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Genetic and Metabolomic Analysis of Phenolic Compounds Associated with *Verticillium longisporum* Resistance in Oilseed Rape (*Brassica napus* L.)

A thesis submitted for the requirement of the doctoral degree
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Justus Liebig University Giessen, Germany

Submitted by

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Dedicated

To my beloved parents whom I lost during my childhood

&

To my brothers and sister who grown up me

&

Last but not the least to my wife for her endless support throughout my stay in Germany

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List of abbreviations and symbols

AUDPC	:	Area under the disease progress curve
BABA	:	*β*-aminobutyric acid
cM	:	centiMorgan
CW	:	Cell wall
DH	:	Doubled haploid
dpi	:	Days post inoculation
Exp.	:	Experiment
ExR53	:	Express 617 x R53
FAOSTAT	:	The statistics division of the Food and Agricultural Organization
g	:	Gravitationnal force
G	:	Guaiacyl
GC	:	Gas chromatography
GC/MS	:	Gas chromatography coupled with mass spectrometry
H	:	*p*-hydroxyphenyl
HPLC	:	High performance liquid chromatography
LC/MS	:	Liquid chromatography coupled with mass spectrometry
LOD	:	Logarithm of the odds
M-inoculated	:	Mock-inoculated
MAS	:	Marker assisted selection
MS/MS	:	Mass spectrometry/Mass spectrometry
MPa	:	Megapascal
M/Z	:	Mass to charge
nm	:	Nanometer
n.s.	:	Not significant
OSR		Oilseed rape
PAL	:	Phenylalanine ammonia lyase
PCR	:	Polymerase chain reaction
QTL	:	Quantitative trail loci
R	:	Pearson correlation
RP	:	Reverse phase
RS	:	Resynthesized
S	:	Syringyl
SNP	:	Single nucleotide polymorphism
SP	:	Soluble phenol
SSR	:	Simple sequence repeat
TLC	:	Thin layer chromatography
VL	:	*Verticillium longisporum*

1 Introduction

1.1 Oilseed rape

Oilseed rape (*Brassica napus*) is the most important oilseed crop in Europe and the second most important worldwide after soybean. Oilseed rape is also the major source of biodiesel in Europe. In 2012 among approximately 12 million hectares of arable land in Germany, rapeseed was cultivated on 1.306 million hectares. Germany is Europe's biggest producer of rapeseed after France producing 4.8 million tons in 2012 (FAOSTAT 2013, faostat.fao.org). *B. napus* originated by spontaneous interspecific hybridization between *B. rapa* L. (syn. *campestris*; genome AA, 2n = 20) and *B. oleracea* L. (CC, 2n = 18) (U, 1935). *B. napus* is considered a "young" species in *Brassica* oil crops and due to an extremely strong bottleneck selection for double-low (00) seed quality (zero erucic acid, low glucosinolate content) during breeding in the last 30 years, current winter oilseed rape cultivars have a relatively narrow genetic basis and are lacking a broad spectrum of disease resistances. Due to rapid increase of area cultivated with oilseed rape as well as lack of resistance in current rapeseed cultivars, infection by pathogens such as *Verticillium longisporum* (VL) has become a major threat to oilseed rape production in Europe (Dunker et al., 2008).

1.2 Rapeseed diseases

Due to intensive cultivation of oilseed rape, disease problems have become a vital consideration in recent breeding programmes. *Brassica* oilseed rape suffers from different types of fungal and viral diseases. Common diseases of rapeseed are stem canker (caused by *Leptosphaeria maculans*), stem rot (caused by *Sclerotinia sclerotiorum)*, Verticillium disease (caused by *Verticillium longisporum*), clubroot (caused by *Plasmodiophora brassicae*), Alternaria leaf spot (caused by *Alternaria brassicae)*, white rust (caused by *Albugo candida*), downy mildew (caused by *Perenospora parasitica*), light leaf spot (caused by *Pyrenopezzia brassicae),* and *Turnip Mosaic Virus* (TuMV) and *Turnip Yellows Virus* (TuYV). Blackleg is the most damaging disease of oilseed rape in Europe and Australia, whereas stem rot disease is particularly important in China. Both blackleg and stem rot diseases play a significant role in Canada. White rust and downy mildew diseases are common on both *B. rapa* and *B. juncea* particularly in India. Black spot caused by *Alternaria brassicae* is one of the most destructive fungal disease affecting *B. napus*, *B. juncea* and *B. rapa* worldwide. Moreover, some diseases have significant local importance. Verticillium disease is a common problem in affected areas of Sweden, northern Germany, Denmark, Great Britain, Poland, France, Southern Russia and

Ukraine, whereas light leaf spot is a particular problem in northern parts of Europe. Clubroot is considered a major disease of oilseed rape in Scandinavian countries and the northern United Kingdom, while substantial yield losses occur due to viral diseases in some parts of China (Snowdon et al., 2006). Global warming might lead to shifts in the future prevalence of oilseed rape pathogens and *V. longisporum* could be particularly favoured under conditions of future global warmings (Siebold et al., 2012).

1.2.1 *Verticillium* species and *Verticillium longisporum*

Verticillium species belong to the phylum Ascomycota. Fungi of the genus *Verticillium* are soilborne and cause vascular diseases in various plant species in temperate and subtropical climates (Pegg & Braddy, 2002). The fungal mycelium is hyaline, septate, multinucleate and simple or branched. Conidia of this fungal species are usually single-celled and ovoid, ellipsoid or elongated in shape. These conidia are borne on phialides. Phialides are the specialized hyphae produced in a whorl around each conidiophore and each of the phialiade bears a bunch of conidia. The name of the Verticillium genus derives from the "verticillate" (= whorled) arrangement of the phialides on the conidiophores (Johansson et al., 2006a). Ten plant pathogenic species belong to this genus of which *V. dahliae* and *V. albo-atrum* are the most wide spread causing billions of dollars in annual crop losses worldwide (Pegg & Braddy, 2002; Klosterman et al., 2011; Inderbitzin et al., 2013). The host range of *V. dahliae* is quite broad which includes many important crop species such as potato, tomato, sunflower and cotton. Woody and tree plants are also infected by this fungus. *V. longisporum* fungus is a new species of the genus *Verticillium* and a recent problem of oilseed rape cultivation in Europe (Evans et al., 2009).

Verticillium disease of oilseed rape was first reported in Sweden in oilseed rape production in the 1960s and has become economically important in the 1970s. Rapid increase of *V. longisporum* incidence in Germany in the major oilseed rape cultivated areas was reported since the 1980s (Dunker et al., 2008). In these regions the fungus can cause yield losses up to 50% (Daebeler et al., 1988; Zeise & Steinbach, 2004).

The fungus responsible for Verticillium disease in oilseed rape is now classified as *Verticillium longisporum* (Karapapa et al., 1997), whereas in earlier reports it is referred to as *V. dahliae*. Since pathologists have been working on this fungus, there was a controversy

concerning the recognition of *V. longisporum* as a separate host-specific species. Isaac (1957) described an exceptional isolate of *V. dahliae* isolated from Brussels sprout which he described as a distinct physiological strain with a restricted host range. Later on, Stark (1961) classified the long-spored *Verticillium* isolates collected from horseradish as *V. dahliae* var. *longisporum*. In 1997, Karapapa and his associates proposed to classify these isolates as *V. longisporum* based on morphological, enzymatic, molecular and virulence characteristics. Karapapa et al. (1997) were also able to clearly differentiate cruciferous *Verticillium* isolates from isolates of *V. dahliae* and *V. albo-atrum*. They also suggested that *V. longisporum* might have emerged from parasexual hybridization between *V. dahliae* and *V. albo-atrum* with a 'near-diploid' state and with an about 1.75 fold nuclear DNA content compared to short-spored isolates. Based on sequence data, Fahleson et al. (2004) proposed that *V. longisporum* should be a distinct species closely related to *V. albo-atrum*. Inderbitzin et al. (2011; 2013) described that *V. longisporum* is an allodiploid hybrid that originated at least three different times involving four different parental lineages representing three different species (two lineages from two unknown species and two lineages from *V. dahliae*). In another study, Tran et al. (2013) also stated that the cruciferous fungal pathogen *V. longisporum* represents an allodiploid hybrid containing long spores and containing almost double the amount of nuclear DNA compared to other *Verticillium* species. Johannson et al. (2005) described that *V. longisporum* can also infect plant species outside the *Brassicaceae* family. This statement contradicts with results of several investigations of Zeise & von Tiedemann (2001; 2002a; 2002b) who showed that *V. longisporum* is a strictly host adapted pathogen specific for *Brassica* species. So, *V. longisporum* differs from other Verticillium species not only in its morphology and molecular pattern but also in its host range.

Fungi belonging to the *Verticillium* genus including *V. longisporum* can survive unfavorable conditions as microsclerotia buried in the soil or resting on crop debris after harvest (Domsch et al., 1980; Inderbitzin et al., 2013). There is no spread by aerial spores, thus dispersal takes place via transfer of crop residues or soil through tillage devices. Microsclerotia germinate most readily at soil water potentials of >-1 MPa and temperatures above 15°C (Soesanto & Termorshuizen, 2001; Dunker & Tiedemann, 2006) and these conditions are also most favorable for mycelia growth, invasion into roots and spread of the fungus within the stem (Pegg & Brady, 2002; Donald & Czeslaw, 1998; Schnathorst, 1981).

1.2.2 Host-pathogen interaction in Verticillium disease

V. longisporum infects the host plants through their roots by penetrating the root epidermis in the root hair zone, and colonizes the xylem of *Brassicaceae* (Eynck et al., 2007) using carbohydrates, amino acids, and minerals present in the xylem sap as nutrient source. After germination of microsclerotia, which is triggered by root exudates of host plants (Mol & Scholte, 1995), the fungal hyphae grow towards the root surface and penetrate the root epidermal cells (Zhou et al., 2006; Eynck et al., 2007). Before entering into the xylem, the fungus traverses the root cortex inter- as well as intra-cellularly. Most of the time of its life cycle *V. longisporum* is confined to the vascular system, which is a nutrient-poor environment to which the fungus is well adapted (Pegg, 1985; Van, 1989). Once the fungus has reached the xylem vessel, the pathogen spreads through hyphae and conidiophores conveyed with the transpiration stream into upper parts of the plant vascular system. Water and nutrient transportation become restricted due to clogging of the xylem vessels. Therefore, it has been speculated that as consequences of water scarcity and inadequate nutrient supply, typical disease symptoms such as wilting, stunting, chlorosis, premature ripening and senescence are developing at the later growing stage (Johansson et al., 2006b). However, wilting symptoms were observed in many different crop species infected by *V. dahliae*, but were not observed in oilseed rape or in *A. thaliana* during the *V. longisporum* infection cycle (Floerl et al., 2008; Eynck et al., 2009a; Florel et al., 2010). In addition, analyses of nutrient status of plants after infection with *V. longisporum* did not reveal nutrient scarcities in these plant species (Florel et al., 2008; Florel et al., 2010). However, severe stunting exhibited in the *V. longisporum* infected plants in the greenhouse (but not in the field) is indicating that the host-pathogen interaction resulted in extensive re-modelling of plant architecture (Florel et al., 2008; Ratzinger et al., 2009; Florel et al., 2010). The pathogen enters a limited saprophytic phase in the later stages of its disease cycle in which microsclerotia are formed in the dying stem parenchyma. Afterwards, with the decomposition of senescence foliage the microsclerotia are released to the soil and thus microsclerotia play a vital role as the primary source of disease in the field (Schnathorst, 1981; Neumann & Dobinson, 2003).

1.3 Plant phenolics

Plant phenolics are secondary metabolites that are synthesized from carbohydrates via the shikimate pathway. Phenylalanine ammonia lyase (PAL) is the key enzyme of the phenylpropanoid pathway which is catalyzing the biosynthesis of phenolics from the aromatic

amino acid phenylalanine. Plants synthesize a greater number of secondary metabolites than animals as they cannot depend on physical mobility to keep away from their predators and consequently developed a chemical defence against such predators (Lattanzio et al., 2006). Phenolics are ubiquitous in the plant kingdom and found in all fruits and vegetables. All the plant parts contain phenolic compounds, but concentrations vary between different tissues of the plant and within different populations of the same plant species. Phenolic contents also vary in plants depending on climatic condition and harvest season (Harbaum, 2007). Plant phenolics comprise a complex mixture, and until now only a small number of plants have been examined systematically for biologically active phenolic compounds. Therefore, the data on plant phenolics are incomplete. Robbins (2003) described that more than 8000 phenolic compounds have been isolated and reported from plant sources which have diverse structural configurations and polarities. Amounts of phenolics in plants can be up to several grams per kilogram fresh weight and they exhibit an enormous variety of chemical structures which include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins (Balasundram, 2006) (Fig. 1). Crozier et al. (2006) stated that phenolics can also be classified based on the arrangement and number of their carbon atoms in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others). These phenolics are commonly conjugated to sugars and organic acids. Flavonoids (mainly flavonols, but also anthocyanins) and the hydroxycinnamic acids are the most prevalent and diverse group of polyphenols in *Brassica* species (Cartea et al., 2011).

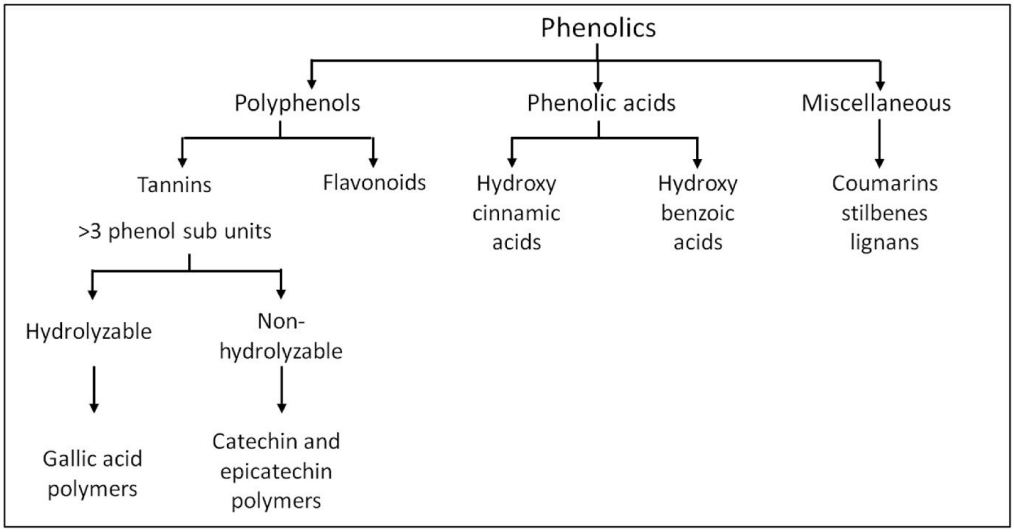

Figure 1: Classification of plant phenolics (from Balasundram et al., 2006).

The polyphenolic flavonoids consist of fifteen carbons with two aromatic rings connected by a three-carbon bridge (C6-C3-C6 carbon skeleton). Flavonoids, which are the most numerous phenolics in the plant kingdom are present in high concentrations in the epidermis of leaves and fruits. Among the flavonoids, flavonols are the most widespread in the plant kingdom. The main flavonols in *Brassica* crops are quercetin, kaempferol and isorhamnetin (Fig. 2), most commonly found as *O*-glycosides.

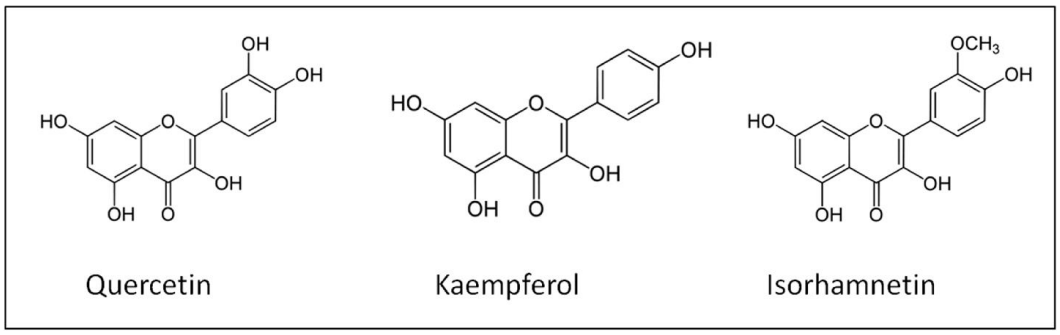

Figure 2: Main flavonols found in *Brassica* crops. From Cartea et al. (2011).

Contrary hydroxycinnamic acids are non-flavonoid phenolic compounds and they are characterized by the C6-C3 structure. These phenolic compounds are abundant in plants and play a role in both structural and chemical plant defence strategies. Derivatives of cinnamic acid can occur freely or as components of cell walls in plants. The most common cinnamic acids in *Brassica* vegetables are *p*-coumaric, sinapic and ferulic acids (Fig. 3), which are often found in conjugation with sugar or other hydroxycinnamic acids (Olsen et al., 2009; Lin et al., 2010).

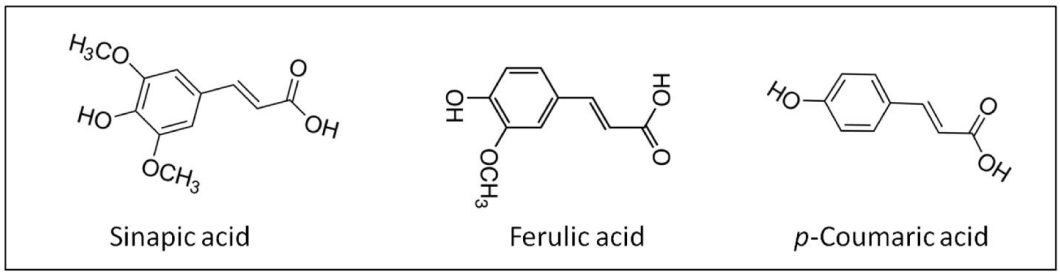

Figure 3: Most common hydroxycinnamic acids in *Brassica* vegetables. From Cartea et al. (2011) .

Phenolics are present in plants as free (e.g. esters of aglycones with sugars and/or organic acids) and bound (cell wall-associated) forms. These compounds are used in plants for pigmentation, growth, reproduction, resistance to pathogens and for many other functions.

Certain phenylpropanoid compounds are polymerized to form lignin which also acts as defensive barriers in plants (Thomma et al., 2001; Gayoso et al., 2010).

1. 4 Lignin and lignin monomer composition in plants

Lignin is a major component of secondary cell walls in plants resulting from the oxidative polymerization of at least two units of the cinnamyl alcohols (monolignols) *p*-coumaryl, coniferyl and sinapyl alcohol, forming *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin monomer units, respectively. The composition of lignin varies across plant lineages and tissue so that gymnosperms generally contain H and mainly G lignin monomers, whereas, angiosperms mostly contain G and S and traces of H lignin monomers (Baucher et al., 2003). Initially lignin monomers and their precursors are synthesized in the cytosol (endoplasmic reticulum) and later transported to the cell wall (Fig. 4). Lignin is deposited in cell walls of tissues as tracheids, veins, fibers of xylem and phloem and sclereids. In case of lignin deposition, each type of lignin in plant tissues is spatially and temporally controlled and first H units are being deposited, followed by G units and finally S units (Bonawitz et al., 2010). Plants lignin rich in H and G residues is mainly deposited in primary cell walls and is strongly cross-linked (Terashima et al., 1988; Chabannes et al., 2001), while lignin rich in G and S units is mainly deposited in secondary cell wall, and the monomers form a linear polymer in which monolignol backbones are mainly linked by *β*-O-4 bonds (Chabannes et al., 2001) (Fig. 4). In general, the S/G ratio increases as the plant matures and the ratio of S-to-G subunits dictates the degree of lignin condensation by allowing for different types of polymeric linkages. Increased G monomer i.e. the lack of S units leads to more highly condensed lignin composed of a greater proportion of biphenyl and other carbon linkages, whereas S subunits are commonly linked through more labile ether bonds at the 4-hydroxyl position (Dixon et al., 1996; Li et al., 2000; Guo et al., 2001)

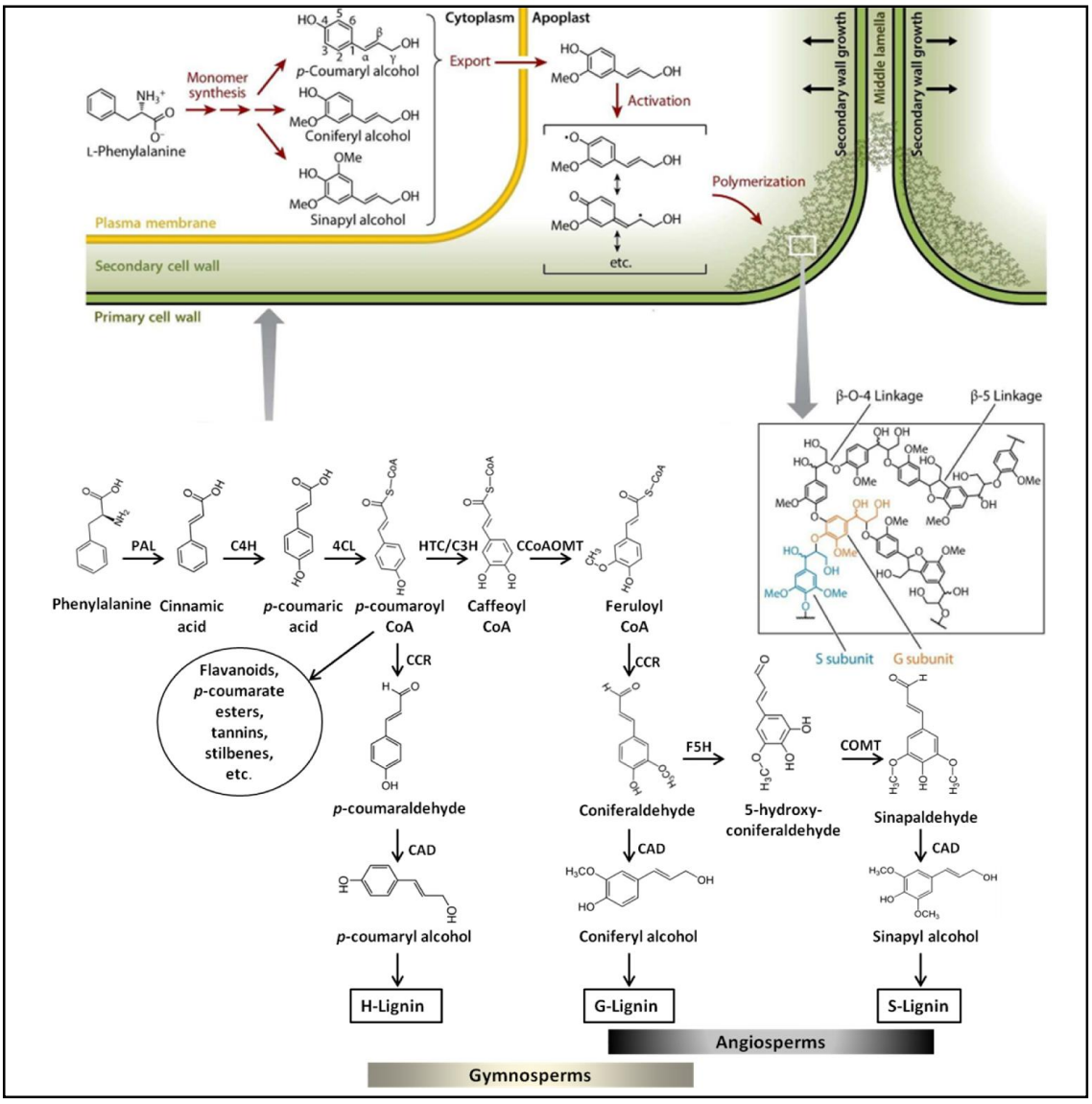

Figure 4: Biosynthesis of monolignols in *Arabidopsis thaliana.* Pathway modified after Bonawitz & Chapple (2010) and Stein (2013). Enzyme names are indicated with standard abbreviations: PAL, Phenylalanine ammonia lyase; C4H, Cinnamate-4-hydroxylase; 4CL, 4-Coumarate:CoA ligase; C3H, Cumarate-3-hydroxylase; HTC, Hydroxycinnamoyl-CoA:Shikimate hydroxycinnamoyl transferase; CCoAOMT, Caffeoyl-CoA O-methyl transferase; CCR, Cinnamoyl-CoA reductase; F5H, Ferulate 5-hydroxylase; COMT, Catechol-O-methyl transferase; CAD, Cinnamoyl alcohol dehydrogenase.

1.5 Role of phenolic compounds in plant disease resistance

Plants are frequently exposed to various biotic and abiotic stressors and therefore have evolved a multi-layered system of defence mechanisms (Eckey-Kaltenbach et al., 1994). Plant cells respond to these biotic and aboitic stimuli by synthesizing a number of secondary metabolites which may protect them against the causal agents. Phenolic compounds belong to

the secondary plant metabolites and in many instances, these substances serve as plant defence mechanisms against predation by insects, herbivores and microorganisms (Cowan, 1999; Beckman, 2000). Compounds that occur constitutively in plants and function as preformed defence metabolites are generally termed as phytoanticipins, and those that are produced in response to pathogen infection are called phytoalexins and are part of the induced defence response (VanEtten et al., 1994; Hammerschmidt, 1999). Phenolic phytoanticipins that inhibit the growth of fungi may include simple phenols, phenolic acids, flavonols, and some isoflavones. Phytoalexins that are induced in response to fungal infection include isoflavonoids, pterocarpans, furocoumarins, flavans, stilbenes, phenanthrenes (reviewed by Latanzio et al., 2001; Chérif et al., 2007).

It is known that resistance of plants to plant pathogenic fungi or bacteria is often a multifactorial process. The accumulation of secondary metabolites especially phenolic compounds can restrict the spread of the pathogen by the formation of biopolymers in plants (e.g. lignin and callose). However, this type of response is only one part of the diverse layers of plant response to pathogen infection. Soluble as well as cell wall-bound phenolic compounds accumulate early after infection in many plant-pathogen systems in both susceptible and resistant interactions. These types of physiological responses may include cell wall thickenings and appositions, such as papillae, as well as the occlusion of plant vessels. From previous investigations it was suggested that biological functions of phenolics range from cell wall reinforcement through esterification, antimicrobial activity, phytohormones, or local and systemic signaling for defence gene induction (Nicholson & Hammerschmidt, 1992; Ryals et al., 1996). Phenolic metabolites may restrict fungal growth as well as disease development in plants through various mechanisms such as inhibition of extracellular fungal enzymes (cellulases, pectinases, laccase, xylanase), inhibition of fungal oxidative phosphorylation, nutrient deprivation (metal complexation, protein insolubilisation), and antioxidant activity in plant tissues (Jersh et al., 1989; Scalbert, 1991). Phenylpropanoid metabolites can be conjugated to various cell wall substances such as polysaccharides, structural proteins or polyamines (Röpenack et al., 1998). Phenolic acids are primarily esterified to the cell wall components, especially to the cell wall polysaccharides and later cross-linked to lignin monomers via ether bonds or directly esterified to the side chain of lignin monomers (reviewed by Santiago et al., 2013). The esterified cell wall phenolic compounds may protect the plants from pathogen invasion (Harbaum, 2007). Cross linking in plant cell wall strengthens the cell wall (Zarra et al., 1999) and is also involved in the

regulation of the cell wall extensibility and elongation processes (Azuma et al., 2005; Hossain et al., 2007).

The role of phenolic metabolites in plant disease resistance has been described by many researchers. In previous studies it was shown that concentrations of phenolic metabolites change in plants upon infection by plant pathogens (De Ascensao & Dubrey, 2003; Eynck et al., 2009b; Obermeier et al., 2013). For example, ferulic acid, 4-hydroxybenzoic acid and *p*-coumaric acid concentrations increased in roots of tomato plants upon elicitation with four elicitors which are produced from *Fusarium* mycelium extract (Mandal & Mitra, 2007, 2008). The amounts of total constitutive and induced soluble phenolics increased in different parts of the capitulum in resistant sunflower lines after inoculation with *Sclerotinia sclerotiorum* compared to susceptible lines (Prats et al., 2003). Soylu (2006) found that accumulation of phenolics, but not camalexin, may be involved in resistance against *Pseudomonas syringae* in *Arabidopsis thaliana*. Plant cell walls respond to fungal pathogens during their invasion by accumulating phenolics and phenolic polymers such as lignin (Coffey & Cassidy, 1984; Carver et al., 1998ab). Induced resistace has been shown in barley against *Blumeria graminis* f. sp *hordei* due to deposition of phenolics in the cell walls (Lyngkjáer & Carver, 1999). Dehydroferulic acid in maize was found to be correlated with resistance to *Fusarium graminearum* (Bily et al., 2003). Upon inoculation with *Verticillium albo-atrum* cell cultures of tomato accumulated up to five fold higher levels of wall-bound phenolics than were found in mock-imoculated cultures (Bernards & Ellis, 1991)

Chérif et al. (2007) described that lipophilic properties and the presence of a hydroxyl group in phenylpropanoids may play a key role in their antifungal activity, allowing respectively the penetration of biological membranes and oxidative phosphorylation uncoupling. Hydroxycinnamaldehydes have been described to be more fungitoxic than hydroxycinnamic acids and hydroxycinnamyl alcohols in *in vitro* culture (Barber et al., 2000). The caffeic acid ester chlorogenic acid, and ferulic acid were shown to be strong inhibitors of different fungal pathogens (Lattanzio et al., 2001). Hydroxybenzoic acids such as 2,5 dihydroxybenzoic acid and 2,5 dimethoxybenzoic acid do inhibit spore germination and mycelial growth of different pathogenic fungi, including *F. oxysporum*, *S. sclerotiorum*, *P. digitatum, G. album* and *B. cinerea* very effectively in *in vitro* assays. These compounds also control disease development of these fungi *in vivo* (Lattanzio et al., 1996). In general, hydroxybenzoic and hydroxycinnamic acids and their aldehydes have been accumulated in different crop species

in response to infection by different plant pathogens and have been suggested to be important factors in disease resistance in plant-microbe interactions. Furthermore, phenolic compounds might be related to diseases susceptibility through the production of mycotoxin and/or through increasing hydrolytic enzyme activity (Caroline et al., 2009). In summary, specific plant phenolics can have a protective function by inhibiting fungal growth and thus disease, but others might have a disease promoting function by increasing mycotoxins levels and hydrolytic enzyme activities.

1.6 Role of lignin in plant disease resistance

Lignin is an end product of the phenylpropanoid pathway and the second most abundant plant polymer after cellulose found in nature. Ros-Barceló (1997) stated that lignification is a terminal process of specialized plant cells capable of forming secondary cell walls such as xylem and phloem cells and their adjacent fibre cells and sclereids. Lignin functions as a genetically inducible physical barrier in plants in response to microbial attack (Jaeck et al., 1992; Ni et al., 1996; Hatfield & Vermerris, 2001). In addition to structural support and defence response in plants, lignin has a role in water transport as a hydrophobic constituent of vascular phloem and xylem cells (Ros-Barcelo, 1997; Inoue et al., 1998).

It is well known that lignification is a common phenomenon in the expression of disease resistance in plants. Lignin synthesis is induced in response to mechanical damage or wounding and many plants respond to invading pathogens with the deposition of lignin and lignin-like material (Boudet et al., 1995). Deviations in lignin composition and augmented lignifications have been described as being the main resistance pathway to fungal invasion in a number of different host-pathogen interactions (Vance et al., 1980; Nicholson & Hammerscmidt, 1992; Dixon & Paiva, 1995). Hückelhoven (2007) found that a lignified cell wall is water resistant and therefore less accessible to fungal cell wall-degrading enzymes. Smit & Dubery (1997) found that synthesis and deposition of lignin and lignin-like polymers increased after exposure of cotton hypocotyls to an elicitor of *V. dahliae*. They also observed that after elicitor treatment lignin polymers increased faster and higher concentration in a resistant cultivar than in a susceptible. The amount of lignin increased significantly in pepper varieties differing in their degree of resistance against *V. dahliae* after inoculation *(*Pomar et al., 2004). Lignin monomer ratio changes in the stem of *Camelina sativa*, a plant species

belonging like *B. napus* to the *Brassicaceae* family, after infection with *Sclerotinia sclerotiorum* (Eynck et al., 2012).

1.7 Plant defence in response to *Verticillium* infection

Until now resistance mechanisms in *Brassica* species against *V. longisporum* are mostly unknown. But such mechanisms are well described in response to *V. dahliae* infection in other plant species. Two different levels of resistance mechanism are described in response to *V. dahliae* infection in host plants. One of them is the rapid deposition of lignin in the endodermis and the surrounding tissues. This type of resistance mechanism is active in prevascular phase where the fungus grows in the root cortex, but is not able to grow further into the vascular system due to lignin acting as a barrier against further outspread of the fungal pathogen (Talboys, 1972; Eynck et al., 2007). On the other hand, resistance in the vascular phase is characterized by quick deposition of suberin and other xylem coating materials (Lee et al., 1992; Lulia, 2005) or vessel occulation by gels, gums or other deposits (Peggs & Braddy, 2002). Both of these mechanisms are involved in the reduction of the spread of the fungus to neighboring host cells. The induction of phytoalexins, PR proteins and phenolic compounds is also involved in the reduction of fungal growth in vascular tissues of infected host plants such as tomato and *A. thaliana* (Williams et al., 2002; Tjamos et al., 2005; Gayoso et al., 2010).

Some responses of *B. napus* in the interaction with *V. longisporum* are similar to the ones described in the interaction of different plant species with *V. dahliae*. Upon *V. longisporum* infection, vascular occlusions through deposition of phenolic compounds and lignin in hypocotyl tissues of rapeseed were found to accompany the resistance phenotype in certain *B. napus* lines (Eynck et al., 2009b). Also, a significant increase in phenylalanine ammonia lyase (PAL) activity in hypocotyl tissues of *β*-aminobutyric acid (BABA) treated oilseed rape plants upon *V. longisporum* infection indicate that the phenylpropanoid pathway plays a crucial role in the resistance reaction (Kamble et al., 2013). Florel et al. (2012) described that lignifications and cell wall materials with an altered composition of carbohydrate polymers increased upon *V. longisporum* infection in the *A. thaliana* leaf apoplast. Phenylpropanoids and lignans were accumulated in leaves of *A. thaliana* upon infection with *V. longisporum* (Götze et al., 2011).

Another study concerning the plants answer to *V. longisporum* infection showed that the jasmonic acid and ethylene signalling pathways, but not the salicylic acid signaling pathway, are involved in the resistance activation in *A. thaliana* studied in mutants deficient in hormone signalling (Johansson et al., 2006a; Pantelides et al., 2010; Fradin et al., 2011). Six apoplast proteins (three peroxidases PRX52, PRX34, P37, serine carboxypeptidase SCPL20, α-galactosidase AGAL2 and a germin-like protein GLP3) which have functions in defence and cell wall modification increased upon *V. longisporum*-infection in *A. thaliana* (Floerl et al., 2012). The above findings are indicating that vascular occlusion through phenolics and lignin, and induction of PR proteins, signals molecules and carbohydrate polymers are involved in *V. longisporum* resistance mechanism in Brassicaceae plants. However, the relevance of all these mechanisms for a successful resistance expression in *B. napus* is unclear.

1.8 Molecular markers, QTL analysis and marker assisted breeding

A molecular marker or DNA marker is a particular sequence of DNA that is identifiable with a certain location within the genome. In molecular biology and biotechnology, molecular markers are used to identify a particular sequence of DNA in a pool of unknown DNA. Henry (2001) described that molecular markers are leading tools for identification of plants and determining the relationships between plants. This identification of plants may be useful in plant breeding, plant production and for intellectual property rights management. In plant breeding, determination of genetic relationships is required in evolutionary and conservation genetic analysis and in selection of germplasm. Within the last two decades, there has been enormous increase worldwide in the use of genetic marker methods to assess genetic variation in crop plants. Molecular techniques may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods. In conventional breeding, the use of DNA markers has also improved the accuracy of crosses and allowed breeders to produce strains with combined traits that were not possible before the introduction of DNA technology (Stuber et al., 1999).

During the last decades, significant progress has been made in the development of molecular markers and their use in quantitative trait loci (QTL) analysis controlling important agronomic traits in all major crops. Collard et al. (2005) defined QTL as the regions within genomes that contain genes associated with a particular quantitative trait. Analysis of QTL is based on detecting an association between marker of a specific genotype and phenotype.

Tanksley (1993) stated that the genes or QTL controlling traits can be detected by genetic linkage analysis, which is based on the principle of genetic recombination during meiosis. A linkage map points out the position and relative distance between markers along chromosomes. There are three major steps required for creating a linkage map: production of a mapping population, identification of polymorphism, and linkage analysis of markers (Collard et al., 2005).

Marker assisted selection (MAS*)* is the specific usage of DNA markers in plant breeding to select for particular traits or genotypes and it is considered a component of the new discipline of molecular breeding (Bertand et al., 2008). Construction of a high-density or well-saturated marker linkage map is the most important step required for a comprehensive genetic study and marker-assisted selection (MAS) approach in any crop as the main objective of QTL mapping in plants. Markers that are close together or tightly-linked to genes of interest have been identified, prior to field evaluation of large numbers of plants. Breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTL (Michelmore, 1995; Young, 1996; Ribaut et al., 1997) and by this way plant breeding is often directly supported by marker assisted selection.

The use of modern molecular genetics tools permits the application of newly available genome sequencing information for crop improvement. Molecular markers have been used to map agronomically important genes in *Brassica* genomes and to aid rapeseed breeding and selection procedures (reviewed by Snowdon & Friedt, 2004). The complete sequencing of the *A. thaliana* genome (The Arabidopsis Initiative 2000), a model crucifer, has also opened the window for detailed comparative investigations of the complex structures of *Brassica* genomes (Quiros et al., 2001; Schmidt et al., 2001). Along with new, much informative and high-throughput marker technologies, the complete sequence information and gene expression data which are now available from Arabidopsis have potential application in the genetic analysis and resistance breeding of *Brassica* crops. Furthermore, the application of haplotype information for allele-trait association studies has also an effective role in *Brassica* breeding.

The greater part of molecular work in oilseed rape breeding until now is based on genetic mapping using different DNA marker systems, in single segregating populations for specific investigations of particular traits of interest. A considerable number of marker technologies have arisen due to development of PCR techniques over the last 20 years, which have

facilitated the generation of high-density molecular maps for all the major *Brassica* crop species (reviewed by Snowdon and Friedt., 2004). DNA markers linked to disease resistance trait have been reported, and a number of them are now successfully integrated into rapeseed resistance breeding programmes such as blackleg/phoma stem canker, clubroot, stem rot and white rust. However, often traditional QTL mapping are rarely directly useful to develop effective markers for map-based cloning of the responsible genes due to their limited resolution. Therefore reports for successful marker-assisted selection for quantitative traits in the literature have been limited. But the information from Arabidopsis together with comparative analysis of its syntenic relationships to *Brassica* genomes may assist in overcoming these limitations in oilseed rape breeding programme (reviewed by Snowdon & Friedt, 2004). In addition, the use of next generation DNA sequencing technology will speed up *Brassica* genome research. These second generation DNA sequencing technologies can produce more than 200 billion nucleotides of sequence data in a single run (Imelfort & Edwards, 2009) and data production continues to increase rapidly. The completed genome sequences of *Arabidopsis*, *Brassica rapa* and the expected forthcoming publication of *B. oleracea* and *B. napus* provides the opportunity to conduct re-sequencing and comparative genomic analysis of individuals and assist in the identification and characterization of sequence variants in oiseed rape (*B. napus*). Edwards & Batley (2009) described that crop genome sequencing data can be useful for genome analysis leading to crop improvement. In recent years, researchers are focusing on single nucleotide polymorphisms (SNPs-GBS marker, genotyping-by-sequencing approaches) in crop species including oilseed rape for genetic variation analysis as well as marker-trait association. Henry (2008) described that simple sequence repeat (SSR) or microsatellite markers are replaced by SNP markers as larger volumes of sequence data became available. Snowdon & Luy (2012) described that genetic analysis of the complex *B. napus* genome using mapping-by-sequencing techniques offers a powerful bridge between genetic map and genome sequences. The eventual progress of the high-throughput genomics technologies provide unparalleled possibilities for gene discovery, complex trait analysis by genome-wide association studies, global gene expression analyses, genomic selection and the implementation of predictive breeding strategies (Snowdon & Luy, 2012).

1.9 Aims of the thesis

Resistance to *V. longisporum* is based on an internal reaction of the plants within hypocotyls associated with deposition of phenolic compounds and lignin. The detailed analysis and identification of the phenolics expression in a *B. napus* mapping population segregating for *V. longisporum* might help to get insights into the mechanisms underlying quantitative resistance. The main objective of the present study was to identify genomic regions involved in *V. longisporum* disease resistance in *B. napus* and to identify metabolites associated with disease resistance or susceptibility. This major objective was addressed by the following sub-objectives.

1. Identification of QTL for *V. longisporum* resistance in mapping populations with different genetic backgrounds and markers which can be used in marker-assisted breeding in a broad range of materials with diverse genetic backgrounds.
2. Investigations of the phenolic metabolic profiles within the hypocotyl of a mapping population segregating for *V. longisporum* resistance.
3. Identification of QTL for soluble and cell wall-bound phenolic compounds in the hypocotyl of a *V. longisporum* infected mapping population.
4. Comparative quantitative trait mapping for resistance and phenolic metabolites, involved in the resistance reaction within the plant hypocotyls.
5. Identification of phenolic metabolites and lignin monomers and their association with Verticillium disease resistance in oilseed rape (OSR) and putative involvement in resistance mechanisms and pathways.

2 Materials and Methods

2.1 Materials

2.1.1 Mapping population and resistance donors

The study was performed using a population of 214 doubled haploid (DH) rapeseed lines which was produced from a cross between an inbred line of the winter OSR cultivar Express 617 and R53, a resynthesized (RS) line. This DH population was produced by Saaten Union BioTec GmbH (Leopoldshöhe, Germany) from plants of an F1 generation of the cross Express 617 x R53 (see Radoev et al., 2008; Basunanda et al., 2010). 'Express 617' is an inbred line derived from the partially *V. longisporum*-resistant German commercial winter OSR cultivar ´Express´ with `00` quality (almost zero seed erucic acid, low seed glucosinolate content). R53 is a resistant RS line with intermediate levels of erucic acid and glucosinolates in seeds developed from interspecific hybridization between a kale (*B. oleracea* var. *sabellica*) and a chinese cabbage (*B. rapa* spp. *pekinensis*). Four other DH populations, i.e. SW08-190001, SW08-190002, DSV-1575 and DSV-1605, were used for marker validation. These populations were produced by the plant breeding companies Lantmännen SW Seed (Svalöv, Sweden) and Deutsche Saatveredelung AG (Lippstadt, Germany) using genetically divergent RS *B. napus* accessions as resistance donors. Details of the pedigree and resistance donors of all populations are listed in Table 1.

Table 1: Overview of oilseed rape (*B. napus*) doubled haploid populations, their parents, resistance sources and pedigrees

DH Population	Susceptible parent		Resistant parent		
	Name	Origin	Name	Origin	Pedigree of RS parent
ExR53-DH[a]	Express 617	Inbred line of cultivar 'Express', Giessen University, Germany	R53	RS *B. napus*, Göttingen University, Germany	Kale x chinese cabbage Early Hybrid G, Germany
SW99-307[b]	307-230-2	Breeding line, Lantmännen SW Seed, Sweden	307-230-1	RS *B. napus*, Lantmännen SW Seed, Sweden	White cabbage x winter turnip rape, Sweden
SW08-190001	MLCH 10 x Honk	Breeding line, Lantmännen SW Seed, Sweden	Credit x BRA1008	RS *B. napus*, Lantmännen SW Seed, Sweden	Winter turnip rape cv. 'Credit' x kale BRA1008

	Susceptible parent		Resistant parent		
	Name	Origin	Name	Origin	Pedigree of RS parent
SW08-190002	MLCH 10 x Honk	Breeding line, Lantmännen SW Seed, Sweden	Credit x HRI8207	RS *B. napus*, Lantmännen SW Seed, Sweden	Winter turnip rape cv. 'Credit' x kale HRI8207, Syria
DSV-1575	OaseE103	Inbred line of cultivar 'Oase', Deutsche Saatveredelung, Germany	S228.8.1	RS *B. napus*, Giessen University, Germany	Pak choi 56515 x kale HRI8207, Syria
DSV-1605	OaseE103	Inbred line of cultivar 'Oase', Deutsche Saatveredelung, Germany	FS94.3	RS *B. napus*, Giessen University, Germany	Spring turnip rape cv. ‚Asko', Germany x white cabbage NIV1428, Russia

Two DH populations (ExR53-DH and SW99-307) with *V. longisporum* resistance from different sources were used for comparative QTL mapping, while the applicability of QTL-linked markers for prediction of resistance was validated in four additional populations derived from a further four genetically diverse resistance donors. RS = resynthesized. [a]Radoev et al., 2008; Basunanda et al., 2010, [b]Rygulla et al., 2008.

2.1.2 Primers and simple sequence repeat (SSR) markers

The following SSR or microsatellite markers have been used in this study. Microsatellite primer pairs and primer sequences are mentioned in table 2. Primer sequences are given in 5'-3' direction. Details on the obtained allele sizes using these markers are listed in supplementary table S2 in Obermeier et al. (2013).

Table 2: Primers used in SSR marker analysis

Marker name	Forward primer sequence	Reverse primer sequence
Na12G12	GAGTGACATCGAAAATCAGATAGC	CCTAAATGGAAAGGCTTGGC
Ol10B02	CACGAACGCGAGAGAGAGAG	TGCATAAGCTCGAAGAGACG
CB10611	GTATCTGCGACAGTGGGA	AGCTTGGCTGTAATGACG
BRMS030	TCAGCCTACCAACGAGTCATAA	AAGGTCTCATACGATGGGAGTG
CB10065	CGGCAATAATGGACCACTGG	CGGCTTTCACGCAGACTTCG
Na12C01	CCAGGTTACTGTTAAAGAATAAGAGAG	ATCGTCTGCGAGTCTCCTTG
Ra2F11	TGAAACTAGGGTTTCCAGCC	CTTCACCATGGTTTTGTCCC
CB10027	CGGCTTGTAAACCTTG	GACTCGAAAATCACTAACAC
Na10D11	GAGACATAGATGAGTGAATCTGGC	CATTAGTTGTGGACGGTCGG
BRMS309	CAAGAGCAAGTTTGAAACAAACGAT	CATCAGTTCTTGATATGCTAGGTA
Na12D10	GCCCTCAAAAAGAGAGTTGC	TTGATGTGGGTGAGGCTAGG
CB10536	ACCTTGTTCTTTGCCTCC	GCAACAGCAAAGACAACC
Ol10E12	TGCTCTGCAAGATATTCCCC	AACCGTCACAGATCCTGTCC
CB10258	ATGATGCCTAGCATGTCC	AAGCTAAAGCGAAAGAAGC
CB10241	CTCTTCGAGACAATGCGT	GGATTGAACAACGTGCAT

2.1.3 Chemicals

The following chemicals were used in HPLC and GC/MS analysis for this study.

Chemicals	Supplier
Gallic acid (HPLC-grade)	Extrasynthese, France
Protocatechuic acid (HPLC-grade)	Sigma-Aldrich, Germany
4-hydroxybenzoic acid (HPLC-grade)	Sigma-Aldrich, Germany
Gentisic acid (HPLC-grade)	Sigma-Aldrich, Germany
Salicylic acid (HPLC-grade)	Roth, Germany
Vanillic acid (HPLC-grade)	Sigma-Aldrich, Germany
Shikimic acid (HPLC-grade)	TransMIT, Germany
Vanillin (HPLC-grade)	Sigma-Aldrich, Germany
p-Coumaric acid (HPLC-grade)	Sigma-Aldrich, Germany
Ferulic acid (HPLC-grade)	Sigma-Aldrich, Germany
Sinapic acid (HPLC-grade)	Sigma-Aldrich, Germany
5-Hydroxyferulic acid (HPLC-grade)	TransMIT, Germany
1,3-Dicaffeoylquinic acid (HPLC-grade)	TransMIT, Germany
Dihydrocaffeic acid (TLC-grade)	TransMIT, Germany
Caffeic acid (HPLC-grade)	Sigma-Aldrich, Germany
trans-Cinnamic acid (HPLC-grade)	Extrasynthese, France
Chlorogenic acid (HPLC-grade)	Sigma-Aldrich, Germany
Rosmarinic acid (HPLC-grade)	TransMIT, Germany
Flavanone (HPLC-grade)	Extrasynthese, France
Naringin (TLC-grade)	TransMIT, Germany
Naringenin (HPLC-grade)	TransMIT, Germany
Phlorizin/ Phloridizin (HPLC-grade)	TransMIT, Germany
Phloretin (HPLC-grade)	TransMIT, Germany
(+) Catechin (HPLC-grade)	TransMIT, Germany
(-) Epicatechin (HPLC-grade)	TransMIT, Germany
(-) Epigallocatechin gallate (HPLC-grade)	TransMIT, Germany
Isoquercitrin (TLC-grade)	TransMIT, Germany
Isorhamnetin (TLC-grade)	TransMIT, Germany
Kaempferol-3-*O*-glucoside (HPLC-grade)	TransMIT, Germany
Kaempferol-7-*O*-glucoside (HPLC-grade)	Extrasynthese, France
Kaempferol (HPLC-grade)	TransMIT, Germany
Kaempferol-3-O-rhamnoside (TLC-grade)	TransMIT, Germany
Quercetin (HPLC-grade)	TransMIT, Germany
Quercetin 3-O-galactoside (Hyperoside) (HPLC-grade)	TransMIT, Germany
Quercetin 7-methylether (Rhamnetin) (HPLC-grade)	TransMIT, Germany
Quercetin 3,7,3‘,4‘-tetramethylether (HPLC-grade)	TransMIT, Germany
7-hydroxyflavonol (HPLC-grade)	Extrasynthese, France
Rutin (HPLC-grade)	TransMIT, Germany
Tangeretin (TLC-grade)	TransMIT, Germany

Cirsimaritin (TLC-grade)	TransMIT, Germany
Nobiletin (TLC-grade)	TransMIT, Germany
Cyanidin chloride (HPLC-grade)	Extrasynthese, France
Procyanidin B1 (HPLC-grade)	Extrasynthese, France
Procyanidin B2 (HPLC-grade)	Extrasynthese, France
Coniferyl alcohol (98%)	Sigma-Aldrich, Germany
Coniferyl aldehyde (98%)	Sigma-Aldrich, Germany
Sinapyl alcohol (Analytical-grade)	Sigma-Aldrich, Germany
Sinapine thiocyanate	Analytical discovery, Germany
Sinapoyl glucose	Seed sinapoyl glucose IPZ, JLU, Giessen
Tetracosane (Analytical-grade)	Sigma-Aldrich, Germany
Boron trifluoride etherate (BF3)	Sigma-Aldrich, Germany
Ethanethiol	Sigma-Aldrich, Germany
Dioxane	Merck/ VWR, Germany
Methylene chloride	Roth, Germany
Sodium bicarbonate	Fluka analytical, Germany
Granular anhydrous sodium sulphate (Na_2SO_4)	Sigma-Aldrich, Germany
Pyridine	Sigma-Aldrich, Germany
N,*O*-bis(trimethylsily)acetamide	Roth, Germany
Acetone	Merck, Germany
Sodium hydroxide (NaOH)	Sigma-Aldrich, Germany
85% Phosphoric acid (H_3PO_4)	Roth, Germany
Ethyl acetate	Roth, Germany
Methanol (HPLC grade)	Roth, Germany
Formic acid (CH_2O_2)	Roth, Germany
Acetonitrile (HPLC grade)	Roth, Germany

HPLC = High performance liquid chromatography, TLC = Thin layer chromatography

2.1.4 Equipments

Equipment	**Supplier**
LI-COR 4200 DNA analyzer	LI-COR Biosciences GmbH, Germany
High performance liquid chromatography (HPLC) machine (D-7000 HPLC system)	Merck-Hitachi, Germany/Japan
Nucleodur sphinx RP column (250 x 4mm, 5 µm)	Macherey-Nagel, Germany
Guard column (10 x 4 mm)	Macherey-Nagel, Germany
UV detector system L-7400	Merck-Hitachi, Germany/Japan
C18 mini columns	Alltech, Germany
Wheaton vial with a Teflon-lined screw cap	Fisher Scientific, Germany
Gas chromatography coupled with mass spectrometry (GC/MS) machine (Trace GC Ultra)	Thermo Fisher Scientific
ITQ 900 ion trap mass spectrometer	Thermo Fisher Scientific
Capillary column (TG-5MS, 30m x 0.32mm x 0.25um)	Thermo Fisher Scientific

2.1.5 Software

Software	Source/Reference
SAS version 9.2	SAS Institute Inc., Cary, NC, USA
JoinMap 3.0	Plant Research International, BV, Wageningen
QGene 4.3.9	Joehanes R, Nelson JC (2008) QGene 4.0, an extensible Java QTL-analysis platform. Bioinformatics 24:2788-2789 (www.qgene.org).
PlabQTL 1.2	University of Hohenheim, Germany
Windows QTL Cartographer 2.5	North Caroline state University, USA
R/qtl	Broman KW, Wu H, Sen Ś, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics 19:889-890 (www.rqtl.org).
D-7000 HPLC system manager	Hitachi, Japan
Xcalibur 2.1 (GC/MS analysis)	Thermo Fisher Scientific

2.2 Methods

2.2.1 Inoculation of plant materials, assessment of disease symptoms and sampling

Plants were inoculated with *V. longisporum* in a greenhouse in 2010 at Göttingen University. Seeds were sterilized with 70% ethanol for 2 minutes and 1% sodium hypochlorite containing 0.1% Tween-20 for 15 minutes under constant shaking. Sterilized seeds were then washed twice with autoclaved water and sown in double-autoclaved silica sand for germination and initial growth in a greenhouse at 22°C temperature with a light regime of 14 h per day. After that seedlings were inoculated by a *V. longisporum* suspension and distilled water (mock treatment) at 14 days after germination according to Eynck et al. (2009a). The inoculation of seedlings was performed by means of a root-deep inoculation for 30 minutes with a mixture of two German *V. longisporum* isolates, VL40 and VL43 (Zeise & von Tiedemann, 2002a) at a spore concentration of 1×10^6 spores/ml. Following inoculation, seedlings were transferred into individual pots which were arranged in a completely random design containing sand/soil (1:1) and grown at same greenhouse conditions. Each experiment included a total of 20-24 *V. longisporum* inoculated and 10-24 mock-inoculated plants for each of the DH lines and parents as well as for the cultivars Falcon, Laser, Express and Lion (Fig. 5). Susceptible *B. napus* cultivars Falcon and Laser and the partial *V. longisporum*-resistant cultivars Express and Lion were included in this experiment as control for the resistance screening. Disease symptoms were evaluated over a time period of 4 weeks using an assessment key with nine classes (Eynck et al., 2009a). For each genotype, the area under the disease progress curve (AUDPC) value was calculated from the disease severity values according to Eynck et al.

(2009a). Also growth reduction was measured at 4 weeks after inoculation from the difference in the means of the height (in cm) from non-inoculated and inoculated plants. Phenotypic data (mean AUDPC and mean growth reduction) were received from the group of Professor Andreas von Tiedemann, Division of Plant Pathology and Crop Protection, Georg August University, Göttingen. Hypocotyls were collected at 28 days post inoculation (dpi) from both mock- and *V. longisporum*-inoculated plants and 24 pooled hypocotyls were immediately frozen in liquid nitrozen and then freeze dried for subsequent extraction of phenolic compounds and lignin monomers.

Figure 5: *V. Longisporum* (left) and mock-inoculated *Brassica napus* plants (right) of the partially resistant reference cultivar 'Express' 28 days post inoculation in a greenhouse screening procedure.

2.2.2 Simple sequence repeat (SSR) marker analysis

Genomic DNA samples were extracted from fresh or freeze-dried leaf material of DH lines according to Doyle & Doyle (1990). The M13-tailing procedure described by Berg & Olaisen (1994) was used for all SSR marker analyses. In this method the fluorescently labeled universal M13 primer 5′-AGGGTTTTCCCAGTCACGACGTT-3′ is added to the PCR reaction, and the forward primer of each SSR is appended with the sequence 5′-TTTCCCAGTCACGACGTT-3′. The PCR fragments are subsequently amplified by the labeled universal primer after the first cycle of amplification. All SSR amplification products were separated using a LI-COR 4200 DNA Analyzer (LI-COR Biosciences) and scored visually. The SSR markers used in the present study derived from numerous published *B. napus* maps (Lowe et al., 2004; Suwabe et al., 2004; Radoev et al., 2008; Rygulla et al.,

2008). See supplementary table S2, Obermeier et al., 2013, for detailed information on the SSR markers successfully mapped to the chromosomes of interest.

2.2.3 Genetic map construction, QTL and statistical analyses

A genetic map was created by using a framework map as a basis published by Radoev et al. (2008) where 191 markers were mapped in 275 DH lines of the mapping population. Raw marker scoring data were received from the group of Prof. Heiko Becker, Division of Plant Breeding, Department for Crop Sciences, Georg August University, Göttingen. In addition, more than 40 SSR primer combinations were selected from different publications and tested for their polymorphisms in a screening with the two parents for subsequent marker saturation of chromosomes C1 and C5. Among the 40 SSR markers 15 markers did show polymorphisms among the parents and were subsequently genotyped in 214 DH lines and added to the framework map using the program JoinMap 3.0 (Stam, 1993).

QTL were identified by composite interval mapping using the software QGene, version 4.3.9 (Joehanes & Nelson, 2008) with automatic forward cofactor selection allowing QTL cofactors instead of markers. The map was scanned at 2 cM intervals and used 2.5 as Log_{10}-likelihood ratio (LOD) value for QTL analysis. A value of $p \leq 0.005$ for type I error was also used as criteria to indicate putative QTL positions. Additive effects and phenotypic variation (R^2) explained by each QTL were also estimated and computed. Simultaneously, confidence intervals were calculated based on the 1-LOD drop-off method according to Lander & Botstein (1989) for each of the QTL. Later on, QTL obtained from QGene software analysis were confirmed by re-analysis using the alternative software packages PlabQTL version 1.2 (Utz & Melchinger, 1996), WindowsQTL cartographer version 2.5 (Wang et al., 2011) and R/qtl (Broman et al., 2003). Statistical analysis for phenotypic data was carried out with SAS version 9.2 (SAS Institute Inc.).

2.2.4 Extraction of soluble and cell wall-bound phenolics from rapeseed hypocotyls

Total soluble phenolics of containing free unconjugated phenolics, glycoside-bound phenolics, and ester-bound phenolics oilseed rape hypocotyls were extracted from 98 DH lines according to Eynck et al. (2009b). Extraction was performed from pooled freeze dried hypocotyl samples of 24 plants (50-100 mg) for each genotype. The principle is shown in

Figure 6. The following steps have been followed for the extraction of soluble phenolics from the mock- and *V. longisporum*–inoculated oilseed rape hypocotyls samples.

- 50-100 mg freeze dried hypocotyl tissues in 2 ml Eppendorf tubes were taken for each of the DH lines and crushed with metal beads in a tissue lyzer machine.
- 1.2 ml of 80% aqueous methanol was added in to the tubes containing crushed samples.
- The samples were incubated for 1 h at room temperature with continuous shaking.
- Samples were centrifuged at 13,000 x *g* for 10 min at 4°C.
- The supernatants were collected from the samples and stored in new 2 ml Eppendorf tubes at 4°C.
- The remaining pellets were extracted again with 1.2 ml of 80% aqueous methanol.
- The supernatants were merged in 2 ml Eppendorf tubes.
- The supernatants containing the extracted soluble phenolics were used for the quantitative analysis by RP-HPLC.

After extraction of soluble phenolic compounds the remaining pellets were used for the extraction of cell wall-bound phenolics according to Eynck et al. (2009b) with minor modifications. The following steps have been followed for the extraction of cell wall-bound phenolics from the mock- and *V. longisporum*-inoculated hypocotyl samples.

- After thoroughly washing the pellets, 1 ml of 1 M NaOH was added to the pellets.
- Samples were incubated for 1 h at 80°C .
- Samples were incubated for another 12 h at room temperature for alkaline hydrolysis.
- Samples were acidified to pH < 4 with 100 µl of 85% phosphoric acid (H_3PO_4).
- 500 µl of ethyl acetate was added and samples incubated on a rotary shaker for 30 minutes.
- Samples were centrifuged at 13,000 x *g* for 5 minutes
- The upper phase was collected.
- The remaining lower phase was extracted again with 500 µl ethyl acetate.
- The combined ethyl acetate phases were evaporated until dryness by using a vacuum evaporator machine.
- The residue pellets containing the extracted cell wall phenolics were resuspended with 800 µl of 80% methanol and used for quantitative analysis by RP-HPLC.

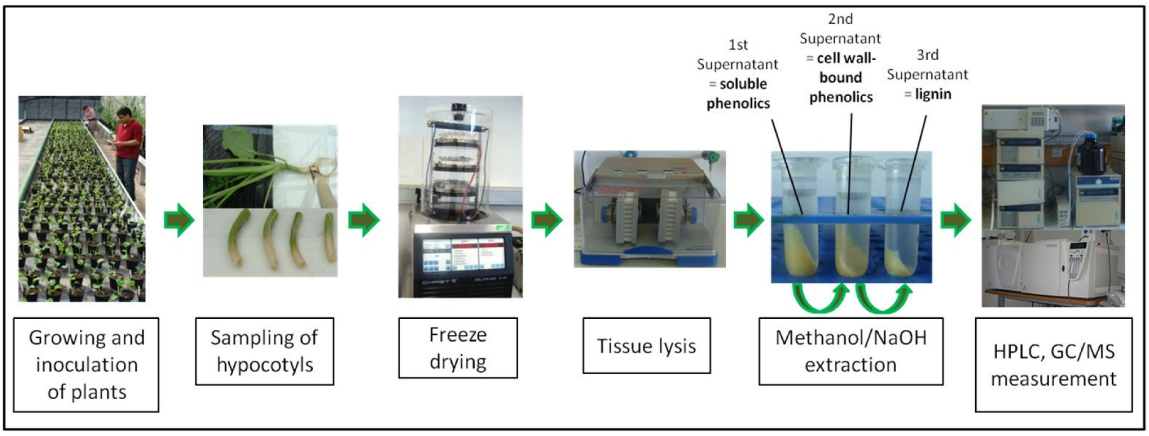

Figure 6: Work flow for the extraction and determination of phenolic compounds in oilseed rape hypocotyls.

2.2.5 Reverse phase-high performance liquid chromatography (RP-HPLC) analysis

Quantitative RP-HPLC analysis for soluble as well as cell wall-bound phenolics was performed using a Merk-Hitachi HPLC machine with a UV detector system L-7400 (Merk-Hitachi). Separation was carried out on a Nucleodur Sphnix RP column (250 x 4mm, 5 µm) containing a guard column (10 x 4 mm) with the same stationary phase from Macherey-Nagel (Germany). The column was maintained at 30°C and run at constant flow rate of 1 ml/min. The mobile phase consisted of two solvents: 4.5% (v/v) formic acid (solvent A) and acetonitrile (solvent B) for a total running time of 50 min with the gradient changed as follows: 20 min with a 95/5, 10 min with a 75/25, followed by 20 min with a 2/98 mix of solvent A and B. The HPLC column was equilibrated with the initial mobile phase (95% A and 5% B) for 30 min (Lipsa et al., 2012). The injected volume was set to 10 µl and samples were injected by an autosampler. Peaks from soluble and cell wall-bound phenolic compounds were detected at 280 nm and designated according to their retention time. The area under each detected peak was compared with the area of the internal standard peak for normalization and relative quantification of each peak size in each genotype. Protocatechuic and gallic acid were used as internal standards for the quantitative analysis of soluble and cell wall-bound phenolics fraction, respectively. Peaks in both phenolics fractions putatively corresponding to external standard were identified by comparison of the retention time with HPLC quality external standards obtained from Roth (Germany, number 5 in table 5), Sigma-Aldrich (Germany, 2-4, 6-10 and 14-18 in Table 5), Extrasynthese (France, 1, 28 and 32-34 in Table 5), TransMIT (Germany, 11-13, 19-21, 24-27 and 29-31 in Table 5) and Analytical Discovery (Germany, 23 in Table 5) (see the Materials chapter for details about the phenolic standards used in this study).

Further fracrtionation has been done for soluble and cell wall-bound phenolics fraction extracted from some selected DH lines into phenolic acids, monomeric and oilgomeric proanthocyanidines and polymeric proanthocyanidine/anthocyanidines through solid phase extraction C18 mini-columns (Alltech) according to Lipsa et al. (2012) and analysed by RP-HPLC.

2.2.6 Liquid chromatography coupled with mass spectrometry (LC/MS) analysis

Authentic standards and fractionated caffeic acid, vanillic acid, *p*-coumaric acid, vanillin, ferulic acid and sinapic acid from cell wall-bound phenolics fraction presumably identified by co-migration with HPLC standard were analysed by LC-MS according to Röhrich et al. (2012) with minor modifications. The LC/MS analysis was conducted with a Dionex Ultimate 3000 UHPLC (Dionex, Idstein, Germany) equipped with a high resolution microTOF-QII mass spectrometer. The chromatographic separation was achieved using a Nucleodur Sphnix RP column (250 x 2mm, 5 µm) from Macherey-Nagel (Germany), maintained at 35°C and run at constant flow rate of 0.25 ml/min. The mobile phase consisted of two solvents: water + 0.1% formic acid (solvent A) and 95% acetonitrile + 0.1% formic acid (solvent B) for a total running time of 50 min with the gradient changed as follows: 20 min with a 95/5, 10 min with a 75/25, followed by 20 min with a 2/98 mix of solvent A and B.

Mass spectrometric analyses of the samples were conducted in the positive ion mode and followed the three-step usual procedure: Mass spectra were recorded at a full scan 50 to 1000 m/z. This was followed by in-source CID (collision-induced dissociation) scan at ionisation energy of 150eV with 50 to 1000 m/z. After that MS/MS experiments on selected precursor ions have been done for the verification of the outcomes from the in-source CID scan at 150eV with <1000 m/z scanning range.

2.2.7 Analysis of the lignin monomer composition by a thioacidolysis procedure

Following the removal of soluble and cell wall-bound phenolic compounds, hypocotyl samples were subjected to thioacidolysis for the extraction of thioethylated lignin monomers according to Eynck et al. (2012) with minor modifications. The principle of the procedure is shown in Figure 7.

- 5 mg of ground, oven-dried extract-free cell wall residue material was transferred into a 5-ml glass Wheaton vial with a Teflon-lined screw cap.

- 1 ml of freshly prepared thioacidolysis reagent [2.5% boron trifluoride etherate, BF3 and 10% ethanethiol in recently distilled dioxane (v/v)] was added to each vial and blanked with nitrogen gas prior to sealing. This step was done in a glove box due to the intese odor of ethanethiol.
- Sample vials were incubated in a dry heating block at 100°C for 4 h with gentle agitation. The progress of reaction was halted by placing the samples at -20°C for 5 minutes.
- 200 µl of tetracosane (2.5 mg/ml methylene chloride) was added to each vial as an internal standard.
- 300 µl of 0.4 M sodium bicarbonate was added to adjust the pH of the reaction to between pH 3 and 4.
- 2 ml of water and 1 ml of methylene chloride were added to each vial to extract the reaction products from the aqueous mixture.
- Samples were recapped, vortexed and allowed to settle for about 5 min for phase-separation of the upper (aqueous) and lower (organic and containing lignin break-down products) phases.
- The organic phase (1.4 ml) was removed, filtered and cleared of residual water by passing through a Pasteur pipette packed with a small tissue-paper plug and about one inch (50 mg) of granular anhydrous Na_2SO_4.
- The cleared samples were evaporated to dryness in a dry heating block at 65°C overnight and resuspended in 2 ml of methylene chloride.
- Derivatization of sample was achieved by combining 50 µl of resuspended sample with 100 µl of pyridine and 400 µl of *N*,*O*-bis(trimethylsily)acetamide.
- After incubation for at least 2 h at 25°C, 1 µl of derivatized sample was analyzed by gas chromatography coupled with mass spectrometry (GC/MS).

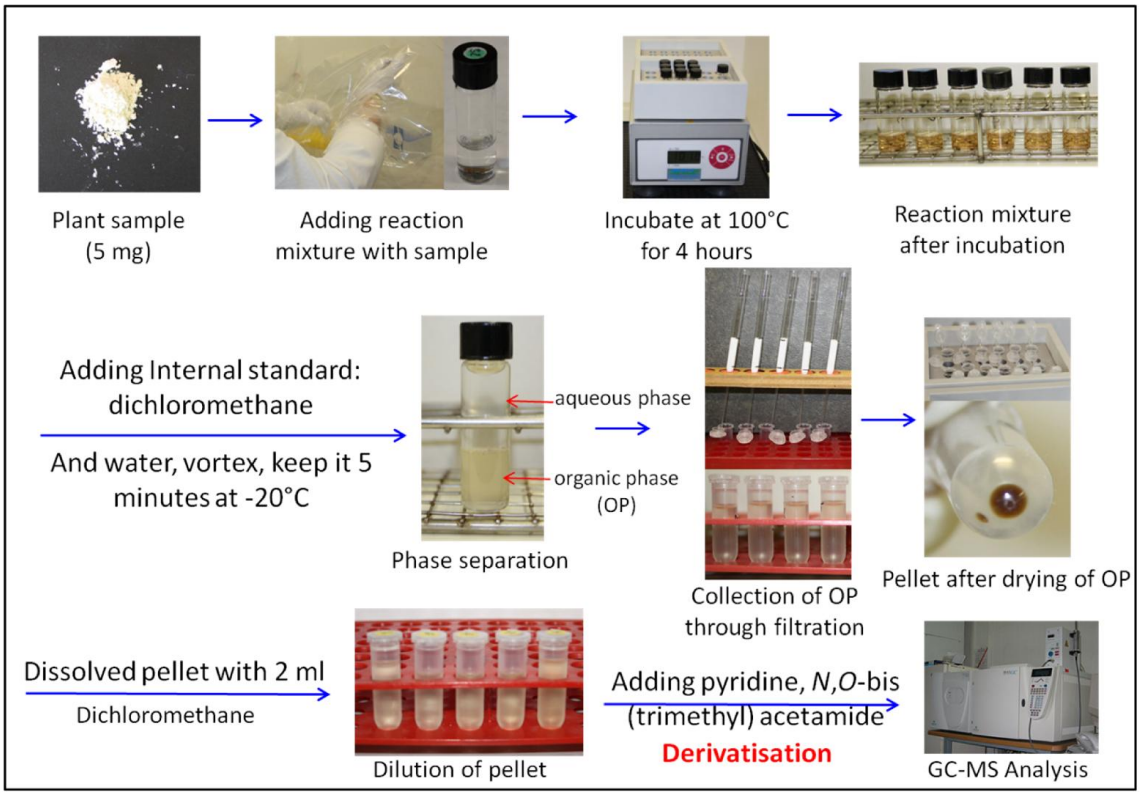

Figure 7: Schematic representation for the analysis of the lignin monomer composition in *B. napus* hypocotyls by thioacidolysis procedure.

2.2.8 Gas chromatography coupled with mass spectrometry (GC/MS) analysis

The GC/MS analysis was conducted with a Trace GC Ultra (Thermo Fisher Scientific) equipped with an ITQ 900 ion trap mass spectrometer (Thermo Fisher Scientific). A capillary column (TG-5MS, 30m x 0.32mm x 0.25um; Thermo Fisher Scientific) was used in the GC system and helium was used as carrier gas at a flow rate of 1 ml/min. The injection technique was splitless for 1 min and the volume of sample injected was 1 µl. Inlet temperature was 250°C, while the oven program consisted of an initial temperature of 130°C, 3 min hold, ramp temperature 3°C/min for 40 min to give a final temperature of 250°C and hold for 5 min. The ion source temperature was 200°C and the transfer line temperature was 250°C. Mass spectra were recorded at ionisation energy of 70eV with 50 to 550 m/z scanning range. Subsequently, thioethylated lignin monomers were identified by comparison of the retention time and mass spectra with GC quality external standards obtained from Sigma-Aldrich. Quantification was carried out by measurement of the relative areas under each peak based on internal standard (tetracosane). Two technical replicates were measured for each of the genotypes

3 Results

3.1 Creating a genetic map for resistance QTL mapping

The genetic map for the Express 617 x R53-DH population (ExR53) used in this study was constructed using simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) markers and has been previously described by Radoev et al. (2008). 15 polymorphic SSR markers mapping to chromosomes C1 and C5 were genotyped in 214 DH lines and added to the framework map using the program JoinMap 3.0 (Stam, 1993). Among the 15 newly added SSR markers, 10 are shown in the genetic maps in Fig. 8 with at least 1 centiMorgan (cM) genetic distance from previously mapped SSR markers. Map distances have been measured via Haldane's function in cM between markers.

3.2 QTL for *V. longisporum* resistance

Calculations for phenotypic data (AUDPC and growth reduction) were done based on four different experiments comprising different subsets of the Express 617 x R53-DH population. In the four experiments a total of 3,146, 1,212, 2,591 and 4,895 plants were monitored, respectively. In experiments 1 to 4 a total number of 100, 32, 82 and 98 DH lines were tested, respectively. In experiment 4 also the two parents were included. A total number of 214 different DH lines were tested in these 4 experiments. In experiment 4 randomly selected DH lines were retested: 52 from experiment 1, 15 from experiment 2 and 31 from experiment 3 and phenotypic data (AUDPC and growth reduction) for QTL analysis were received from Prof. Andreas von Tidemann, Division of Plant Pathology and Crop Production, Georg August University, Göttingen, Germany.

In the three independent resistance tests 1, 3 and 4, significant and stable major QTL with LOD scores larger than 2.5 for both resistance-related traits, AUDPC and growth reduction, were found at the same position on chromosome C5 (Fig. 8). No significant resistance-related QTL were detected in experiment 2, presumably due to the low number of DH lines tested (n = 32). When analyzed using composite interval mapping (CIM) with forward cofactor selection in the software QGENE these resistance QTL explained between 16 and 34% of the phenotypic variance for the different traits and datasets (see supplementary Table S8). Similar results were obtained using different CIM approaches implemented in the software packages PlabQTL, R/qtl and Windows QTL Cartographer.

Besides the major QTL on chromosome C5, an additional minor QTL was identified on chromosome C1 for AUDPC values in experiments 1 and 3, and for growth reduction in experiment 3 (Fig. 8; supplementary Table S8). No clear evidence for epistatic interactions between digenic loci or main effect QTL was found using PlabQTL or R/qtl.

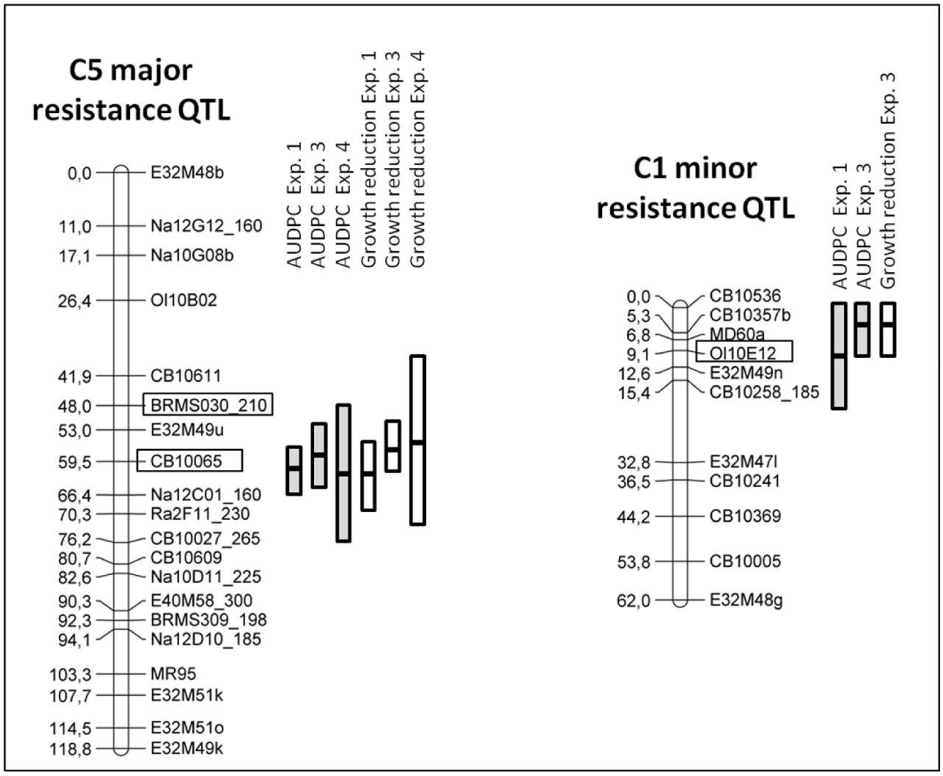

Figure 8: Quantitative trait loci for *V. longisporum* resistance on chromosomes C5 and C1 in the *B. napus* DH population ExR53. Blocks indicate confidence intervals of the QTL. Labelled markers (boxed) were used for resistance QTL validation tests. Exp. = experiment. Phenotypic data (AUDPC and growth reduction) was provided by Professor Andreas von Tiedemann, Division of Plant Pathology and Crop Protection, Georg August University, Göttingen, Germany.

3.3 Resistance QTL validation

From 55 publicly available SSR markers mapped on chromosomes C1 and C5 in 18 other mapping populations, 13 markers were located 10 cM either side of the QTL peaks in ExR53-DH. The percentage of phenotypic variation explained by three selected marker alleles derived from the QTL regions on C1 and C5 with mean AUDPC values in the two mapping populations and in four genetically diverse DH populations are shown in Table 3.

Two of the DH populations used for QTL validation tested together in one resistance screening are genetically similar half-sister populations, SW08-190001 and SW08-190002,

which only differ in the C genome resistance donor (BRA 1008 or HRI 8207) of their respective resynthesized rapeseed parent (Table 1). Although on average these DH populations should be about 75% genetically identical, their normalized AUDPC values in fact show contrasting associations with different marker alleles (for details see the Table 2 from Obermeier et al., 2013). Whereas SW08-190001 significantly associates only with marker Ol10E12 derived from the minor QTL on C1 in one of the two experiments, SW08-190002 shows a significant association only with the maker CB10065 derived from the major QTL region on C5 in one of the two experiments. In addition, in the DSV-1575 mapping population a significant correlation of mean AUDPC values with the marker alleles of CB10065 was observed.

Table 3: The percentage of variation in the resistance reaction explained by marker alleles flanking *V. longisporum* resistance QTL regions calculated based on simple linear regression of marker alleles with AUDPC

Population used for	Name of population	n	Ol10E12_285 QTL on C1	BRMS030_210 QTL on C5	CB10065_198 QTL on C5
QTL mapping	ExR53	Exp. 1: 100	11.8**	15.3**	29.5**
		Exp. 2: 32	ns	ns	ns
		Exp. 3: 82	10.4**	10.9**	16.3**
		Exp. 4: 98	ns	16.9**	17.8**
	SW99-307	Exp. 1: 163	ns	6.0**	mm
		Exp. 2: 163	ns	7.6**	
		Exp. 3: 163	ns	6.4**	
QTL validation	SW08-190001	Exp. 1: 25	17.9*	ns	ns
		Exp. 2: 19	ns	ns	ns
	SW08-190002	Exp. 1: 25	ns	mm	ns
		Exp. 2: 12	ns		54.7*
	DSV-1575	Exp. 1: 37	ns	10.2*	13.9*
		Exp. 2: 26	ns	18.6**	13.4*
	DSV-1605	Exp. 1: 39	ns	21.7**	12.2**
		Exp. 2: 29	ns	ns	ns

Marker bands (size in bp indicated in marker name after underscore) were scored in a 1-0 binary format and used in regression analysis with disease scores calculated as AUDPC values in DH populations representing diverse resistance donors. For details on the population pedigrees see table 1 (in material section). Mapping data for SW99-307 was obtained from Rygulla et al. 2008. mm = monomorphic marker, ns = not significant, * = significant at 0.1 level, ** = significant at 0.05 level.

3.4 Soluble and cell wall-bound phenolic compounds in the hypocotyls of mock- and *V. longisporum*-inoculated oilseed rape

To evaluate the effects of *V. longisporum* infection on the composition of free soluble and cell wall-bound phenolic compounds, pooled hypocotyl samples were collected from 98 DH lines of the mapping population from experiment 4. For the soluble phenolics fractions in total 43 and 49 HPLC peaks with definite retention times were identified in the chromatograms from the mock- and *V. longisporum*-inoculated DH lines, respectively (see Figure 9 as a typical example). One peak was detected only in the extracts from the mock treatment, but was absent in the extracts from the *V. longisporum* treatment. Seven peaks only appeared in the extracts from the DH lines after *V. longisporum*-inoculation, but were absent in the extracts from the mock-treated DH lines. Forty-two HPLC peaks were common in the soluble phenolics fraction from the mock- as well as from the *V. longisporum*-inoculated plant lines (Table 4; for exclusive peaks see supplementary Table S2 in the appendix).

For the cell wall-bound phenolics fractions in total 36 HPLC peaks with defined retention times were identified in the chromatograms from both treatments, mock- and *V. longisporum*-inoculated. Thirty-two HPLC peaks were detected to be common in the cell wall-bound fraction from the mock- as well as from the *V. longisporum*-inoculated plant lines. In addition, four HPLC peaks were only present in the cell wall-bound fraction from the mock treatment and four HPLC peaks were only present in the cell wall-bound fraction from the *V. longisporum* treatment (Table 4; for exclusive peaks see supplementary Table S2 shown in the appendix).

Table 4: Total number of HPLC phenolics peaks present in soluble and cell wall-bound phenolics fractions from 98 DH lines

Fraction used for HPLC analysis	Treatment	Total number of HPLC peaks	Number of exclusive peaks
Soluble phenolics	Mock	43	1
Soluble phenolics	VL	49	7
Cell wall-bound phenolics	Mock	36	4
Cell wall-bound phenolics	VL	36	4

VL = *Verticillium longisporum*

3.5 Identification of individual phenolic compounds by co-migration with standard compounds

A set of 49 commercially available HPLC quality standard substances from different groups of phenolics, from simple phenylpropanoids to more complex flavonoids, were used to putatively identify unknown RP-HPLC peaks from the soluble and cell wall-bound fractions by comparison of the retention times and further confirmation by co-injection (see supplementary table S1 in the appendix for standard phenolic comounds). From these 49 phenolic substances 33 substances were separated well in the applied RP-HPLC protocol including members from all different phenolic groups (Table 5). The phenolic compounds that could not be separated well within the applied RP-HPLC protocol were mainly members of the groups of very simple phenolics (e.g. shikimic acid and trans-cinnamic acid) or members of the groups of highly complex phenolics like flavanones, flavones or flavonols and their glycosides (however, 10 out of 23 tested from the last mentioned groups were well separated using the applied RP-HPLC protocol). In total 22 peaks were identified that co-migrated with one of the 34 standard substances in the extracts from the soluble and/or from the cell wall-bound phenolics fractions (summarized in Table 5, suppnementary Table S1 shown in the appendix).

In the soluble phenolics fraction 15 peaks were identified that co-migrated with one of the 32 standard substances (Table 5). Compounds identified by co-migration in RP-HPLC in soluble phenolics fraction can be assigned to eight quite diverse groups of phenolics including the hydroxybenzoic acid/aldehyde (vanillin; see compound no 7 in Table 5), phenylpropanoids/ hydroxycinnamic acids/aldehydes (*p*-coumaric acid (8), ferulic acid (9), sinapic acid (10) caffeic acid (14) and coniferyl aldehyde (15) in Table 5), monolignol (sinapyl alcohol (17) in table 5), phenylpropanoid esters (chlorogenic acid (18) and rosmarinic acid (19) in Table 5), flavanone/flavanone glycosides (naringin (20) and Phlorizin (21) in Table 5), sinapate esters (sinapoyl glucose (22) and sinapine (23) in Table 5), flavanol (catechin (24) in Table 5) and proanthocyanidin (procyanidin B1 (33) in Table 5). Most of them were detected in both, the soluble phenolics fractions from the mock- and from the *V. longisporum*-inoculated DH lines (Fig. 9; summarized in Table 5).

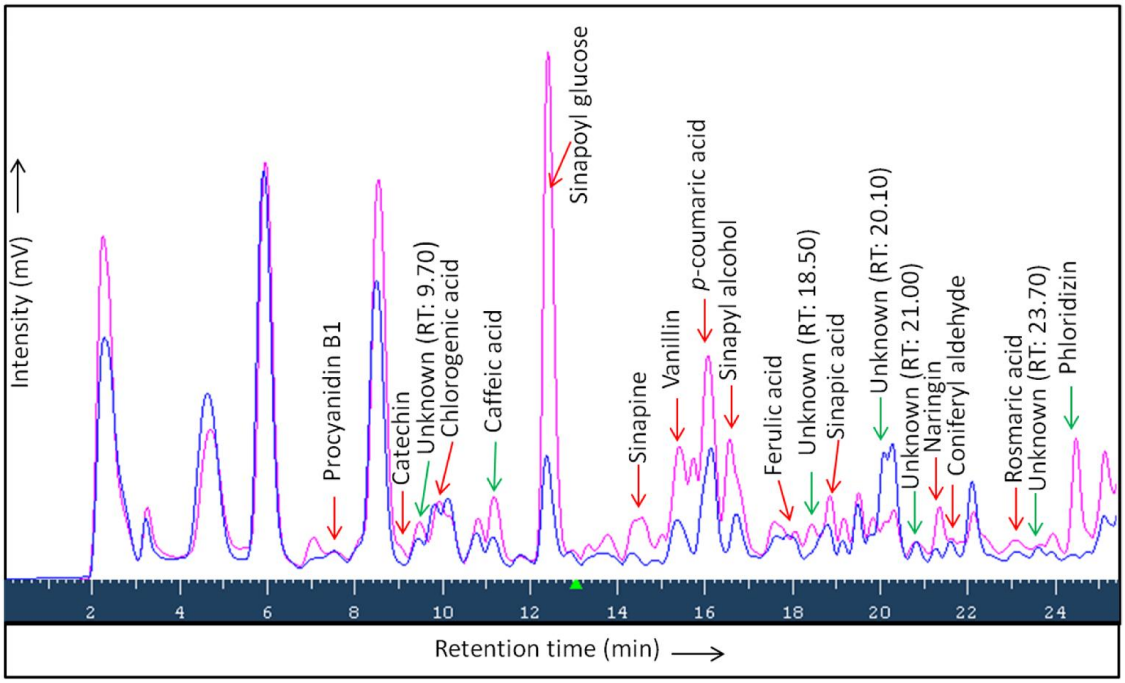

Figure 9: Example of a HPLC profile for the soluble phenolics fraction in DH line 138 (blue = mock-inoculated; pink = *V. longisporum*-inoculated). Some peaks are identified based on retention times of external standards (red arrows). HPLC peak areas that produce QTL which are co-localizing with QTL for *V. longisporum* resistance and which are also significantly correlated with AUDPC are indicated by green arrows (see chapter 3.11 for metabolic QTL from soluble phenolics).

In the cell wall-bound fraction 15 peaks were identified that co-migrated with one of the 32 standard substances (Fig. 10, Table 5). Similarly to the compounds extracted from the soluble fraction, they also can be assigned to six different diverse groups of phenolics such as hydroxybenzoic acids/aldehydes (see Table 5 for details: (2) protocatechuic acid, (3) 4-hydroxybenzoic acid, (4) gentisic acid, (5) salicylic acid, (6) vanillic acid, (7) vanillin), phenylpropanoids/hydroxycinnamic acids/aldehydes ((8) *p*-coumaric acid, (9) ferulic acid, (10) sinapic acid, (11) 5-hydroxy ferulic acid, (14) caffeic acid), phenylpropanoid esters ((18) chlorogenic acid, (19) rosmarinic acid), flavanone ((20) naringin) and anthocyanidin ((32) cyaniding chloride) (Fig. 10; summarized in Table 5). The identities of *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, vanillic acid and vanillin were reconfirmed by LC-MS analysis in this study.

From eight exclusive peaks only present in the mock- or only present in the *V. longisporum* treatment of the soluble phenolics fraction one was identified from the soluble phenolics fraction of the *V. longisporum* treated DH lines as (+) catechin (see compound number 24 in table 5). From 8 total exclusive peaks only present in the mock- or only present in the *V.*

longisporum treatment of the cell wall-bound phenolics fraction one was identified from the cell wall-bound phenolics fraction of the *V. longisporum* treated DH lines as chlorogenic acid (see compound number 18 in table 5) (for details of the exclusive peaks see supplementary Table 2 in the appendix).

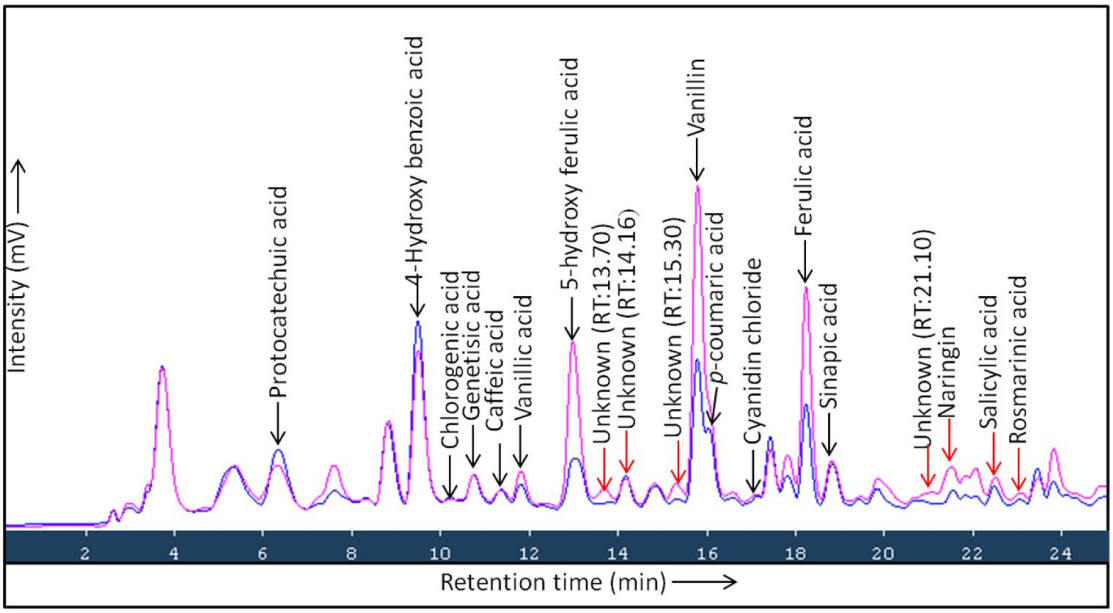

Figure 10: Example of a HPLC profile for the cell wall-bound phenolics fraction in DH line 103 (mock-inoculated: blue and *V. longisporum*-inoculated: pink). Some peaks are identified based on retention times of external standards (black arrows). HPLC peak areas that produce QTL which are co-localizing with QTL for *V. longisporum* resistance and which are also significantly correlated with AUDPC are indicated by red arrows (see chapter 3.12 for metabolic QTL from cell wall-bound phenolics).

In addition, further fractionation of soluble and cell wall-bound phenolics fraction extracted from some selected DH lines revealed that most of the peaks belong to the phenolic acid fraction and only one of approximately 45 peaks from soluble phenolics fraction and four of approximately 36 peaks from cell wall-bound phenolics fraction were also present in the monomeric and oligomeric proanthocyanidin fraction. There was no peak in the fraction of polymeric proanthocyanidin/anthocyanidins.

Table 5: Summary on the co-migration of commercially available HPLC quality standard substances with HPLC peaks in phenolic extracts from soluble (S) and cell wall-bound (CW) fractions and from mock- (M) and *V. longisporum*-(VL) inoculation treatments of DH lines (n = 98)

groups of compounds	names of tested standard substances (code number)	numbers co-migrating/ numbers tested	numbers co-migrating in S/CW fractions	numbers co-migrating in M/VL treatments	code numbers of standard substances co-migrating with peaks in fraction-treatment combinations: S-M	S-VL	CW-M	CW-VL
hydroxybenzoic acid/aldehyde	(1) gallic acid, (2) protocatechuic acid, (3) 4-hydroxybenzoic acid, (4) gentisic acid, (5) salicylic acid, (6) vanillic acid, (7) vanillin	6/7	1/6	1/1 (S) 6/6 (CW)	(7)	(7)	(2), (3), (4), (5), (6), (7)	(2), (3), (4), (5), (6), (7)
phenylpropanoid/ hydroxy-cinnamic acid/aldehyde	(8) *p*-coumaric acid, (9) ferulic acid, (10) sinapic acid, (11) 5-hydroxyferulic acid, (12) 1,3-dicaffeoylquinic acid, (13) dihydrocaffeic acid, (14) caffeic acid, (15) coniferyl aldehyde	6/8	5/5	5/5 (S) 5/5 (CW)	(8), (9), (10), (14), (15)	(8), (9), (10), (14), (15)	(8), (9), (10), (11), (14)	(8), (9), (10), (11), (14)
monolignol	(16) coniferyl alcohol, (17) sinapyl alcohol	1/2	1/0	1/1 (S)	(17)	(17)	-	-
phenylpropanoid ester	(18) chlorogenic acid, (19) rosmarinic acid	2/2	2/2	2/2 (S) 1/2 (CW)	(18), (19)	(18), (19)	(19)	(18), (19)
flavanon/ flavanone glycoside	(20) naringin, (21) phlorizin (phloridizin)	2/2	2/1	2/2 (S) 1/1 (CW)	(20), (21)	(20), (21)	(20)	(20)
sinapate ester	(22) sinapoyl glucose (23) sinapine	2/2	2/0	2/2 (S)	(22), (23)	(22), (23)	-	
flavonol/ flavonol glycoside	(24) (+) catechin, (25) (-) epicatechin, (26) (-) epigallocatechin gallate, (27) isoquercitrin, (28) kaempferol-7-O-glucoside, (29) kaempferol-3-O-rhamnoside, (30) quercetin 3-O-galactoside (hyperoside), (31) rutin	1/8	1/0	0/1 (S)	-	(24)	-	-
anthocyanidin	(32) cyaniding chloride	1/1	0/1	1/1 (CW)	-	-	(32)	(32)
proanthocyanidin	(33) procyanidin B1, (34) procyanidin B2	1/2	1/0	1/1 (S)	(33)	(33)	-	-
all groups		22/34	15/15					

3.6 Total soluble and cell wall-bound phenolics and their correlation with *V. longisporum* resistance

The total concentration of phenolics estimated by the sum of the peak areas from all HPLC peaks in the mock-inoculated cell wall-bound phenolics fraction showed no significant correlation with resistance measured as AUDPC. In contrast, mock treatment for the soluble fraction showed significant correlation with AUDPC (Table 6). In addition, significant correlations for the total phenolics concentrations with AUDPC were found for the two *V. longisporum* treatments. However, the variance in AUDPC explained by the total concentration of phenolics was with 43% much higher in the cell wall-bound fraction compared with 7% in the soluble fraction (Table 6).

Table 6: Total phenolics concentrations in the hypocotyls of the oilseed rape mapping population ExR53-DH correlated with area under the disease progress curve (AUDPC)

Fraction used for HPLC analysis	Treatment	Correlation for sum of peak areas with AUDPC: R (R^2)
Soluble phenolics	Mock	0.27 (7%)
Soluble phenolics	VL	0.21 (4%)
Cell wall-bound phenolics	Mock	n.s.
Cell wall-bound phenolics	VL	0.66 (43%)

Pearson correlation (R) significant for n = 98 DH lines at $p \leq 0.05$, n.s. = not significant, VL = *Verticillium longisporum*.

Relative concentrations of soluble phenolic compounds for individual DH lines increased 1.1-fold up to 4.0-fold upon infection and 2.1-fold on average. Relative concentration of cell wall-bound phenolic compounds for individual DH lines increased 0.6-fold up to 11.5-fold upon infection and 2.2-fold on average. Thus, the average fold-change upon *V. longisporum*-infection is quite similar, but the range of relative fold-changes within the 98 DH lines is much broader for the cell wall-bound compared to the soluble phenolics fraction. This is also apparent for the total concentrations in frequency distribution plots in Figure 11 and 12. Frequencies for classes of total phenolics concentrations showed a shift to higher concentrations after *V. longisporum* infection for total soluble as well as for total cell wall-bound phenolics concentrations within the DH population (Fig. 11 & Fig. 12). In both cases, for soluble as well as for cell wall-bound phenolics, total concentrations covered a small range of concentration classes in the mock treatment, but showed a much broader range of different concentration classes in the *V. longisporum* treatment indicating a genotype-specific accumulation exclusively after inoculation.

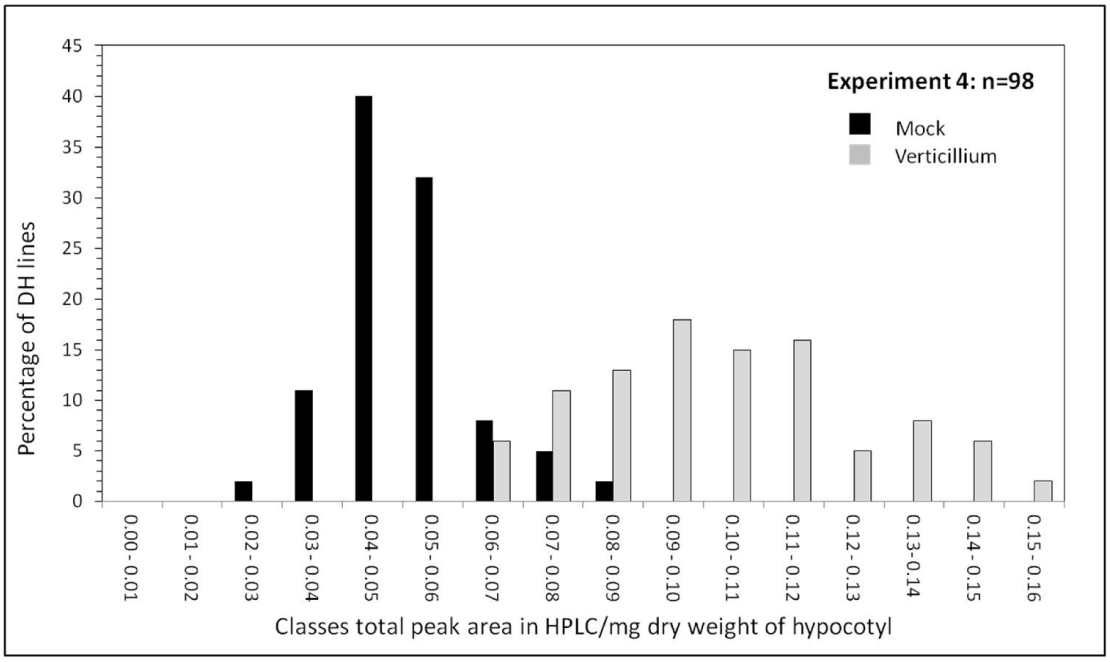

Figure 11: Frequency distribution for classes of total soluble phenolics concentrations in the hypocotyls of mock- and *V. longisporum*-inoculated DH lines of the oilseed rape mapping population 'Express 617 x R53' in greenhouse screening experiment 4 (n = 98 DH lines).

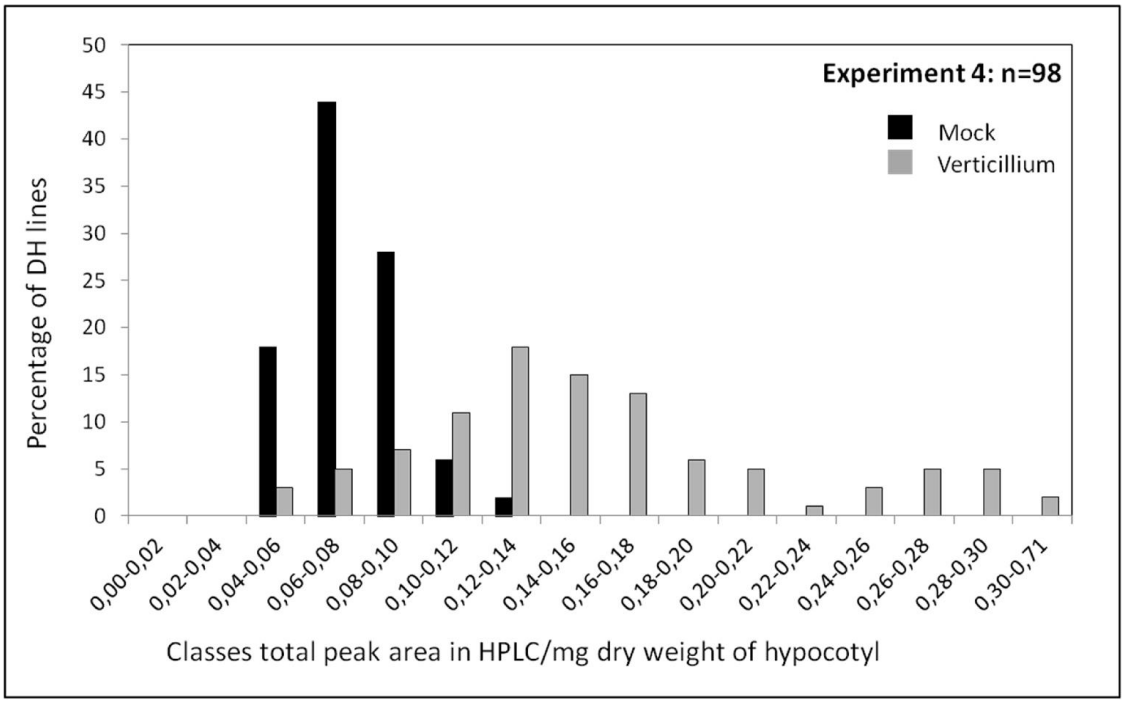

Figure 12: Frequency distribution for classes of total cell wall-bound phenolics concentrations in the hypocotyls of mock- and *V. longisporum*-inoculated DH lines of the oilseed rape mapping population 'Express 617 x R53' in greenhouse screening experiment 4 (n = 98 DH lines).

3.7 Individual soluble phenolics and their correlation with *V. longisporum* resistance

To identify individual HPLC peaks presumably representing individual phenolic compounds that were associated with resistance the normalized HPLC peak area per mg of dry weight of hypocotyl samples analyzed from each genotype was used for estimation of metabolite concentrations in the hypocotyl tissues. The Pearson correlation was calculated with the mean AUDPC values as measures of resistance for the mapping population of 98 DH lines for different fractions (soluble/cell wall-bound phenolics) and treatments (mock- and *V. longisporum*-inoculated).

Six HPLC peaks out of 43 from the mock- and 22 peaks out of 49 from the *V. longisporum*-inoculated treatment for the soluble phenolics fraction were significantly correlated with mean AUDPC and mean growth reduction (Table 7; supplementary Table S3 and S4 shown in the appendix). The concentrations of individual soluble phenolic compounds showed a weak to medium correlation with phenotype, i.e. resistance measures ($R^2 \leq 37\%$).

Among the correlated phenolic metabolites such as ferulic acid, sinapic acid, phlorizin and some other unknown phenolic compounds from soluble phenolics fraction were up-regulated in resistant DH lines upon *V. longisporum* infection, whereas vanillin, rosmarinic acid and some other unknown phenolic metabolites were down-regulated in resistant genotypes upon infection with *V. longisporum*. In addition, caffeic acid and ferulic acid from mock-inoculated soluble phenolics fraction were down-regulated in the hypocotyls of resistant oilseed rape genotypes in response to *V. longisporum* infection (Table 7; supplementary Table S3 shown in the appendix). The concentrations of catechin and sinapoyl alcohol from soluble phenolics fraction were not correlated with AUDPC, but they showed negative correlation with plant growth reduction (Supplementary Table S3 and S4 shown in the appendix).

Table 7: Correlation between area under the disease progress curve (AUDPC) and concentrations of soluble phenolic compounds in the hypocotyls of the *V. longisporum*- and mock-inoculated mapping population ExR53-DH sorted by correlation coefficient R

Retention time (RT) in HPLC	Identified phenolics by external standard	Group of Phenolic compounds	Treatment	Correlation with mean AUDPC-R(R^2)	Regulation in resistance genotyes
RT: 21.0	unknown	-	VL	0.61** (0.37)	down
RT:2.50	unknown	-	VL	0.41** (0.17)	down
RT:18.11	ferulic acid	hydroxycinnamic acid	Mock	0.40** (0.16)	down
RT:11.30	caffeic acid	hydroxycinnamic acid	Mock	0.39** (0.15)	down
RT:9.70	unknown	-	VL	-0.34** (0.12)	up

RT:11.97	unknown	-	Mock	-0.33** (0.11)	up
RT:18.50	unknown	-	VL	0.33** (0.11)	down
RT:23.70	unknown	-	VL	0.33** (0.11)	down
RT:15.50	vanillin	hydroxybenzoic acid	VL	0.32** (0.10)	down
RT:5.75	unknown	-	VL	-0.30** (0.09)	up
RT:23.11	rosmarinic acid	caffeic acid ester	VL	0.30** (0.09)	down
RT:2.70	unknown	-	Mock	0.30** (0.09)	down
RT:3.50	unknown	-	VL	0.27** (0.07)	down
RT:24.50	phlorizin	flavanone glycoside	VL	-0.26** (0.07)	up
RT:7.28	unknown	-	VL	0.26** (0.07)	down
RT:20.10	unknown	-	VL	0.26** (0.07)	down
RT:20.82	unknown	-	VL	-0.25* (0.06)	up
RT:7.28	unknown	-	Mock	0.25* (0.06)	down
RT:19.80	unknown	-	VL	-0.24* (0.06)	up
RT:18.11	ferulic acid	hydroxycinnamic acid	VL	-0.22* (0.05)	up
RT:4.95	unknown	-	VL	-0.21* (0.04)	up
RT:18.85	sinapic acid	hydroxycinnamic acid	VL	-0.21* (0.04)	up
RT:19.20	unknown	-	VL	-0.21* (0.04)	up

Only phenolic compounds that are significantly correlated with AUDPC, where SP = soluble phenolic compound. * =significant at 0.05 level, ** = significant at 0.01 level, n = 98 DH lines and VL = *Verticillium longisporum.*

3.8 Individual cell wall-bound phenolics and their correlation with *V. longisporum* resistance

In case of the cell wall-bound phenolics fractions, 3 HPLC peaks out of 36 from the mock- and 29 peaks out of 36 from the *V. longisporum*-inoculated treatment were significantly correlated with mean AUDPC and growth reduction (Table 8; supplementary Table S3 and S4 shown in the appendix). A large number of the *V. longisporum*-inoculated cell wall-bound individual phenolics showed a medium to strong correlation with AUDPC ($R^2 \leq 59\%$; table 8; supplementary Table S3 shown in the appendix). Some of these correlated phenolic compounds such as gentisic acid, caffeic acid, *p*-coumaric acid, naringin, rosmarinic acid and some other unknown phenolic compounds were negatively correlated with AUDPC indicating that these compounds were up-regulated in the resistant DH lines upon *V. longisporum* infection (Table 8; supplementary Table S3 shown in the appendix). In contrast, protocatechuic acid, chlorogenic acid, vanillic acid, vanillin, 5-hydroxy ferulic acid, ferulic acid, sinapic acid and some unknown phenolic compounds were positively correlated with AUDPC meaning that these compounds were up-regulated in the susceptible DH lines after inoculation with *V. longisporum*. In addition, identified cell wall-bound salicylic acid and 4-hydroxybenzoic acid were not correlated with AUDPC, but they were negatively correlated with plant growth reduction (Supplementary Table S3 and S4 shown in the appendix).

Table 8: Correlation between area under the disease progress curve (AUDPC) and concentrations of cell wall-bound phenolic compounds in the hypocotyls of the *V. longisporum*- and mock-inoculated mapping population ExR53-DH sorted by correlation coefficient R

Retention time (RT) in HPLC	Identified phenolics by external standard	Group of Phenolic compounds	Treatment	Correlation with mean AUDPC-R(R^2)	Regulation in resistance genotypes
RT:15.30	unknown	-	VL	0.77** (0.59)	down
RT: 21.10	unknown	-	VL	0.71** (0.50)	down
RT:15.75	vanillin	hydroxybenzoic acid	VL	0.70** (0.49)	down
RT:11.82	vanillic acid	hydroxybenzoic acid	VL	0.70** (0.49)	down
RT:13.00	5-hydroxy ferulic acid	hydroxycinnamic acid	VL	0.65** (0.42)	down
RT:17.82	unknown	-	VL	0.65** (0.42)	down
RT:7.62	unknown	-	VL	0.61** (0.37)	down
RT:17.45	unknown	-	VL	0.55** (0.30)	down
RT:18.75	sinapic acid	hydroxycinnamic acid	VL	0.53** (0.28)	down
RT:6.28	protocatechuic acid	hydroxybenzoic acid	VL	0.52** (0.27)	down
RT:11.45	caffeic acid	hydroxycinnamic acid	VL	-0.49** (0.24)	up
RT: 3.00	unknown	-	VL	0.49** (0.24)	down
RT:13.70	unknown	-	VL	0.48** (0.23)	down
RT:14.20	unknown	-	VL	-0.47** (0.22)	up
RT:23.10	rosmarinic acid	caffeic acid ester	VL	-0.44** (0.19)	up
RT:20.62	unknown	-	VL	-0.43** (0.18)	up
RT:8.82	unknown	-	VL	-0.42** (0.18)	up
RT:18.20	ferulic acid	hydroxycinnamic acid	VL	0.41** (0.17)	down
RT:19.80	unknown	-	VL	-0.38** (0.14)	up
RT:10.75	unknown	-	VL	0.36** (0.13)	down
RT:7.25	unknown	-	Mock	0.36** (0.13)	down
RT:6.32	protocatechuic acid	hydroxybenzoic acid	Mock	-0.34** (0.12)	up
RT:22.05	unknown	-	VL	0.32** (0.10)	down
RT:23.80	unknown	-	VL	0.29** (0.08)	down
RT:21.50	naringin	flavanone glycoside	VL	-0.27** (0.07)	up
RT:10.07	chlorogenic acid	caffeic acid ester	VL	0.27** (0.07)	down
RT:21.80	unknown	-	Mock	0.27** (0.07)	down
RT:23.40	unknown	-	VL	0.25* (0.06)	down
RT:7.28	unknown	-	Mock	0.25* (0.06)	down
RT:16.05	*p*-coumaric acid	hydroxycinnamic acid	VL	-0.24* (0.06)	up
RT:19.80	unknown	-	VL	-0.24* (0.06)	up
RT:16.52	unknown	-	VL	-0.22* (0.05)	up
RT:10.40	gentisic acid	hydroxybenzoic acid	VL	-0.20** (0.04)	up
RT:12.30	unknown	-	VL	-0.20* (0.04)	up

Only cell wall-bound phenolic compounds that are significantly correlated with AUDPC, where CW = cell wall-bound phenolic compound, * =significant at 0.05 level, ** = significant at 0.01 level, n = 98 DH lines and VL = *Verticillium longisporum*.

3.9 Relevance of soluble and cell wall-bound phenolics with regard to *V. longisporum* resistance

A high ratio of individual HPLC peaks (81%) from the *V. longisporum*-inoculated cell wall-bound phenolics fraction showed a significant correlation of their concentrations with mean AUDPC ($R^2 \leq 59$ %), whereas only 37% of the individual HPLC peaks from the *V.*

longisporum-inoculated soluble phenolics fraction showed significant correlations of their concentrations with mean AUDPC ($R^2 \leq 37\%$).

Importance of the phenolic compounds has been judged according to their correlation percentage with resistance measures. Among the 10 individual HPLC peak areas which showed the highest correlations with mean AUDPC (R^2 = 30-59%) nine were from the cell wall-bound phenolics fraction and only one was from the soluble phenolics fraction (Table 9; supplementary Table S3 shown in the appendix). All of these 10 were only correlated with AUDPC in the data set from the *V. longisporum*-inoculated population, but not in the data set from the mock-inoculated population. The 10 HPLC peak areas showing the highest correlation with mean AUDPC was always reduced in the resistant genotypes. From these 10 HPLC peaks 4 could be identified using external standard substances as vanillic acid, vanillin, 5-hydroxyferulic acid and sinapic acid. All of these 4 cell wall-bound compounds are members of the simple phenolics groups of hydroxybenzoic or hydroxycinnamic acids.

Table 9: Detailed characteristics on ten HPLC peaks from hypocotyl phenolic compounds showing highest correlation of their normalized peak area with mean AUDPC

Retention time (RT) in HPLC	Identified phenolics by External standard	Group of Phenolic compounds	Treatment	Fraction	Correlation with mean AUDPC-R(R^2)	Regulation in resistance genotypes
RT:15.28	unknown	-	VL	CW	0.77** (0.59)	down
RT:21.10	unknown	-	VL	CW	0.71** (0.50)	down
RT:15.75	vanillin	hydroxybenzoic acid	VL	CW	0.70** (0.49)	down
RT:11.82	vanillic acid	hydroxybenzoic acid	VL	CW	0.70** (0.49)	down
RT:13.00	5-hydroxy ferulic acid	hydroxycinnamic acid	VL	CW	0.65** (0.42)	down
RT:17.82	unknown	-	VL	CW	0.65** (0.42)	down
RT:7.62	unknown	-	VL	CW	0.61** (0.37)	down
RT: 21.0	unknown	-	VL	SP	0.61** (0.37)	down
RT:17.45	unknown	-	VL	CW	0.55** (0.30)	down
RT:18.75	sinapic acid	hydroxycinnamic acid	VL	CW	0.53** (0.28)	down

Here, ** = significant at 0.01, n = 98 DH lines, SP = soluble phenolic compound, CW = cell wall-bound phenolic compound and VL = *Verticillium longisporum*. Soluble phenol labelled in grey.

3.10 Preformed and *V. longisporum*-induced changes of phenolic compounds in oilseed rape hypocotyls

Phenolics in plants might be classified into two major groups: (i) preformed constitutive expressed phenolics and (ii) induced phenolics synthesized in response to physical damage, infection or other abiotic or biotic stresses. Induced phenolics may also be constitutively synthesized but, additionally, their synthesis may be enhanced under biotic or abiotic stress (Lattanzio et al., 2006). Preformed antibiotic compounds that occur constitutively in healthy plants without the need for infection with plant pathogens or are produced after infection soley from preexisting constituents and function as preformed defence metabolites are generally defined as phytoanticipins (Van Ettern et al., 1995).

Only eight preformed phenolics (5 from soluble and 3 from cell wall-bound phenolics fraction) out of 79 constitutively expressed phenolics in both fractions were correlated with resistance measure (mean AUDPC). Caffeic acid, ferulic acid and protocatechuic acid were identified as preformed phenolics and showed significant correlation with *V. longisporum* reistance reaction in oilseed rape (supplementary Table S3 shown in the appendix).

In contrast, *V. longisporum*-induced changes of 42 phenolic compounds (17 from the soluble and 25 from the cell wall-bound phenolics fraction) showed correlation with mean AUDPC. These correlated phenolic compounds did also preexist in the hypocotyls of healthy oilseed rape plants and their expression pattern changed upon *V. longisporum* infection and showed correlation with resistance reaction. Ferulic acid, sinapic acid, vanillin, sinapyl alcohol, rosmarinic acid and phlorizin from the soluble phenolics fraction induced by *V. longisporum*-inoculation, whereas protocatechuic acid, 4-hydroxybenzoic acid, gentisic acid, salicylic acid, vanillic acid, vanillin, caffeic acid, *p*-coumaric acid, 5-hydroxyferulic acid, ferulic acid, sinapic acid, naringin and rosmarinic acids induced by *V. longisporum*-inoculation in the cell wall-bound phenolics fraction (supplementary Table S3 shown in the appendix).

Upon *V. longisporum* infection seven phenolic compounds were exclusively expressed (3 from the soluble and 4 from the cell wall-bound phenolics fraction) in oilseed rape hypocotyls and showed significant correlation with resistance measures (mean AUDPC and mean growth reduction) (Table 10). Among the 7 exclusively expressed phenolics in *V. longisporum*-inoculated data set 2 were identified as catechin (from soluble phenolics fraction) and chlorogenic acid (from cell wall-bound phenolics fraction). Soluble catechin was not

correlated with AUDPC but showed correlation with growth reduction, whereas cell wall-bound chlorogenic acid showed positive correlation with measures for resistance reaction (Table 10).

Table 10: Phenolic compounds exclusively expressed in *V. longisporum*-induced oilseed rape hypocotyls sorted by correlation coefficient R

Retention time (RT) in HPLC	Identified phenolics by external styndard	Treat-ment	Fraction	Corelation with mean AUDPC, R ($R^{2)}$	Correlation with mean growth Reduction, R (R^2)	Regulation in resistance genotypes
RT:21.10	unknown	VL	CW	0.71** (0.50)	0.66** (0.43)	down
RT:21.00	unknown	VL	SP	0.61** (0.37)	0.54** (0.29)	down
RT:13.70	unknown	VL	CW	0.48** (0.23)	0.50** (0.25)	down
RT:22.05	unknown	VL	CW	0.32** (0.10)	0.38** (0.14)	down
RT:10.10	chlorogenic acid	VL	CW	0.27** (0.07)	0.21* (0.04)	down
RT:9.20	catechin	VL	SP	n.s.	0.23* (0.05)	down
RT:21.85	unknown	VL	SP	n.s.	0.23** (0.05)	down

Here, SP = soluble phenolic compound, CW = cell wall-bound phenolic compound, * = significant at .05, ** = significant at 0.01, n.s. = not significant, n = 98 DH lines, and VL = *Verticillium longisporum.*

3.11 QTL for soluble phenolics and co-localization with QTL for *V. longisporum* resistance

For the individual soluble phenolic compounds, 34 QTL with a LOD score threshold of 2.5 were detected for a total number of 43 RP-HPLC peaks from mock-inoculated DH lines, and 37 QTL for 49 peaks from *V. longisporum*-inoculated lines. Four major regions were identified in the DH line set for the mock-inoculation treatment on chromosomes A9, C1, C6 and C8 showing overlapping confidence intervals for at least 3 QTL involved in the concentrations of individual soluble phenolic compounds in the hypocotyl. In the DH line set for the *V. longisporum*-inoculation treatment two major regions were identified on chromosomes C1 and C5 (for details see supplementary Table S5 and S6 in the appendix).

Further analysis focused on the phenolic compounds that exhibited a significant correlation with AUDPC and explained at least 5 % of the phenotypic variation in AUDPC. These were 9 compounds from the *V. longisporum*-inoculated DH line data set and 3 compounds from the mock-inoculated DH line data set (see supplementary Table S5 and S6 in the appendix). From these 12 significantly with AUDPC correlated phenolic compounds 7 produced QTL with a

LOD score of 2.5 which showed at least one QTL co-localizing with the QTL for AUDPC on chromosome C1 or C5 (Fig. 13; Table 11; detailed data in supplementary Table S5 and S6; summarized in supplementary Table S8 shown in the appendix). Six of these soluble phenolic compounds where QTL also co-localized with the QTL for AUDPC on C1 or C5 were detected only in the *V. longisporum*-inoculated data set. These 6 compounds explained 12 to 17% of the phenotypic variation in AUDPC (Table 11). In contrast, only one phenolic compound identified as caffeic acid (at retention time 11.30 min; Table 7) was detected in the mock-inoculated data set on C1 where a QTL for this compound also co-localized with the QTL for AUDPC. In this case 12% of the phenotypic variation of AUDPC was explained by the variation in caffeic acid concentration in the mock-inoculated data set (Table 11; supplementary Table S5 shown in the appendix).

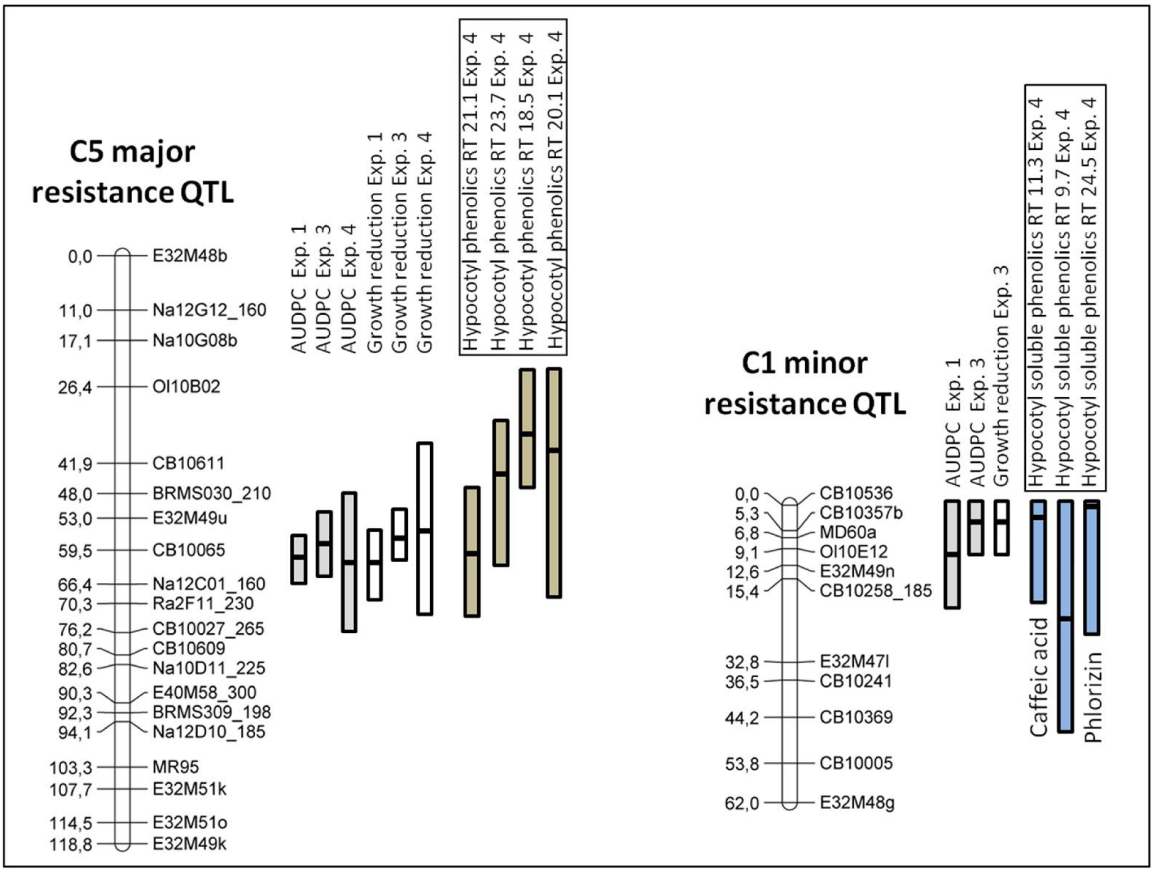

Figure 13: Comparison of quantitative trait loci for *V. longisporum* resistance and soluble phenolic metabolites in the hypocotyl localized on chromosomes C5 and C1. Blocks indicate confidence intervals of the QTL. Exp. = experiment and RT = retention time of peak in RP-HPLC.

Correlation of the concentrations for lignin precursors in soluble phenolics fractions such as *p*-coumaric acid, sinapic acid (from mock- and *V. longisporum*-inoculated data set) and

ferulic acid (from *V. longisporum*-inoculated data set) with AUDPC were not observed. But ferulic acid from the mock-inoculated data set showed a significant correlation with AUDPC (Table 7; supplementary Table S3 shown in the appendix). But, the constitutive concentration of the lignin precursor caffeic acid significantly correlated with AUDPC, and a QTL for the constitutive expression of caffeic acid in the hypocotyl co-localized with the minor QTL for *V. longisporum* resistance on C1 (Fig. 13; Table 11). In addition, QTL for phlorizin (at retention time 24.50 min) from the *V. longisporum*-inoculated data set also showed co-ocalized QTL on C1 (Fig. 13 ; Table 11). Phenolic compound at retention time 9.70 min from *V. longisporum*-inoculated data set is yet not identified which was correlated with mean AUDPC and co-localized with the minor *V. longisporum* resistance QTL on C1. Furthermore, four other soluble phenolic compounds from *V. longisporum*-inoculated data set were not identified until now which were also correlated with AUDPC and co-localized with the major resistance QTL on C5 (Fig. 13; Table 11).

Table 11: QTL for soluble phenolic compounds in the hypocotyls of the mock- and *V. longisporum*-inoculated mapping population ExR53-DH sorted by correlation coefficient R

Compound with their RT (min)	Treatment	Correlation with mean AUDPC: R (R^2)	Chromosome	QTL peak, position, cM	Phenotypic variation, (R^2)
Unknown (21.0)	VL	0.61 (0.37)	C5	60	14.0
Caffeic acid (11.30)	Mock	0.39 (0.15)	C1	4	12.0
Unknown (9.70)	VL	-0.34 (0.12)	C1	24	14.0
Unknown (23.70)	VL	0.33 (0.11)	C5	42	17.0
Unknown(18.50)	VL	0.32 (0.10)	C5	34	14.0
Unknown(20.10)	VL	0.26 (0.07)	C5	34	13.0
Phlorizin (24.50)	VL	-0.26 (0.07)	C1	0	14.0

Only compounds that co-localize with resistance QTL on C1 and C5 and are significantly correlated with AUDPC are listed. RT = retention time, LOD = logarithm of the odds, cM = centiMorgan, VL = *Verticillium longisporum* (n = 98 DH lines).

3.12 QTL for cell wall-bound phenolics and co-localization with QTL for *V. longisporum* resistance

For the individual cell wall-bound phenolic compounds, 15 QTL with a LOD threshold of 2.5 were detected for a total number of 36 HPLC peak areas for the mock-inoculated DH lines, and 10 QTL from 36 peak areas for the *V. longisporum*-inoculated DH lines. One major region was identified on chromosome C5 for the *Verticillium*-inoculation treatment and one major region on chromosome A3 for the mock-inoculation treatment showing overlapping

confidence intervals for four QTL involved in the concentrations of individual cell wall-bound phenolic compounds in the hypocotyl (for details see supplementary Table S7 in the appendix).

Among the 10 QTL from the *V. longisporum*-inoculated treatment, 5 QTL were co-localizing with the major resistance QTL for *V. longisporum* resistance on chromosome C5. In addition, two *V. longisporum* inoculated cell wall-bound phenolic compounds resulted in metabolite QTL that co-localize with the minor resistance QTL on chromosome C1 (Fig. 14; Table 12; detailed data in supplementary Table S7; summarized in supplementary Table S8). These 7 cell wall-bound metabolites showed a medium to strong correlation with AUDPC and explained 11-17% of the phenotypic variation in AUDPC (Table 12). No QTL was detected in the mock-inoculated cell wall-bound phenolics fraction overlapping with the QTL for AUDPC on chromosome C5 or C1 (see supplementary Table S7 in the appendix).

Table 12: QTL for cell wall-bound phenolic compounds in the hypocotyls of the *V. longisporum*-inoculated mapping population ExR53-DH sorted by correlation coefficient R

Compound with their RT (min)	Correlation with mean AUDPC: R (R^2)	Chromosome	QTL peak, position, cM	Phenotypic variation, (R^2)
Unknown (15.3)	0.77 (0.59)	C5	54	12.0
Unknown (21.10)	0.72 (0.52)	C5	64	13.0
Unknown (13.70)	0.52 (0.25)	C5	36	12.0
Unknown (14.20)	-0.48 (0.23)	C5	74	17.0
Rosmarinic acid (23.10)	-0.45 (0.21)	C5	42	13.0
Naringin (21.5)	-0.27 (0.07)	C1	0	15.0
Salicylic acid (22.50)	-0.25 (0.06)	C1	10	11.0

Only compounds that co-localize with resistance QTL on C1 and C5 and are significantly correlated with AUDPC are listed. RT = retention Time, cM = centiMorgan, VL = *Verticillium longisporum* and n = 98 DH lines.

Three cell wall-bound phenolics peaks which showed co-localized QTL for resistance from the *V. longisporum*-inoculated data set were identified as rosmarinic acid, naringin and salicylic acid based on their retention times and co-migration with standard compounds (retention times 23.10 min, 21.50 min and 22.50 min respectively) (Fig. 14; Table 12). The QTL for rosmarinic acid co-localized with the major QTL for *V. longisporum* resistance on chromosome C5, whereas the QTL for naringin and salicylic acid co-localized with the minor QTL for *V. longisporum* resistance on chromosome C1 (Fig.14; Table12). Four other cell wall-bound phenolic compounds extracted from the *V. longisporum*-inoculated genotypes

which also correlated with AUDPC and co-localized with the major resistance QTL on C5 have been not identified yet (Fig 14; Table 12). No QTL were detected for the *V. longisporum*-induced and constitutively expressed cell wall-bound fractions for *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid although they showed medium ($R^2 \leq 28\%$) correlation with mean AUDPC (Supplementary Table S7 and S3 shown in the appendix).

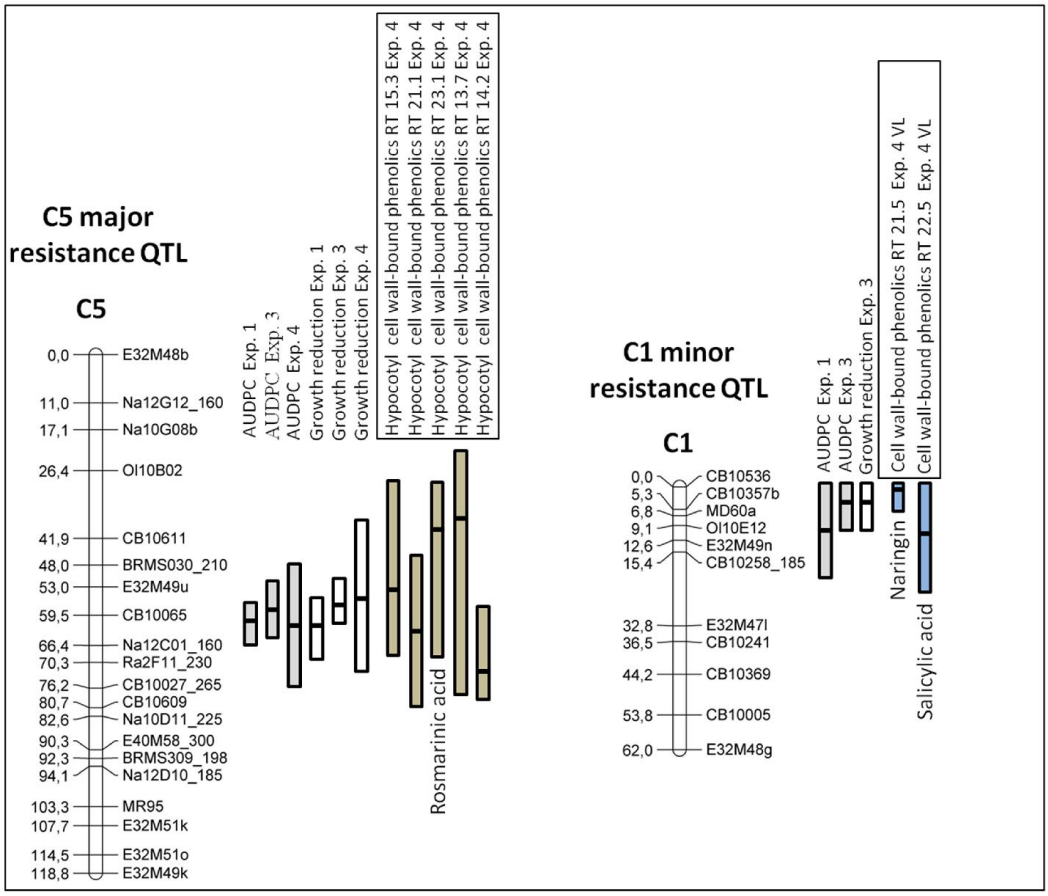

Figure 14: Comparison of quantitative trait loci for *V. longisporum* resistance-related traits and cell wall-bound phenolic metabolites in the hypocotyl localized on chromosomes C5 and C1. Blocks indicate confidence intervals of the QTL. Exp. = experiment and RT = retention time of peak in RP-HPLC.

Among the identified cell wall-bound phenolic peaks vanillic acid and vanillin showed the highest correlations with disease resistance measures (mean AUDPC and mean growth reduction) in the *V. longisporum*-inoculated data set. There were no QTL for these cell wall-bound phenolic metabolites detected applying a LOD score of >2.5. However, these compounds showed co-localized QTL on chromosome C5 for *V. longisporum* resistance in OSR applying a LOD score threshold of 1.8 (Fig. 15).

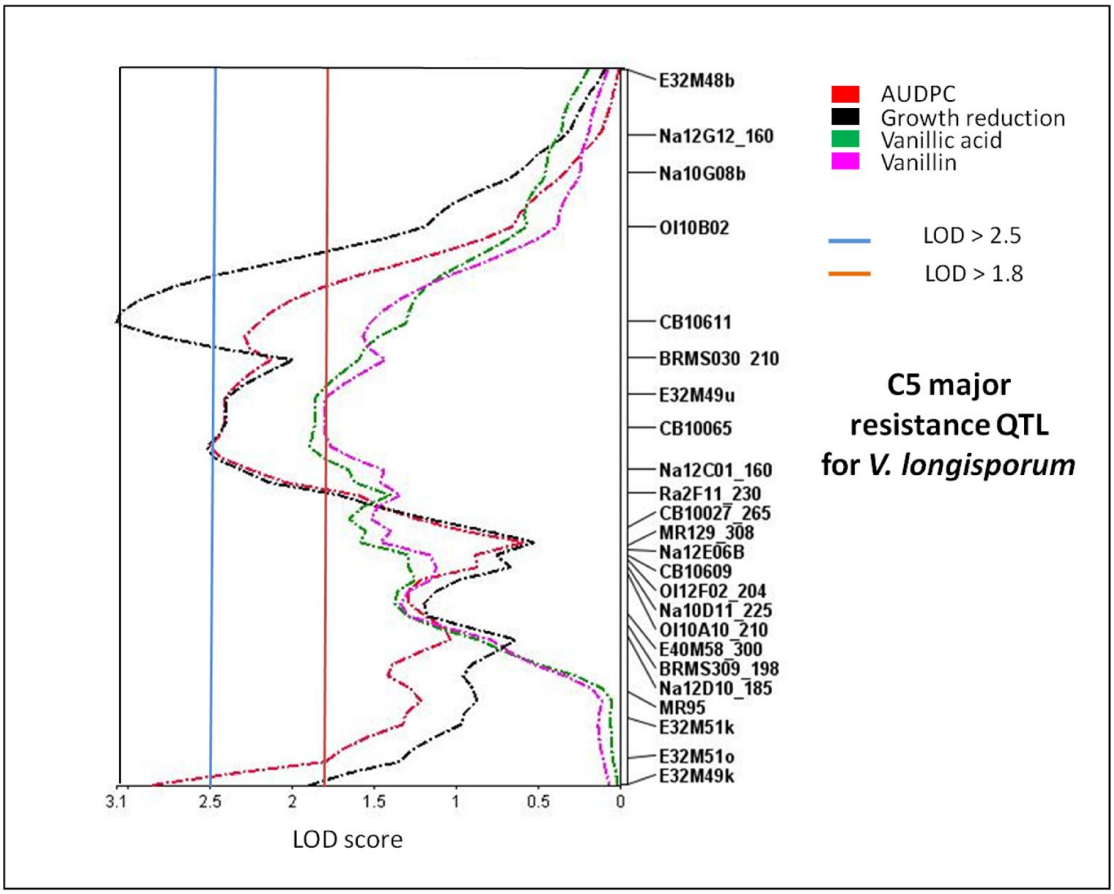

Figure 15: Comparison of quantitative trait loci for *V. longisporum* resistance and phenolic metabolites (cell wall-bound vanillic acid and vanillin) in the hypocotyl localized on chromosome C5. AUDPC and growth reduction showed *V. longisporum* resistance QTL on chromosome C5 at LOD >2.5, whereas vanillic acid and vanillin from *V. longisporum*-inoculated data set showed co-localized QTL for *V. longisporum* resistance on chromosome C5 at LOD >1.8.

3.13 Lignin monomer composition changes associated with *V. longisporum* infection

After removal of soluble and cell wall-bound phenolic compounds, oven dried hypocotyl samples were used for the extraction of lignin monomers applying the thioacidolysis method. Then the recovered thioethylated monomers were analysed by gas chromatography coupled with mass spectrometry (GC/MS). Mainly the uncondensed arylglycerol-beta-aryl ether-linked (β-*O*-4 ether linked) H, G and S monomers of lignin are cleaved from the polymer and their abundances can be determined by this method (Robinson & Mansfield, 2009; Rolando et al., 1992). In this approach, lignin monomers were detected as the thioethylated products of erythro- and threo- isomers corresponding to each *p*-hydroxycinnamyl alcohol (Fig. 16, peaks G1, G2 and S1, S2) and the *p*-hydroxycinnamyl aldehydes (Fig. 16, peaks G3 and S3) (Eynck et al., 2012; Rolando et al., 1992). Discrete measurements for each of the lignin monomers

were only possible for the G- and S-derived monomers, as the H-derived monomers were present only at trace levels in all samples.

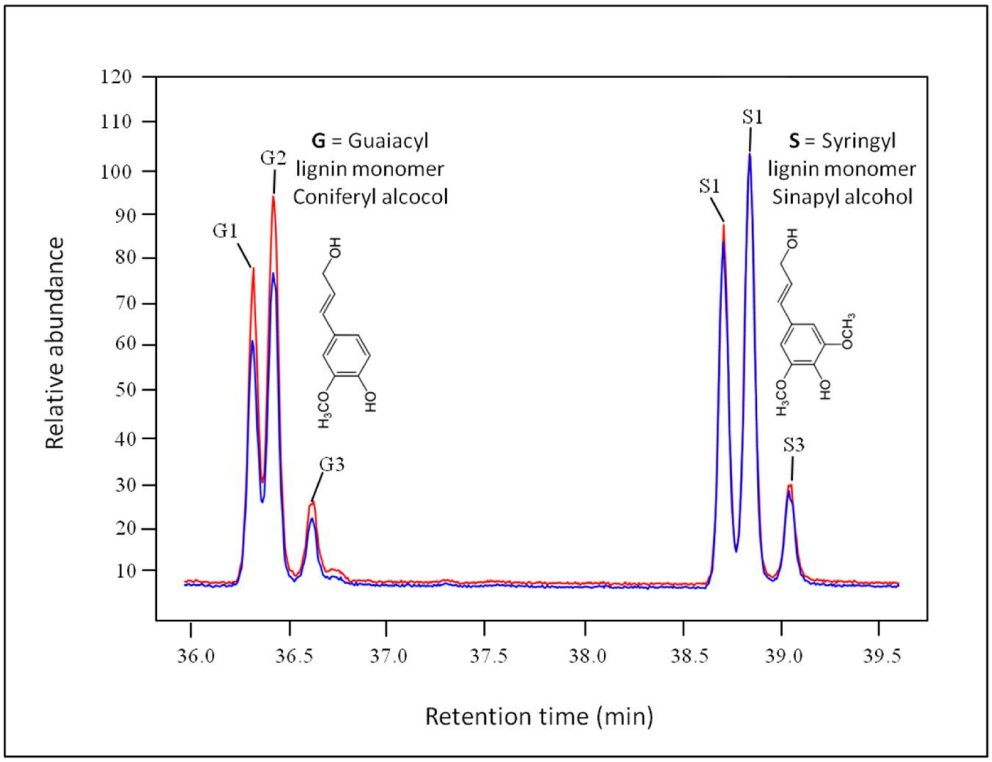

Figure 16: Thioacidolysis products of hypocotyls tissue of oilseed rape line DH106 (blue line: mock-inoculated and red line: *V. longisporum*-inoculated). G1, erythro-guaiacyl-CHR-CHR-CH_2R; G2, threo-guaiacyl-CHR-CHR-CH_2R; G3, guaiacyl-CH_2-CHR-CHR_2; S1, erythro-syringyl-CHR-CHR-CH_2R; S2, threo-syringyl-CHR-CHR-CH_2R; S3, syringyl-CH_2-CHR-CHR_2.

Hypocotyl tissues of mock- and *V. longisporum*-inoculated DH lines were analysed in order to detect potential changes in lignin monomer yield and monomer ratios in response to an infection with *V. longisporum*. Analysis of thioethylated products showed that *V. longisporum* infection had a clear influence on lignin monomer composition in oilseed rape hypocotyls at 28 days after post inoculation (dpi). Upon fungal infection β-*O*-4-linked thioethylated monomers especially S and G units were significantly reduced in susceptible genotypes compared to the resistant genotypes and controls (Fig. 17 and 18). S lignin monomers decreased more than the G lignin monomers in compatible host-pathogen interaction in susceptible DH lines. Only a slight increase in G and S lignin subunits were observed in the *V. longisporum*-inoculated resistant lines. G/S ratios increased in pathogen inoculated resistant DH lines, consequent to the reduction of S units in susceptible genotypes upon *V. longisporum* infection (Fig. 17).

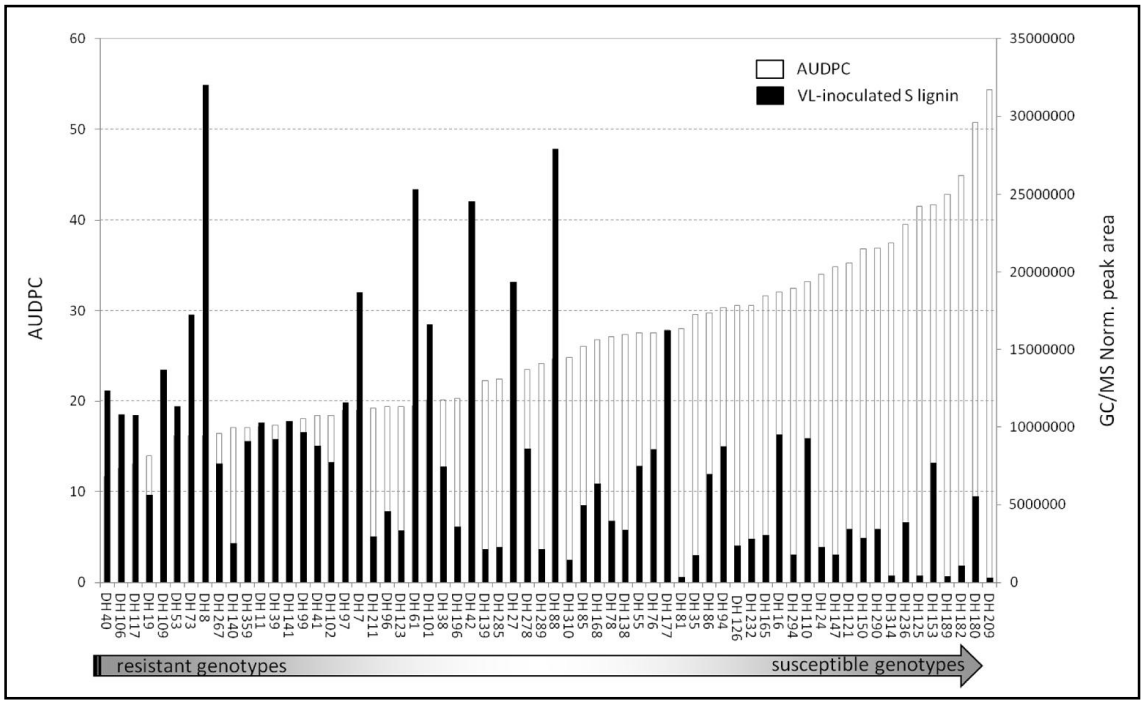

Figure 17: The β-*O*-4 linked S lignin monomer (syringyl lignin) concentration decreases in the cell wall in susceptible genotypes after infection with *V. longisporum* in the mapping population ExR53 (mean from 2 technical replicates). Genotypes are sorted by resistance to susceptibility reaction (AUDPC). Pearson correlation (R = -0.46) significant for n = 64 DH lines at $p \leq 0.001$.

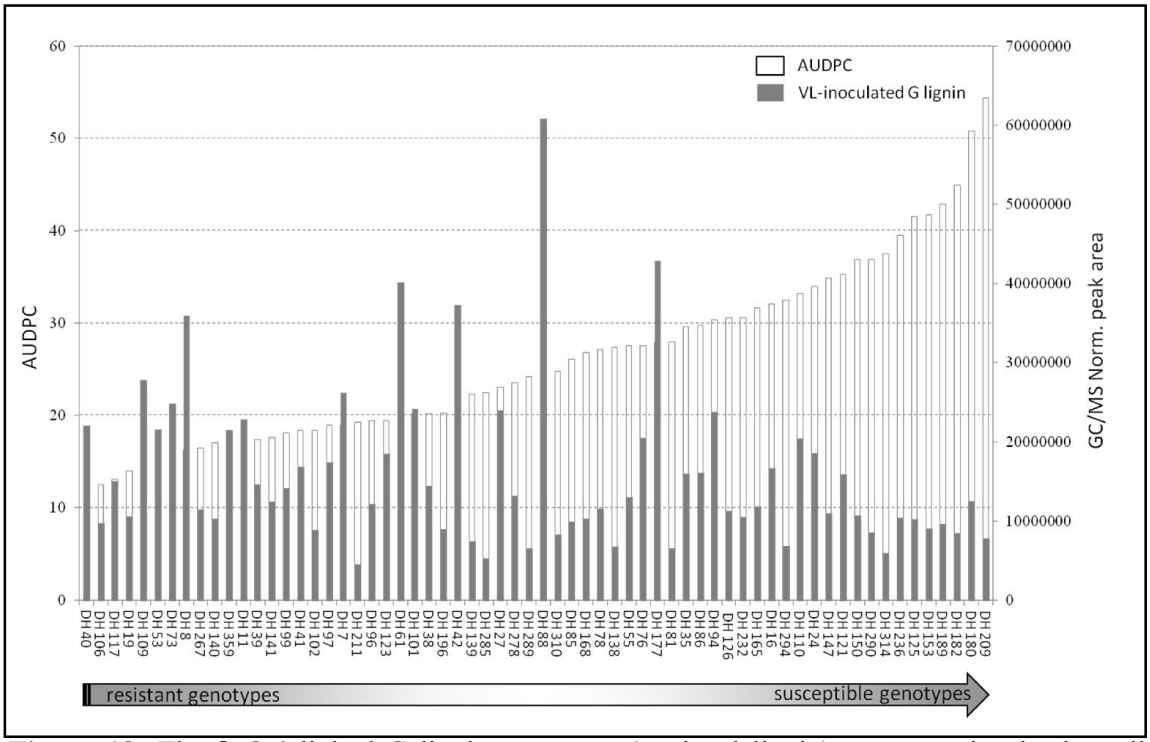

Figure 18: The β-*O*-4 linked G lignin monomer (guaiacyl lignin) concentration in the cell wall decrease in susceptible genotypes after *V. longisporum* infection in the mapping population ExR53 (mean of 2 technical replicates). Genotypes are sorted by increasing AUDPC (susceptibility). Pearson correlation (R = -0.46) significant for 64 DH lines at $p \leq 0.05$.

To investigate the effects of *V. longisporum* infection on the lignin monomer composition, a correlation of individual lignin monomer composition and their ratio with resistance measured as AUDPC were calculated for the mapping population and the different treatments (mock- and *V. longisporum*-inoculated). G and S lignin monomers were negatively correlated with AUDPC meaning that these monomers were down-regulated in the susceptible DH lines and up-regulated in the resistance lines after infection with *V. longisporum* (Fig. 17 and 18; Table 13). S lignin monomer concentrations showed a medium correlation (R = -0.46, R^2 = 21%; n = 64) with mean AUDPC, whereas G lignin monomer concentrations showed a weak but significant correlation (R = -0.28, R^2 = 7.8%; n = 64) with mean AUDPC upon *V. lingisporum* infection (Table 13). Accordingly, G/S ratios also showed a medium correlation (R = 0.58, R^2 = 34%; n = 64) with mean AUDPC (Table 13). After removing of all the potential artifacts 64 DH lines out of 98 DH lines have been used for the correlation study and these 64 DH lines were selected based on lower standard deviation (SD <10%) obtained from the two technical replicates. In addition, very similar correlation values (R^2 = 17-21% for the correlation between S lignin concentrations from *V. longisporum* inoculated data set and mean AUDPC) were observed when including also 91 DH lines with only one technical replicate measurement or when observed 73 DH lines with 2 technical replicates considering the higher standard deviation of uneven measurements (see supplementary Table S9 and S10). Seven DH lines were not included in this study due to lack of initial materials for the thioacidolysis extraction.

Table 13: Correlation between area under the disease progress curve (AUDPC) and concentrations and ratios of lignin monomers in the oilseed rape hypocotyls (n = 64 DH lines)

	AUDPC	Guaiacyl (G) lignin mock-inoculated	Syringyl (S) lignin mock-inoculated	Guaiacyl (G) lignin VL-inoculated	Syringyl (S) lignin VL-inoculated	Mock-inoculated G/S	VL-inoculated G/S
AUDPC	1						
Guaiacyl (G) lignin mock-inoculated	n.s.	1					
Syringyl (S) lignin mock-inoculated	n.s.	0.95***	1				
Guaiacyl (G) lignin VL-inoculated	-0.28*	0.38**	0.35**	1			
Syringyl (S) lignin VL-inoculated	-0.46***	0.43***	0.45***	0.86***	1		
Mock-inoculated G/S	n.s.	n.s.	n.s:	n.s.	n.s.	1	

VL-inoculated G/S	0.58***	n.s.	n.s.	-0.26*	-0.44***	n.s.	1

Pearson correlation (R) significant for n = 64 DH lines (mean from 2 technical replicates) at ≤ 0.05 (*), ≤ 0.01 (**), and at ≤ 0.001(***), n.s. = not significant, VL = *Verticillium longisporum* and AUDPC = area under the disease progress curve. R value from the correlation between G lignin and S lignin concentrations and G/S ratios with the mean AUDPC are labelled in grey.

4. Discussion

Verticillium longisporum is an important soilborne pathogen causing a serious disease in oilseed rape. The Verticillium disease has been an increasing threat to rapeseed production for the last decades especially in Northern and Central Europe. Resistance breeding is the most promising approach for the long term control of this and other diseases due to the lack of fungicides providing effective field control of *V. longisporum.* No complete but partial, quantitative resistance is known against *V. longisporum* in *B. napus*. The genetic basis of resistance to the systemic spread of this fungal pathogen in *B. napus* and other *Brassica* species is not clear. Histochemical studies have shown that phenolic compounds including soluble phenolics, cell wall-bound phenolics as well as lignin in the hypocotyl may play an important role in the inhibition of systemic spread of *V. longisporum* in oilseed rape. Owing to that, genetic analysis of phenolic metabolites associated with resistance against *V. longisporum* might help to identify candidate genes playing a role in *V. longisporum* resistance in *B. napus*.

4.1 Chromosomal regions on C1 and C5 are involved in *V. longisporum* resistance in oilseed rape

Resistance to *V. longisporum* infection in *B. napus* has been described to be polygenic or multifactorial (Happstadius et al., 2003; Rygulla et al., 2007a; Rygulla et al., 2007b; Rygulla et al., 2008; Eynck et al., 2009a). In an earlier study, major quantitative trait loci have been identified for *V. longisporum* resistance on chromosomes C4 and C5 and minor QTL on A6 and C8 in a DH mapping population, SW99-307, produced from resynthesized rapeseed (Rygulla et al., 2008). In the present study a different DH population, Express 617 x R53 has been used for resistance mapping to dissect the genetic basis of *V. longisporum* resistance of commercial and resynthesized rapeseed material. This was approached by genetic analysis using a cross of an inbred line from a commercial cultivar exhibiting partial resistance, Express 617, and a resynthesized rapeseed line, R53, of entirely different origin exhibiting pronounced resistance. A major QTL for *V. longisporum* resistance has been identified on chromosome C5 and a minor QTL on chromosome C1 in the mapping population 'Express 617 x R53' in this study applying a traditional QTL mapping approach (Fig. 8). The allele linked to resistance at the detected major QTL on C5 originated from the C genome donor of the resistant RS rapeseed parent R53, whereas the allele linked to resistance at the minor QTL on C1 originated from Express 617 (see Supplementary Table S3 from Obermeier et al.,

2013). Phenotypic difference in the AUDPC and the degree of disease-induced growth reduction in the mapping population Express 617 x R53 showed similar, continuous frequency distributions in four resistance screening experiments with different subsets of the population (see Supplementary Fig. S3 from Obermeier et al. 2013). In addition, the ExR53 population also showed a transgressive segregation for resistance that was demonstrated to be due to recombination of additive alleles contributed by both parents, the inbreed line Express 617 and the resynthesized rapeseed line R53, without clear evidence for epistatic interaction. This emphasizes the value of useful genetic markers for combination of different resistance QTL from different genetic backgrounds, including less resistance genotypes.

In a different study using the mapping population SW99-307 the resistance was derived from a white cabbage (*B. oleracea* ssp. *oleracea* convar. *capitata*), which had been crossed with a low erucic acid winter turnip rape (*B. rapa* ssp. *oleifera*) to generate a synthetic *B. napus* in Sweden in 1976 (Rygulla et al., 2008). In contrast, the resistance donor 'R53' used in the present study was produced at Göttingen University from a cross between a kale (*B. oleracea* ssp. *oleracea* convar. *acephela*) and a chinese cabbage (*B. rapa* ssp. *pekinensis*) (Radoev et al. 2008). It was demonstrated in this study that markers derived from 'R53' that flank the major QTL for *V. longisporum* resistance on C5 also flank the major QTL for the same trait found on C5 in the former study. This chromosomal region therefore appears to play an important role in the expression of *V. longisporum* resistance from very different C genome genetic backgrounds. Therefore molecular markers linked to resistance alleles in this region might be broadly applicable in marker-assisted breeding for *V. longisporum* resistance. Combination with markers linked to the minor resistance QTL on chromosome C1, which represents yet another genetic origin for resistance against *V. longisporum*, has the potential for a marker-assisted pyramiding strategy to assist the effective combination of *V. longisporum* resistance from RS rapeseed donors with partial resistance in elite breeding lines.

Phenotypic correlations between the AUDPC values normalized based on the reaction of reference cultivars from different greenhouse experiments, and between greenhouse and field experiments, suggest that QTL influencing resistance to artificial inoculation in the greenhouse are relevant for field resistance against *V. longisporum*. Effective molecular markers derived from resistance QTL are therefore an extremely valuable resource for breeding of new oilseed rape cultivars with effective field resistance against this important disease.

4.2 Markers derived from resistance QTL are useful for *V. longisporum* resistance breeding in oilseed rape

Other than the Express 617 x R53 mapping population four additional DH populations with known pedigrees were used for the purpose of marker validation in this study. The QTL validation experiment showed that a number of selected QTL-derived markers were polymorphic in numerous, genetically diverse breeding populations. The marker CB10065 derived from the major QTL region on C5 was found polymorphic and showed significant association with AUDPC within the populations SW08-190002, DSV-1575 and DSV-1605 in one of the two experiments. On the other hand, the marker O110E12 derived from the minor QTL on C1 showed significant association only with the SW08-190001 population in one of the two experiments. The pedigree information of these *B. napus* mapping populations in table 3 indicates that the A and C genome contributing to these populations originated from genetically diverse parents. These results are suggesting that the markers allow differentiation between different C genome resistance donors and the resistance QTL on C1 and C5. Comparison of the marker-AUDPC association of SW08-190002 with population DSV-1575, which is genetically diverse but shares the same C genome resistance donor HRI 8207, indicates that the marker CB10065 is also significantly related to resistance. This concurs with the pedigree information in table 3 and confirms that CB10065 can be effectively used to select for this resistance QTL in the different genetic backgrounds tested here. These markers now can be helpful for breeders to distinguish the common resistance QTL on chromosomes C1 and C5 and combine them in new oilseed rape breeding lines and cultivars with increased quantitative resistance to *V. longisporum*.

On the other hand, QTL and associated markers identified in biparental mapping populations are rarely directly useful in marker-assisted selection, requiring a more comprehensive verification that they are effective in a large number of different commercially valuable genetic backgrounds. Due to limited polymorphism of individual QTL-linked markers in different genotypes, a suite of additional markers within a narrow window spanning the QTL are often required for QTL validation and establishment of a broadly applicable marker-assisted selection strategy (Akhtar et al., 2010). However, the tested markers and some additional markers flanking the identified QTL regions are currently used and have been experienced by a number of German oilseed rape breeding companies in their recent breeding programms to be useful for *V. longisporum* resistance breeding (personal information, Dr. Christian Obermeier, Dept. of Plant Breeding, Justus Liebig University, Giessen, Germany).

4.3 *V. longisporum*-induced changes in cell wall-bound phenolics are strongly associated with *V. longiaporum* resistance

It has been documented that phenolic metabolites accumulate in plants challenged by fungal pathogens (von Ropenack et al., 1998; de Ascensao & Dubery, 2003; Mandal & Mitra, 2007). They also have been shown to be involved in resistance mechanisms of plants against fungal pathogens (reviewed by Lattanzio et al., 2006; Dixon et al., 2002; Nicholson & Hammerschmidt, 1992). In plants soluble and cell wall-bound phenolic compounds are present. These compounds are hydroxycinnamic acids and aldehydes, hydroxybenzoic acids, and hydroxybenzaldehydes (Harbaum, 2007). In the present study 15 HPLC peaks from the cell wall-bound phenolics fraction and 15 HPLC peaks from soluble phenolics fraction in the hypocotyls of oilseed rape were putatively identified by co-migration with external standards. The detected compounds represent metabolites from the core phenylpropanoid and branching pathways involved in biosynthesis of phenolics. They include metabolites classified as members of the groups of hydroxycinnamic acids, hydroxybenzoic acids and aldehydes, monolignols, phenypropanoid esters, sinapte esters, flavanone glycosides, anthocyanidins and proanthocyanidins.

The large diversity of simple and complex phenolics expressed in healthy and *V. longisporum*-infected *B. napus* plants makes it difficult to come to a clear conclusion about their relevance for and their involvement in resistance mechanisms by comparing their expression patterns in single contrasting genotypes as it has been done in the past in a number of studies on *Verticillium*-plant species interactions (e.g. Pomar et al., 2004; Eynck et al., 2009b; Gayoso et al., 2010). In contrast, in this study a mapping population of 98 genotypes which was genetically well characterized and segregating for *V. longisporum* resistance was investigated to be able to statistically validate and rank the associations of the large diversity of identified individual phenolic metabolites with resistance. In addition, it was possible to map the genetic regions involved in the expression of these cell wall-bound phenolic metabolites and co-localize the metabolite QTL with genetic regions involved in resistance expression. Cell wall-associated plant defence has been described to be important in constitutive and in induced basal resistance, but genetic evidence for the importance of phenylpropanoids and other phenolics in cell wall-associated defence has been rare and it has been poorly understood whether phenolics function by a toxic or by a cell wall-strengthening principle (Hückelhoven, 2007). It has been documented before that the crucial resistance reaction involving phenolic metabolite expression is taking place within the hypocotyl tissue

of *B. napus* (Eynck et al., 2009b). For this reason pooled samples of hypocotyl tissues from *V. longisporum*- and mock-inoculated plants of the mapping population were investigated and data from metabolite profiling was applied for correlation analyses and genetic mapping. The *V. longisporum*-inoculated soluble and cell wall-bound phenolics were found to play clearly different roles in the interaction.

The total *V. longisporum*-inoculated soluble phenolics concentrations showed a significant, but only weak correlation with resistance measured as mean AUDPC values (coefficient of determination, R^2 = 4%). In contrast, a significant, medium correlation (R^2 = 43%) was detected between the total *V. longisporum*-inoculated cell wall-bound phenolics concentrations and the mean AUDPC values. Also individual phenolic metabolites showed a higher medium to strong correlation with AUDPC for most metabolites from the *V. longisporum*-inoculated cell wall-bound phenolics fraction ($R^2 \leq 59\%$) compared to a weak to medium correlation for most of the metabolites from the *V. longisporum*-inoculated soluble phenolics fraction ($R^2 \leq 37\%$).

A large number of individual HPLC peaks (81%) from the *V. longisporum*-inoculated cell wall-bound phenolics fraction showed significant correlations of their concentrations with mean AUDPC. In contrast, only a smaller number of individual *V. longisporum*-inoculated soluble phenolic peaks (37%) showed significant correlations with mean AUDPC. In addition, among the 10 phenolic compounds showing the highest correlation of their concentrations with mean AUDPC ($R^2 > 30\%$) nine were from the *V. longisporum*-induced cell wall-bound fraction, whereas only 1 was detected in the *V. longisporum*-inioculated soluble fraction. Thus, one of the major outcomes of the present study is that changes induced by *V. longisporum*-inoculation in the phenolics patterns which are associated with the resistance expression are mainly occurring on the cell wall level of the hypocotyl tissue, and not on the cytoplasmatic and vacuole level represented by the soluble phenolics fraction.

4.4 Preformed phenolics play a minor role in the resistance interaction of *V. longisporum* with oilseed rape

Biological functions of phenolics include diverse areas such as cell wall reinforcement, antimicrobial activity, plant hormone activity, defense signalling and scavenging of reactive oxygen species (Lattanzio et al. 2006; Nicholson & Hammerschmidt et al., 1992).

Phenylpropanoids and simple phenolics can be conjugated with each other to form complex polymers (e.g. lignin) and with a diversity of other substances (e.g. carbohydrates, proteins) via ester, ether, and other types of bounds. Phenolics in plants might be classified into two major groups such as preformed constitutive expressed phenolics and induced phenolics synthesized in response to abiotic or biotic stresses. Preformed antibiotic compounds that occur constitutively in healthy plants (sometimes also named phytoanticipins) are mainly simple phenols, phenolic acids, flavonols and dihydrochalcones and are likely to represent inbuilt chemical barriers which extend to a broad range of potential pests and pathogens (Lattanzio et al., 2006).

The preformed cell wall-bound total phenolics concentrations in the hypocotyls of the *B. napus* population detected in the present study did not show a significant correlation with resistance (mock-inoculation treatments). In contrast, the preformed total soluble phenolics concentrations exhibited a weak correlation with mean AUDPC (R^2 = 7%). In total 8 individual preformed phenolics in the soluble and cell wall-bound fraction (from a total of 55 with significant correlations from all treatments) showed a weak correlations with mean AUDPC (maximum R^2 = 16%). The 3 identified preformed phenolics which are correlated with resistance all belong to the classes of simple phenols or phenolic acids which have been described in the literature to have direct antifungal activity to a wide range of fungal and bacterial plant pathogens (Panizzi et al., 2002; Lattanzio et al., 2006; Vio-Michaelis et al., 2012). This is consistent with the general characteristics of the group of phytoanticipins. The two preformed phenolics with the highest correlations of their concentrations with AUDPC, ferulic acid and caffeic acid, are synthesized within the core phenylpropanoid biosynthesis pathway. Caffeic and ferulic acid have been repeatedly reported to have one of the highest direct antifungal effects compared with other simple hydroxybenzoic acids and hydroxycinnamic acids in *in vitro* inhibition tests against a number of bacterial and fungal plant pathogens (e.g. Aziz et al., 1998; Amborabé et al., 2002; el Modafar & el Boustani, 2005). However, in this study the concentrations of caffeic and ferulic acid were found to be reduced in the soluble fraction of the resistant genotypes. Thus a direct antifungal effect of these preformed phenylpropanoid metabolites is unlikely to be responsible for the reduced invasion of *V. longisporum* into the hypocotyls of the resistant genotypes. Rather an indirect action of these preformed metabolites is plausible to take place in the *V. longisporum*-rapeseed interaction e.g. through a channeling into other branching pathways and conversion into other yet unidentified phenolic compounds or from the cytoplasma or vacuole into other

cell compartments like the cell wall. This has been observed in this study for the preformed concentrations of protocatechuic acid, a member of the hydroxybenzoic acid group, where the concentration in the cell wall of non-infected plants (mock) was correlated with resistance and the concentration was increased in the resistant non-infected genotypes. The water soluble protocatechuic acid is one of the first simple benzoic acid which has been described to directly inhibit the growth of the spores of the smudge fungus *Colleotrichum circinans* by diffusing into infection drops on the surface of the outer scale of onions and preventing germination and penetration of the fungus (Walker & Stahmann, 1955). However, in the *V. longisporum*-oilseed rape interaction a correlation with resistance was detected in the cell wall-bound fraction, but not in the soluble fraction. This also suggests a different mechanism in the *V. longisporum*-oilseed rape interaction than in the *C. circinans*-onion interaction not involving a direct antifungal activity of protecatechuic acid in the soluble fraction. Also, the constitutive expression of protocatechuic acid explains here with 12% only a small fraction of the phenotypic variation in the resistance expression of *B. napus* against *V. longisporum* and represents a minor effect. Interestingly, for the constitutively expressed caffeic acid explaining with 15% also only a minor fraction of the resistance reaction (similarly also to ferulic acid), a QTL was identified which co-localizes with a minor resistance QTL for *V. longisporum* resistance on chromosome C1. In summary, preformed soluble and cell wall-bound phenolics obviously play a minor role in the resistance interaction of *V. longisporum* with *B. napus*.

4.5 *V. longisporum*-induced cell wall esterified phenolics are weakly associated with *resistance* interaction in oilseed rape

A considerable amount of phenolic compounds are located in the plant cell wall. These phenolics are primarily present as structural components of cell walls and are esterified with other cell wall components. Harbaum (2007) described that phenolic esters in plant cell walls especially with hydroxycinnamic and hydroxybenzoic acids and aldehydes may construct cross-links with cell wall polysaccharides by means of dimer formation and these esterifications and dimer formations determine the biodegradability of the cell walls. The esterified phenolic compounds in the cell wall may well protect pathogen infestation into the plants.

In this study, individual phenolic compounds were detected that were present in both, the mock- and *V. longisporum*-inoculated plants, but were expressed in different concentrations in resistant and susceptible genotypes upon *V. longisporum* infection. *V. longisporum*-induced changes of individual soluble and cell wall-bound phenolics showed a medium to strong correlation ($R^2 \leq 59\%$) with mean AUDPC, whereas preformed individual phenolics from the mock treatment showed a weak correlation with resistance measures ($R^2 \leq 16\%$). The concentrations of some of the identified cell-wall bound as well as soluble phenolics expressed in hypocotyl tissue were found to be negatively correlated with the resistance reaction (mean AUDPC), whereas others showed a positive correlation. A negative correlation with mean AUDPC values means that in resistant lines upon *V. longisporum* infection on average a higher concentration is measured than in susceptible lines. The compound concentrations negatively correlated with mean AUDPC mostly exhibited a weaker correlation with AUDPC ($R^2 \leq 24\%$) compared to most of the positively with mean AUDPC correlated (down-regulated) compound concentrations ($R^2 \leq 59\%$).

Higher average concentrations in resistant lines than in susceptible lines (negative correlation with mean AUDPC, up-regulated) were found for the *V. longisporum*-inoculation treatments for some cell wall esterified phenolics such as *p*-coumaric acid, caffeic acid and caffeic acid ester (rosmarinic acid), and some unknown phenolic compounds. In contrast, cell wall-bound chlorogenic acid (a caffeic acid ester) showed a positive correlation with resistance measures.

For all of these four identified esterified phenolic compounds some reports exist on their direct antibiotic effects in *in vitro* cultures in soluble form against bacterial and fungal pathogens (Bais et al., 2002; Lattanzio et al., 2006; Chakraborty et al., 2007; Báidez et al., 2007; Chong et al., 2009; Merkl et al., 2010). However, in this study these phenolics were mostly found to be correlated with resistance in the cell wall-bound fraction, but not in the soluble fraction, which might also suggest a slightly different mode of action. For example, Fry (1987) described that *p*-coumaric acid is involved in cell wall esterification which enhances plant resistance against cell wall degrading fungal enzymes. Upon *V. dahliae* infection, *p*-coumaric acid increased in roots in the cell wall of resistant tomato plants when compared with the control plants of both lines or with inoculated susceptible plants (Gayoso et al., 2010). Gayoso et al. (2010) also observed that this up-regulation of *p*-coumaric acid in the cell wall is related to a maximum peroxidase activity in roots of inoculated resistant tomato plants compared to the roots of susceptible plants. It is also known that enhanced

activity of peroxidase enzymes often is associated with resistance phenomena such as lignin synthesis (Hammerschmidt et al., 1982), activity of phenylalanine ammonia lyase (PAL), and phenolics accumulation (Tena & Valbuena, 1983; Pollock & Drysdale, 1976; Rathmell, 1973). Florel et al. (2008) described that 12 proteins were up-regulated after *V. longisporum* infection in rapeseed leaf apoplasts which include the pathogen defence-related enzymes peroxidase, *β*-1,3-gluconase, PR4 and endochitinase. In plants, ferulic acid and *p*-coumaric acid have a very important role in the maintenance of cell walls and it mediates the cross-linking of lignins to polysaccharides in cell walls of gramineous plants (Pan et al., 1998). Caffeic acid may be produced in root and stem tissues partly to strengthen the cell wall in response to the structural damage caused by *V. dahliae* in potato (Ahmed et al., 2013). In general, caffeic acid and *p*-coumaric acid have also been reported to be accumulated in different crops in response to infection by other fungi (Ahmed et al., 2013; Gayso et al., 2010; Mandal & Mirta, 2008, 2007; Kuc' et al., 1956) and have been suggested to be important factors in disease resistance in plant-microbe interactions. Interestingly, all of these compounds showing higher concentrations in the cell wall fraction upon *V. longisporum* infection are either simple hydroxycinnamic acids (*p*-coumaric acid or caffeic acid) synthesized within the basic part of the core phenylpropanoid pathway (see Fig. 22) or are derivates of these two hydroxycinnamic acids branching into the hydroxycinnamic ester biosynthesis pathways (rosmarinic acid, chlorogenic acid). The pattern of up- and down-regulation in the soluble and cell wall fraction in these pathways suggests that coumaric acid or *p*-coumaroyl-CoA are key compounds which get diverted from the basic part of the core phenylpropanoid pathway in the resistant or the susceptible interaction into different substrates (e.g. rosmarinic acid instead of chlorogenic acid, Fig. 22). The biosynthesis of *p*-coumaroyl-CoA is regulated by the 4-Coumarate: CoA ligase (4CL) gene within the core phenylpropanoid pathway. 4-Coumarate: CoA ligase (4CL), the last enzyme of the general phenylpropanoid biosynthesis pathway, catalyzes the formation of CoA esters of *p*-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid, and sinapic acid (Hu et al., 1998; Lee et al., 1997; see Fig. 19). The 4CL enzyme is very important for the diversion of phenylpropanoids and appears to be the most conspicuously affected enzyme by fungal infection (reviewed by Nicolson & Hammerschmidt, 1992). Following biosynthesis from the upper part of the phenylpropanoid pathway (see Fig. 22) these compounds then will be eventually relocated to the cell wall and esterifiied changing the wall-bound phenolics and putatively also the glycosylation pattern in resistant and susceptible genotypes. Interestingly, for the *V. longisporum*-induced rosmarinic acid expressison (a caffeic acid ester) explaining

with 19% also only a medium fraction of the resistance reaction, a QTL was identified which co-localizes with a major resistance QTL for *V. longisporum* resistance on chromosome C5. Petersen et al. (2009) described that caffeic acid esters, i.e. rosmarinic acid and chlorogenic acid, are widely found in the plant kingdom and presumably accumulate in plants as defence metabolites.

Cell wall chlorogenic acid (a caffeic acid ester) was increased in susceptible oilseed rape DH lines upon *V. longisporum* infection. This result is in agreement with previous findings for other host-pathogen interactions. After infection with fungal pathogens chlorogenic acid accumulates sooner in susceptible potato cultivars compared to resistant cultivars and controls (Gans, 1978; Henderson & Friend, 1979; Friend et al., 1981). Friend et al. (1981) described that chlorogenic acid might be functioning as a reservoir for the caffeoyl moiety in the phenylpropanoid pathway and could be pushed to the synthesis of other phenolics possibly involved in suppression of the pathogens. The lack of chlorogenic acid accumulation in resistant genotypes might also correspond to the direct conversion of phenylpropanoid CoA esters to other phenolics more important in the resistance reaction. However, this *V. longisporum*-induced caffeic acid ester showed a rather weak correlation (R^2 = 9%) with resistance measures and can thus not be considered to represent a major factor in the defence response.

The above results are suggesting that esterified phenolics such as caffeic acid, caffeic acid ester (rosmarinic acid) and *p*-coumaric acid which show an increase in their concentration in the cell wall upon *V. longisporum*-infection in resistant genotypes are involved directly or indirectly in resistance expression, but are not key compounds for *V. longisporum* resistance expression in *B. napus* indicated by their low R^2 (below 25%, Table 8). These phenolics play a minor role in the resistance interaction of *V. longisporum* with *B. napus*. Changes induced by *V. longisporum*-inoculation of these phenolic metabolites might be related to cell wall esterification, which enhances plant resistance against cell wall degrading fungal enzymes. They might also be involved in antioxidant activity, increased H_2O_2 levels and peroxidase activity in resistant lines. In addition, these phenolic compounds might have direct inhibitory effects on growth and/or germination of *V. longisporum*. The pattern of expression of caffeic acid esters which are converted from *p*-coumaryl CoA in the hypocotyl of susceptible and resistant oilseed rape genotypes might be regulated by the 4CL gene in the core phenylpropanoid pathway.

4.6 Flavonoids play a minor role in the resistance interaction of *V. longisporum* and oilseed rape

Flavonoids are secondary metabolites synthesized via the phenylpropanoid and polyketide pathways having significant and different functions in flowering plants. These metabolites are used for floral pigments, UV protectants, and resistance to pathogens or insects and for many other purposes in plants.

In this study two flavonoids (flavanone glycosides), naringin and phlorizin, were identified in the cell wall-bound and soluble phenolics fraction, respectively, by comparison of the retention time with HPLC quality external standards and further confirmed by co-injection with the same standards. The concentrations of these identified flavanone glycosides expressed in hypocotyl tissue were found to be negatively correlated with the resistance measures (mean AUDPC and mean growth reduction), indicating that higher concentrations in resistant genotypes upon *V. longisporum* infection. These flavanone glycosides are derived from different closely related branches of the flavonoid pathways. The pattern of up- and down-regulation in the cell wall and soluble fraction in these pathways suggests that CoA-ester of coumaric acid is the key precursor of these flavonoids which get converted from the basic part of the core phenylpropanoid pathway (see Fig. 19 & Fig. 22).

Phlorizin and naringin are both closely related members of the diverse flavonoid group synthesized in a highly branched pathway. Most of the flavonoid biosynthetic enzymes are recovered in the 'soluble' cell fractions (Koes et al., 2005). Phlorizin is a mono-glucoside of phloretin (a dihydrochalcone). Similar to naringin which is a di-glucoside of naringenin (a flavanone) their biosynthesis is thought to occur through closely related side branches of the flavonoid biosynthesis pathway starting in both cases with the activity of the enzyme chalcone synthetase (CHS). CHS is considered the gatekeeper of flavonoid biosynthesis playing an important role in regulating the whole pathway (Dao et al., 2011). Whereas *p*-coumaroyl-CoA is the precursor for the naringenin chalcone leading to further flavonoid formation up to the key compound naringenin and the derived di-glucoside naringin, the slightly modified *p*-dihydrocoumaroyl-CoA is required for the biosynthesis of dihydrochalcones such as phloretin and the derived mono-glucoside phlorizin (Fig. 19 & Fig. 22) (Gosch et al., 2010a). Both phlorizin and naringin and its aglycons have been repeatedly reported to be associated with resistance against a number of plant pathogens and their direct antifungal properties have been documented (Lattanzio et al., 2006; Hussain et al., 2009; Gosch et al., 2010a).

The relevance of both of these resistance-related flavonoids, phlorizin and naringin, for direct or indirect involvement in resistance is further confirmed by the detected co-localization of QTL for plozizin and naringin with the minor resistance QTL for *V. longisporum* on C1. This makes members of the early phenylpropanoid core pathway and the flavonoid pathway and especially from the CHS mulitgene family or regulatory genes for this flavonoid key enzyme interesting candidate genes for involvement in resistance expression of *B. napus* against *V. longisporum*. In *B. napus* four genomic copies of the CHS gene have been identified (Qu et al., 2013). Chalcone synthase has been reported as a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway. Besides being part of the plant developmental program, the CHS gene expression is induced in plants under stress conditions such as UV light, bacterial or fungal infection (Dao et al., 2011). Also flavonoids have been shown to accumulate in the hypocotyl-root transition zone in *A. thaliana* and to be involved in growth regulation and auxin transport/metabolism (Winkel-Shirley 2001, 2002). In plants an increase of CHS activity causes a high accumulation of the flavonoid level that inhibits auxin transport and inhibitors of auxin transport could increase the resistance of plants to fungal pathogens, e.g. of tomato plants to *Fusarium oxysporum* (Dao et al., 2011; Davis, 1954). On the other hand, the UDP-glycosyl transferase (UGT) enzyme might be an interesting candidate genes which regulate the precursors of phlorizin and narinzin in the flavonoid pathway (Fig. 19). The gene UDP-glucose: sinapate glucosyl transferase expressed in seeds of *B. napus* has been mapped on chromosome C1 (Mittasch et al., 2010) and this QTL might also be overlapping with the QTL for phlorizin and naringin on chromosome C1 found in this study.

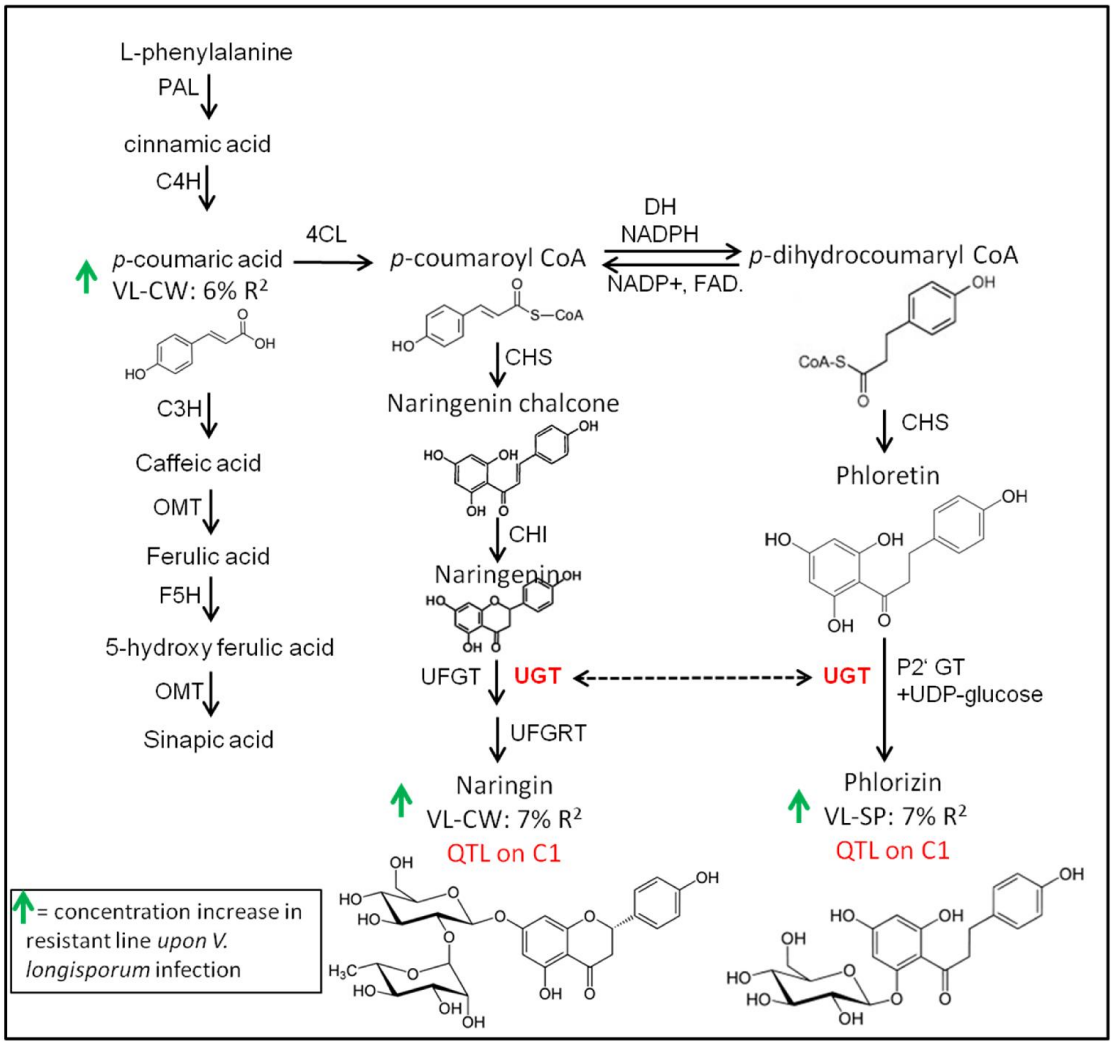

Figure 19: Naringin and phlorizin biosynthesis pathway originated from core phenylpropanoid pathway. Modified after Gosch et al., 2010ab, Winkel, 2006 and Lewinsohn et al., 1989. QTL: quantitative trait loci. C1: chromosome one. Solid arrows: direct conversion of the respective compound. Dashed arrow: identity of cDNA nucleotide sequence of 50% in apple and enzymes use the same substrate. Enzyme names are indicated with standard abbreviations:PAL, Phenylalanine ammonia-lyase; C4H, Cinnamate -4-hydroxylase ; C3H, Cumarate-3-hydroxylase; OMT, O-Methyl transferase; F5H, Ferulate 5-hydroxylase; 4CL, 4-Coumarate: CoA ligase; CHS, Chalcone synthase; CHI, Chalcone isomerase; UGT, UDP-glycosyl transferase; UFGT, UDP-glucose:flavanone glucosyl transferase; P2' GT, UDP-glucose:phloretin 2'-*O*-glycosylsyl transferase; UFGRT, UDP-rhamnose:flavanone rhamnosyl transferase.

In the present study, resistant *B. napus* genotypes always showed reduced relative growth (R^2 = 8%) and concentrations of both flavonoids, phlorizin as well as naringin, were found to be negatively correlated with resistance as well as with relative growth. However, the up-regulation of both, phlorizin in the soluble fraction and naringin in the cell wall fraction in resistant genotypes after *V. longisporum* infection explained only 4% and 7% of the resistance

reaction, respectively. Metabolites from the flavonoid group should thus not be considered to represent major factors contributing to resistance expression of *B. napus* to *V. longisporum*.

4.7 Phytohormones play a minor role in *V. longisporum* resistance expression

Phytohormones are chemical regulators produced by plants to regulate not only growth and development but also have a role in various biotic and abiotic stress. Not unexpectedly, hydroxybenzoic acids functioning as plant hormones and defence signals to resistance against *V. longisporum* were detected, in this case salicylic acid and gentisic acid. Dao et al. (2011) described that the accumulation of flavonoids and isoflavonoids and expression of the chalcone synthese (CHS) enzyme are involved in the salicylic acid defence pathway. In this study, the flavonoids phlorozin and naringin, were accumulated in the hypocotyl of resistant oilseed rape genotypes upon *V. longisporum* infection. Vertcillium-infection changes the abundance of several hundreds of soluble metabolites in the *A. thaliana* leaf apoplast including signalling and defence compounds such as glycosides of salicylic acid, lignans and dihydroxybenzoic acid as well as oxylipins (Floerl et al., 2012). In another study, jasmonic acid and abscisic acid levels remained unchanged, whereas salicylic acid and its glucoside increased in the xylem sap of *B. napus* shoots after infection with *V. longisporum* (Ratzinger et al., 2009). Salicylic acid is also known to cross-talks with auxin by repressing auxin signaling and reducing susceptibility in plants (Vlot et al., 2009) which might also partly explain the growth reduction seen in this experiments in resistant genotypes. Salicylic acid also showed direct antifungal activity against the fungus *Entypa lata* in *in vitro* studies (Lattanzio et al., 2006). However, salicylic acid was not detected in the soluble fraction in this study as in all the reports mentioned above, but in the cell wall-bound fraction. This suggests a different mode of action here. The QTL for the *V. longisporum*-inoculated cell wall-bound salicylic acid also co-localized with the minor *V. longisporum* resistance QTL on chromosome C1, indicating that salicylic acid may play a role in the activation of the inducible defences in oilseed rape.

Gentisic acid is known to induce specific sets of pathogen-related (PR) genes in tomato which are not induced by salicylic acid (Bellés et al., 1999; Vlot et al., 2009) which strongly indicates that salicylic acid and gentisic acid play complementary signaling roles in the activation of the inducible defence system. In this metabolomic investigation gentisic acid also was detected only in the cell wall and the concentrations of cell wall-bound gentisic acid showed a negative correlation with mean AUDPC, indicating that resistant genotypes with

higher gentisic acid levels in the cell wall fraction of the hypocotyl show less necrotic/chlorotic symptoms on leaves upon *V. longisporum* infection. This result might corroborate the findings of Bellés et al. (1999) who described that gentisic acid signaling may be restricted to non-necrotizing reactions of the host plant. But it is not clear whether this induced gentisic acid functions as an antifungal compound or as a component of the signaling system in this study. Bellés et al. (2006) described that gentisic acid showed an additional signal to salicylic acid for the activation of plant defences in cucumber and *Gynura* plants. Gentisic acid could acts as a pathogen-induced signal which is additional to salicylic acid, for activation of plant defence genes in tomato (Bellés et al. 1999). This signal molecule also showed inhibitory activity against fungi *in vitro* (Lattanzio et al., 1994; Merkel et al., 2010).

The unexpected finding, that these two plant hormones showed a significant correlation with resistance or growth reduction only in the cell wall fraction but not in the soluble fraction raises questions. In a few previous reports it has been described that these plant hormones can be conjugated with cell wall carbohydrates and their expression changed upon pathogen infection. Vernooij et al. (1994) described salicylic acid as a precursor of gentisic acid. The latter is found not only as a free molecule, but mostly as a glucose conjugate in plants. Usually salicylic acid forms glucosylsalicylic acid by conjugating with glucose in plants, and gentisic acid is thought to conjugate likewise (Yalpani et al., 1993; Belles et al., 1999). Resistant potato cultivars with higher amounts of conjugated salicylic acid showed field resistance to late blight (*Phytophthora infestans*) compared to susceptible one (Coquoz et al., 1995). In another study, higher amounts of conjugated salicylic and gentisic acids were found in four potato cultivars compared to free salicylic and gentisic acid and their expression changed upon infection by potato virus Y^{NTN}(PVY^{NTN}) (Krecic-Stres et al.; 2005). The reports on localization of these compounds in the cell wall fraction and strong association with resistance or growth control in this cellular compartment are not well described so far.

However, it also should be noted that salicylic acid and gentisic acid are both members of the groups of simple hydroxybenzoic acids which do not only act as plant hormones, but are precursors in diversed biosynthesis pathways and also might represent degradation products of a number of complex phenolics compounds. Thus salicily acid and gentisic acid might not only act as a hormones here, but mitgth be involved in cross-linking with cell wall compounds or by-products of resistance or susceptibility related plant cell wall degradation triggered by fungal infection. However, compared with other detected phenolics they clearly play a minor

role in the resistance host-pathogen interaction of *V. longisporum* and rapeseed indicated by their low R^2 (4 % and 6%) within this study.

4.8 Reduction of syringyl lignin monomer concentration is associated with a susceptible rapeseed-*V. longisporum* interaction

In the present study the lignin composition changed with regard to monomer ratios in *B. napus* hypocotyls upon *V. longisporum* infection. Differences in lignin monomer composition between lignin formed during normal development of healthy plants and resistant-related lignin in infected plants has been described in several studies of interaction with a variety of pathogens (Vance et al., 1980; Menden et al., 2007). The current study is the first investigation on lignin monomers composition for rapeseed plants infected by *V. longisporum*. Lignin monomer subunits concentrations such as H, G and S lignin have been shown to be specifically increased or decreased depending on the host-pathogen interaction under investigation (Hammerschmidt et al., 1985; Lange et al., 1995; Hawkins & Boudet, 2003; Pomar et al., 2004; Menden et al., 2007; Gayoso et al., 2010; Eynck et al., 2012).

In this thioacidolysis analysis of rapeseed monolignol composition in the hypocotyl, remarkable changes were detected in susceptible DH lines compared to the resistant lines. At 28 dpi the G/S ratio increased in the inoculated resistant rapeseed DH lines and this is the consequence of the reduction of β-*O*-4 linked S and G lignin in susceptible lines. S lignin decreased more than G lignin in susceptible lines. These results are similar to the findings by Pomar et al. (2004) who observed that upon *V. dahliae* infection β-*O*-4 linked thioethylated lignin monomers were significantly reduced in diseased pepper plants compared to the controls and S lignin decreased more than G lignin. They concluded that thioethylated monomer concentrations decreased in infected plants because the linear β-*O*-4 lignin fraction was further cross-linked and insolubilized in response to infection. Also Gayoso et al. (2010) reported that upon *V. dahliae* infection β-*O*-4 linked total G lignin increased slightly, whereas total S lignin decreased conspicuously in one susceptible tomato genotype. The authors also found that the guaiacyl/syringyl ratio increased at 16 dpi after inoculation with *V. dahlia*e as a consequence to the reduction of S units in the lignins of inoculated plants.This might be due to a lower incorporation of syringyl groups within the lignin polymerization process. In susceptible genotypes this results in a lower number of β-*O*-4 bonds and, consequently, in a lower proportion of the linear fraction of lignins in secondary cell walls. Jung & Deetz (1993) described that S units in lignins are more linear polymer than G units and consequently better

protects large areas of secondary cell walls from degradation. The Arabidopsis mutant C4H:F5H which has a higher content of syringyl lignin monomers compared to guaiacyl lignin monomers showed reduced *V. longisporum* growth upon infection, whereas the Arabidopsis *fah1-2* mutant which is devoid of sinapate and its esters (defect in the syringyl lignin synthesis due to an lack of the F5H (ferulate 5-hydroxylase) enzyme showed a higher susceptibility to *V. longisporum* upon infection (König, 2011; König et al., 2014). This finding also is in agreement with the result of this study in *B. napus* revealing that DH lines with a lower amount of S lignin monomers showed a higher susceptibility to *V. longisporum.* Also in this experiment sinapic acid (sinapate, a precursor of S lignin) in the soluble phenolics fraction showed a negative correlation with mean AUDPC, meaning lower concentrations in susceptible lines after *V. longisporum* inoculation. Menden et al. (2007) described that resistant genotypes of wheat with lignin rich in syrngyl units showed defence response against *Puccinia graminin* f. sp. *tritici* i.e. the lack of syringyl lignin in susceptible genotypes showed susceptibility to this *Puccinia graminin* f. sp. *tritici* pathogen. But these results are not supported by König et al. (2011; 2014) who described that free coniferyl alcohol (coniferin), but not sinapyl alcohol (precursor of syringyl lignin) plays a role in restriction of *V. longisporum* in *in vitro* growth assays. However, in this study it was found that the syringyl alcohol lignin and not lignification plays a minor role in the defence of oilseed rape against *V. longisporum* indicated by its low R^2 (up to 21%).

König et al. (2014) described that soluble sinapoyl glucose (a sinapate ester) accumulates in leaves of Arabidopsis at early stages of infection and suggested an important role in of sinapoyl glucose in the resistance reaction to *V. longisporum*.

In the present study sinapate esters (sinapoyl glucose and sinapine) have been detected in the soluble phenolics fraction, but do not show any statistically significant correlation with the resistance reaction in the hypocotyl of oilseed rape. The resistance reaction takes place in internally in the hypocotyl of rapeseed plants (Eynck et al., 2009b), and the reaction in the leave might also be expected to be substantially different from the reaction in the hypocotyl. In addition, in this study the metabolome profile was studied in the late stage of infection at 28 days post inoculation whereas in the study of König et al. (2014) the resistance reaction in Arabidopsis was studied in the early stages of infection at 10 days after inoculation. *A. thaliana* is not a natural host of *V. longisporum*. The results of König et al. (2014) might thus

also indicate once more that the transfer of knowledge from the model plant Arabidopsis to the crop plant *B. napus* can be limited.

Schneider et al. (2003) found that the 4CL4 gene is indeed able to convert sinapic acid to the corresponding CoA ester, sinapoyl CoA which is a precursor of sinapoyl alcohol (see Fig. 22). The 4CL1 and 4CL2 genes are strong candidates for a function in monolignol biosynthesis during developmental lignifications (Raes et al., 2003) and these findings are indicating that the activity of the 4CL gene family regulates the lignin biosynthesis and lignin monomer composition in plants. In another study, Moura et al. (2010) described that CCoAOMT, COMT and CAD genes are more useful target genes for manipulation to reduced and change the lignin composition in plants compared to the PAL, C4H, C3H and 4CL genes. The enzyme F5H from the core phenylpropanoid pathway, is another promising candidate gene for the expression of *V. longisporum* resistance in *B. napus*. F5H is converting ferulic acid to 5-hydroxy ferulic acid , a precursor of syringyl lignin biosynthesis (Fig. 22) and thus it has been speculated based on results from a Arabidopsis mutant study to be an important candidate gene for resistance expression. F5H also produces precursors of sinapate and its esters which have been shown in the same mutant study to play a minor role in *V. longisporum* resistance in Arabidopsis (see above, König, 2011; König et al., 2014). However, in this study it was found that the direct product of F5H, 5-hydroxy ferulic acid, and not the substrate of F5H, ferulic acid, shows a strong correlation with the resistance reaction (R^2 = 42%) (Fig. 22). Thus the conclusion of König et al. (2014) that sinapate esters and coniferyl alcohol (coniferin) accumulation are important factors in resistance expression needs to be modified. It is rather likely that a diverting non-lignin biosynthesis and non-sinapate ester biosynthesis pathway is involved in the interaction. This could for example be the modification of ester-linked 5-hydroxy ferulic acid residues in the cell wall of resistant genotypes by cross-linking with other biomolecules.

In summary, lignin composition has been demonstrated to change with regard to monomer ratios upon *V. longisporum* infection. The reduction of syringyl lignin is associated with susceptible host-pathogen interaction, but plays a minor role in resistance reaction of *V. longisporum* with *B. napus*.

4.9 Simple hydroxycinnamic and hydroxybenzoic acids and aldehydes are strongly associated with *V. longisporum* resistance expression

Some of the *V. longisporum*-induced changes in cell wall-bound hydroxybenzoic acids and aldehydes such as vanillic acid, vanillin and protocatechuic acid showed a strong positive correlation with AUDPC ($R^2 \leq 49\%$) meaning a lower concentration of these compounds in the cell walls from hypocotyls of resistant OSR genotypes upon infection. In addition, the vanillin concentration is also lower in the soluble fraction of resistant genotypes after *V. longisporum* infection (positive correlation with mean AUDPC, $R^2 = 10\%$). Similarly, the concentrations of cell-wall-bound hydroxycinnamic acids such as 5-hydroxy ferulic acid (also see discussion in 4.8), ferulic acid and sinapic acid is lower in resistant genotypes and higher in susceptible genotypes upon *V. longisporum* infection showing a medium to strong positive correlation with mean AUDPC ($R^2 \leq 42\%$).

All these simple hydroxycinnamic and hydroxybenzoic acids and aldehydes exhibiting a strong positive correlation regarding their concentration with mean AUDPC ($R^2 \leq 59\%$) have common methoxy group (O-CH3) in their molecular structure and derived from late phenylpropanoid pathway (see Fig. 20 and Fig. 22).

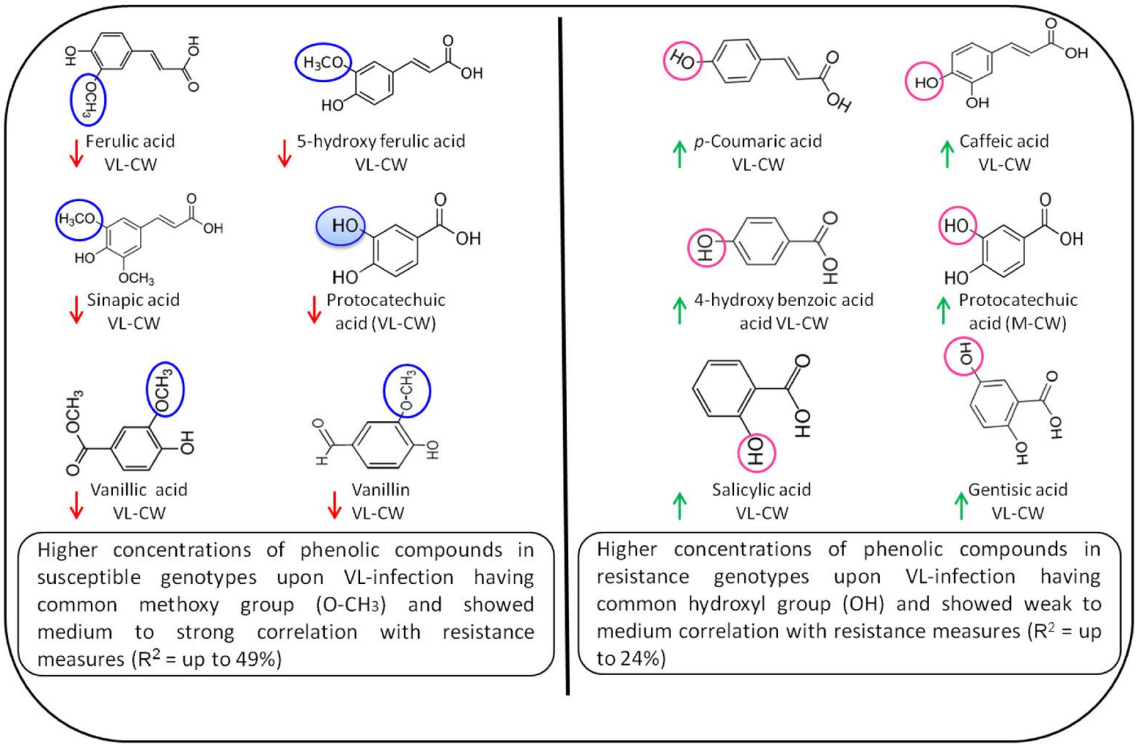

Figure 20: Molecular structures of cell wall-bound hydroxycinnamic and hydroxybenzoic acids and aldehydes associated with *V. longisporum* (VL) resistance in *B. napus*. Green arrow:

higher concentration in resistant lines upon VL-inoculation. Red arrow: Lower concentration in resistant lines compared to suceptibles upon VL-inoculation. M = Mock. Source: Own drawings.

A number of plant pathogenic microorganism including ascomycetous fungi have been reported to produce lignocelullolytic enzymes to degrade cell walls of plants. Fungi can degrade lignin by secreting enzymes classified as either phenol oxidases (laccase) or different classes of peroxidases (Dashtban et al. 2010). Laccase or ligninolytic peroxidases oxidize the lignin polymer, thereby generating aromatic radicals which might also lead to demethoxylation (Martinez et al. 2005). This could explain the pattern of higher concentration of phenolics without methoxy groups in susceptible genotypes (Fig. 20 right side) and might reflect the succesful invasion and cell wall degradation in susceptible genotypes. Thus, these resistance-correlated changes for simple hydroxybenzoic acids and hydroxycinnamic acids might be an indirect effect of the ability of the resistant genotype to withstand or inhibit degradation of the cell wall matrix by the fungus. Common break-down products of phenolics and lignin include vanillin, vanillic acid, and protocatechuic acid all of which have been detected at higher concentration in susceptible compared to resistant genotypes after inoculation (Fig. 20).

Another possible explanation for the oberserved differences in the hydroxycinnamic acid and benzoic acid concentrations extracted from the cell wall of susceptible and resistant genotypes upon inoculation could be due to the nature of bonds in the cell wall. Under the applied extraction conditions in this study, ester bridges in the cell wall are broken, whereas phenolic ether bonds are unaffected (Iiyama et al., 1994). The above results might also are indicate that in resistant genotypes the amount of ether bonds and cross-linking of certain phenolics with other cell wall components increase. On the other hand, phenolic compounds in susceptible genotypes might have more ester bonds of the observed phenolics and less cross linked ether bonds compared to the resistant genotypes. This would suggests a change in bonding types or the addition of different bonding types in the cell wall fraction of the hypocotyls for the susceptible compared to the resistant interaction of rapeseed with *V. longisporum* (Fig. 21).

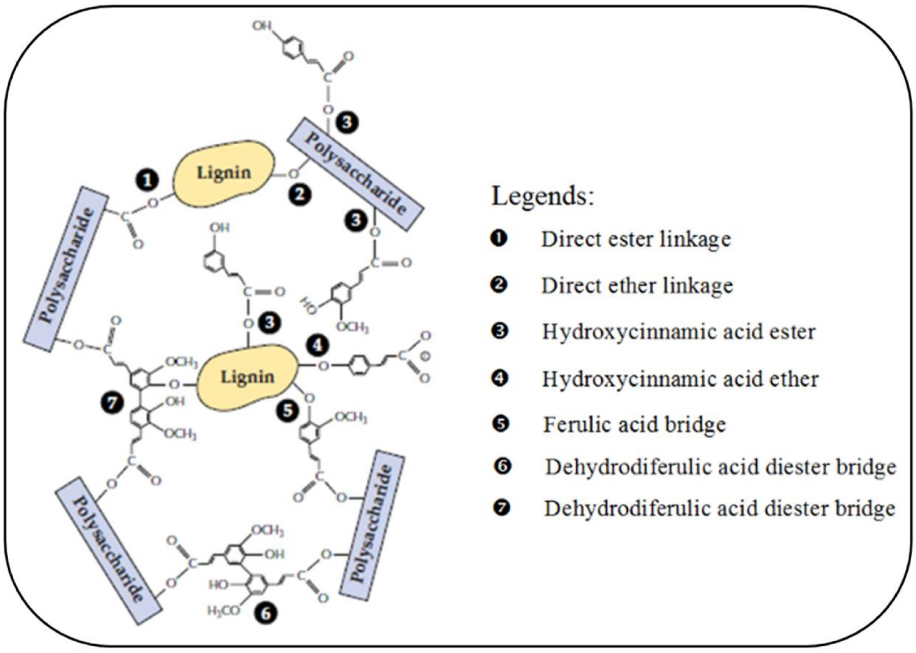

Figure 21: Schematic diagram showing possible covalent cross-links between/among wall polymers (Adapted from Carpita & McCann, 2000). With the applied extraction protocol ester linkage bounds (1,3,6,7) are broken, but not ether linkage bounds (2,4,5) which might result in detection of a different composition of hydroxycinnamic acid composition when binding types are changed differentially in susceptible and resistant genotypes.

This is the first report on the particular importance of simple hydroxybenzoic acids and hydroxycinnamic acids in resistance expression in rapeseed against *V. longisporum* in hypocotyl tissue which was statistically confirmed by analyzing a segregating *B. napus* mapping population (98 genotypes). In previous studies these compounds were only shown to exhibit antifungal effect in *in vitro* cultures (López-Malo et al., 1995; Panizzi et al., 2002; Lattanzio et al., 2006; Vio-Michaelis et al., 2012) or in a limited number of genetically diverse genotypes of different crops in response to infection by other fungi (Nicholson & Hammerschmidt, 1992; Chérif et al., 2007; Mandel & Mitra, 2007, 2008; Caroline et al., 2009; Gayoso et al., 2010; Ahmad et al., 2013). The comparatively high R^2 values indicate that these *V. longisporum*-induced changes in phenolic compound concentrations belonging to the group of simple hydroxycinnamic acids and hydroxyenzoic acids and aldehydes play a more important role in *V. longisporum* resistance/susceptibility expression in *B. napus* than the complex flavonoid and caffeic acid ester derivates discussed above. However, it reamins open whether the higher concentrations of these phenolic compounds in susceptible genotypes were a response or effect of the higher colonization by *V. longisporum* in the tissues that is advantageous to the pathogen, or indicating the up-regulation, but less efficient conversion of

these compounds in susceptible genotypes compared to resistant into more effective defence reaction.

Transgenic tobacco and sugarcane with strong ectopic expression of *Pseudomonas fluorescens* hydroxycinnamoyl-CoA hydratase-lyase (HCHL) enzyme which is involved in the production of vanillin and *p*-hydroxybenzoic acid in plants showed adverse phenotypes such as chlorosis, leaves senescence, stunting, collapsed xylem vessels, low pollen production and decrease of biomass (Mayer et al., 2001; McQualter et al., 2005; Merali et al., 2007). This HCHL enzyme also exists in *Verticillium* species (information obtained by personal communication from Dr. Steve Klosterman, USDA ARS, Salinas, CA 93905, USA) and the above adverse phenotypes caused by HCHL enzyme might also corroborate the findings in this study: susceptible genotypes which are rich in cell wall-bound vanillin and vanillic acid showed leaf chlorosis, premature leaf senescence, distorted xylem system and severe stunting of plant symptoms upon *V. lonsiporum* infection. Phenolic compounds correlated with disease susceptibility might also be related to mycotoxin production and/or increase in hydrolytic enzyme activity. In an earlier study, Wu et al. (2008abc) showed that mycotoxin production (probably predominantly fusaric acid) increased when cinnamic acid, coumaric acid or vanillic acid is applied to *F. oxysporum* f. sp. *niveum* cultures. Wu et al. (2008abc) also observed that the activity of hydrolytic enzymes such as pectinase, cellulase and amylase were increased in presence of cinnamic acid and vanillic acid. The activity of cell wall degrading enzymes such as pectinase, proteinase and cellulase were also stimulated at different concentrations of ferulic acid in liquid culture of *F. oxyporum* f. sp. *niveum* (Wu et al., 2010). Cell wall degrading enzymes (pectinase and cellulase) of plant pathogenic fungi promote the infection process in many plant diseases. These hydrolytic enzymes are responsible for the hydrolytic cleavage of cell wall constitute polymers (pectin, cellulose) and thus facilitate the penetration of the fungus in to the plant (Fuchs et al., 1965).

The above results are indicating that simple hydroxycinnamic and hydroxybenzoic acids and aldehydes which show increased concentrations in the cell wall upon *V. longisporum*-infection in susceptible genotypes are key compounds for the identification of fungal and/or plant enzymes or genes involved in the susceptible oilseed rape-*V. longisporum* interaction. Changes of these phenolic metabolites induced by *V. longisporum*-inoculation might be related to disease promoting function by increasing hydrolytic enzyme activities.

4.10 Chromosomal regions on C1 and C5 are involved in synthesis or modification of phenolics

Over 30 QTL were detected for soluble phenolic compounds in the hypocotyl of the mapping population in the mock- as well as in the *V. longisporum* inoculated data collected in the present study. In only about half of the cases, the QTL were located in the same genomic region, suggesting a strong *V. longisporum*-induced activation of different genomic regions for synthesis or modification of soluble phenolic compounds. There were four major regions (on A9, C1, C6, C8) involved in constitutive soluble phenolic compound concentrations and two major regions (on C1, C5) involved in *V. longisporum*-induced soluble phenolic compound concentrations. One major region on C1 was detected in the mock- as well as in the *V. longisporum*-inoculated data set. In addition, one major genomic region was also detected on chromosome C5 involved in synthesis or modification of *V. longisporum*-induced cell wall-bound phenolics in oilseed rape hypocotyls.

Until now, only a small number of research articles has been published on the genetic mapping of phenolic compounds in rapeseed. However, within the same major region on C5 an overlapping QTL for acid detergent lignin (ADL) concentration in seeds was mapped in the sister DH population Express 617 x 1012-98 (data provided by Dr. Christian Obermeier, Plant Breeding Department, Justus Liebig University, Giessen, Germany). Also, within the minor QTL region on C1 two overlapping QTL for phenolic compounds, sinapic acid and sinapoyl-glucose concentrations in seeds, have been mapped and the gene UDP-glucose:sinapate glucosyltransferase has been identified in the same region (Mittasch et al., 2010). These results are supporting the findings of this study that the chromosomal regions on C1 and C5 are involved in synthesis or modification of phenolics in oilseed rape.

4.11 Co-localized QTL from phenolics might help to identify putative resistance genes involved in *V. longisporum* resistance in oilseed rape

Seven QTL out of 25 QTL were detected in the cell wall-bound phenolics fraction which co-localized with the major and minor QTL for *V. longisporum* resistance on chromosome C5 or C1. Seven of these QTL for phenolic compounds co-localized with the QTL for AUDPC on C1 or C5 also exhibited a medium to strong correlation of their concentrations with mean AUDPC values ($R^2 \leq 59\%$). In addition, from a total of 71 QTL detected for the conentrations of soluble phenolic compounds, 18 co-localized with the major and minor QTL for *V.*

longisporum resistance on C1 and C5. As many as 38 percent of these phenylpropanoid compounds (seven out of 18) also showed significant correlations of their concentrations with AUDPC. This considerable number of co-localized metabolic QTL are indicating that they do not co-localized by chance, but might be associated with *V. longisporum* resistance in oilseed rape. Most of these co-localizing QTL where in addition the concentrations of the respective phenolic compounds are significantly correlated with resistance (R^2 of more than 5%) were detected in the *V. longisporum-inoculated* data set (13 out of 14). The variation in concentration of these 13 phenolic compounds explained up to 17% of the phenotypic variation in mean AUDPC.

Rosmarinic acid, naringin and salicylic acid from *V. longisporum*-induced cell wall-bound phenolics fraction showed co-localized QTL on chromosome C5 amd C1 for *V. longisporum* resistance in oilsed rape. These phenolic compounds were accumulated in resistance genotypes upon *V. longisporum* inection and showed a weak correlation ($R^2 \leq 19\%$) with resistance measures (mean AUDPC and mean growth reduction). This result indicates that these compounds might not be directly involved in resistance reaction, but rather might be precursors or derivates of compounds involved in resistance or might be due to pleitoropic effects of genes involved in the resistance interaction. In addition, the QTL for the constitutive caffeic acid concentration in the soluble phenolics fraction co-localized with the minor resistance QTL on C1. Caffeic acid is a key component of the phenylpropanoid pathway and major lignin precursor (Dixon & Reddy, 2003). Constitutive caffecic acid concentration in the hypocotyl showed a positive correlation with AUDPC ($R^2 = 15\%$), meaning lower constitutive concentration of caffeic acid in the resistant genotypes compared to the susceptible genotypes upon infection. This might suggest that caffeic acid was more effectively incorporated in resistant lines into other phenolic compounds or cellular structures which are part of preformed physical or physiological barriers to infection, e.g. in lignin of cell walls within the vascular system. This is in agreement with biochemical and microscopical investigations of a susceptible and resistant *B. napus* line by Eynck et al. (2009b) who described that at earlier time points of infection preformed soluble and cell wall-bound phenolics appear to limit the extent of infection and colonization by the fungal pathogen, whereas *de novo* formation of lignin and lignin-like polymers become more important at later stages of infection. The QTL for phlorizin (flavanone glycoside) in soluble phenolics fraction also co-localized with the minor resistance QTL on C1 and the plorizin concentration increased in resistant genotypes after infection with *V. longisporum*. The caffeic

acid ester (rosmarinic acid), flavanone glycosides (phlorizin and naringin) and the plant hormone salicylic acid of this study showed a weak correlation with resistance measures and should thus not be considered to represent major factors contributing to resistance expression of *B. napus* to *V. longisporum*. These *V. longisporum*-induced phenolic compounds might be related to cell wall esterification or these phenolic compounds might have direct inhibitory effects on growth and/or germination of *V. longisporum*. In addition, flavonoids especially flavanone glycosides might also trigger the salicylic acid defence pathway.

In summary, the *V. longisporum*-induced regulation of individual soluble and cell wall-bound phenolic compounds in the hypocotyl of resistant genotypes, and their correlation and co-localization with QTL for resistance-related traits, described in this study indicates that genes from the phenylpropanoid and its branching pathways should be considered as promising candidate genes for expression of *V. longisporum* resistance in oilseed rape. Therefore, identification of unknown phenolic metabolite peaks strongly correlating with *V. longisporum* resistance and co-localizing with resistance QTL by mass spectroscopy might allow the identification of further putative genes and the evaluation of the role they play in this host-pathogen interaction.

4.12. Conclusions on metabolome expression patterns associated with *V. longisporum* resistance in oilseed rape

The above results provide evidence that *V. longisporum* infection has a clear influence on the concentrations of phenolic compounds and lignin monomer compositions in OSR hypocotyls. The concentrations of some of the identified phenolic metabolites are positively correlated with resistance, whereas the concentrations of others are negatively correlated with resistance reaction of *B. napus* to *V. longisporum*. The following general patterns of metabolite expression associated with resistance have been observed:

- *V. longisporum* resistance expression in *B. napus* is strongly associated with the cell wall-bound phenolics fraction, but not with the soluble phenolics fraction (cytoplasma/vacuole) or with the lignin fraction (compare coefficients of determination R^2 for metabolites from these fractions in Fig. 22).
- Preformed phenolics play a minor role in the resistance interaction of *B. napus* and *V. longisporum* (see metabolites labeled with circles in Fig. 22)

- *V. longisporum*-induced changes in the phenolics concentrations play a key role in this host-parhogen interaction (see metabolites labeled with arrows in Fig. 22).
- The detected metabolites correlated with resistance are members of very diverse groups of simple and complex phenolics as summarized in Figure 22.
- Esterification of the cell wall caused by key metabolites (*p*-coumaric acid, caffeic acid) of the early phenylpropanoid biosynthesis pathway and their derivates like caffeic acid esters (rosmarinic and chlorogenic acid), phytohormones (salicylic and gentisic acid) and glycosylated flavonoids (phlorizin, naringenin) reveal QTL co-localizing with resistance QTL on C1 and C5, but are minor factors in resistance expression indicated by their low R^2 (below 19%. Fig. 22, upper part).
- Sinapate esters (sinapoyl glucose and sinapoyl choline/sinapine) in OSR hypocotyls were not correlated with the resistance reaction to *V. longisporum* (Fig. 22, lower part).
- Contrary to common assumptions in the literature, it was statisitcally validated that the change in lignin monomer composition of *B. napus* in response to *V. longisporum* infection plays a minor role in resistace reaction (Fig. 22).
- A so far unreported aspect of the resistance interaction is the strong correlation and key role of the concentrations of simple phenolics belonging to the group of hydroxycinamic acids (ferulic acid, 5-hydroxyferulic acid and sinapic acid) and their hydroxybenzoic acid derivates (vanillin, vanillic acid, protocatechiuc acid) with *V. longisporum* resistace (R^2 = up to 59%, Fig. 22, lower part).
- Putatively these simple phenolics might be highly expressed in the susceptible genotypes due to

(1) effective degradation of cell wall components during spread of the fungal pathogen in susceptible genotypes .

(2) a lack of conversion of these metabolites into compounds involved in effective defence reactions.

(3) effective cross-linking of these metabolites with other cell wall components in resistant genotypes only.

- From the metabolite expression pattern putative candidate genes involved in key interactions of the resistance reaction were identified as the 4CL and F5H gene families from the core phenylpropanoid and the CHS and UGT genes form the flavanone glycoside pathways (see Fig. 19 & Fig. 22).

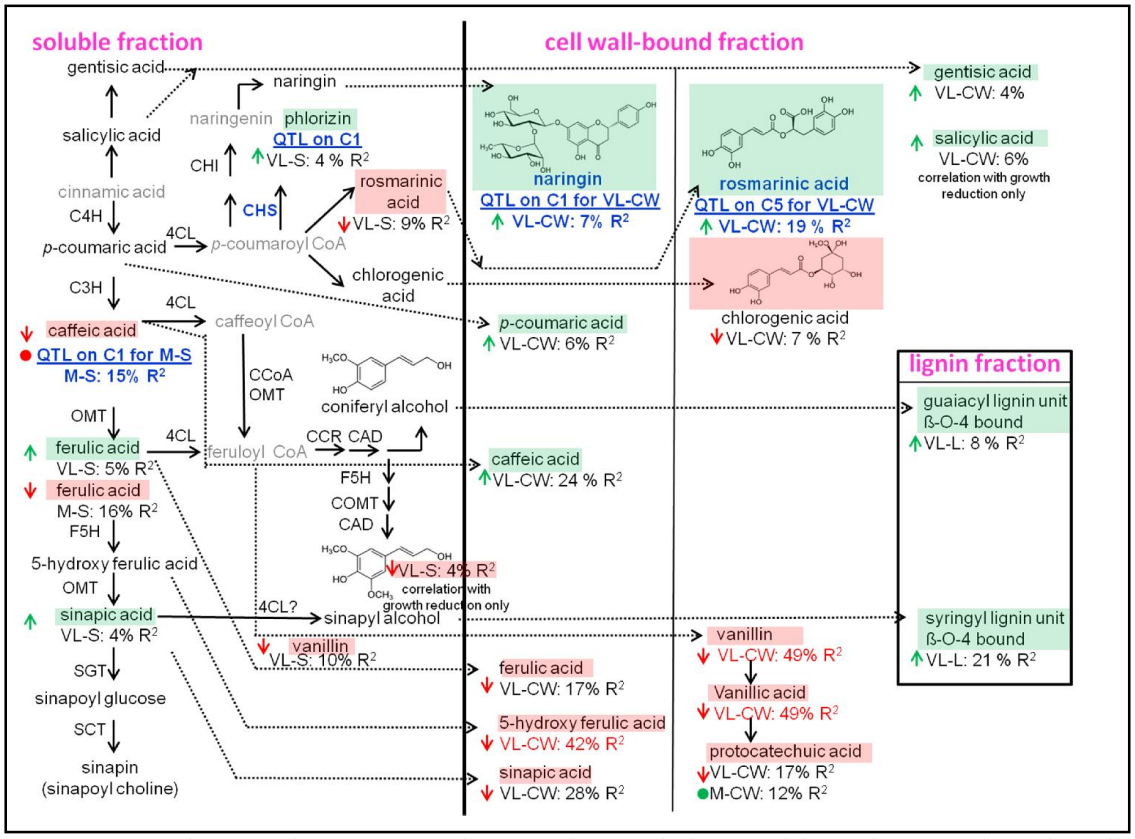

Figure 22: Changes in concentrations and correlations with resistance for phenolic metabolites in oilseed rape hypocotyl in response to *V. longisporum* infection. Phenylpropanoid and related pathways in plants and cellular localizations are shown (adapted from Edues et al. 2012, Vanholme et al. 2010, Gosch et al. 2010, Petersen et al. 2009, Rastogi & Dwivedi 2008). Green arrow: concentration increase in resistant lines / decrease in susceptible lines upon VL-inoculation. Red arrow: concentration increase in susceptible lines / decrease in resistant lines upon VL-inoculation. Red circle: lower concentration in mock-inoculated resistant lines (soluble fraction), Solid arrows : direct conversion of the respective compound. Dashed arrows: putative transportation of the respective compound. Green circle: lower concentration in mock-inoculated resistant lines (cell wall-bound phenolics fraction). Enzyme names are indicated with standard abbreviations: C4H, Cinnamate -4-hydroxylase; 4CL, 4-Coumarate: CoA ligase; CHS, Chalcone synthase; CHI, Chalcone isomerase; C3H, Cumarate-3-hydroxylase; OMT, O-Methyl transferase; F5H, Ferulate 5-hydroxylase; CCR, Cinnamoyl-CoA reductase; CAD, Cinnamoyl alcohol dehydrogenase; SGT, UDP-glucose: sinapate glucosyl transferase; SCT, Sinapoylglucose: choline sinapoyl tranferase; COMT, Catechol-O-Methyltransferase; CCoAOMT, CoA 3-O-methyltransferase . Compound not tested in this study shown in grey. ? = not well accepted.

5 Summary

V. longisporum is an important soilborne pathogen causing Verticillium disease in oilseed rape. The aim of the study was to develop new markers and obtain insights into putative mechanisms and pathways involved in the *V. longisporum* resistance reaction in oilseed rape. A genetic approach was used to identify quantitative trait loci (QTL) for *V. longisporum* resistance and metabolic traits potentially influencing resistance in a *B. napus* mapping population. The composition of soluble phenolics, cell wall phenolics and lignin fractions from hypocotyls of the mapping populations were determined by RP-HPLC and GC/MS. Their patterns of expression, correlation with resistance expression and genetic co-localization with resistance loci were investigated in order to understand their biological relevance.

A major and minor QTL for *V. longisporum* resistance in oilseed rape was identified on chromosomes C1 and C5 of *B. napus*. These loci were also found to be involved in expression of simple and complex phenolics involved in the resistance expression. QTL-linked markers were identified which were validated and shown to be useful for *V. longisporum* resistance breeding in oilseed rape. It was shown based on correlation analyses using 98 DH lines that resistance expression for *V. longisporum* is strongly associated with cell wall-bound simple and complex phenolics, but only to a limited extent with the soluble phenolics fraction (representing the cytoplasm and vacuole). Ranking of identified phenolics and tissue fractions based on statistical evaluation revealed that *V. longisporum*-induced changes of phenolics and not preformed phenolics play a major role in the resistance host-pathogen interaction. Phenolics involved in cell wall esterification (*p*-coumaric acid, caffeic acid) and their derivates such as caffeic acid esters (chlorogenic acid, rosmarinic acid), phytohormones (salicylic acid, gentisic acid) and glycosylated flavonoids (naringin and phlorizin) play a minor role in oilseed rape-*V. longisporum* resistance interaction. Surprisingly and contrary to common assumptions in the literature, statistical evaluation revealed that also the lignin monomer composition only plays a minor role for *V. longisporum* resistance. As shown for the first time, *V. longisporum* resistance expression in oilseed rape is, however, strongly associated with simple phenolics belonging to the group of hydroxycinamic acids (ferulic acid, 5-hydroxyferulic acid and sinapic acid) and their hydroxybenzoic acid derivates (vanillin, vanillic acid, protocatechiuc acid). The relevance of these compounds was further confirmed by co-localization of the metabolite QTL with the major and minor resistance QTL. Based on the pattern of metabolite expression and integration with published pathway data, a number of genes from the core phenylpropanoid and its branching pathways (4CL,

F5H, CHS, UGT) are suggested as candidates to be involved in *V. longisporum* resistance of rapeseed.

6 Zusammenfassung

Verticillium longisporum ist ein bedeutendes bodenbürtiges Pathogen, das die Verticillium-Krankheit in Raps („Rapswelke“) verursacht. Das Ziel dieser Arbeit war die Entwicklung neuer genetischer Marker für Verticillium-Resistenz und die Aufdeckung involvierter Mechanismen und Signalwege, die ggf. zu einer Resistenzreaktion von Raps gegen *V. longisporum* führen. Zunächst wurde ein genetischer Ansatz verfolgt, um *quantitative trait loci* (QTL) und mögliche metabolische Eigenschaften der *B. napus* Resistenz gegen *V. longisporum* in einer Kartierungspopulation zu identifizieren. Im weiteren wurde die Zusammensetzung löslicher Phenole, Zellwand-gebundener Phenole und der Lignin-Fraktion aus Hypokotylen wurde mittels RP-HPLC und GC/MS untersucht. Deren Expressionsmuster, Korrelation mit der Resistenz-Ausprägung und genetische Kolokalisationen mit Resistenzloci wurden analysiert.

Auf den Chromosomen C1 und C5 wurden ein Major- bzw. ein Minor-QTL für *V. longisporum*-Resistenz identifiziert. Des Weiteren konnte ein Zusammenhang dieser Loci mit der Expression einfacher und komplexer Phenole gezeigt werden. QTL-gekoppelte Marker wurden identifiziert, validiert und als nützlich für eine marker- bzw. metabolom-gestützte Resistenzzüchtung gegen *V. longisporum* bei Raps eingestuft. Korrelationsanalysen anhand von 98 DH-Linien zeigten, dass die Resistenzausprägung stark mit der Akkumulation zellwand-gebundener Phenole assoziiert ist, aber nur im geringen Ausmaß mit in der Vakuole und im Zytoplasma befindlichen löslichen Phenolen assoziiert ist. Statistische Auswertungen zeigten, dass eine Veränderung *V. longisporum*-induzierter Phenole und nicht der präformierten Phenole eine wichtige Rolle in der Wirt-Pathogen-Interaktion spielen. Phenole (*p*-Cumarsäure, Kaffeesäure), die für die Zellwand-synthese notwendig sind, Kaffesäure-Derivate (Chlorogensäure, Rosmarinsäure), Pflanzenhormone (Salizylsäure, Gentisinsäure) und glykosilierte Flavonoide (Naringin, Phlorizin) hingegen spielen zwar eine signifikante, aber doch untergeordnete Rolle für die Resistenzausprägung in Raps. Darüber hinaus konnte in dieser Studie nachgewiesen werden, dass entgegen anderen Angaben in der Fachliteratur, die Ligninmonomer-Zusammensetzung nur eine marginale Rolle bei der Resistenz gegen *V. longisporum* spielt. Erstmalig wird hier gezeigt, dass eine starke Assoziation zwischen Resistenzausprägung und einfachen Phenolen aus der Gruppe der Hydroxyzimtsäuren (Ferulasäure, 5-Hydroxyferulasäure und Sinapinsäure) und Hydroxybenzoesäure-Derivaten (Vanillin, Vanillinsäure, Protocatechusäure a) gegeben ist. Die Wichtigkeit dieser Verbindungen konnte durch Kolokalisation der Metaboliten-QTL mit dem Major- und Minor-

Resistenz-QTL bestätigt werden. Basierend auf Metabolitenexpressionsmustern und Integration von publizierten Stoffwechselwegsdaten konnten Kandidatengene des zentralen Phenylpropanoid- und abzweigender Stoffwechselwege (4CL, F5H, CHS, UGT) als relevant für die *B. napus – V. longisporum*-Interaktion identifiziert werden.

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8 Appendix

Supplementary table S1: Standard phenolic compounds used in HPLC analysis for the identification of HPLC peaks in phenolic extracts from soluble (S) and cell wall-bound (CW) fractions and from mock- (M) and *V. longisporum*-(VL) inoculation treatments of DH lines (n = 98)

Phenolic compounds	Group	Soluble phenolics (mock)	Soluble phenolics (VL)	Cell wall phenolic (Mock)	Cell wall phenolics (VL)	Source
Gallic acid	hydroxybenzoic acid	-	-	-	-	Extrasynthese
Protocatechuic acid	hydroxybenzoic acid	-	-	+	+	Sigma-Aldrich
4-hydroxybenzoic acid	hydroxybenzoic acid	-	-	+	+	Sigma-Aldrich
Gentisic acid	hydroxybenzoic acid	-	-	+	+	Sigma-Aldrich
Salicylic acid	hydroxybenzoic acid	-	-	+	+	Roth
Vanillic acid	hydroxybenzoic acid	-	-	+	+	Sigma-Aldrich
Shikimic acid	hydroxybenzoic acid	nd	nd	nd	nd	TransMIT
Vanillin	vanillic aldehyde/ phenylpropanoid aldehyde	+	+	+	+	Sigma-Aldrich
Coniferyl aldehyde	phenylpropanoid aldehyde	+	+	-	-	Sigma-Aldrich
p-Coumaric acid	hydroxycinnamic acid	+	+	+	+	Sigma-Aldrich
Ferulic acid	hydroxycinnamic acid	+	+	+	+	Sigma-Aldrich
Sinapic acid	hydroxycinnamic acid	+	+	+	+	Sigma-Aldrich
5-Hydroxyferulic acid	hydroxycinnamic acid	-	-	+	+	TransMIT
1,3-Dicaffeoylquinic acid	hydroxycinnamic acid	-	-	-	-	TransMIT
Dihydrocaffeic acid	hydroxycinnamic acid	-	-	-	-	TransMIT
Caffeic acid	hydroxycinnamic acid	+	+	+	+	Sigma-Aldrich
trans-Cinnamic acid	cinnamic acid	nd	nd	nd	nd	Extrasynthese
Chlorogenic acid	hydroxycinnamic acid /Caffeic acid ester	+	+	-	+	Sigma-Aldrich
Rosmarinic acid	phenolic acid/	+	+	+	+	TransMIT

	caffeic acid ester					
Flavanone	flavanone	nd	nd	nd	nd	Extrasynthese
Naringin	flavanone/ flavanone glycoside	+	+	+	+	TransMIT
Naringenin	flavanone	nd	nd	nd	nd	TransMIT
Phlorizin/ Phloridizin	flavanone glycoside/ dihydrochalcone	+	+	-	-	TransMIT
Phloretin	dihydrochalcone	nd	nd	nd	nd	TransMIT
Coniferyl alcohol	monolignol	**?**	**?**	-	-	Sigma-Aldrich
Sinapyl alcohol	monolignol	+	+	-	-	Sigma-Aldrich
Sinapoyl glucose	sinapate ester	+	+	-	-	From oilseed rape sinapoyl glucose
Sinapine thiocyanate	sinapate ester	+	+	-	-	Analyticon discovery GmbH
(+) Catechin	flavanol	-	+	-	-	TransMIT
(-) Epicatechin	flavanol	-	-	-	-	TransMIT
(-) Epigallocatechin gallate	flavanol	-	-	**?**	**?**	TransMIT
Isoquercitrin	flavonol	-	-	-	-	TransMIT
Isorhamnetin	flavonol	nd	nd	nd	nd	TransMIT
Kaempferol-3-*O*-glucoside	flavonol	nd	nd	nd	nd	TransMIT
Kaempferol-7-*O*-glucoside	flavonol	-	-	-	-	Extrasynthese
Kaempferol	flavonol	nd	nd	nd	nd	TransMIT
Kaempferol-3-O-rhamnoside	flavonol	-	-	-	-	TransMIT
Quercetin	flavonol	nd	nd	nd	nd	TransMIT
Quercetin 3-O-galactoside (Hyperoside)	flavonol	-	-	-	-	TransMIT
Quercetin 7-methylether (Rhamnetin)	flavonol	nd	nd	nd	nd	TransMIT
Quercetin 3,7,3‘,4‘-tetramethylether	flavonol	nd	nd	nd	nd	TransMIT
7-hydroxyflavonol	flavonol	nd	nd	nd	nd	Extrasynthese
Rutin	flavonol	-	-	-	-	TransMIT
Tangeretin	flavonol	nd	nd	nd	nd	TransMIT
Cirsimaritin	flavone	nd	nd	nd	nd	TransMIT
Nobiletin	flavone	nd	nd	nd	nd	TransMIT
Cyanidin chloride	anthocynidin	-	-	+	+	Extrasynthese
Procyanidin B1	proanthocyanidin	+	+	-	-	Extrasynthese

Procyanidin B2	proanthocyanidin	-	-	-	-	Extrasynthese

Here: + = present, - = absent, nd = not detected and ? = not confirm. In total, 49 standards used in this experiment belong to hydroxybenzoic acids (7), hydroxycinnamic acids (7), phenylpropanoid esters (2), cinnamic acid (1), flavanone (3) dihydrochalcone/flavanone glycoside (2), phenypropanoid alcohol/monolignol (2), sinapate esters (2), phenylpropanoid aldehyde (2), flavanol (3) flavonol (13) flavone (2), anthocyanidin (1) and proanthocyanidin (2).

Supplementary table S2: Exclusive HPLC peaks present in soluble and cell wall-bound phenolics fractions in the hypocotyls of the *V. longisporum*- and mock-inoculated mapping population Express 617 x R53-DH

Retention time (RT) in HPLC	Identified phenolics by external standard	Treat-ment	Frac-tion	Correlation with mean AUDPC-R(R^2)	Correlation with mean growth reduction-R(R^2)	Regulation in resistance genotype	Co-localization with resistance QTL
RT: 9.20	catechin	VL	SP	n.s.	0.23* (0.05)	down	No
RT:15.82	unknown	VL	SP	n.s.	n.s.	-	No
RT:20.30	unknown	VL	SP	n.s.	n.s.	-	No
RT:21.0	unknown	VL	SP	0.61** (0.37)	0.54** (0.29)	down	yes, C5
RT:21.85	unknown	VL	SP	n.s.	0.23** (0.05)	down	no
RT:22.74	unknown	VL	SP	n.s.	n.s.	-	no
RT:23.32	unknown	VL	SP	n.s.	n.s.	down	no
RT:24.70	unknown	Mock	SP	n.s.	n.s.	-	No
RT:10.10	chloro-genic acid	VL	CW	0.27** (0.07)	0.21* (0.04)	down	No
RT:13.70	unknown	VL	CW	0.48** (0.23)	0.50** (0.25)	down	yes, C5
RT:21.10	unknown	VL	CW	0.71** (0.50)	0.66** (0.43)	down	yes, C5
RT:22.05	unknown	VL	CW	0.32** (0.10)	0.38** (0.14)	down	No
RT: 7.25	unknown	Mock	CW	0.36** (0.13)	0.28** (0.08)	down	No
RT:11.05	unknown	Mock	CW	n.s.	n.s.	-	No
RT:21.80	unknown	Mock	CW	0.27** (0.07)	n.s	down	No
RT:24.05	unknown	Mock	CW	n.s.	n.s.	-	No

Phenolic compounds present only in the mock-inoculated data set or present only in the *V. longisporum*-inoculated data set for the soluble as well as for the cell wall-bound phenolics fractions, where CW = cell wall-bound phenolic compound , SP = soluble phenolic compound, R = Pearson correlation, * = significant at 0.05, ** = significant at 0.01, n.s. = not significant, n = 98 DH lines and VL = *Verticillium longisporum.*

Supplementary table S3: Correlation between mean area under the disease progress curve (AUDPC) and concentrations of phenolic compounds estimated by HPLC in the hypocotyls of the *V. longisporum* (VL)- and mock (M)-inoculated mapping population Express 617 x R53-DH

Retention time (RT) in HPLC	Identified phenolics by external standard	Group of phenolic compounds	Treatment	Fraction	Correlation with mean AUDPC-R	Regulation in resistance genotypes	Co-localization with resistance QTL
RT:15.30	unknown	-	VL	CW	0.77**	down	yes, C5
RT:21.10	unknown	-	VL	CW	0.71**	down	yes, C5
RT:15.75	vanillin	hydroxybenzoic acid	VL	CW	0.70**	down	No
RT:11.82	vanillic acid	hydroxybenzoic acid	VL	CW	0.70**	down	no
RT:13.00	5-hydroxy - ferulic acid	hydroxycinnamic acid	VL	CW	0.65**	down	no
RT:17.82	unknown	-	VL	CW	0.65**	down	no
RT:7.62	unknown	-	VL	CW	0.61**	down	no
RT:21.0	unknown	-	VL	SP	0.61**	down	yes, C5
RT:17.45	unknown	-	VL	CW	0.55**	down	no
RT:18.75	sinapic acid	hydroxycinnamic acid	VL	CW	0.53**	down	no
RT:6.28	protocate-chuic acid	hydroxybenzoic acid	VL	CW	0.52**	down	no
RT:11.45	caffeic acid	hydroxycinnamic acid	VL	CW	-0.49**	up	no
RT: 3.00	unknown	-	VL	CW	0.49**	down	no
RT:13.70	unknown	-	VL	CW	0.48**	down	yes, C5
RT:14.20	unknown	-	VL	CW	-0.47**	up	yes, C5
RT:23.10	rosmarinic acid	caffeic acid ester	VL	CW	-0.44**	up	yes, C5
RT:20.62	unknown	-	VL	CW	-0.43**	up	no
RT:8.82	unknown	-	VL	CW	-0.42**	up	no
RT:18.20	ferulic acid	hydroxycinnamic acid	VL	CW	0.41**	down	no
RT:2.50	unknown	-	VL	SP	0.41**	down	no
RT:18.11	ferulic acid	hydroxycinnamic acid	M	SP	0.40**	down	no
RT:11.30	caffeic acid	hydroxycinnamic acid	M	SP	0.39**	down	yes, C1
RT:19.80	unknown	-	VL	CW	-0.38**	up	no
RT:10.75	unknown	-	VL	CW	0.36**	down	no
RT:7.25	unknown	-	M	CW	0.36**	down	no
RT:6.32	protocate-chuic acid	hydroxybenzoic acid	M	CW	-0.34**	up	no
RT:9.70	unknown	-	VL	SP	-0.34**	up	yes, C1
RT:11.97	unknown	-	M	SP	-0.33**	up	no
RT:18.50	unknown	-	VL	SP	0.33**	down	yes, C5
RT:23.70	unknown	-	VL	SP	0.33**	down	yes, C5
RT:22.05	unknown	-	VL	CW	0.32**	down	no

RT:15.50	vanillin	hydroxybenzoic acid	VL	SP	0.32**	down	no
RT:5.75	unknown	-	VL	SP	-0.30**	up	no
RT:23.11	rosmarinic acid	caffeic acid ester	VL	SP	0.30**	down	no
RT:2.70	unknown	-	M	SP	0.30**	down	no
RT:23.80	unknown	-	VL	CW	0.29**	down	no
RT:21.50	naringin	flavanone glycoside	VL	CW	-0.27**	up	yes, C1
RT:10.07	chlorogenic acid	caffeic acid ester	VL	CW	0.27**	down	no
RT:21.80	unknown	-	M	CW	0.27**	down	no
RT:3.50	unknown	-	VL	SP	0.27**	down	no
RT:24.50	phlorizin	flavanone glycoside	VL	SP	-0.26**	up	yes, C1
RT:7.28	unknown	-	VL	SP	0.26**	down	no
RT:20.10	unknown	-	VL	SP	0.26**	down	yes, C5
RT:20.82	unknown	-	VL	SP	-0.25*	up	no
RT:23.40	unknown	-	VL	CW	0.25*	down	no
RT:7.28	unknown	-	M	SP	0.25*	down	no
RT:16.05	*p*-coumaric acid	hydroxycinnamic acid	VL	CW	-0.24*	up	no
RT:19.80	unknown	-	VL	SP	-0.24*	up	no
RT:16.52	unknown	-	VL	CW	-0.22*	up	no
RT:18.11	ferulic acid	hydroxycinnamic acid	VL	SP	-0.22*	up	no
RT:4.95	unknown	-	VL	SP	-0.21*	up	no
RT:18.85	sinapic acid	hydroxycinnamic acid	VL	SP	-0.21*	up	no
RT:19.20	unknown	-	VL	SP	-0.21*	up	no
RT:10.40	gentisic acid	hydroxybenzoic acid	VL	CW	-0.20**	up	no
RT:12.30	unknown	-	VL	CW	-0.20*	up	no
RT:9.50	4-hydroxy - benzoic acid	hydroxybenzoic acid	VL	CW	n.s.	-	no
RT:22.50	salicylic acid	hydroxybenzoic acid	VL	CW	n.s.	-	yes, C1
RT:9.20	catechin	flavanol	VL	SP	n.s.	-	no
RT:14.72	unknown	-	VL	SP	n.s.	-	no
RT:16.60	sinapyl alcohol	monolignol	VL	SP	n.s.	-	no
RT:21.85	unknown	-	VL	SP	n.s.	-	no
RT: 3.50	unknown	-	M	SP	n.s.	-	no

Here, CW = cell wall-bound phenolic compound , SP = soluble phenolic compound, R = Pearson correlation, * = significant at 0.05, ** = significant at 0.01, n.s. = not significant, n = 98 DH lines, M = Mock and VL = *Verticillium longisporum*. 8 preformed phenolics, 42 *V. longisporum*-induced changes of phenolics and 7 exclusively expressed phenolics are correlated with mean AUDPC. Compounds not correlated with mean AUDPC but correlated with mean growth reduction are labelled in grey.

Supplementary table S4: Correlation between mean growth reduction and concentrations of phenolic compounds estimated by HPLC in the hypocotyls of the *V. longisporum* (VL)- and mock (M)-inoculated mapping population Express 617 x R53-DH

Retention time (RT) in HPLC	Identified phenolics by external standard	Group of phenolic compounds	Treat-ment	Frac-tion	Correlation with growth reduction-R(R^2)	Regulat-ion in resistan-ce genotype-s	Co-localizat-ion with resistanc-e QTL
RT:15.30	unknown	-	VL	CW	0.71** (0.50)	down	yes, C5
RT:21.10	unknown	-	VL	CW	0.66** (0.43)	down	yes, C5
RT:15.75	vanillin	hydroxybenzoic acid	VL	CW	0.63** (0.40)	down	no
RT:11.82	vanillic acid	hydroxybenzoic acid	VL	CW	0.62** (0.38)	down	no
RT:13.00	5-hydroxy-ferulic acid	hydroxycinnamic acid	VL	CW	0.58** (0.34)	down	no
RT:17.82	unknown	-	VL	CW	0.56** (0.31)	down	no
RT:14.20	unknown	-	VL	CW	-0.54** (0.29)	up	yes, C5
RT:7.62	unknown	-	VL	CW	0.54** (0.29)	down	no
RT:21.0	unknown	-	VL	SP	0.54** (0.29)	down	yes, C5
RT:13.70	unknown	-	VL	CW	0.50** (0.25)	down	yes, C5
RT:18.20	ferulic acid	hydroxycinnamic acid	VL	CW	0.47** (0.22)	down	no
RT:20.62	unknown	-	VL	CW	-0.46** (0.21)	up	no
RT:23.10	rosmarini-c acid	caffeic acid ester	VL	CW	-0.46** (0.21)	up	yes, C5
RT:11.45	caffeic acid	hydroxycinnamic acid	VL	CW	-0.44** (0.19)	up	no
RT:18.75	sinapic acid	hydroxycinnamic acid	VL	CW	0.44** (0.19)	down	no
RT:8.82	unknown	-	VL	CW	-0.43** (0.18)	up	no
RT:17.45	unknown	-	VL	CW	0.42** (0.18)	down	no
RT:2.50	unknown	-	VL	SP	0.42** (0.18)	down	no
RT:23.70	unknown	-	VL	SP	0.42** (0.18)	down	yes C5
RT:9.70	unknown	-	VL	SP	-0.40** (0.16)	up	yes, C1
RT:6.28	protocate-chuic acid	hydroxybenzoic acid	VL	CW	0.40** (0.16)	down	no
RT:11.30	caffeic acid	hydroxycinnamic acid	M	SP	0.40** (0.16)	down	yes, C1
RT:22.05	unknown	-	VL	CW	0.38** (0.14)	down	no
RT:18.50	unknown	-	VL	SP	0.37** (0.14)	down	yes , C5
RT:19.80	unknown	-	VL	CW	-0.36** (0.13)	up	no
RT:6.32	protocate-chuic acid	hydroxybenzoic acid	M	CW	-0.35** (0.12)	up	no
RT:3.00	unknown	-	VL	CW	0.35** (0.12)	down	no
RT:23.80	unknown	-	VL	CW	0.33** (0.11)	down	no

RT:16.52	unknown	-	VL	CW	-0.32** (0.10)	up	no
RT:5.75	unknown	-	VL	SP	-0.32** (0.10)	up	no
RT:10.75	unknown	-	VL	CW	0.32** (0.10)	down	no
RT:20.10	unknown	-	VL	SP	0.32** (0.10)	down	yes, C5
RT:11.97	unknown	-	M	SP	-0.31** (0.10)	up	no
RT:15.50	vanillin	hydroxybenzoic acid	VL	SP	0.30** (0.09)	down	no
RT:2.70	unknown	-	M	SP	0.30** (0.09)	down	no
RT:4.95	unknown	-	VL	SP	-0.29** (0.08)	up	no
RT:24.50	phlorizin	flavanone glycoside	VL	SP	-0.29** (0.08)	up	yes, C1
RT:3.50	unknown	-	VL	SP	0.29** (0.08)	down	no
RT:21.47	naringin	flavanone glycoside	VL	CW	-0.28** (0.08)	up	yes, C1
RT:7.25	unknown	-	M	CW	0.28** (0.08)	down	no
RT:10.42	gentisic acid	hydroxybenzoic acid	VL	CW	-0.26** (0.07)	up	no
RT:18.11	ferulic acid	hydroxycinnamic acid	M	SP	0.26* (0.07)	down	no
RT:16.05	*p*-coumaric acid	hydroxycinnamic acid	VL	CW	-0.25* (0.06)	up	no
RT 22.50	salicylic acid	hydroxybenzoic acid	VL	CW	-0.25* (0.06)	up	yes, C1
RT:18.11	ferulic acid	hydroxycinnamic acid	VL	SP	-0.25* (0.06)	up	no
RT:7.28	unknown	-	VL	SP	0.25* (0.06)	down	no
RT:19.80	unknown	-	VL	SP	-0.24* (0.06)	up	no
RT:20.82	unknown	-	VL	SP	-0.24* (0.06)	up	no
RT:9.50	4-hydroxy-benzoic acid	hydroxybenzoic acid	VL	CW	-0.23* (0.05)	up	no
RT: 9.20	catechin	flavanol	VL	SP	0.23* (0.05)	down	no
RT:21.85	unknown	-	VL	SP	0.23* (0.05)	down	no
RT:18.85	sinapic acid	hydroxycinnamic acid	VL	SP	-0.22* (0.05)	up	no
RT:23.11	rosmarini-c acid	caffeic acid ester	VL	SP	0.22* (0.05)	down	no
RT:19.20	unknown	-	VL	SP	-0.21* (0.04)	up	no
RT:3.50	unknown	-	M	SP	-0.21* (0.04)	up	no
RT:10.07	chloroge-nic acid	caffeic acid ester	VL	CW	0.21* (0.04)	down	no
RT:16.60	sinapyl alcohol	monolignol	VL	SP	0.21* (0.04)	down	no
RT:14.72	unknown	-	VL	SP	0.20* (0.04)	down	no
RT:12.30	unknown	-	VL	CW	n.s.	-	no
RT:23.40	unknown	-	VL	CW	n.s.	-	no
RT:21.80	unknown	-	M	CW	n.s.	-	no
RT:7.28	unknown	-	M	SP	n.s.	-	no

Here, CW = cell wall-bound phenolic compound, SP = soluble phenolic compound, R = Pearson correlation, * =significant at 0.05, ** = significant at 0.01, n.s. = not significant, n = 98 DH lines, M = Mock and VL = *Verticillium longisporum*. 7 preformed phenolics, 44 *V. longisporum*-induced changes of phenolic and 7 exclusively expressed phenolics are correlated with growth reduction. Compounds not correlated with mean growth reduction but correlated with mean AUDPC are labeled in grey.

Supplementary table S5: QTL for soluble phenolics in the hypocotyl of a mock-inoculated mapping population Express 617 x R53-DH

Retentio-n time (RT) in HPLC	HPLC peak present in	*B. napus* chrom-osome	QTL peak position (cM)	Confidence interval (cM)	Phenotypic variation, R^2	Co-localizati-on with resistanc-e QTL	Correla-tion with mean AUDPC-R(R^2)
RT:19,5	both treatments	A1	78	70-92	0.157	no	n.s.
RT:16.2 (*p*-coumari-c acid)	both treatments	A2	4	0-20	0.14	no	n.s.
RT: 13.6	both treatments	A3	64	50-76	0.115	no	n.s.
RT:16.8	both treatments	A3	96	76-106	0.133	no	n.s.
RT:21.6	both treatments	A3	106	84-112	0.179	no	n.s.
RT:19.8	both treatments	A5	36	18-54	0.144	no	n.s.
RT:19.5	both treatments	A5	74	46-92	0.14	no	n.s.
RT:7.28	both treatments	A6	48	38-62	0.211	no	0.25* (0.06)
RT:22.1	both treatments	A9	24	14-36	0.14	no	n.s.
RT:23.9	both treatments	A9	24	14-36	0.168	no	n.s.
RT:17.2	both treatments	A9	28	0-84	0.115	no	n.s.
RT:21.6	both treatments	A10	114	96-120	0.123	no	n.s.
RT:11.3 (caffeic acid)	both treatments	C1	4	0-20	0.124	**yes, C1**	0.39** (0.15)
RT:18.8	both treatments	C1	18	0-30	0.126	no	n.s.
RT:23.6	both treatments	C1	18	0-28	0.214	no	n.s.
RT:23.9	both treatments	C1	26	2-42	0.138	no	n.s.
RT:14.5	both treatments	C3	118	84-142	0.132	no	n.s.
RT:16.8	both treatments	C3	124	110-134	0.20	no	n.s.
RT:22.1	both treatments	C3	148	124-174	0.116	no	n.s.
RT:11.0	both treatments	C4	32	8-40	0.147	no	n.s.
RT:12.0	both treatments	C5	74	32-94	0.119	no	n.s.
RT:14.7	both treatments	C6	22	14-32	0.38	no	n.s.

RT:15.0	both treatments	C6	32	18-50	0.152	no	n.s.
RT:21.3	both treatments	C6	38	24-48	0.385	no	n.s.
RT:20.8	both treatments	C6	48	34-62	0.233	no	n.s.
RT:22.1	both treatments	C6	70	54-84	0.23	no	n.s.
RT:18.1 (ferulic acid)	both treatments	C6	84	28-92	0.121	no	0.40** (0.16)
RT:10.3	both treatments	C7	18	6-24	0.28	no	n.s.
RT:11.0	both treatments	C7	52	40-62	0.199	no	n.s.
RT:18.1 (ferulic acid)	both treatments	C7	94	76-110	0.138	no	0.40** (0.16)
RT:12.6	both treatments	C8	26	12-42	0.117	no	n.s.
RT:16.2 (*p*-coumaric acid)	both treatments	C8	26	20-46	0.128	no	n.s.
RT:8.7	both treatments	C8	38	28-50	0.245	no	n.s.
RT:12.6	both treatments	C9	44	22-58	0.192	no	n.s.

QTL with a LOD score ≥ 2.5 which explain at least 5 % of the phenotypic variation. Peaks putatively identified by co-migration of standard. Phenotypic variation of HPLC peak area explained by the QTL, R^2 (QGene, $R^2 \geq 5\%$) . Here, n = 98 DH lines, R = Pearson correlation, n.s. = not significant * = significant at 0.05,** = significant at 0.01 and both treatments = mock and *V. longisporum* treatment.

Summary of the table

Total number of HPLC peaks = 43

Total number of QTL = 34

Maximum number of QTL for one HPLC Peak = 3

Number of QTL for HPLC peak concentrations co-localizing with QTL on C1 and C5 for AUDPC = 1

Major regions with overlapping QTL involved in phenylpropanoid content

Supplementary table S6: QTL for soluble phenolics in the hypocotyl of a *V. longisporum*-inoculated mapping population Express 617 x R53-DH

Retenti-on time in HPLC	HPLC peak present in	*B. napus* chromo-some	QTL peak position (cM)	Confidence interval (cM)	Phenotypic variation, R^2	Co-localizati-on with resistanc-e QTL	Correlati-on with AUDPC-R(R^2)
RT:18.5	both treatments	A2	122	104-142	0.118	no	0.33** (0.11)
RT:9.2 (catechi-n)	only VL-inoculated	A2	160	150-160	0.126	no	n.s.
RT:16.6 (sinapo-yl alcohol)	both treatments	A3	93	70-108	0.136	no	n.s.
RT:13.5	both treatments	A5	38	20-52	0.15	no	n.s.
RT:19.6	both treatments	A5	56	44-76	0.212	no	n.s.
RT:7.3	both treatments	A6	16	4-36	0.273	no	0.26** (0.07)
RT:9.2 (catechi-n)	only VL-inoculated	A6	50	38-62	0.14	no	n.s.
RT:19.6	both treatments	A9	74	38-104	0.131	no	n.s.
RT:20.8	both treatments	A9	64	44-94	0.137	no	-0.25* (0.06)
RT:21.4	both treatments	C1	0	0-4	0.137	no	n.s.
RT:24.5 (phloriz-in)	both treatments	C1	0	0-28	0.14	**yes, C1**	-0.26** (0.07)
RT:9.7	both treatments	C1	24	0-50	0.144	**yes, C1**	-0.34** (0.12)
RT:23.9	both treatments	C1	44	14-54	0.134	no	n.s.
RT:11.0	both treatments	C2	2	0-10	0.111	no	n.s.
RT:17.7	both treatments	C2	6	0-18	0.112	no	n.s.
RT:24.5 (phloriz-in)	both treatments	C2	20	12-32	0.13	no	-0.26** (0.07)
RT:21.0	only VL-inoculated	C2	32	14-32	0.139	no	0.61** (0.37)
RT:14.5	both treatments	C3	32	20-44	0.118	no	n.s.
RT:13.5	both treatments	C4	30	14-38	0.113	no	n.s.
RT:15.8	only VL-inoculated	C4	128	114-136	0.112	no	n.s.
RT:18.5	both	C5	34	22-48	0.143	**yes, C5**	0.33**

	treatments						(0.11)
RT:20.1	both treatments	C5	34	22-70	0.134	**yes, C5**	0.26** (0.07)
RT:23.7	both treatments	C5	42	32-62	0.167	**yes, C5**	0.33** (0.11)
RT:21.0	only VL-inoculated	C5	60	48-74	0.14	**yes, C5**	0.61** (0.37)
RT:19.2	both treatments	C5	72	62-80	0.111	no	-0.21* (0.04)
RT:19.6	both treatments	C5	106	96-118	0.125	no	n.s.
RT:14.7	both treatments	C6	32	16-44	0.13	no	n.s.
RT:21.4	both treatments	C6	36	18-52	0.178	no	n.s.
RT:17.9	both treatments	C6	78	66-82	0.119	no	n.s.
RT:9.2 (catechi -n)	only VL-inoculated	C6	88	72-104	0.158	no	n.s.
RT:10.3	both treatments	C7	20	6-24	0.235	no	n.s.
RT:17.9	both treatments	C7	42	28-54	0.137	no	n.s.
RT:11.0	both treatments	C7	48	16-66	0.163	no	n.s.
RT:16.2 (*p*-coumari -c acid)	both treatments	C7	94	74-110	0.136	no	n.s.
RT:19.2	both treatments	C8	84	68-94	0.12	no	-0.21* (0.04)
RT:12.6	both treatments	C9	60	54-68	0.191	no	n.s.
RT:11.0	both treatments	C9	102	94-108	0.149	no	n.s.

QTL with a LOD score ≥ 2.5 which explain at least 5 % of the phenotypic variation. Peaks putatively identified by co-migration of standard. Phenotypic variation of HPLC peak area explained by the QTL, R^2 (QGene, $R^2 \geq 5\%$). VL = *Verticillium longisporum*, n = 98 DH lines, R = Pearson correlation, n.s. = not significant * = significant at 0.05, ** = significant at 0.01 and both treatments = mock and *V. longisporum* treatment.

Summary of the table

Total number of HPLC peaks = 49

Total number of QTL = 37

Maximum number of QTL for one HPLC Peak = 3

Number of QTL for HPLC peak concentrations co-localizing with QTL on C1 and C5 for AUDPC = 6

Major regions with overlapping QTL involved in phenylpropanoid content

Supplementary table S7: QTL for cell wall-bound phenolics in the hypocotyl of a mock- and *V. longisporum*-inoculated mapping population Express 617 x R53-DH

Retention time of HPLC peak	HPLC peak present in	*B. napus* chrom-osome	QTL peak position (cM)	Confidence interval (cM)	Phenotypic variation, R^2	Co-localiza-tion with resistance QTL	Correla-tion with AUDPC-R(R^2)
RT:21.10	VL-inoculated	A6	56.0	48-74	0.113	no	0.71** (0.50)
RT:21.50 (naringin)	VL-inoculated	C1	0.0	0.0-6.0	0.152	**yes, C1**	-0.27** (0.07)
RT:22.50 (salicylic acid)	VL-inoculated	C1	10	0.0-28	0.109	**yes, C1**	n.s.
RT:22.50 (salicylic acid)	VL-inoculated	C2	18.0	12-30	0.124	no	n.s.
RT:8.82	VL-inoculated	C5	86.0	76-94	0.137	no	-0.42** (0.18)
RT:10.10 (chlorogenic acid)	VL-inoculated	C5	30.0	14-42	0.109	no	0.27** (0.07)
RT:13.70	VL-inoculated	C5	36.0	20-80	0.118	**yes, C5**	0.48** (0.23)
RT:14.20	VL-inoculated	C5	74.0	58-82	0.166	**yes, C5**	-0.47** (0.22)
RT:15.30	VL-inoculated	C5	54.0	32-68	0.117	**yes, C5**	0.77 ** (0.59)
RT:21.10	VL-inoculated	C5	64.0	46-80	0.125	**yes, C5**	0.71** (0.50)
RT:23.10 (rosmarinic acid)	VL-inoculated	C5	42.0	32-70	0.131	**yes, C5**	-0.44** (0.19)
RT:17.80	mock-inoculated	A1	24.0	12-42	0.134	no	n.s.
RT:3.00	mock-inoculated	A2	90.0	74-106	0.171	no	n.s.
RT:9.50 (4-hydroxy-benzoic acid)	mock-inoculated	A3	46.0	30-64	0.143	no	n.s.
RT:11.05	mock-inoculated	A3	62.0	40-72	0.114	no	n.s.
RT:17.80	Mock-inoculated	A3	46.0	34-62	0.187	no	n.s.
RT:23.38	mock-inoculated	A3	46.0	34-66	0.154	no	n.s.
RT:13.10 (5-hydroxy-ferulic acid)	mock-inoculated	A7	30.0	24-30	0.117	no	n.s.
RT:11.45 (caffeic acid)	mock-inoculated	A10	116.0	98-120	0.116	no	n.s.

RT:18.75 (sinapic acid)	mock-inoculated	C1	20.0	4-28	0.16	no	n.s.
RT:7.25	mock-inoculated	C3	116.0	112-178	0.113	no	0.36** (0.13)
RT:17.10	mock-inoculated	C6	80.0	68-96	0.116	no	n.s.
RT:12.32	mock-inoculated	C7	34.0	12-54	0.13	no	n.s.
RT:11.85 (vanillic acid)	mock-inoculated	C8	126.0	104-134	0.113	no	n.s.
RT:12.32	mock-inoculated	C9	56.0	38-60	0.164	no	n.s.
RT:18.20 (ferulic acid)	mock-inoculated	C9	80.0	72-108	0.112	no	n.s.
RT:15.35	mock-inoculated	C9	16.0	0.0-38	0.124	no	n.s.

QTL with a LOD score ≥ 2.5 which explain at least 5 % of the phenotypic variation. Peaks putatively identified by co-migration of standard. Phenotypic variation of HPLC peak area explained by the QTL, R^2 (QGene, $R^2 \geq 5\%$). VL = *Verticillium longisporum*, n = 98 DH lines, R = Pearson correlation, n.s. = not significant and ** = significant at 0.01.

Summary of the table

V. longisporum-inoculated

Total number of HPLC peaks: 36

Total number of QTL (LOD 2.5): 11

Maximum number of QTL for one HPLC peak: 2

Number of QTL for HPLC peak concentrations co-localizing with QTL on C1 and C5 for AUDPC = 7

Major regions with overlapping QTL involved in phenylpropanoid content

Mock-inoculated

Total number of HPLC peaks: 36

Total number of QTL (LOD 2.5): 16

Maximum number of QTL for one HPLC peak: 2

Number of QTL for HPLC peak concentrations co-localizing with QTL on C1 and C5 for AUDPC = 0

Major regions with overlapping QTL involved in phenylpropanoid content

Supplementary table S8: Quantitative trait loci for *V. longisporum* resistance-related traits in mapping population Express 617 x R53-DH

Experiment number	Number of DH lines	Treatment (VL= *V. longisporum*-inoculated)	*V. longisporum* resistance-related trait	Chromosome	LOD	Peak position (cM)	Confidence interval (cM)	Left flanking marker (position, cM)	Right flanking marker (position, cM)	R^2 (%)	Additive effects
1	100	VL	AUDPC	C1	3.4	10	0-22	Ol10E12 (9.1)	E32M49n (12.6)	14.6	-5.034
		VL	AUDPC	C5	9.0	62	56-66	CB10065 (59.5)	Na12C01 (66.4)	33.8	7.867
		Mock-VL	Growth reduction	C5	5.9	62	56-70	CB10065 (59.5)	Na12C01 (66.4)	23.8	7.909
3	82	VL	AUDPC	C1	2.5	4	0-6	CB10536 (0.0)	CB10357b (5.3)	13.2	-4.603
		VL	AUDPC	C5	7.2	58	52-64	E32M49u (54.0)	CB10065 (59.5)	33.2	8.897
		Mock-VL	Growth reduction	C1	3.4	4	0-6	CB10536 (0.0)	CB10357b (5.3)	17.3	-7.458
		Mock-VL	Growth reduction	C5	6.6	58	54-62	E32M49u (54.0)	CB10065 (59.5)	31.1	11.789
4	98	VL	AUDPC	C5	4.2	62	42-70	CB10065 (59.5)	Na12C01 (66.4)	17.7	5.512
		Mock-VL	Growth reduction	C5	4.4	56	33-70	E32M49u (54.0)	CB10065 (59.5)	19.0	7.517
		VL (SP)	RT 21.0	C5	3.2	60	48-74	CB10065 (59.5)	Na12C01 (66.4)	14.0	0.271
		Mock (SP)	RT 11.3 (caffeic acid)	C1	2.8	4	0-20	CB10536 (0.0)	CB10357b (5.3)	12.4	-0.081
		VL (SP)	RT 9.7	C1	3.3	24	0-50	CB10528_185 (15.4)	E32M47I (32.8)	14.4	0.437
		VL (SP)	RT 23.7	C5	3.9	42	32-62	CB10611 (41.9)	BRMS030_210 (48.0)	16.7	0.146
		VL (SP)	RT 18.5	C5	3.2	34	22-48	Ol10B02 (26.4)	CB10611 (41.9)	14.3	0.693
		VL (SP)	RT 20.1	C5	3.0	36	22-70	Ol10B02 (26.4)	CB10611 (41.9)	13.4	0.393
		VL (SP)	RT 24.5 (phlorizin)	C1	3.2	0	0-28	CB10536 (0.0)	CB10357b (5.3)	14.0	0.477
4	98	VL (CW)	RT 15.30	C5	2.6	54	32-68	E32M49u (53.0)	CB 10065(59.5)	12.0	0.626
		VL (CW)	RT 21.10	C5	2.9	64	46-80	CB 10065(59.5)	Na12CO1_160 (66.4)	13.0	0.426
		VL (CW)	RT 13.70	C5	2.7	36	20-80	Ol10B02 (26.4)	CB10611 (41.9)	12.0	0.849
		VL (CW)	RT 14.20	C5	3.9	74	58-82	Ra2F11_230 (70.3)	CB 10027_265_(76.2)	17.0	- 0.583
		VL (CW)	RT 23.10 (rosmarinic acid)	C5	3.0	42	32-70	Ol10B02 (26.4)	CB10611 (41.9)	13.0	- 0.231
		VL (CW)	RT 21.50 (naringin)	C1	3.5	0	0-6	CB10536 (00.0)	CB10357b (05.3)	15.0	1.768
		VL (CW)	RT 22.50 (salicylic acid)	C1	2.5	10	0-28	CB10536 (00.0	E32M471	11.0	0.402

Only significant QTL detected in at least two experiments at a LOD score threshold of 2.5 are shown. LOD = Logarithm of odds; cM = centiMorgan; R^2 = percentage of phenotypic variation explained. AUDPC = area under the disease progress curve, SP = Soluble phenolics fraction, CW = cell wall-bound phenolics fraction and RT = retention time in min measured by HPLC of soluble & cell wall-bound phenolics fraction from hypocotyl samples.

Supplementary table S9: Correlation between area under the disease progress curve (AUDPC) and concentrations and ratios of lignin monomers in the oilseed rape hypocotyls

	AUDPC	Guaiacyl (G) lignin mock-inoculated	Syringyl (S) lignin mock-inoculated	Guaiacyl (G) lignin VL-inoculated	Syringyl (S) lignin VL-inoculated	Mock-inoculated G/S	VL-inoculated G/S
AUDPC	1						
Guaiacyl (G) lignin mock-inoculated	n.s.	1					
Syringyl (S) lignin mock-inoculated	n.s.	0.92***	1				
Guaiacyl (G) lignin VL-inoculated	n.s.	0.20*	n.s.	1			
Syringyl (S) lignin VL-inoculated	-0.41***	0.39***	0.43***	0.61***	1		
Mock-inoculated G/S	n.s.	n.s.	-0.25*	0.21*	n.s.	1	
VL-inoculated G/S	0.55***	n.s.	n.s.	n.s	-0.42***	n.s.	1

Pearson correlation (R) significant for n = 91 DH lines (one technical replicate) at ≤ 0.05 (*), at ≤ 0.001(***), n.s. = not significant, VL = *Verticillium longisporum*, AUDPC = area under the disease progress curve. R value from the correlation between S lignin concentrations and G/S ratios with the mean AUDPC are labelled in grey.

Supplementary table S10: Correlation between area under the disease progress curve (AUDPC) and concentrations and ratios of lignin monomers in the oilseed rape hypocotyls

	AUDPC	Guaiacyl (G) lignin mock-inoculated	Syringyl (S) lignin mock-inoculated	Guaiacyl (G) lignin VL-inoculated	Syringyl (S) lignin VL-inoculated	Mock-inoculated G/S	VL-inoculated G/S
AUDPC	1						
Guaiacyl (G) lignin mock-inoculated	n.s.	1					
Syringyl (S) lignin mock-inoculated	n.s.	0.95***	1				
Guaiacyl (G) lignin VL-inoculated	-0.31***	0.37**	0.34**	1			
Syringyl (S) lignin VL-inoculated	-0.46***	0.39***	0.43***	0.86***	1		
Mock-inoculated G/S	n.s.	n.s.	n.s.	n.s.	n.s.	1	
VL-inoculated G/S	0.54***	n.s.	n.s.	0.24*	-0.43***	n.s.	1

Pearson correlation (R) significant for n = 73 DH lines (mean from 2 technical replicates) at ≤ 0.05 (*), ≤ 0.01 (**), and at ≤ 0.001(***), n.s. = not significant, VL = *Verticillium longisporum* and AUDPC = area under the disease progress curve. R value from the correlation between G lignin and S lignin concentrations and G/S ratios with the mean AUDPC are labelled in grey.

Erklärung

„Ich erkläre: die vorgelegte Dissertation habe ich selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen, February 15, 2014 **Muhammed Ali Hossain**

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Oilseed rape plants (cv. Express)

Verticillium longisporum-inoculated Mock-inoculated (control)

édition scientifique
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