Verticillium Suppression Is Associated with the Glucosinolate Composition of Arabidopsis thaliana Leaves

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Abstract

The soil-borne fungal pathogen Verticillium longisporum is able to penetrate the root of a number of plant species and spread systemically via the xylem. Fumigation of Verticillium contaminated soil with Brassica green manure is used as an environmentally friendly method for crop protection. Here we present a study focused on the potential role of glucosinolates and their breakdown products of the model plant Arabidopsis thaliana in suppressing growth of V. longisporum. For this purpose we analysed the glucosinolate composition of the leaves and roots of a set of 19 key accesses of A. thaliana. The effect of volatile glucosinolate hydrolysis products on the in vitro growth of the pathogen was tested by exposing the fungus to hydrated lyophilized plant tissue. Volatiles released from leaf tissue were more effective than from root tissue in suppressing mycelial growth of V. longisporum. The accesses varied in their efficacy, with the most effective suppressing mycelial growth by 90%. An analysis of glucosinolate profiles and their enzymatic degradation products revealed a correlation between fungal growth inhibition and the concentration of alkenyl glucosinolates, particularly 2-propenyl (2Prop) glucosinolate, respectively its hydrolysis products. Exposure of the fungus to purified 2Prop glucosinolate revealed that its suppressive activity was correlated with its concentration. Spiking of 2Prop glucosinolate to leaf material of one of the least effective A. thaliana accesses led to fungal growth suppression. It is suggested that much of the inhibitory effect observed for the tested accesses can be explained by the accumulation of 2Prop glucosinolate.

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Plants have evolved inducible and preformed defence mechanisms to counteract pathogen attacks. Production of secondary metabolites with antimicrobial properties is a preformed defence mechanism. A known group of constitutive natural plant compounds are glucosinolates found mainly in Capparales and almost exclusively in Brassicaceae family including economically important crops as well as in the model plant A. thaliana [21]. Nitrogen- and sulfur-containing glucosinolates, derived from chain elongated and glucosidated amino acids, represent a diverse set of secondary metabolites [22]. In their intact form, they appear to be relatively inactive, but upon hydrolysis, they display a range of herbivore- and pathogen-suppressing activity [23,24]. Their degradation is catalysed by myrosinase and is regulated by proteins which control the synthesis of isothiocyanates (ITCs), nitriles and thiocyanates, among others [25]. The unstable aglycone that is produced upon glucosinolate degradation by myrosinase is converted into ITCs by default. However, the nature of the hydrolysis products is mainly defined by the structure of the glucosinolate side chain and depends on the plant species [21]. In Arabidopsis, depending on the glucosinolate side chain, hydrolysis conditions, and presence of specific protein factors, the formation of nitriles and epithionitrites can be favoured. This shift is
controlled by nitrile-specifier proteins [26], epithiospecifier proteins ESP [27], thiocyanate-forming proteins [20] and epithiospecifier modifier proteins [29]. The anti-fungal activity of oils purified from mustard was discovered as early as the 1930s [30], and a wealth of data has since confirmed these early findings [31].

While some of their fungicidal activity has been ascribed to non-volatile degradation products, most of it derives from volatile products, including 2-propenyl ITC (2Prop-ITC), 3-butenyl ITC (3But-ITC) and benzyl ITC [31].

The antifungal activity of volatile glucosinolate breakdown products, mainly ITCs, are assumed as driving compounds in biofumigation where crop residues (particularly those of Brassica spp.) have high glucosinolate content are incorporated into the soil for control of soil-borne pathogens [32,33]. The hydrolysis of glucosinolates in the residue is an important component of this control, acting against fungi [34,35,36,37,38,39,40], bacteria [41,42] and nematodes [43,44]. Although it has been established that the severity of the disease caused by a number of pathogens may be limited by the host's glucosinolate composition, this has been no systematic attempt until now to determine the extent to which this genetic variation in glucosinolate composition affects pathogen growth within the plant. Here, our research formed an analysis of genetic variation with respect to the volatile glucosinolate breakdown product composition from the leaves and roots of A. thaliana. As a bioassay, we hypothesize that the glucosinolate profile correlates with disease suppression of V. longisporum in a set of 19 key accessions of A. thaliana accessions.

**Experimental Procedures**

**Cultivation of Verticillium spp.**

A. thaliana plants were inoculated with either one of two V. longisporum isolates 43-3 [46] or VD-1 [47] or V. dahliae isolate GU060637 (kindly provided by Valerie Grimault, GEVES, Angers, France). The fungi were cultivated at 25°C in the dark on potato dextrose agar (PDA) (VWR International GmbH, Germany). Conidial suspensions were prepared by inoculating 500 mL sucrose sodium nitrate medium with five mm diameter plugs excised from a PDA plate, and shaking the culture at room temperature for three weeks.

**Plant material, growth and inoculation method**

The 19 A. thaliana (L.) Heynh. accessions investigated were Bur-0, Can-0, Col-0, Ctr-1, Edi-0, Hi-0, Kn-0, Ler-0, Mt-0, No-0, Oyt-0, Po-0, Rsch-4, Sl-2, Tsu-0, Wil-2, Ws-0, Wu-0 and Zu-0, which together make up the set of parents used by Kover et al. [48] to create a MAGIC (Multiparent Advanced Generation Intercross) population (kindly provided by L. Westphal, IBP Halle, Germany). All plants were grown in sand watered with nutrient solution, as described by Gibeaut et al. [49], and were first equilibrated in 2 M acetic acid, then pre-treated by the addition of two 1 mL aliquots of 6 M imidazole-formate (Carl Roth GmbH, Germany). Prior to sample loading, the column was first equilibrated in 2 M acetic acid, then pre-treated by the addition of two 1 mL aliquots of 6 M imidazole-formate (Carl Roth GmbH, Germany) in 30% v/v formic acid, followed by two washes with 1 mL deionized water. The column was washed twice with 1 mL 20 mM sodium acetate buffer pH 4.0 (Sigma-Aldrich Chemie GmbH, Germany), and 75 µL purified Helix pomatia aryl sulfatase (Roche Diagnostics GmbH, Germany) was added and left to stand for 12 h. Desulfo compounds were eluted with 1 mL of 20 mM sodium acetate buffer pH 4.0, followed by two washes with 1 mL deionized water. Desulfo-glucosinolate quantification was carried out by HPLC (Merck HPLC pump L-7100, DAD detector L-7455, automatic sampler AS-7200 and HPLC-Manager-Software D-7000) using a Spherisorb ODS2 column (Bischoff, Germany).
Analysis of glucosinolate hydrolysis products derived from leaf tissue or purified 2Prop glucosinolate

For the determination of enzymatically formed breakdown products of the GSL, the method of Lambrick et al. (2001) was adapted. Either one mL of water was added to 30 mg of lyophilized plant tissue in centrifugal tubes and left for 30 min at room temperature for glucosinolate hydrolysis or 0.4 or 0.8 mg of purified 2Prop glucosinolate dissolved in the sterile filtrated citrate buffer described in 6.3 was hydrolysed for 2 h or 24 h by adding 0.1 U thiglucosidase. Next, 2 mL of methylene chloride (Carl Roth GmbH, Germany; GC Ultra Grade) and 100 mL of 2 mM benzonitrile in methylene chloride as internal standard (Sigma-Aldrich Chemie GmbH, Germany; ≥99.9%) were added and the tubes were sealed. After shaking for 20 sec and centrifugation for 5 min, the methylene chloride layer was removed and filtered through a small column of anhydrous sodium sulfate (VWR International GmbH, Germany; ≥99%) to remove residual water. The remaining aqueous layer was re-extracted with 2 mL of methylene chloride. The dried extracts were combined, concentrated under nitrogen gas flow to 300 μL and transferred into a vial. Samples were analyzed by gas chromatography-mass spectrometry detection (GC-MS) using an Agilent 6890 A Series GC System (Agilent Technologies, Germany) with a Gerstel Multi Purpose Sampler MPS2 (Gerstel GmbH & Co. KG, Germany) and an Agilent 5973 Network MSD. The GC was equipped with an Optima 5 MS column (Macherey-Nagel, Germany, 30 m x 0.25 mm x 0.25 μm film). After splitless injection of 1 μL of the sample at 190°C, analytes were separated, using helium as carrier gas (1.8 mL/min), and a temperature gradient starting at 60°C (3 min) and raising up to 300°C with 3°C/min. After holding this temperature for 7 min, the temperature increased to 230°C with 9°C/min and then with 35°C/min to 310°C. The temperature of the transfer line was 310°C, the ion source of the MSD was set to 230°C. Mass spectra were acquired in the EI mode (70 eV) in the full scan mode (TIC) for the plant tissue samples (m/z 30–350) or in the selected ion monitoring mode (SIM) for the hydrolysed 2Prop glucosinolate samples (Quantifier ions: m/z 41 for 2Prop-CN, m/z 99 for 2Prop-ITC and m/z 103 for the internal standard benzonitrile). Analytes were identified by comparing mass spectra and retention times with those of authentic standards and with literature data [53,54]. Analyte content was calculated using benzonitrile as internal standard and the response factor (RF) of each compound relative to benzonitrile. The RF were experimentally determined for 2Prop-ITC (RF_{TIC} = 1.07, RF_{SIM} = 3.07), 3-hutencnitrile (2-Prop-CN; RF_{TIC} = 3.70, RF_{SIM} = 7.32), 4-pentenitrile (3But-CN; RF_{TIC} = 2.45), and 3-(methylthio)propyl ITC (3MTP-ITC; RF_{TIC} = 1.07) [all purchased from Sigma-Aldrich Chemie GmbH, Germany]; 3-hydroxypropionitrile (RF_{TIC} = 7.67; Thermo Fischer Scientific, Belgium), 3But-ITC (RF_{TIC} = 1.06) and 4-pentenyl ITC (4-Pent-ITC, RF_{TIC} = 1.14) (both purchased from TCI Deutschland GmbH, Germany), 4-(methylthio)butyl ITC (4MTB-ITC; RF_{TIC} = 0.76; Santa Cruz Biotechnology, Germany), and for 4-(methylsulfinyl)butyl ITC (4MSOB-ITC; RF_{TIC} = 3.01; Enzo Life Sciences GmbH, Germany). For those compounds that were commercially not available, the RF of the chemically most similar compound was used: For the epitionitriles of 2Prop and 3-But glucosinolate the RF of the corresponding ITC was used, diastereometric 3-hydroxy-4,5-epithiopentyl nitrile (2OH3But-ITC) and 5-vinyl-1,3-oxazolidine-2-thione (OZT) were calculated with the RF of 3-But-ITC. The corresponding nitriles of 3-(methylthio)propyl (3-MTP) glucosinolate, 4-(methylthio)butyl (4-MTB) glucosinolate and 4-(methylsulfinyl)butyl (4MSOB) glucosinolate were calculated with the RF of the analogous ITC. The degradation products of β-(methylthio)octyl (8MTO) glucosinolate were calculated with the RF of 4MTB-ITC and all sulfanyl nitriles and ITC were calculated with the RF of 4MSOB-ITC. For the quantification of degradation products of the 3-hydroxypropyl glucosinolate the RF determined for 3-hydroxypropionitrile was utilized. The limit of detection ranged between 0.9 μM (4Pent-ITC) and 15.5 μM (3-hydroxypropionitrile).

DNA extraction and qRT-PCR analysis

Extraction of DNA from infected plant material was performed following Tinker et al. [55], with the inclusion of an additional DNA purification procedure [56]. The integrity and quantity of the DNA were assessed photometrically using a NanoDrop ND-1000 device (PeqLab GmbH, Germany).

The abundance of fungal DNA present in the plant material was estimated by a PCR based on the primer pair VDS1 (5'-CAC ATT CAG TTC AGG AGA CGG A-3') and VDS2 (5'-CCT TCT ACT GGA GTA TTT CGG-3'), which specifically amplifies a 521 bp product from a template of either V. dahliae [57] or V. longisporum DNA. The amplicon was generated by imposing an initial denaturation of 95°C/3 min, followed by 40 cycles of 95°C/20 s, 66°C/20 s, 72°C/60 s. The template DNA was diluted tenfold in sterile water to an approximate concentration of 10 ng μL⁻¹. Two primer pairs were selected as A. thaliana reference genes based on geNORM [58] analysis of expression stability and previous evaluation [59]. The two reference genes selected were a gene encoding a pentatricopeptide repeat (At5g58540; 5'-AAG ACA GTA AGG GTG CAA CCT TAC T-3', 5'-GTT TTT GAG TTG TAT TTG TCA GAG AAA G-3', amplicon 61 bp in length), and one encoding the mitosis-associated protein YLS8 (At5g08290; 5'-TTA CTG TTG CTT TTG TTC ATC ATG TTC GAA GCA AGT-3', amplicon 66 bp in length). Both amplicons were generated by imposing an initial denaturation of 95°C/3 min, followed by 40 cycles of 95°C/10 s, 60°C/30 s. The template DNA was diluted 100 fold in sterile water to an approximate concentration of 1 ng μL⁻¹. The PCR efficiency of the primer pairs, as estimated from a template dilution series, was respectively 97% for VDS, 98% for YLS8 and 99% for PPR. A CFX96 real-time System driven by CFX Manager software v2.1 was used for qRT-PCR, in reactions based on SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA). Each 6 μL reaction was composed of 3 μL 2× SsoAdvanced, 1 μL diluted DNA and 1 μL of each gene-specific primer (2 μM) and was replicated three times per biological sample. Primer specificity was assessed by inspection of the melting curve after cycle 40 and agarose gel electrophoresis of the amplicon. The Cq values of
individual well traces were determined using the regression model implemented in the CFX Manager software. The data were analyzed using qbasePLUS software v2.3 (Biogazelle NV, Belgium) applying the following parameters: primer amplification efficiency: 100%, normalization strategy: two reference targets [60].

Data analysis

All chemical and microbial data were checked to be normally distributed and showed homogeneity of variances before the analysis of variances (ANOVA’s) were calculated using Tukey’s HSD test at $p < 0.001–0.05$.

Results and Discussion

Genetic variation in *A. thaliana* for the suppression of *in vitro* growth of *V. longisporum*

When *V. longisporum* 43-3 was exposed to plant material extracted from the 19 different *A. thaliana* accessions, its growth was more noticeably retarded by the presence of lyophilized leaf rather than root tissue (Fig. 1). The leaf tissue-induced reduction in growth reached 92% of the non-treated control, whereas the maximum extent of the suppression induced by the presence of root tissue was only 58%. The six accessions whose leaf tissue induced the most substantial growth reduction (>50%) were Bur-0, Can-0, Edi-0, Hi-0, Ws-0 and Wu-0. The most efficacious root tissues (reducing fungal growth by >30%) were those prepared from Edi-0, Hi-0, Ws-0, Wu-0 and Zu-0. The apparent presence of genetic variation in *A. thaliana* for the ability to suppress fungal growth mirrors equivalent variation demonstrated in other Brassica spp. [61,62]. The extent of the inhibition is also comparable to that observed against *Sclerotinia sclerotiorum* [63], *Leptosphaeria maculans* [64], *Xanthomonas campestris* [41] and *V. dahliae* [65,66].

Genetic variation for glucosinolate composition among *A. thaliana* accessions

The profile of compounds emitted by intact *A. thaliana* leaves is dominated by terpenes and various aromatic compounds, but wounding induces a shift towards that of glucosinolate hydrolysis products [67]. For this reason, our focus was to obtain the glucosinolate profiles of the 19 *A. thaliana* accessions. These profiles are known to be affected by both genetic and environmental
factors [68,69,70], and vary between plant organs [71,72] and over development [73]. A total of 20 distinct glucosinolates was identified and quantified, of which 16 were aliphatic (alkenyl, hydroxyalkenyl, hydroxyalkyl, thioalkyl and sulfinylalkyl glucosinolates) and four indole (Tables S1 and S2). All 20 compounds have previously been detected in *A. thaliana* [68,74].

The aliphatic glucosinolate concentration in the leaf tissue was tenfold that in the root tissue, while the indole glucosinolates were equally represented in both tissues (Fig. 2); a similar partitioning was obtained in the Col-0 accession [71,73]. The glucosinolate composition varied from accession to accession. While some compounds (particularly the indole glucosinolates) were present in all 19 accessions, most of the aliphatic ones were accession-specific (Tables S1 and S2). The aliphatic glucosinolate concentration in the leaf tissue varied from 5 μmol g⁻¹ dry weight (DW) in Mt-0 to 54 μmol g⁻¹ DW in Can-0, while the indole glucosinolate concentration lay between 3.3 μmol g⁻¹ DW (Ct-1) and 12 μmol g⁻¹ DW (Can-0). In root tissue, the range in aliphatic glucosinolate concentration was 1.4–7.8 μmol g⁻¹ DW (for, respectively, Oy-0 and Zu-0), while that for the indole glucosinolates was higher abundant with 2.5–12.7 μmol g⁻¹ DW (Ct-1 and Tsu-0) (Fig. 2). A similar range both with respect to composition and quantity has also been demonstrated in 82 different *B. rapa* cultivars [62], and in 39 [68] and 96 *A. thaliana* accessions [74].

A hierarchical clustering was performed to group accessions on the basis of their glucosinolate profile. This analysis delivered three major clusters, the first comprising accessions Can-0, Edi-0, Bur-0, Ws-0, Hi-0 and Wu-0, which preferentially accumulated the alkenyl glucosinolates 2Prop, 3-butenyl (3But) and 4-pentenyl glucosinolate (4Pent) (with 2Prop being the most abundant); the second group featured those accumulating hydroxyalkyl glucosinolates (Kn-0, Ler-0, Rsch-4, No-0, Tsu-0, Ct-1, Wil-2), and the third those with an elevated level of methylsulfinylalkyl and indole glucosinolates (Mt-0, Col-0, Oy-0, Po-0, Si-2) (Fig. 3). There was a correlation between an accession’s ability to accumulate alkenyl glucosinolates and the suppression by its leaf tissue of *Verticillium* sp. growth. Leaf tissue prepared from Can-0 was the most effective for inhibiting fungal growth, and this accession also accumulated the most 2Prop glucosinolate; as a result, the hypothesis was that a hydrolysis product of 2Prop glucosinolate is the major agent of anti-fungal activity.

**V. longisporum** growth is affected by the formation of 2Prop-ITC

A subset of ten Arabidopsis accessions was selected for chemical analysis based on contrasting leaf glucosinolate patterns to identify glucosinolate hydrolysis products with an inhibitory effect on *Verticillium* growth. Accessions included alkenyl glucosinolate accumulators with strongest antifungal effects (Bur-0, Can-0, Hi-0,
and Wu-0) as well as accessions being rich in hydroxyalkenyl, hydroxylalkyl, methylthioalkyl and methylsulfynalkyl glucosinolates that showed low antifungal activity (Kn-0, Ler-0, Po-0, Rsch-4, Wil-2, and Zu-0). A total of 20 different glucosinolate hydrolysis products was identified and quantified in lyophilized plant tissue of the 20 accessions. The predominant hydrolysis product in Can-0, Hi-0, Kn-0, Ler-0, Po-0, Rsch-4, and Wil-2 was 2Prop-ITC with concentrations in the range of 0.13–0.24 μmol g⁻¹ DW for, respectively, Po-0 and Wil-2, and 0.15–0.35 μmol g⁻¹ DW for, respectively, Kn-0, Ler-0, Po-0, Rsch-4, and Wil-2. The application of 4 mg 2Prop glucosinolate, matching the same amount in 1 g leaf material of Bur-0, resulted in a growth reduction of 97% as the main degradation product. After 2 h of hydrolysis time 91% of the 2Prop glucosinolate (0.4 mg level) were recovered as ITC, formed 2Prop-ITC as main glucosinolate hydrolysis product. Therefore, the inhibitory effect of 2Prop glucosinolate was tested for the V. longisporum isolate 43-3. Fungal growth was significantly inhibited by concentrations of 0.4 and 4.0 mg 2Prop glucosinolate per plate in a dose-dependent manner (Fig. 4 A). The application of 4 mg 2Prop glucosinolate, matching the same amount in 1 g leaf material of Bur-0, resulted in a growth reduction of 97% as compared to the 54% inhibition obtained by the leaf material of the same accession. The GC-MS analysis of the hydrolysed 2Prop glucosinolate revealed that 3But-CN was the main hydrolysis product upon myrosinase-driven breakdown in Zu-0, whereas Bur-0 formed slightly more 3But-ITC than 2Prop-ITC. The main degradation product upon myrosinase-driven breakdown in Zu-0 was also 3But-ITC, but this accession also formed OZT in substantial amounts.

Accessions Kn-0, Ler-0, Po-0, Rsch-4, and Wil-2 revealed a high level of 3-hydroxypropyl ITC (3OH-ITC), ranging from 13–44.3 μmol g⁻¹ DW for, respectively, Po-0 and Kn-0. Epithionitriles, being formed only in presence of the ESP from alkenyl or hydroxyalkenyl glucosinolates [27] were detected in hydrolysed leaf tissues of Bur-0, Can-0, Wu-0 and Zu-0, but not in those from Hi-0. Nitrile production usually is accompanied by the formation of ITC, however hydrolysed Kn-0 leaf tissue was absent of the nitrile deriving from 3OH-ITC, although it was detected in all other hydroxyalkyl rich accessions.

Alkenyl accumulating Arabidopsis accessions, that were able to restrict growth of V. longisporum, formed 2Prop-ITC as main glucosinolate hydrolysis product. Therefore, the inhibitory effect of hydrolysis products of purified 2Prop glucosinolate was tested for the V. longisporum isolate 43-3. Fungal growth was significantly inhibited by concentrations of 0.4 and 4.0 mg 2Prop glucosinolate per plate in a dose-dependent manner (Fig. 4 A). The application of 4 mg 2Prop glucosinolate, matching the same amount in 1 g leaf material of Bur-0, resulted in a growth reduction of 97% as compared to the 54% inhibition obtained by the leaf material of the same accession. The GC-MS analysis of the hydrolysed 2Prop glucosinolate (Fig. 4 B) confirmed the formation of 2Prop-ITC as the main degradation product. After 2 h of hydrolysis time 91% of the 2Prop glucosinolate (0.4 mg level) were recovered as ITC, those concentrations declining to 63% within the next 22 h. Low alkenyl-accumulating A. thaliana accessions showed no ability to suppress fungal growth in the bioassay (see Fig. 1). The pure 2Prop glucosinolate was added to leaf material of Osy-0 in order to complement this deficiency in fungitoxicity (Fig. 5).

Table 1. Breakdown products formed by hydrolysis of glucosinolates in the leaf tissue of selected Arabidopsis thaliana accessions.

| Breakdown products | Bur-0 | Can-0 | Hi-0 | Kn-0 | Ler-0 | Po-0 | Rsch-4 | Wil-2 | Wu-0 | Zu-0 |
|-------------------|-------|-------|------|------|-------|------|--------|-------|------|------|
| Alkenyl hydrolysis products |       |       |      |      |       |      |        |       |      |      |
| 2Prop-CN | 0.05±0.05 | 0.12±0.03 | 0.06±0.00 | n.d. | n.d. | 0.05±0.03 | n.d. | n.d. | 0.16±0.10 | n.d. |
| 2Prop-ITC | 5.30±0.84 | 9.21±1.47 | 19.74±0.51 | n.d. | n.d. | n.d. | n.d. | 16.60±6.04 | 2.39±0.31 |       |
| 2Prop-EPT | 0.54±0.36 | 1.06±0.13 | n.d. | n.d. | n.d. | n.d. | n.d. | 1.12±0.87 | 0.17±0.11 |       |
| 3But-CN | 0.16±0.07 | 0.16±0.02 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.18±0.04 | n.d. |       |
| 3But-ITC | 6.82±0.78 | 0.25±0.04 | n.d. | n.d. | n.d. | n.d. | n.d. | 6.44±1.00 | n.d. |       |
| 3But-EPT | 0.65±0.38 | 0.02±0.00 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.29±0.18 | n.d. |       |
| 4Pent-ITC | 0.30±0.06 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.27±0.02 | n.d. |       |
| Hydroxylalkenyl hydrolysis products |       |       |      |      |       |      |        |       |      |      |
| Ep2OH3But-EPT | 0.04±0.05 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.49±0.26 | n.d. |       |
| OZT | 0.19±0.23 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 2.63±0.35 | n.d. |       |
| Methylthioalkyl hydrolysis products |       |       |      |      |       |      |        |       |      |      |
| 3MTP-CN | n.d. | 0.05±0.01 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |       |
| 3MTP-ITC | n.d. | 0.45±0.14 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.09±0.04 | n.d. |       |
| 4MTB-ITC | n.d. | 0.08±0.03 | n.d. | n.d. | n.d. | 0.17±0.04 | n.d. | n.d. | n.d. |       |
| 8MT-CN | n.d. | 0.03±0.00 | n.d. | n.d. | 0.47±0.11 | n.d. | n.d. | 0.02±0.02 | n.d. |       |
| 8MT-ITC | 0.14±0.03 | 0.64±0.05 | 0.14±0.05 | n.d. | 0.21±0.10 | 0.48±0.08 | 0.36±0.13 | 0.09±0.02 | 0.40±0.15 | 0.35±0.02 |
| Methylsulfynalkyl hydrolysis products |       |       |      |      |       |      |        |       |      |      |
| 3MSOP-ITC | n.d. | 0.37±0.11 | n.d. | n.d. | n.d. | 0.29±0.04 | 0.15±0.26 | n.d. | n.d. |       |
| 4MSOB-ITC | n.d. | n.d. | n.d. | n.d. | n.d. | 2.54±0.31 | n.d. | n.d. | n.d. |       |
| 8MSO-CN | 0.22±0.10 | 0.32±0.02 | n.d. | 0.17±0.02 | n.d. | 0.15±0.01 | 0.11±0.03 | n.d. | n.d. |       |
| 8MSO-ITC | 0.54±0.13 | 0.27±0.01 | 0.16±0.04 | 0.84±0.25 | 0.29±0.23 | 0.51±0.03 | 0.43±0.21 | 0.30±0.03 | n.d. | 0.17±0.11 |
| Hydroxylalkyl hydrolysis products |       |       |      |      |       |      |        |       |      |      |
| 3OHP-CN | n.d. | n.d. | n.d. | n.d. | 0.76±0.05 | 0.70±0.28 | 0.74±0.48 | 0.76±0.21 | n.d. | n.d. |
| 3OHP-ITC | n.d. | n.d. | n.d. | n.d. | 44.34±1.96 | 38.93±2.28 | 13.00±2.32 | 37.17±12.79 | 32.31±0.23 | n.d. |

Quantities shown in μmol g⁻¹ DW, derived from the mean of three batches of plants (each n = 50) and two technical replicates per sample. Errors denote standard deviation.
Fungal growth was not significantly affected by the presence of Oy-0 leaf material. However, when 0.4 or 4 mg 2Prop glucosinolate were added to the lyophilized Oy-0 leaf material, the fungal growth rate decreased to 11 and 14%, respectively, as compared to the non-treated control. This indicates that 2Prop glucosinolate greatly contributes to the growth suppression observed for alkenyl-accumulating plant accessions. Thus, present data suggest that 2Prop-ITC can provide protection against fungal pathogen infection. The abundance of 2Prop-ITC has been correlated with fungicidal activity in several *Brassica* spp. [75,76,77], while the exposure to purified 2Prop-ITC is strongly inhibitory over the growth of both * Fusarium oxysporum* [36], *Phymatotrichopsis omnivora* [78] and *V. dahliae* [79]. Transcriptional analysis in *A. brassicicola* points to oxidative damage and redox imbalance being the result of exposure [80].

**Differential systemic colonization by *Verticillium* ssp.**

The accessions accumulating 2Prop glucosinolate were those whose leaf tissue most strongly inhibited the growth of *V. longisporum*. It was therefore of interest to contrast two accessions differing in their ability to accumulate 2Prop in their leaf with respect to their capacity to resist the systemic spread of the pathogen *in planta*. Since genetic mapping of resistance against *V. longisporum* infection has already been carried out in a population derived from a cross between Ler-0 and Bur-0 [20], these two accessions represented an appropriate choice of material. The extent of fungal colonization in the root and leaf tissue of the two
Bacteria is clearly therefore a complex one.

Factors might contribute to the suppression of fungal spread enzymes loose activity after freeze-drying. Hence, also other between lyophilized and fresh plant material since modifying Note, however, that glucosinolate hydrolysis products differ seems to represent an important mechanism for plant resistance, accumulation of alkenyl glucosinolates in the leaf tissue thus probably acting to inhibit the systemic spread of the pathogen.

Bur-0. The implication is that resistance is determined by an interaction occurring in the shoot, as also suggested in recent studies on Verticillium interactions with Bur-0/Ler-0 (high/low alkenyl) [20] or Ws-0/Ler-0 (high/low alkenyl) [81]. The studies on interaction occurring in the shoot, as also suggested in recent

Figure 5. The effect of increased 2Prop glucosinolate concentrations in the low 2Prop glucosinolate accumulating Arabidopsis thaliana accession Oy-0 on the growth of Verticillium longisporum 43-3. Data represent the mean of five technical replicates per biological sample and error bars represent the standard error. Significant differences between the control mycelia and those exposed to Oy-0 volatiles spiked with 2Prop glucosinolates are indicated by asterisks (*: p<0.001).

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Figure 6. Systemic spread of Verticillium longisporum 43-3, VD-1 and V. dahliae GU060637 within the leaf and root of Arabidopsis thaliana accessions Ler-0 and Bur-0, as measured by qRT-PCR, five weeks after inoculation. Data represent the mean of three batches consisting of five plants each, measured in technical triplicates via qRT-PCR. Bars denote standard deviations. Significant differences between Ler-0 and Bur-0 are indicated by asterisks (*: p<0.001).

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Supporting Information

Table S1 Glucosinolates present in the leaf of a range of Arabidopsis thaliana accessions. Quantities shown in \( \mu \)mol g\(^{-1}\) DW, derived from the mean of three batches of plants (each n = 50) and two technical replicates per sample. Errors denote standard deviation. 2Prop: 2-propenyl, 3But: 3-butenyl, 4Pent: 4-pentenyl, 2OH3But: (2R)-2-hydroxy-3-butenyl, Epi2OH3But: (2S)-2-hydroxy-3-butenyl, 8MTO: 8-(methylthio)octyl, 8MSOO: 8-(methylsulfinyl)octyl, 4MSOB: 4-(methylthio)butyl, 7MTH: 7-(methylthio)heptyl, 3MTP: 3-(methylthio)propyl, 3MSOP: 3-(methylsulfinyl)propyl, 3MOSB: 3-(methylsulfinyl)butyl, 5MSOP: 5-(methylsulfinyl)pentyl, 6MOSO: 6-(methylsulfinyl)hexyl, 7MSOH: 7-(methylsulfinyl)heptyl, 8MSOO: 8-(methylsulfinyl)octyl, 3OH: 3-hydroxypropyl, 13M: 3-indolylmethyl, 4OH13M: 4-hydroxy-3-indolylmethyl, 1MO13M: 1-methoxy-3-indolylmethyl, 4MO13M: 4-methoxy-3-indolylmethyl glucosinolate, n.d. not detected.

(DOCX)

Table S2 Glucosinolates present in the root tissue of a range of Arabidopsis thaliana accessions. Quantities shown in \( \mu \)mol g\(^{-1}\) DW, derived from the mean of three batches of plants (each n = 50) and two technical replicates per sample. Glucosinolate abbreviations as used in Table S1. n.d.: not detected.

(DOCX)

Concluding Remarks

We have reported here that key A. thaliana accessions vary with respect to their accumulation of glucosinolates in the leaf and root tissue, and that the accumulation of 2Prop glucosinolate in the leaf can explain much of the inhibitory effect of leaf tissue on the in vitro growth of V. longisporum. In order to further assess the biofumigation potential of 2Prop glucosinolate for crop protection, effectiveness should be investigated under field conditions.

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Glucosinolate abbreviations as used in Table S1. n.d.: not detected.
Author Contributions
Conceived and designed the experiments: KW FSH AK MS SR RG. Performed the experiments: KW FSH. Analyzed the data: KW FSH. Contributed reagents/materials/analysis tools: MS SR RG. Wrote the paper: KW FSH AK MS SR RG.

References
1. Koike ST, Subbarao KV, Davis RM, Gordon TR, Hubbard JC (1994) Verticillium wilt of cauliflower in California. Plant Disease 78: 1116–1121.
2. Dunker S, Keunecke H, Steinbach P, von Tiedemann A (2008) Impact of Verticillium longisporum on yield and morphology of winter oilseed rape (Brassica napus) in relation to systemic spread in the plant. Journal of Phytopathology 156: 690–707.
3. Fredin EF, Thomma B (2006) Physiology and molecular aspects of Verticillium wilt diseases caused by V. dahliae and V. albo-atrum. Molecular Plant Pathology 7: 81–96.
4. Zhou L, Hu Q, Johansson A, Dixelius C (2006) Verticillium longisporum and V. dahliae: infection and disease in Brassica napus. Plant Pathology 55: 137–144.
5. Klosterman SJ, Subbarao KV, Kang SC, Veronese P, Gold SE, et al. (2011) Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. PLoS Pathogens 7.
6. McFadden HG, Chapple R, de Feyer R, Dennis E (2001) Expression of pathogenesis-related genes in cotton stems in response to infection by Verticillium dahliae. Physiological and Molecular Plant Pathology 58: 119-131.
7. Hill MK, Lyon K, Lyon BR (1999) Identification of disease resistance genes expressed in Gossypium hirsutum upon infection with the wilt pathogen Verticillium dahliae. Plant Molecular Biology 40: 289–296.
8. Xu L, Zhu LF, Tu LL, Guo XP, Long L, et al. (2011) Differential gene expression in cotton defence response to Verticillium dahliae by SSH. Journal of Phytopathology 159: 66-615.
9. van Esse HP, Fredin EF, de Groot PJ, de Wit P, Thomma B (2009) Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct.
10. Tischner R, Koltermann M, Hesse H, Plath M (2010) Early responses of Arabidopsis thaliana to infection by Verticillium longisporum. Physiological and Molecular Plant Pathology 74: 419-427.
11. Johnson T, Konig S, Singh S, Brass-Stromeyer S, Bischoff M, et al. (2012) Molecular activation and production of tryptophan-derived secondary metabolites in Arabidopsis roots contributes to the defense against the fungal vascular pathogen Verticillium dahliae. Molecular Plant 5: 1389–1402.
12. Wang F, Ma YP, Yang CL, Zhao PM, Yao Y, et al. (2011) Proteomic analysis of the sea-island cotton roots infected by wilt pathogen Verticillium dahliae. Proteomics 11: 4296–4309.
13. Roelf S, Majcherzyck A, Possienke M, Feussner K, Tappe H, et al. (2012) Verticillium longisporum infection affects the leaf apoplastic proteome, metabolome, and cell wall properties in Arabidopsis thaliana. PLoS ONE 7:
14. Zhao FA, Fang WP, Xie DY, Zhao YM, Tang ZJ, et al. (2012) Proteomic analysis of the sea-island cotton roots infected by wilt pathogen Verticillium dahliae. Plant Journal 70: 360-376.
15. Kover PX, Valdar W, Trakalo J, Scarcelli N, Ehrenreich IM, et al. (2009) A multiparent advanced generation inter-cross to fine-map quantitative traits in Arabidopsis thaliana. Mycological Research 106: 570–578.
16. de Jonge R, van Esse HP, Maruthachalam K, Bolton MD, Santhanam P, et al. (2011) Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. PLoS Pathogens 7.
Leaf Glucosinolates Affect Verticillium Growth

66. Uppal AK, El Hadrami A, Adam LR, Tenuta M, Daayf F (2008) Biological control of potato Verticillium wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. Biological Control 44: 90–100.

67. Rohloff J, Bones AM (2003) Volatile profiling of Arabidopsis thaliana – Putative olfactory compounds in plant communication. Phytochemistry 66: 1941–1955.

68. Kliebenstein DJ, Kroymann J, Brown P, Fugit A, Pedersen D, et al. (2001) Genetic control of natural variation in Arabidopsis glucosinolate accumulation. Plant Physiology 126: 811–825.

69. Textor S, Gregerson J (2009) Herbivore induction of the glucosinolate-myrosinase defense system: major trends, biochemical bases and ecological significance. Phytochemistry Reviews 8: 149–170.

70. Verkerk R, Schreiner M, Krumbein A, Ciska E, Holt B, et al. (2009) Glucosinolates in Brassica vegetables: The influence of the food supply chain on intake, bioavailability and human health. Molecular Nutrition & Food Research 53: S219–S265.

71. Brown PD, Tokuhashi JG, Reichelt M, Gregerson J (2003) Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. Phytochemistry 62: 471–481.

72. Sarsby J, Towers MW, Stain C, Cramer R, Koroleva OA (2012) Mass spectrometry imaging of glucosinolates in Arabidopsis flowers and siliques. Phytochemistry 77: 110–118.

73. Petersen BL, Chen SX, Hansen CH, Olsen CE, Halkier BA (2002) Composition and content of glucosinolates in developing Arabidopsis thaliana. Planta 214: 562–571.

74. Chan EKF, Rose HC, Kliebenstein DJ (2010) Understanding the evolution of defense metabolites in Arabidopsis thaliana using genome-wide association mapping. Genetics 183: 991–1007.

75. Kirkegaard JA, Wong PTW, Desmarchelier JM (1996) In vitro suppression of fungal root pathogens of cereals by Brassica species. Plant Pathology 45: 593–603.

76. Olivier C, Vaughn SF, Mizubuti ESG, Loria R (1999) Variation in allyl isothiocyanate production within Brassica species and correlation with fungicidal activity. Journal of Chemical Ecology 25: 2687–2701.

77. Mayton HS, Olivier C, Vaughn SF, Loria R (1996) Correlation of fungicidal activity of Brassica species with allyl isothiocyanate production in macerated leaf tissue. Phytopathology 86: 267–271.

78. Hu P, Wang AS, Eunledow AS, Hollister EB, Rothlisberger KL, et al. (2011) Inhibition of the germination and growth of Physiomonticola annuae (cotton root rot) by oiled meal and isothiocyanates. Applied Soil Ecology 49: 69–75.

79. Down GJ, Harris DC, Murray RA (2007) Destruction of Verticillium dahliae in soil following the addition of sulphur-containing volatile compounds potentially produced from Brassica tissues. Agriculture Industry 3: 293–294.

80. Sellam A, Dzago A, Guillermete T, Hudhomme P, Simonneau P (2007) Transcriptional responses to exposure to the brassicaceous defense metabolites camalexin and allyl-isothiocyanate in the necrotrophic fungus Alternaria brassicicola. Molecular Plant Pathology 8: 195–206.

81. Stevenson LA, Okori P, Dieulain C (2001) An investigation of the susceptibility of Arabidopsis thaliana to isolates of two species of Verticillium. Journal of Phytopathology 149: 395–401.