoter activity locally restricted to vascular elements, thus supporting this hypothesis (Figure 15B).

Candidates for elicitors are phytotoxins and other molecules causing disease symptoms that have been identified in crude extracts from *V. dahliae* or *V. albo-atrum* (Fradin and Thomma, 2006). Active compounds can be protein-lipopolysaccharide complexes, glycoproteins, and/or cell-wall-degrading enzymes. Also Nep1-like peptides, which induce necrosis and ethylene synthesis in *Fusarium* species (Bailey et al., 1997) have been found in *V. dahliae* and could play the role of an elicitor. Alternatively, the transported signal could be a plant-derived molecule generated in the roots upon *V. longisporum* infection. For instance, expression of a fungal cutinase leads to expression of a number of defense genes independent of the stress hormones SA, JA and ET (Chassot et al., 2007). Transcription of the *V. longisporum*-induced peroxidases At2g37130 and At5g64120 was also up-regulated in these plants. In addition, the presumed function of the genes induced upon expression of cutinase (peroxidases, LTPs) is similar to the presumed function of the VliGs, which are identified here. Thus, cutin or suberin monomers or other cell-wall-derived molecules may act as elicitors. By analyzing plants expressing *FIREFLY LUCIFERASE* gene under the control of selected VliG promoters as biosensors, first attempts were made to identify the transported signal eliciting VliG expression. Fungal culture extracts (kindly provided by AG

Karlovsky) were fed through petioles of P_{PER21}:*luciferase* transgenic plants. After three days the leaves treated with the aqueous fraction started a chlorosis and seven days post-treatment the expression of the *FIREFLY LUCIFERASE* gene under the control of selected VliG promoters was detectable (Figure 16). The putative elicitor(s) remained in the aqueous fraction after extraction with ethyl acetate and n-butanol indicating that it/they must be highly polar. Interestingly, the yellowing of the leaf occurred after 3 days, whereas the P_{PER21}:*luciferase*-expression was detected only after 7 days. This suggests that *V. longisporum* produces a factor that acts directly on the chloroplasts, causing chlorosis, and that maybe another factor, which might be produced by the plant, leads to the VliG expression. The xylem sap of an infected *Brassica napus* plant (kindly provided by AG Tiedemann) also induced the yellowing and a very weak P_{PER21}:*luciferase*-expression in one of the two tested plants. Thus, it can be concluded, that the same elicitor(s) that induce the symptoms in *Arabidopsis* is present in a *Verticillium*-infected *B. napus* plant. It might be that this elicitor also induces the *Verticillium* disease in *B. napus*.

4.7 *Arabidopsis* initiates defense response at 5 dpi

4.7.1 Interpretation of 5 dpi transcriptome profiling by their presumed functions

To get further insights into the gene expression involved in early, fungal-induced plant response, a transcriptome profiling was done at 5 dpi. In the following section, the presumed functions of *V. longisporum*-induced genes at 5 dpi (VliG5s) are discussed regarding their role in the potential defense responses. Out of 47 up-regulated genes at 5 dpi, 12 or 25.5 %, corresponded to genes involved in cell wall modification (group 1, Table 4). The up-regulation of genes with homologies to expansins (Cosgrove et al., 2002) and hydrolases suggests that cell wall loosening processes were initiated. It might be that this is the reason for the symptom “stunting”.

The second group (Table 4) contains genes that are related to the synthesis or signaling of phytohormones. Five of the 47 up-regulated VliG5s (9 %) are linked to the phytohormones jasmonic acid (JA). Their activation is typical for a classical defense reaction to necrotrophic pathogens like *Botrytis cinerea* (Glasebroock, 2005) or after wounding (*VSP2, LOX2, CORI3*; Suza and Staswick, 2008). It is thus intriguing that these genes are up-regulated at such an early time point of infection, despite the fact that *Verticillium* is in its parasitic stage and does not kill the infected tissue.

Group 3 “signal transduction” (group 3, Table 4) contains 9 of the 47 up-regulated VliG5s (19%). Two of the up-regulated transcription regulators are related to the circadian clock of *Arabidopsis*. *APRR5* encodes a pseudo-response regulator. After mutating this gene various circadian-associated biological events, such as the flowering time in the long-day photoperiod conditions, were affected (Nakamichi et al., 2007; Matsushika et al., 2007). *PCL*1 (*PHYTOCLOCK*1) encodes a GARP protein essential to the *Arabidopsis* circadian clock (Onai and Ishiura, 2005). The GARP motif contains a nuclear localization signal (Onai and Ishiura, 2005). Changes in these circadian processes may lead to the symptom of “early flowering”, which is induced in Col-0 plants upon infection with *Verticillium* (Veronese et al., 2003).

Two AP2 domain-containing transcription factor family proteins are up-regulated five days post *Verticillium*-infection (group 3, Table 4). The AP2 domain was first identified as a repeat motif within the *Arabidopsis* AP2 protein, which is involved in flower development (Jofuku et al., 1994; Elliott et al., 1996). AP2 domain proteins are part of the AP2/ERF superfamily of transcription factors. The AP2/ERF proteins have important function in the transcriptional regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli. Nakano et al. (2006) did a genome-wide analysis of the ERF gene family in Arabidopsis and rice. Phylogenetic analyses were performed, as well as exon/intron and protein motif structural analyses of the ERF family genes. The two AP2 domain-containing transcription factor family proteins that are up-regulated five days post *Verticillium*-infection (group 3, Table 4), were classified in two different phylogenetic groups. At4g28140 (AtERF#054) belongs to the group Ia (Nakano et al., 2006). The functions of these genes are unknown. At1g21910 (AtERF#012) was classified in group IIb. All of the genes in this group contain the CMII-1 motif in the C-terminal region adjacent to the AP3/ERF domain. This motif is similar to the CMIII-1 motif found in group III, but as to date the functions of these genes are also unknown (Nakano et al., 2006).

Another transcription factors deserve special mentioning: *WRKY38*. The *Verticillium*-induced *WRKY38* gene belongs to the type III WRKY transcription factors. *WRKY38* exhibits an SA- and NPR1 dependent induction in response to the bacterial pathogen *Pseudomonas syringae* and after a treatment with benzothiadiazole S-methylester (BTH; a functional analog of SA) (Kim et al., 2007, Wang et al., 2006). *WRKY38* seems to be required for a full induction of SAR (Wang et al., 2006). *BT4*

(BTB AND TAZ DOMAIN PROTEIN 4) is a *Verticillium*-induced protein related to Ca^{2+}-signaling that also responds rapidly to treatments with H_2O_2 and SA (Du and Poovaiah, 2004). Thus, the up-regulation of *WRKY38* and *BT4,* after *Verticillium* infection, suggests that a parasitic growing fungus in the xylem leads to an activation of a common biotrophic defense pathway with SA.

Group 4 (Table 4) contains *Verticillium*-induced genes that are up-regulated in response to biotic or abiotic stress. A member of this group is the disease resistance family protein gene At2g34830. Ramonell et al. (2005) found that At2g34830 is induced after infection with the powdery mildew *Erysiphe cichoracearum*. A loss of function mutation for this gene leads to an enhanced susceptibility to the powdery mildew.

In summary, *Arabidopsis* seems to simultaneously initiate defense response against necrotrophic (group 1, Table 4) and biotrophic pathogens (group 2 and 3, Table 4). Since the fungus changes its status from a parasitic to a saprophytic during the infection, both defense strategies are likely to be required. This idea is further supported by the *Verticillium*-induced expression of the transcription factor *MYB73*. When conducting an expression analysis of the *MYB* transcription factor superfamily in *Arabidopsis,* Yanhui et al. (2006) found that most of the *MYB* genes are responsive to stress or hormone treatments and many of them are activated in a very stimulus-specific manner. However, *MYB73* is an exception. In contrast to the treatment-specific activation of other *MYB* genes, all tested treatments (ABA, ET, GA, IAA, JA, SA, and salt stress) led to an induction of *MYB73*, suggesting that it is a general factor playing a role in stress response. Since *Arabidopsis* seems to activate genes that are typically involved in diverse plant-pathogen interactions, thus mounting a general defense response, it appears logical that the "generalist" MYB73 is also induced by *Verticillium* infection.

4.7.2 VliG5s expression pattern analysis with the "Genevestigator V3 – mutant analyzer"

Another possibility to analyze the results of the transcriptome profiling at 5 dpi is an investigation with the Genevestigator V3 software. As described above, the Genevestigator V3 software suite (https://www.genevestigator.ethz.ch) provides categorized quantitative information about genes contained in large microarray databases. It is possible to analyse the expression of the VliG5s in different mutants/transgenic lines or in response to different stimuli (Zimmermann et al., 2005).

Several mutants and transgenic *Arabidopsis* lines that express at least four *V. longisporum*-induced genes at 5 dpi (VliG5s) were identified using the Genevestigator V3 (Figure 20). Some of these mutants (*ang4, axr1-12, elo2,* and *elo3)* showed the typical *Verticillium*-induced pathophenotype "stunted leaves growth". *ANG4* is an allel of *HUB1*. *HUB* knockdown mutants showed pale leaf coloration, modified leaf shape, and reduced rosette biomass (Fleury et al., 2007). Lincoln et al. (1990) described plants with decreased plant height, defects in root gravitropism, hypocotyls elongation, and fertility as a result of a mutation in the *AXR1* gene. This means, that an induction of genes after *Verticillium* infection and after mutation of *ANG4,* and *AXR1-12* leads to the same phenotype (reduction in rosette biomass and hypocotyls elongation) (Figure 20). Therefore, it can be speculated that the symptom "stunted leaves growth" is induced at this early time point of the infection. This idea is supported by an investigation of *elo2* and *elo3* mutant plants (Nelissen et al., 2005). In comparison to the wildtype *elo* mutants exhibit a "narrow leaves" phenotype due to the reduced cell number and missing transition between petiole and leaf lamina. This means that another mutant that was found by the Genevestigator V3, has a connection to an altered leaf phenotype.

Another transgenic line, identified due to the presence of many up-regulated VliG5s, is the 35S::MBF1c plant (Figure 20). It is an over-expressor of the MBF1c protein (multiprotein bridging factor 1c), which is a key regulator of thermotolerance in *Arabidopsis* (Suzuki et al., 2008). The MBF1c functions upstream of SA, trehalose, and ethylene, and thus plays a role in SA mediated defense responses, suggesting that SA plays a role in *Verticillium*-induced defense response.

Several VliG5s, such as lipoxigenase (At1g72520), TCH4 (At5g57560), and AOC3 (At3g25780), are up-regulated in yet another mutant, *aba1-1* (ABA deficient). *ABA1* encodes for a zeaxynthin epoxidase that functions in ABA biosynthesis (Xiong et al., 2002). Its expression is induced by osmotic stress. Since ABA-impaired *Arabidopsis* mutants share up-regulated genes with *Verticillium*-infected plants it seems that no ABA-synthesis is required to induce the corresponding VliG5s after the *Verticillium* infection (Figure 20). Supporting this interpretation, no ABA-inducible genes were activated upon *Verticillium* infection (Figure 10).

The gene expression analysis of the *ctr1* mutant plant showed a down-regulation of many VliG5s (Figure 20). CTR1 is known to be a negative regulator of the ethylene signaling in *Arabidopsis* (Huang et al., 2003; Yoo et al., 2008). Yoo et al. (2008)

demonstrated that CTR1 MAPKKK activity negatively regulates downstream MAPK activities, which play a positive role in ethylene signaling. In the absence of ethylene, CTR1 directly or indirectly inactivates MKK9-MPK3/6 and probably activates downstream MAPKs to promote EIN3 (ETHYLENE INSENSITIVE3) degradation. EIN3 is a nuclear protein which induces the ethylene response pathway in *Arabidopsis* (Chao et al., 1997). Ethylene inactivates CTR1 for MKK9-MPK3/6 activation and stabilizes EIN3, leading to ethylene signaling cascade (Yoo et al., 2008). In the *ctr1* mutant there is no negative regulation by CTR1 and the plant behaves comparable to a constant ET-treatment. Down-regulation of the majority of VliG5s in this mutant suggests that ET somehow influence the *Verticillium*-mediated defense response at 5 dpi.

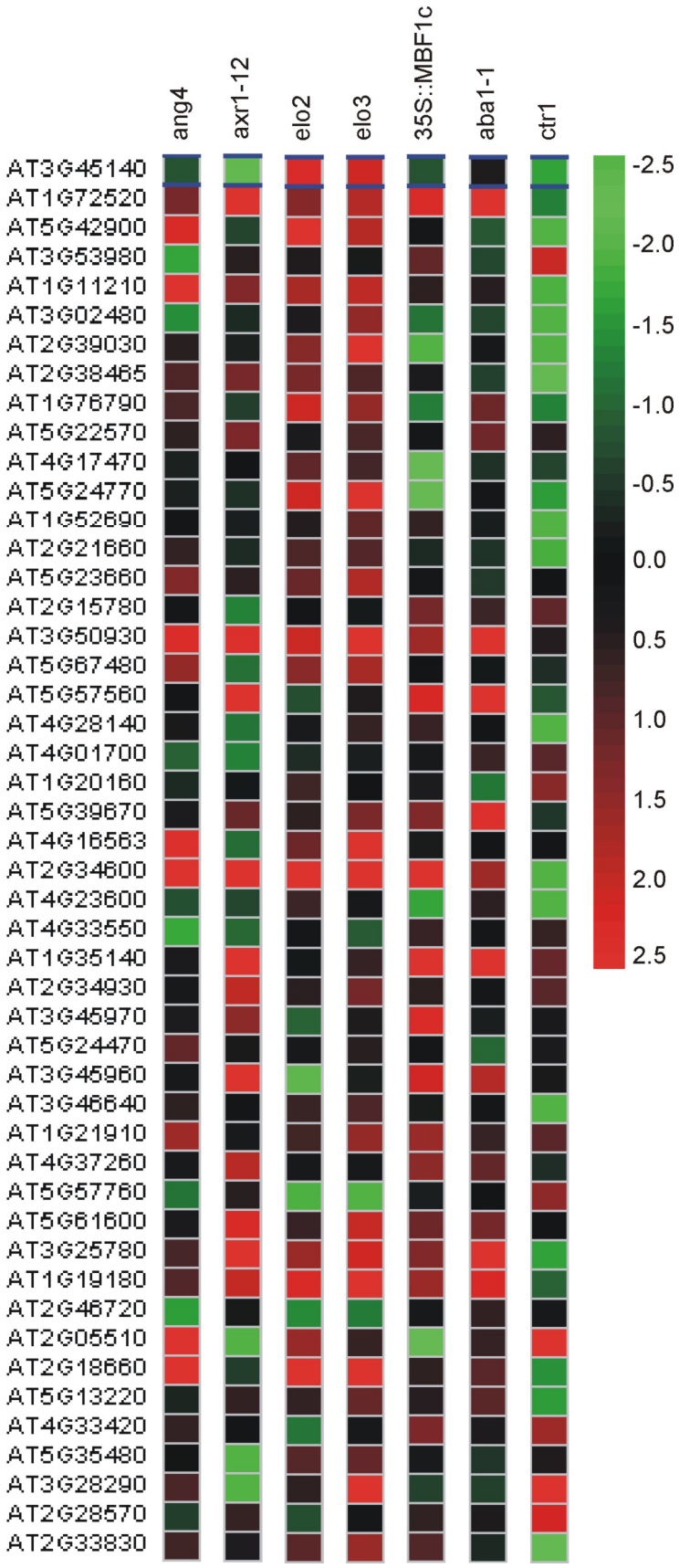


Figure 20: Genevestigator analysis with selected mutants of VliG5s
Adapted from Genevestigator V3. Genes whose expression is affected upon infection of *Arabidopsis* with *V. longisporum* 43 (at 5 dpi) were analyzed by the Genevestigator V3 (https://www.genevestigator.ethz.ch). Presented are those mutants or transgenic lines that differentially express more than 4 VliG5s. Descriptions of the genes are listed in Table 4, page 60. Color code: lower expression levels are light-green; higher expression levels are light-red (log_2 scale).

To summarize the Genevestigator-mutant-analyses it was possible to show that the symptom, "stunted leaves growth", seems to be initiated at this early time point after infection (*ang4, axr1-12, elo,* and *elo3)*. It was also concluded, that ABA (*aba1*) does not seem to play a role in and ET (*ctr1*) somehow influence the initiated defense reaction. In contrast, SA (35S::MBF1c) might be involved in *Verticillium*-mediated defense response. These findings confirm the results of the transcriptome analysis with TAIR, described above (4.7.1).

4.7.3 VliG5s expression pattern analysis with the "Genevestigator V3 – stimuli analyzer"

The response of the VliG5s to different stimuli was analysed by the Genevestigator V3 software (Zimmermann et al., 2005) (Figure 21). Many of the genes that were up-regulated at 5 days after infection with *V. longisporum,* were also induced by different treatments aimed at activation of the classical plant defense responses (Figure 21). This means that these treatments induced the same set of genes as an infection with *Verticillium*. An interesting result gives the treatment with $AgNO_3$, an ethylene-signaling inhibitor. The inhibition of the ethylene signaling by $AgNO_3$ leads to the opposite gene expression compared to the mutant analyses of the *ctr1* mutant (4.7.2). That means the treatment with $AgNO_3$ further supports the results of the mutant analyses (4.7.2). $AgNO_3$ leads to the up-regulation of genes that are down-regulated in the *ctr1* mutant. While *ctr1* mimics constant ethylene activation (Figure 20), $AgNO_3$ elicits the opposite response (Figure 21).

Another interesting finding is that many of the VliGs are also up-regulated after the treatment with elongation factor Tu (EF-Tu) (Figure 21), the most abundant bacterial protein (Kunze et al., 2004). *Arabidopsis* plants specifically recognize the N-terminus of the protein, and an N-acetylated peptide comprising the first 18 amino acids, termed elf18, is fully active as an inducer of defence responses (Kunze et al., 2004). Therefore, initiation of basal defense response in *Arabidopsis* at early stages after *Verticillium* infection can, well be, speculated.

In summary, the results of the mutant- and treatment-analysis (Figure 20 and Figure 21) by Genevestigator software suggest that the up-regulated VliG5s seem to reflect the initiating of a defense response. This confirms the results of the interpretation of the 5 dpi transcriptome profiling by their presumed gene functions (4.7.1).

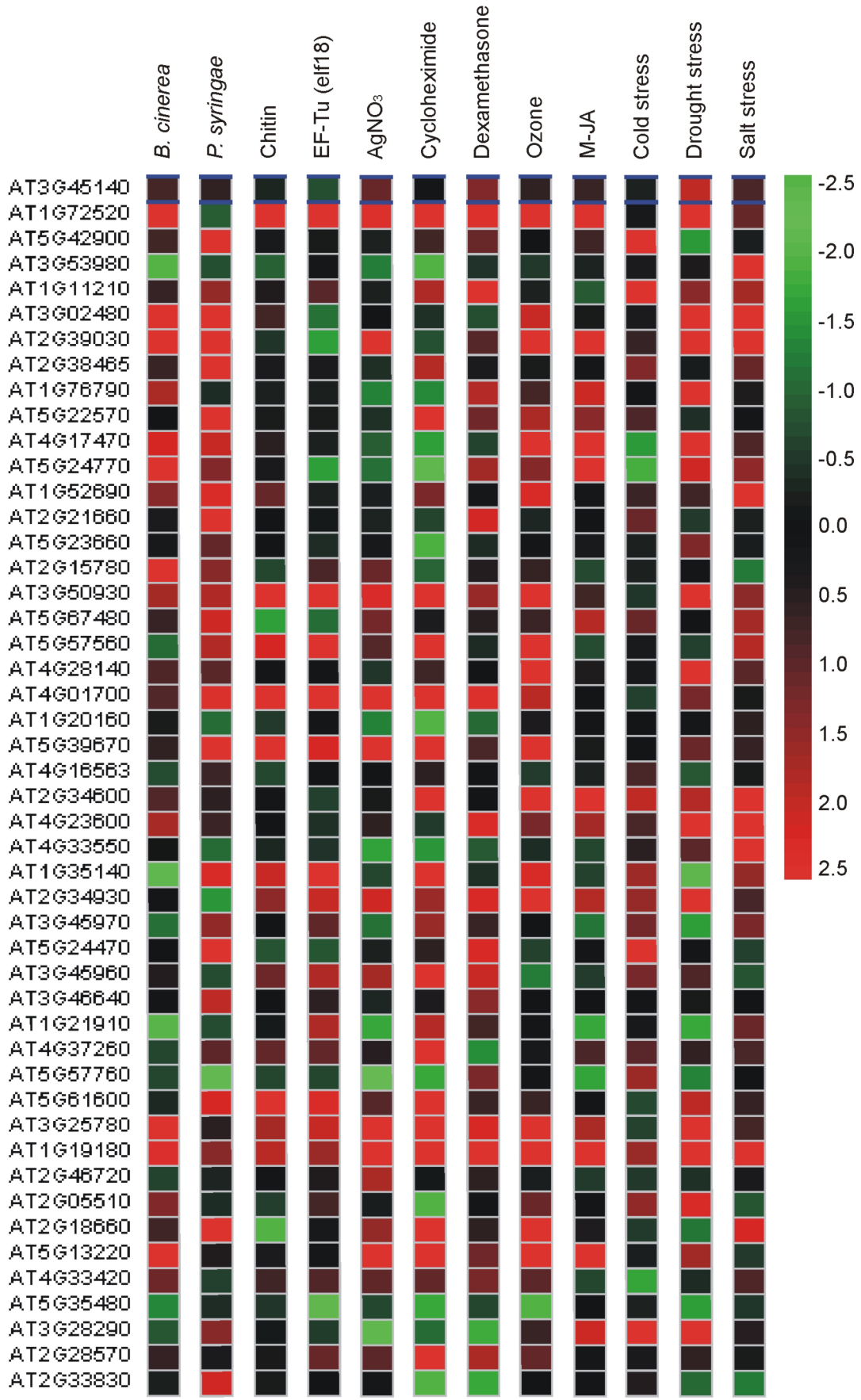


Figure 21: Genevestigator analysis with selected treatments of VliG5s
Adapted form Genevestigator V3. Genes whose expression is affected 5 dpi upon infection of *Arabidopsis* with *V. longisporum* 43 (at 5 dpi) were analyzed by the Genevestigator V3 (https://www.genevestigator.ethz.ch). Presented are those treatments that induce more than 10 VliG5s.

Descriptions of the genes are listed in Table 4, page 60. Color code: lower expression levels are light-green; higher expression levels are light-red (log_2 scale). Among the tested stimuli were treatments of seedling with cycloheximide (blocks the *de novo* protein-biosyntheses), ozone (fumigation for 6 hours), chitin, $AgNO_3$ (an ethylene inhibitor), and methyl-jasmonate (M-JA). Adult rosettes were subjects to treatments with *Pseudomonas syringae, Botrytis cinerea* as well as cold, drought, and salt stress.

4.8 Comparison of *V. longisporum*-induced genes at 5 and 18 dpi

By comparison of the transcriptome profiles at 5 and 18 dpi only four genes that were up-regulated at 5 dpi were also up-regulated at 18 dpi (At3g53980, *LTP(1)*; At4g33420, class III peroxidase *ATP*32; At4g33550, *LTP(3)*; and At1g20160, *SFP*) (Table 3 and Table 4). To compare both transcriptome profiles with respect to the presumed function of the up-regulated genes an analysis-tool at the "The *Arabidopsis* Information Resource" (TAIR; Rhee et al., 2003) web page (http://www.arabidopsis.org/) was used. TAIR provides a possibility to analyze a set of genes with regard to their functional categorization by the gene ontology (GO) annotation. This analysis showed that the biological processes "response to abiotic or biotic stimulus" and "response to stress" were over-represented at 5 dpi compared to the not-treated transcriptome of *A. thaliana* (Table 5). At 18 dpi, the process "transport" was detected in addition to those already over-represented at 5 dpi (Table 5). Interestingly, the process "transcription" was completely abolished at 18 dpi.

Table 5: Functional categorization of the *V. longisporum*-induced genes at 5 and 18 dpi
Comparison of both transcriptome profiles (5 and 18 dpi). The analysis with a tool of the TAIR web page showed which biological processes of *Arabidopsis* were over- or underrepresented. Source: http://www.arabidopsis.org/tools/bulk/go/index.jsp (Feb. 2008)

Functional Categorization by annotation for GO Biological Process[a]	Whole genome data[b]	Array at 5 dpi[c]	Array at 18 dpi[c]
other metabolic processes	21	15	13
other cellular processes	21	15	10
unknown biological processes	20	11	19
protein metabolism	8	2	7
transport	4	2	13
developmental processes	4	2	1
response to abiotic or biotic stimulus	4	14	13
other biological processes	4	18	9
cell organization and biogenesis	4	4	2
transcription	4	5	0

response to stress	3	12	13
signal transduction	2	1	1
electron transport or energy pathways	2	1	0
DNA or RNA metabolism	1	1	0

[a] The first column states the annotated biological processes; [b] the second shows the percentage of annotated genes for the indicated process in the whole genome of *Arabidopsis* [% of all annotated genes]; [c] the third and forth columns show the percentage of annotated genes for the indicated process that are induced after infection with *V. longisporum* [% of all genes induced after infection] at 5 and 18 dpi, respectively. Light-grey color indicates an over-represented process; dark-grey color indicates an under-represented process.

In contrast to the transcriptome profile at 18 dpi (no transcriptional activity of any up-regulated gene), transcription regulators encoding genes induced at 5 dpi can be assigned to two categories of regulatory proteins. First of them includes transcription regulators that correspond to the circadian clock of *A. thaliana* (*APRR5* and *PCL1*). It is possible that these transcription regulators initiate the symptom "early flowering", which has been described for infected plants growing under long day conditions (Veronese et al., 2003). Second category includes transcription regulators that seem to be involved into phytohormone SA mediated defense response (*WRKY38, MYB73, AP2* domain-containing and *BT4*). These findings can by summarized in the following way:

A. thaliana ecotype Col-0 activates some defense associated transcription genes and regulators (group 2, Table 4) upon infection with *V. longisporum*, but it is not capable of inhibiting the fungal propagation (Figure 5D). At 18 dpi (Table 3), none of the defense related genes were induced and the analysis with the Genevestigator (Figure 9) found no up-regulated VliGs in the corresponding treatments. This suggests that the up-regulation of genes involved in plant defense against pathogens is only transient and is blocked by the fungus.

5 Summary

Verticillium longisporum is a soil-borne fungal pathogen causing vascular disease predominantly in oilseed rape. The pathogen enters its host through the roots and maintains a parasitic life stage in the xylem before invading other tissues late in the infection cycle. *Arabidopsis thaliana* was used as a model plant to characterize the response of the aerial parts of the plant to the infection. Whole genome transcriptome profiling experiments of *Arabidopsis* Col-0 plants were performed at 5 and 18 dpi, respectively. At 5 dpi, no symptoms were detected, but a number of genes related to jasmonic acid (JA) -biosynthesis or -signal transduction were already expressed. However, this initial defense reaction did not influence the pathophenotype and the expression of *Verticillium*-induced genes (VliGs) at 18 dpi as revealed by analysis of an *Arabidopsis* mutant deficient in JA biosynthesis. More than one fourth (5 dpi) and one third (18 dpi) of the VliGs encode apoplastically localized proteins involved in cell wall modifications and potential defense responses. Their expression was dependent on the specific fungal isolate. The identified VliGs provide a useful tool to elucidate the contribution of the induced genes to the disease phenotype and the defense response. Moreover, they might help to identify the elicitor(s) as suggested by preliminary experiments. They demonstrated that fractionated extracts from fungal cultures induced expression of a VliG when applied to the petioles of detached leaves.

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