The Vascular Pathogen *Verticillium longisporum* Does Not Affect Water Relations and Plant Responses to Drought Stress of Its Host, *Brassica napus*

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ABSTRACT

Verticillium longisporum is a host-specific vascular pathogen of oilseed rape (*Brassica napus* L.) that causes economic crop losses by impairing plant growth and inducing premature senescence. This study investigates whether plant damage through *Verticillium* stem striping is due to impaired plant water relations, whether *V. longisporum* affects responses of a susceptible *B. napus* variety to drought stress, and whether drought stress, in turn, affects plant responses to *V. longisporum*. Two-factorial experiments on a susceptible cultivar of *B. napus* infected or noninfected with *V. longisporum* and exposed to three watering levels (30, 60, and 100% field capacity) revealed that drought stress and *V. longisporum* impaired plant growth by entirely different mechanisms. Although both stresses similarly affected plant growth parameters (plant height, hypocotyl diameter, and shoot and root dry matter), infection of *B. napus* with

V. longisporum did not affect any drought-related physiological or molecular genetic plant parameters, including transpiration rate, stomatal conductance, photosynthesis rate, water use efficiency, relative leaf water content, leaf proline content, or the expression of drought-responsive genes. Thus, this study provides comprehensive physiological and molecular genetic evidence explaining the lack of wilt symptoms in *B. napus* infected with *V. longisporum*. Likewise, drought tolerance of *B. napus* was unaffected by *V. longisporum*, as was the level of disease by drought conditions, thus excluding a concerted action of both stresses in the field. Although it is evident that drought and vascular infection with *V. longisporum* impair plant growth by different mechanisms, it remains to be determined by which other factors *V. longisporum* causes crop loss.

Additional keywords: gas exchange.

Drought is the most adverse abiotic factor currently limiting crop productivity in many regions of the world (Anjum et al. 2011). It affects photosynthesis and assimilates partitioning and other metabolic processes that cause reduced growth, wilting, premature senescence, and, ultimately, yield losses. Drought stress in oilseed rape has caused delayed growth (Din et al. 2011) and prolonged flowering, leading to significant reductions in yield (Müller et al. 2010) and seed oil content (Sinaki et al. 2007).

Because the vascular system is crucial for plant water supply and provides vascular pathogens a compartment for colonizing their hosts, the interaction of drought stress with vascular diseases is of particular importance. Previous research has demonstrated controversial types of this interaction. Cross-resistance induced by drought stress decreased infection of alfalfa with *Verticillium alfalfae* (Pennypacker et al. 1991) and of tomato with *V. dahliae* (Arbogast et al. 1999). The major known mechanisms by which drought can enhance disease resistance in plants include production of reactive oxygen species, induction of plant defense genes (PR-5 and PDF1.2), or enhanced hormonal activity regulating callose deposition (Mauch-Mani and Mauch 2005; Ramegowda et al. 2013). Conversely, drought has been also shown to aggravate vascular plant diseases by increasing host susceptibility. Increased severity of infection with *V. dahliae* at decreased water supply has been reported in eggplant (Bletsos et al. 1999). In addition to drought stress predisposing plants to pathogen infection, infection with vascular diseases may, in turn, affect plant water relations and, thereby, response to drought. Vascular plugging and occlusions are well-known systemic plant responses to infection with vascular pathogens, which may significantly compromise vessel functions with regard to the transport of water and nutrients, leading

to reduced growth and irreversible damage through wilt. This may be associated with significant reductions in gas exchange and related plant syntheses, as has been demonstrated for *V. dahliae* wilt in pepper (Goicoechea et al. 2000), potato (Bowden et al. 1990), and cotton (Hampton et al. 1990). In contrast, there are also several examples where infection with *Verticillium* spp. did not cause any drought stress in plants such as pepper (Goicoechea et al. 2000), potato (Haverkort et al. 1990), *Arabidopsis* (Reusche et al. 2014), and sunflower (Sadras et al. 2000).

V. longisporum is a vascular pathogen of *Brassica napus* that causes premature senescence, potentially resulting in substantial yield losses (Dunker et al. 2008). *V. longisporum* is a host-specialized (Novakazi et al. 2015; Zeise and von Tiedemann 2002) and recently evolved (Inderbitzin et al. 2011) vascular pathogen inducing partial vessel plugging with pectic substances and accumulation of polyphenols and lignin in the xylem parenchyma (Eynck et al. 2009). In contrast to most other diseases induced by *Verticillium* spp., no wilt symptoms have been reported from *V. longisporum*-infected *B. napus* (Depotter et al. 2016). However, a comprehensive study is lacking to demonstrate whether this is due to masking of wilt symptoms by senescence which may be coincident with advanced stages of disease development or whether, in fact, yield losses in *B. napus* are not due to impaired plant water relations. Moreover, it is unknown whether vascular plugging by occlusions, which has been demonstrated previously in the hypocotyl of *B. napus* upon infection with *V. longisporum* (Eynck et al. 2009), may affect plant tolerance to drought.

Here, we present, for the first time, a comprehensive study exploring whether infection with *V. longisporum* compromises plant water relations in a susceptible variety of *B. napus*, whether drought stress affects plant responses to *V. longisporum*, and whether vascular infection by *V. longisporum* compromises drought-stress tolerance of *B. napus*.

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MATERIALS AND METHODS

Plant material and drought-stress treatments. Seed of the *V. longisporum*-susceptible winter oilseed rape ‘Falcon’ (NPZ)

were double surface sterilized with 70% ethanol for 2 min and rinsed with autoclaved tap water. Seeds were sown in multipot trays containing autoclaved silica sand and soil (1:1) and grown in a climate-controlled chamber (20°C, 16 h of light, and 60% relative humidity). Two weeks after sowing, at growth stage (GS) 12 (Meier 2001), temperature and day length were gradually decreased to 4°C and 10 h, respectively, and seedlings were subjected to vernalization for 13 weeks. Before inoculation, seedlings were acclimatized for 3 days at 10°C and 16 h of light. After inoculation, seedlings were transplanted to plastic pots (1.5 liter) filled with substrate, as described below.

First, the moisture content at field capacity (FC) of a soil/sand mix (3:1), hereafter called substrate, was determined according to Grzesiak et al. (2013). Briefly, plastic cylinders (2.5 cm in radius and 20 cm in height) with a drainage plastic net at the bottom were filled up to 3 cm with gravel (20 to 50 mm in diameter) in six replications and placed for 15 min in a container filled with tap water. Cylinders were covered with aluminum foil and placed on a wire grid for 15 min to drain excess water. Subsequently, weight was measured using an analytical balance (W_{t1}). Then, 150 g of oven-dried (105°C for 24 h) substrate was added to each cylinder and cylinders were placed in the water container for 2 h. Additional water was gently poured on the surface of the substrate until saturation. Cylinders were covered on the top and then placed on a wire grid and on absorbent paper for 15 min to drain the excess water. Then, cylinders were weighed a second time (W_{t2}). Substrate moisture content at this stage was assumed to be equal to FC and calculated as $[(W_{t2} - \text{substrate oven dry weight}) - (W_{t1})]$. This water status was considered the soil moisture at 100% FC and maintained throughout the experiment as the control. Accordingly, 60 and 30% FC were adjusted and referred to as moderate (60% FC) and severe (30% FC) drought-stress treatments, respectively.

The combination of the two experimental factors, disease (mock-inoculated and *V. longisporum*-inoculated) and watering levels (100, 60, and 30% FC), resulted in six treatments (Table 1). Treatments were arranged in a completely randomized design with three biological replicates, each composed of five plants independently grown in separate pots. Mock- or *V. longisporum*-inoculated plants supplied with water at 100% FC were used as the control. Plants were uniformly and regularly watered until three weeks after inoculation. Starting from 21 days postinoculation (DPI), water regimes in pots of both mock- and *V. longisporum*-inoculated plants were adjusted, considering the initial pot weight and weekly plant biomass increments (Grzesiak et al. 2013). Accordingly, plant biomass increment was determined weekly from 15 plants randomly selected from each treatment. For this purpose and other analyses that involved destructive sampling (leaf proline and relative water content; polymerase chain reaction [PCR]), at least 105 extra plants/treatment were included. Roots were carefully washed under running tap water and total fresh weight was determined. Water consumption in each pot was monitored daily by weighing the pots and adding the required amount of water, equivalent to the sum of pot weight loss and daily plant biomass increment, to maintain individual FC levels.

Fungal isolate, inoculation procedure, and assessment of disease and biometric traits. Inoculum was produced from a virulent isolate of *V. longisporum* (VL43) and inoculation was performed by root dip inoculation of vernalized seedlings (GS14), as described previously (Eynck et al. 2009). After inoculation, seedlings were transferred to pots filled with substrate (see above) and grown for 7 weeks under greenhouse conditions at 24°C, 16 h of light, and 70% relative humidity. Throughout the experiment period, a liquid nutrient mixture was equally supplied to all treatments. Disease severity was assessed weekly according to (Zeise 1992) and the net area under the disease progress curve (AUDPCnet) was determined as described previously (Eynck et al. 2009). Relative stunting was recorded as percentage of plant height of inoculated plants relative to mock-inoculated plants. Similarly,

multiple shoot induction (MSI) was determined by recording the number of primary shoot branches relative to mock control plants. *V. longisporum* colonization of hypocotyls was quantified by quantitative PCR (qPCR) analysis of three biological replicates each consisting of five independently grown plants, according to the method we developed recently (Knüfer et al. 2016). Plant dry matter (DM) yield and stem thickness were determined at the end of the experiment, 28 days after initiation of drought stress treatments (49 DPI). Stem thickness was measured with a digital caliper at the upper hypocotyl close to the stem base. Shoot (above the hypocotyls) and root dry weight were determined after oven drying (70°C for 24 h) of samples.

Measurement of physiological parameters. Photosynthesis rate (A), transpiration rate (E), and stomatal conductance of CO₂ (gs) were measured using a portable infrared gas analyzer LCpro+ (ADC BioScientific Ltd.). Measurements were performed in situ on the upper third fully developed leaf 1 week (28 DPI) and 4 weeks (49 DPI) after the start of drought-stress treatments, representing inflorescence emergence and end of flowering stages, respectively. The former time point marks sufficient acclimatization of transplanted seedlings, successful colonization of roots by the pathogen, and significant accumulation of infection responses in the plant (Eynck et al. 2009; Obermeier et al. 2013) and a critical time for systemic spread of *V. longisporum* in *B. napus* (Zhou et al. 2006). The second time point is the stage at which substantial natural senescence of leaves and branches starts, a symptom which overlaps with *V. longisporum*-induced damage. Furthermore, this time point marks the flowering stage, a GS at which *B. napus* is more prone to drought stress (Müller et al. 2010). Accordingly, this time point was considered as the last time point for the unbiased assessment of disease and physiological parameters. All measurements referred to a leaf surface area of 6.25 cm². Greenhouse conditions for photosynthetically active radiation measurement were set to 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 70% relative humidity, and CO₂ concentration of 500 $\mu\text{mol mol}^{-1}$ of air. Water use efficiency (WUE) was calculated from the ratio of A and E.

Relative leaf water content (RWC) was determined gravimetrically. Leaf discs (10 cm²) were excised from the third upper fully expanded leaf (excluding the midrib) and fresh weight was determined. To obtain turgid weight, leaf discs were immersed in double-distilled water for 3 h in the dark. Dry weight was obtained after oven drying overnight at 70°C. Percent RWC was calculated as described by Jones (2006).

Accumulation of leaf free proline content was determined according to Bates et al. (1973), with slight modifications. Briefly, the third upper fully expanded leaves were collected, immediately frozen in liquid nitrogen, lyophilized for 72 h (freeze dryer ALPHA 1-4; Martin Christ Gefriertrocknungsanlagen GmbH), and ground with a mixer mill (MM 200; Retsch GmbH). Subsequently, 100 mg of dry leaf powder were homogenized in 2 ml of 3% sulfosalicylic acid for 10 min at room temperature. Subsequently, 500 μl of the supernatant was amended with equal volumes of acid ninhydrin solution and glacial acetic acid in a water bath for 1 h at 100°C. To stop the reaction, tubes were placed on ice and proline was extracted by adding 800 μl of toluene into 1,200 μl of the reaction mixture. For quantification, 200 μl of the chromophore containing toluene was

TABLE 1. Experimental treatments and descriptions of independent variables^a

Number	Disease	Drought (%FC)	Treatment
1	Mock-inoculated	100	M1
2	Mock-inoculated	60	M2
3	Mock-inoculated	30	M3
4	<i>V. longisporum</i> -inoculated	100	VL1
5	<i>V. longisporum</i> -inoculated	60	VL2
6	<i>V. longisporum</i> -inoculated	30	VL3

^a FC = field capacity, 100% FC = optimal watering, 60% FC = moderate drought stress, and 30% FC = severe drought stress.

loaded on 96-well microtiter plates and absorbance was measured in triplicate at 520 nm using a Microplate Spectrophotometer (μ Quant; Bio-Tek Instruments). Toluene was used as a blank and concentration of proline in plant samples was determined by referring to the standard curve produced from known concentrations of L-proline treated the same way as leaf extract samples.

Characterization of drought-responsive genes. Genes encoding functional and regulatory proteins involved in drought stress tolerance or responses in *B. napus* were selected from previous studies. Gene-specific primers were either obtained from the literature or designed using primer3 software (Table 2). Optimization of PCR conditions was carried out in a gradient PCR in 25- μ l reaction volumes containing 10 ng of template DNA, 10 $\times$ *Pfu* buffer with MgSO₄ (2 mM), dNTP mix (0.2 mM each), 25 pmol of each primer, and 0.5 U of recombinant *Pfu* DNA polymerase (Thermo Fisher Scientific). Specific amplification of genes was verified on 1.2% (wt/vol) Tris-borate-EDTA agarose gel. Further confirmation of amplicon specificity was performed by cloning and sequence analysis of partial gene fragments (Table 3). Briefly, PCR products were purified from gel, cloned into pBluescript SK⁻ vector, and transferred to *Escherichia coli* DH5- α competent cells. Subsequently, plasmid DNA from transformed cells was isolated following the alkaline lysis procedure and presence of the correct insert in plasmid DNA was verified by colony PCR and *Pvu*II (Thermo Fisher Scientific) restriction digest analysis. Prior to sequencing, purification of miniprep plasmid DNA samples was performed using a SureClean kit (Bioline GmbH). Purified plasmid clones were sequenced using

the universal T7 promoter forward and reverse primers (Eurofins MWG Operon) and nucleotide sequence identity was verified by comparison with known gene sequences available at the National Center for Biotechnology Information GenBank database using blastn search (Table 3).

RNA was isolated from leaf samples collected from the third upper fully expanded leaf 7 days after the start of drought stress treatment (28 DPI), where effects of drought stress became apparent as demonstrated by physiological data. Leaf samples collected from five well-watered (100% FC) and drought-stressed (30% FC) plants (five plants per replication) were ground in liquid nitrogen and total RNA was isolated from 100 mg of tissue using TRI-Reagent (Sigma-Aldrich). RNA quality and concentration were measured using an Epoch microplate spectrophotometer (Epoch; BioTek Inc.). cDNA was synthesized from 1 μ g of total RNA samples using the QuantiTect Reverse Transcription Kit (Qiagen).

Real-time PCR was set up in 10 μ l of total reaction volume consisting of 5 μ l of Absolute Blue SYBR Green Fluorescein Mix (final 1 $\times$), 1 μ l of cDNA (10-fold dilution), and 1 μ l (10 pmol) of each forward and reverse gene-specific primers. Reverse-transcription (RT)-qPCR was performed with Absolute Blue SYBR Green Fluorescein Mix (ABgene) in a CFX384 real-time PCR detection system (Bio-Rad Laboratories, Inc.) with three technical replicates. The amplification conditions for all genes were 95°C for 15 min to activate Thermo-Start DNA polymerase; followed by 35 cycles of denaturation (95°C for 1 min), annealing (64°C, 30 s), and extension (72°C, 20 s); with a final elongation step of 10 min at 72°C. Melting curve analysis was performed from 65 to 95°C with a heating rate of 0.5°C per 5 s and continuous fluorescence measurement. To further ensure amplification specificity, RT-qPCR products were resolved on 1% agarose gel and the size of drought-responsive (DR) gene fragments was verified by comparison with a molecular weight marker. The relative expression of DR genes obtained from three biological replicates was determined according to Pfaffl (2001).

Data analysis. Because disease data were relative values derived from comparison with the respective mock-inoculated treatments and represented one factor, the influence of watering regimes on disease parameters was analyzed by one-way analysis of variance (ANOVA). Main and interactive effects of pathogen and drought stress on physiological and biometric parameters were determined by factorial ANOVA. Posthoc comparisons were made with Tukey's honestly significant difference test at a 5% level of significance. A *t* test with 95% confidential intervals was employed to analyze gene expression data. All ANOVA and *t* test analyses were performed using STATISTICA 12 (StatSoft, Inc.). The relationships among disease, physiological, and biometric parameters were analyzed with the Pearson product-moment correlation analysis (PROC CORR), which was performed with SAS 9.4 (SAS Institute, Inc.). Partial and combined effects of pathogen and drought-stress parameters on disease severity and physiological and biometric

TABLE 2. Sequences of primer pairs used in reverse-transcription polymerase chain reaction

Gene name	Primer sequence (5'-3')	Source
<i>BnActin</i>	GCCCAGAGTCTTGTTCAG (F) GAACCACCGATCCAGACACT (R)	This study
<i>BnCBF17</i>	AATGATCATGGCATGAACATGGC (F) ATCCTCGTCCATGTAAACCC (R)	Gao et al. 2002
<i>BnCIPK1</i>	GACCGTGGAAAGGACTCCATGAAGC ATC (F) TCTGGCGGCTTCCAGATGATGT (R)	This study
<i>DREB2-23</i>	GAGCTGTCCGAAGAAACCTG (F) ATAACTCCAAAGGGACACGTC (R)	This study
<i>BnHb6</i>	ATGATGAAGAGATTAAGCAGTTCA GA (F) GTGATCCTCCGTCTGCTCCA (R)	Yu et al. 2005
<i>BnP5CS1</i>	CGATTTGGACTTGGTGCTGA (F) GCCATCCTCTCTAGTCTC (R)	Xue et al. 2009
<i>BnP5CS2</i>	CCATTATCTTCTCTCTCAC (F) AACAAGCTGTGCTCCAA CC (R)	Xue et al. 2009
<i>BnLEA4-1</i>	ATGCAGTCGATGAAGGAAACAGC (F) CCGGCGGAGGATATACTGGATAA (R)	This study
<i>ME-leaN4</i>	GGCAAGGACAAGACTTCCCA (F) CGGATCAGTGCTCTGAGTAG (R)	Park et al. 2005

TABLE 3. Summary of drought-responsive gene partial sequence matches obtained by blastn searches of the GenBank

Gene name ^a	Best match; accession number	Identity (%)	E-value
<i>BnActin</i>	<i>Brassica napus</i> actin (ACT) mRNA, partial cds; DQ370142.1	98	2e-106
<i>BnCBF17</i>	<i>B. napus</i> clone 662 CBF-like protein mRNA, complete cds; AF370733	100	2e-90
<i>CIPK1</i>	<i>B. napus</i> CBL-interacting protein kinase (CIPK1) mRNA, partial cds; GU189585.1	98	2e-24
<i>BnDREB2-23</i>	<i>B. napus</i> DREB2-2 mRNA, complete cds; AY444874.1	99	0.0
<i>BnHb6</i>	<i>B. rapa</i> subsp. <i>pekinensis</i> hb-6-like protein mRNA, complete cds; AF268422.1	97	2e-148
<i>BnP5SC1</i>	<i>B. napus</i> Δ 1-pyrroline-5-carboxylate synthetase A mRNA, complete cds; AF314811.1	98	4e-82
<i>BnP5SC2</i>	<i>B. napus</i> Δ 1-pyrroline-5-carboxylate synthetase B mRNA, complete cds; AF314812.1	100	1e-69
<i>BnLEA4-1</i>	<i>B. napus</i> group 1 late embryogenesis abundant protein mRNA, complete cds; AY572958.1	97	0.0
<i>ME-leaN4</i>	<i>B. napus</i> ME-leaN4 mRNA for late embryogenesis-abundant protein, complete cds; AB083361.1	97	0.0

^a Gene annotations: *BnActin* = *B. napus* actin, *BnCBF17* = *B. napus* C-repeat binding factor, *CIPK1* = *B. napus* calcineurin B-like-interacting protein kinase, *BnDREB2-23* = *B. napus* drought-responsive element binding factor, *BnHb6* = *B. napus* homeodomain leucine-zipper, *BnP5SC1* = *B. napus* Δ 1-pyrroline-5-carboxylate synthetase A, *BnP5SC2* = *B. napus* Δ 1-pyrroline-5-carboxylate synthetase B, *BnLEA4-1* = *B. napus* group 4 late embryogenesis abundant protein, and *ME-leaN4* = *B. napus* group 3 late embryogenesis abundant protein.

traits were determined by the PROC REG stepwise multiple linear regression procedure implemented in SAS 9.4.

RESULTS

Disease symptoms caused by *V. longisporum*. Two weeks after inoculation, symptoms induced by *V. longisporum* in the greenhouse on susceptible *B. napus* Falcon consisted of either half-sided or interveinal chlorosis with dark veins, shriveling and deformation of leaves, severe stunting, and induction of multiple shoot branching (Fig. 1). One week later (21 DPI), senescence of lower leaves and branches and discoloration on hypocotyl cross sections became visible. At plant maturity (84 DPI), microsclerotia were produced in the pith and typical symptoms of vascular discoloration occurred (Fig. 1D). In the field, disease symptoms are different and become visible only at crop maturity stages, when the fungus proliferates from systemically colonized xylem into the entire shoot parenchyma (BBCH 80). Typical yellow to brown to gray longitudinal stripes on main stems which have recently given this disease the name *Verticillium* stem striping (Depotter et al. 2016) are shown in Figure 1G. Shortly before harvest, stem, branch, or even root epidermal tissues start peeling off, ultimately leading to disintegrated pith filled with dark microsclerotia

(Fig. 1H). A unique and striking feature of *V. longisporum* disease phenology in oilseed rape is the entire lack of wilting symptoms on infected plants in both the greenhouse and field (Fig. 1).

Effects of drought stress and *V. longisporum* on oilseed rape growth. To better understand mechanisms of how *V. longisporum* damages its host and how drought conditions may modulate these effects, alterations in plant growth (stem thickness and DM) of differentially watered mock- and *V. longisporum*-infected plants were assessed. The significant effect of *V. longisporum* ($P = 0.000$) on stem thickness was evident at all watering regimes (Supplementary Table S1). *V. longisporum* alone reduced stem thickness by 14%. Together with drought, the combined stress increased stem diameter reduction to 27 to 30%. Stem growth reduction due to *V. longisporum* was more pronounced at well-watered (100 and 60% FC) than drought (30% FC) conditions (Fig. 2).

Significant effects of *V. longisporum* ($P = 0.000$), drought ($P \leq 0.012$), and their interaction ($P \leq 0.032$) were also recorded on shoot and root dry biomass. Under optimal growth conditions, more than half of the shoot DM was reduced by infection (Fig. 2). This was substantially higher compared with the sole effect of drought, which caused 5.1 and 40.7% biomass reductions at 60 and 30% FC treatments, respectively. The differential effects of *V. longisporum* and drought showed even

Fig. 1. Typical symptoms of *Verticillium* stem striping in *Brassica napus*. **A–F**, Symptoms on inoculated plants in greenhouse conditions at 28 (A and B) and 49 (C, D, E, and F) days postinoculation (DPI). **G and H**, Symptoms on naturally infested plants in the field after growth stage BBCH 89. **A**, One-sided leaf chlorosis at 28 DPI. **B**, Irregularly shaped or interveinal chlorosis on leaf with dark veins. **C**, Leaf shriveling and deformation. **D**, Hypocotyl cross-section showing vascular discoloration. **E**, Stunting: control (left) and inoculated plant (right). **F**, Multiple shoot induction. **G**, Yellow, brown, or gray longitudinal lesions on main stem. **H**, Stem longitudinal section showing peeling off of epidermis and disintegrated dark pith filled with masses of black microsclerotia (arrows).

more contrast with root DM. Accordingly, compared with the single effect of *V. longisporum*, which caused 73.9% root reduction, the sole effects of moderate and severe drought were 15.4- and 0.7-fold lower, respectively. Similar to well-watered plants, *V. longisporum*-induced shoot DM reduction in drought-stressed plants was also significant, resulting in comparable losses at 60% FC (63.2%) and 30% FC (51.4%) treatments. Conversely, no effect of drought stress on root DM of infected plants was observed (Fig. 2). This is possibly due to the stronger effect of *V. longisporum* than drought stress on growth. In general, simultaneous presence of *V. longisporum* and drought stress causes larger agronomic damage than the effects of each single stress factor.

Drought-stress effects on *V. longisporum* disease severity. Disease progress as measured by AUDPCnet values showed similar, drought-independent increments over time at all watering levels, indicating that drought stress added at 21 DPI caused no significant alteration (Supplementary Table S2) of disease development (Fig. 3A, B and C). Similarly, molecular analysis of disease severity by qPCR revealed a drought-independent increase of plant colonization with *V. longisporum* over time (Fig. 3D). Likewise, assessment of stunting, typically induced by *V. longisporum* in controlled environmental conditions, indicated that infection induced a significant reduction of plant height (up to 75%) regardless of watering regimes and crop developmental stage (Fig. 3E). This pattern of effects was finally confirmed by MSI, which was solely induced by *V. longisporum*, an effect not found due to different watering levels (Fig. 3F).

Fig. 2. Single and combined effects of infection with *Verticillium longisporum* and drought stress on **A**, dry biomass yield and **B**, stem thickness of a *V. longisporum*-susceptible *Brassica napus* cultivar 28 days after initiation drought stress (49 days postinoculation). FC = field capacity, 100% FC = optimal watering, 60% FC = moderate drought stress, 30% FC = severe drought stress, M = mock-inoculated control, and VL = *V. longisporum* infected. Normally watered (100% FC) mock- or *V. longisporum*-inoculated plants were used as control. Means obtained from 30 plants are presented. Bars indicate standard deviations. Different letters on bars with the same letter case indicate significant differences (Tukey's honestly significant difference test; $P = 0.05$).

Gas exchange and WUE. Leaf gas exchange measurements (E, gs, and A) revealed significant effects ($P \leq 0.001$) of water supply, whereas *V. longisporum*, considered either as a single factor or in its interaction with drought, had no significant impact on any of these parameters (Supplementary Table S3), which was largely consistent at both time points analyzed (28 and 49 DPI) (Table 4; Fig. 4). At 7 weeks after initiation of drought treatments (49 DPI), when significant disease severity was evident, average E in normally watered control and infected plants were nearly the same, with 1.76 and 1.63 mmol m⁻² s⁻¹, respectively. At 60 and 30% FC, similar rates of reduction in E were recorded in infected and noninfected plants, resulting in 0.84 and 0.36 mmol m⁻² s⁻² in the control and 0.56 and 0.52 mmol m⁻² s⁻² in infected plants, respectively (Fig. 4E). Similarly, plant infection by *V. longisporum* did not aggravate drought-induced reduction of gs (Fig. 4B and F) or A (Fig. 4C and G) at any of the watering conditions. Watering at 60 and 30% FC substantially reduced A but this was unaffected by plant infection with *V. longisporum*, except for 60% FC at the early time point.

Drought stress generally caused an increase of WUE. Interestingly, addition of the pathogen did not significantly alter this effect (Fig. 4D and H). Nevertheless, the rate of drought-induced increase in WUE seemed to be dependent on plant age because, in infected older plants (49 DPI), WUE was more strongly enhanced by reduced watering levels than in younger (28 DPI) plants (Fig. 4D and H).

Leaf water content and proline accumulation. Drought-induced reduction in leaf water content (LWC) was apparent but significant ($P = 0.001$) reductions (up to 12%) were only observed in plants exposed to severe drought conditions. Consistent with the effects on gas exchange parameters and WUE, plant infection by *V. longisporum* did not significantly affect LWC and no interaction of both stresses was observed (Fig. 5B).

Leaf proline contents in fully watered mock and inoculated plants a week after exposure to drought stress (28 DPI) were exactly the same at 3.3 μmol/g. Regardless of infection, moderate drought stress caused more than threefold proline increase. Whenever severe drought stress was applied, proline content was increased up to 15-fold, again regardless of plant infection with *V. longisporum*. At 49 DPI, the most pronounced effect was caused by drought. In general, analysis of leaf proline content revealed strong responses (3- to 15-fold increases) to drought stress but no consistent effects of *V. longisporum* were recorded, indicating proline to be a sensitive marker of low water supply in plants but not of vascular infection with *V. longisporum* (Fig. 5A).

Expression of DR genes. The expression of DR genes in mock- and *V. longisporum*-inoculated plants was analyzed by comparing transcript levels in severely drought-stressed (30% FC) with well-watered (100% FC) plants. Before RT-qPCR analysis, sequence identity of eight DR genes was verified by sequencing of cloned PCR products (Table 3). Analysis of relative gene expression normalized to *BnActin* (Pfaffl 2001) showed up to sixfold increased gene expression induced by severe drought treatment. *P5SC1* (which regulates proline biosynthesis and transcription factors *DREB2-23* and *CIPK1*, which control the expression of stress-responsive genes via the abscisic acid (ABA)-independent pathway) showed the highest levels of expression. Compared with the aforementioned three genes, however, expression of *P5SC2*, *CBF17*, and *HB6* was relatively lower, at about a onefold level of increase. In contrast, none of the tested genes showed significant transcriptional differences between mock- and *V. longisporum*-inoculated plants (Fig. 6). RT-qPCR amplification for the two late embryogenesis abundant genes (*BnLEA4-1* and *ME-lea14*) was not successful.

Regression analysis for effects of *V. longisporum* and drought stress on disease severity and physiological and biometric plant responses. Stepwise regression analysis revealed specific and independent effects of *V. longisporum* ($r^2 = 0.87$ to 0.99) on disease severity and of drought ($r^2 = 0.65$ to 0.95) on physiological stress responses of *B. napus*, whereas no significant inverse relationships were found. Both stress factors exerted negative effects on plant growth

(biometric) parameters. However, inhibition of plant growth by *V. longisporum* was much stronger than by severe drought (Table 4).

Correlation of disease parameters with physiological and biometric plant responses. There was a high and significant ($P = 0.000$) correlation among all four disease parameters ($r = 0.89$ to 0.99). Although these parameters were unrelated to any of the physiological parameters related to plant drought stress, they were in a significantly negative relationship with the four biometric variables (Table 5). Among these, plant height was most negatively affected, corroborating the pronounced stunting effect induced by *V. longisporum* in *B. napus*.

DISCUSSION

***V. longisporum* induces a nonwilt vascular syndrome in *B. napus*.** Since the discovery of *V. longisporum* as a vascular, host-specialized pathogen on members of the Brassicaceae family (Zeise and von Tiedemann 2002), it has been noted that wilting symptoms that are commonly induced by xylem-invading *Verticillium* spp. or *Fusarium oxysporum* are lacking in oilseed rape. Instead, symptoms induced by *V. longisporum* in the greenhouse comprise chlorosis, stunting, dark veins, induction of multiple shoots, leaf deformation, and shriveling or curling of leaves (Fig. 1), whereas brown stripes on stems and premature ripening are the typical disease

symptoms in the field (Depotter et al. 2016; Knüfer et al. 2016). The lack of wilting symptoms in oilseed rape prompted the present study with the aim to confirm such phenotypic observations by analysis of physiological parameters and expression of genes related to plant water relations. This aspect also has considerable agronomic significance as to whether *V. longisporum* may affect WUE and enhance sensitivity of *B. napus* to drought stress.

The present study provides clear physiological and molecular evidence that *V. longisporum* lacks an effect on water relations in *B. napus*, in contrast to the effects of drought stress. Failure of *V. longisporum* to induce wilt phenotypes has been previously observed in *B. napus* (Dunker et al. 2008; Floerl et al. 2008) and *Arabidopsis* (Floerl et al. 2010; Reusche et al. 2012). In addition to various known genetic, morphological, and host range differences (Stevenson et al. 2002; Zeise and von Tiedemann 2002), this is a further important difference which separates *V. longisporum* from its related species *V. dahliae*. Therefore, the name ‘wilt’ used thus far for the disease induced by *V. longisporum* (Happstadius et al. 2003; Stevenson et al. 2002; Tyvaert et al. 2014) is inadequate and should be abandoned, and the disease renamed as *Verticillium* stem striping, as proposed recently (Depotter et al. 2016).

Drought stress and *V. longisporum* impair plant growth by different mechanisms. Drought stress has the potential to

Fig. 3. *Verticillium longisporum* disease severity in susceptible *Brassica napus* Falcon plants under optimal watering and moderate or severe drought-stress conditions, as determined by molecular and phenotypic disease parameters. **A, B, and C,** Net area under the disease progress curve (AUDPC) values generated from weekly disease index measurements. Mean net AUDPC $\pm$ standard deviations 28 days after initiation of drought-stress treatments (49 days postinoculation [DPI]) are shown. **D,** Quantitative polymerase chain reaction quantification of fungal DNA in hypocotyl. **E,** Relative stunting in inoculated plants relative to the plant height of mock-inoculated plants. **F,** Fold increase in multiple shoot induction relative to mock-inoculated plants. FC = field capacity, 100% FC = optimal watering, 60% FC = moderate drought stress, 30% FC = severe drought stress, VL = *V. longisporum*, DM = dry matter, and MSI = multiple shoot induction. Means obtained from 30 plants are presented. Bars indicate standard deviations. Values with different letters indicate significant differences (Tukey's honestly significant difference test; $P = 0.05$); ns = not significant.

alter plant responses to pathogens in various ways, which may result in either increased or decreased disease severity (Ramegowda and Senthil-Kumar 2015). In the present study, disease levels, as quantified by AUDPC obtained from weekly disease scorings, revealed no significant interaction of disease progress with drought stress at either early or late plant GS (Table 4). Likewise, fungal DNA in plants, relative stunting, and the induction of multiple shoots were not affected by exposure to different intensities of drought stress, indicating no interaction between infection with *V. longisporum* and water relations in the plants. A similar induction of branching by *V. longisporum* in *B. napus* has been reported previously (Zeise 1992). Häffner et al. (2010) also identified two quantitative trait loci that control *V. longisporum*-induced excessive branching in *Arabidopsis*.

Although the watering level did not significantly affect any of the disease parameters, its effect on plant growth parameters was significant and similar to infection with *V. longisporum*. All biometric measurements such as plant height, stem thickness, and shoot and root DM responded negatively to either drought stress or infection with *V. longisporum* (Table 4). The decline in plant DM or stem thickness induced by reduced water supply was similar in inoculated and healthy plants, again indicating an independent action of the two stresses. It is well known that *B. napus* plants exposed to drought stress display reduced plant growth (Khalili et al. 2012). However, although such effects of drought stress and vascular infection on plant biometric parameters were apparently similar, the underlying physiological mechanisms appear to be different and independent from each other. This is verified by a conspicuous lack of correlation of disease variables with any of the physiological parameters analyzed (Table 5), whereas these parameters showed significant responses to drought (Table 4).

Physiological responses of *B. napus* to *V. longisporum* and drought stress. A common characteristic of vascular wilt pathogens is impairment of water-conducting functions of xylem vessels, resulting in physiological effects similar to those induced by drought (Hashem et al. 1998; Naderikharaji et al. 2008; Sangtarash et al. 2009). However, the present study reveals differential responses of physiological parameters to drought stress and infection with *V. longisporum*. A clear and significant negative correlation with

reduced watering levels was found for E, gs, A, and LWC, indicating that these parameters are suitable determinants of drought stress in oilseed rape. LWC is considered a reliable indicator of the plant water status and its adaptation to drought stress (Jones 2006), and previous studies with *B. napus* reported up to 40% decrease under drought-stress conditions (Khalili et al. 2012). Accordingly, the impact of drought on *B. napus* LWC was particularly significant under severe stress conditions.

Likewise, WUE and leaf proline content significantly increased at increasing water shortage. Accumulation of free proline has multiple positive roles in stress adaptation, recovery, and signaling as a response to a wide range of biotic and abiotic stresses in different organisms. It is involved in intracellular osmotic adjustment between cytoplasm and vacuole, protects chloroplasts, and regulates plant growth and development during stress conditions (Szabados and Savaure 2010). In pepper plants, proline accumulation has been considered a sensor of wilt damage caused by *V. dahliae* (Goicoechea et al. 2000).

Interestingly, none of the physiological determinants of plant water relations or the proline content were affected by *V. longisporum*, and a factorial ANOVA for single and interactive effects revealed that *V. longisporum* did not cause any significant changes in these physiological variables either alone or via interaction with drought. Consequently, there were no additive negative effects recorded for *V. longisporum* and drought stress on water relations and growth in *B. napus*.

Water-conducting functions of xylem vessels may be impaired either directly by accumulation of mycelium or due to host reactions resulting in xylem occlusions such as tyloses or embolism (Yadeta and Thomma 2013). Examples are Panama disease of banana caused by *F. oxysporum* f. sp. *cubense* (Li et al. 2015), Pierce's disease of grapevine, alfalfa dwarf disease caused by the bacterial pathogen *Xylella fastidiosa* (Pérez-Donoso et al. 2007), or Dutch elm disease caused by *Ophiostoma* spp. (Santini and Faccoli 2015). From earlier histological studies (Knüfer 2013), it is known that *V. longisporum* is constricted to a relatively small number of functional vessels in *B. napus*, which probably limits the potential of an overall impairment of plant water transport. In addition, pathogen-derived phytotoxins that have been demonstrated to act as

TABLE 4. Stepwise multiple linear regression analysis of effects of *Verticillium longisporum* (VL) and severe drought stress (SDS) on disease expression and physiological and biometric traits of *Brassica napus* Falcon at 7 and 28 days after initiation of drought treatments (28 and 49 days postinoculation [DPI], respectively)^a

Parameters ^b	28 DPI				49 DPI			
	<i>b</i>		Partial <i>r</i> ²		<i>b</i>		Partial <i>r</i> ²	
	VL	SDS	VL	SDS	VL	SDS	VL	SDS
Disease								
AUDPC	33.5***	NS	0.97***	NS	73.5***	NS	0.97***	NS
DNA	700.5***	NS	0.90***	NS	967.7***	NS	0.92***	NS
RS	71.2***	NS	0.99***	NS	65.6***	NS	0.99***	NS
MSI	3.64***	NS	0.87***	NS	5.5***	NS	0.95***	NS
Physiological								
E	NS	-1.5***	NS	0.83***	NS	-1.3***	NS	0.80***
gs	NS	-0.23***	NS	0.93***	NS	-0.95***	NS	0.73***
A	NS	-9.7***	NS	0.92***	NS	-6.4***	NS	0.88***
WUE	NS	4.2***	NS	0.88***	NS	5.9*	NS	0.49*
RWC	NS	-8.2**	NS	0.65**	NS	-10.2***	NS	0.71***
LPC	NS	39.1***	NS	0.95***	NS	26.2***	NS	0.96***
Biometric								
PH	-44.4***	NS	0.92***	NS	-81.5***	-22.6**	0.90***	0.07**
HD	ND	ND	ND	ND	-1.54***	-1.03*	0.29***	0.64*
SDM	-8.4***	-1.6*	0.92***	0.03*	-7.9***	-5.2***	0.65**	0.28**
RDM	-2.2***	-0.95**	0.78***	0.05**	-2.9***	-1.3*	0.74***	0.13*

^a Analyses: *b* = coefficient of determination and *r*² = partial regression coefficient. NS = not significant; ND = not determined; and *, **, and *** indicate significant at *P* = 0.05, 0.01, and 0.001, respectively. Analysis was performed using mean data obtained from fully irrigated (100% field capacity [FC]) and severely stressed (30% FC) treatments (PROC REG procedure, SAS 9.4; SAS Institute, Inc.).

^b AUDPC = net area under disease progress curve value, DNA = *V. longisporum* DNA in hypocotyl, RS = relative stunting, MSI = multiple shoot induction, E = transpiration rate, gs = stomatal conductance of CO₂, A = photosynthesis rate, WUE = water use efficiency, RWC = relative leaf water content, LPC = leaf proline content, PH = plant height, HD = hypocotyl diameter, SDM = shoot dry matter, and RDM = root dry matter.

wilting factors in various *Verticillium* diseases (Palmer et al. 2005; Wang et al. 2004) have, thus far, not been found to be produced by *V. longisporum* (Aroud 2013). Therefore, we conclude that the lack of impairment of water relations by this pathogen is due to failure to colonize and occlude a sufficiently large proportion of the xylem (Eynck et al. 2009), even at high disease severity levels, and a lack of wilting toxins.

The *V. longisporum*-induced slight reductions of gas exchange and WUE, mainly observed at full irrigation (100% FC), were not significant (Fig. 4). The consistency among disease parameters and their impact on plant growth but not on physiological parameters responsive to drought stress was further confirmed by Pearson correlation analysis (Table 5) that showed a significant and positive relationship ($r = 0.89$ to 0.97) among phenotypic

Fig. 4. Changes in gas exchange and water use efficiency (WUE) in a *Verticillium longisporum*-susceptible *Brassica napus* cultivar subjected to *V. longisporum* infection or drought stress under greenhouse conditions. **A–D**, One week after initiation of drought treatments (28 days postinoculation [DPI]). **E–H**, Four weeks after initiation of drought treatments (49 DPI). E = transpiration rate, g_s = stomatal conductance of CO_2 , and A = photosynthesis rate. Normally watered (100% field capacity [FC]) mock- or *V. longisporum*-inoculated plants were used as control. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress. Mean data obtained from 30 plants are presented. Different letters on bars indicate significant differences (Tukey's honestly significant difference test; $P = 0.05$). M = mock control, noninoculated, and VL = *V. longisporum*-infected. Treatments followed by the numbers 1, 2, and 3 refer to watering at 100, 60, and 30% FC, respectively; SE = standard error and SD = standard deviation.

Fig. 5. Single and combined effects of infection with *Verticillium longisporum* and drought stress on **A**, leaf proline and **B**, relative water content of a *V. longisporum*-susceptible *Brassica napus* cultivar 7 and 28 days after initiation of moderate or severe drought-stress treatments at 28 and 49 days postinoculation (DPI), respectively. FC = field capacity, 100% FC = optimal watering, 60% FC = moderate drought stress, 30% FC = severe drought stress, Mock = noninoculated control, VL = *V. longisporum* infected, and DM = dry matter. Normally watered mock- or *V. longisporum*-inoculated plants were used as control. Mean data obtained from 30 plants are presented. Bars indicate standard deviations. Different letters on bars with the same letter case indicate significant differences (Tukey's honestly significant difference test; $P = 0.05$).

Fig. 6. Reverse-transcription polymerase chain reaction analysis of expression of drought-inducible genes in a *Verticillium longisporum*-susceptible *Brassica napus* cultivar subjected to drought stress or infection with *V. longisporum*. Total RNA was extracted from leaf samples collected 7 days after exposure to drought stress (28 days postinoculation). Data show the fold changes in gene expression in severely drought stressed (30% field capacity) versus optimally watered (100% field capacity) mock-infected (black bars) and *V. longisporum*-infected (gray bars) plants. Expression levels were normalized against *BnActin* according to Pfaffl (2001). Error bars represent standard deviations of means obtained from three independent biological replicates each consisting of 10 plants. Mock = control noninoculated, VL = *V. longisporum* infected, DREB2-23 = drought-responsive element binding factor, P5SC1 = $\Delta 1$ -pyrroline-5-carboxylate synthetase A, CIPK1 = calcineurin B-like-interacting protein kinase, P5SC2 = $\Delta 1$ -pyrroline-5-carboxylate synthetase B, HB6 = homeodomain leucine-zipper, and CBF17 = C-repeat binding factor. A significant change in gene expression between control and infected plants was detected for no gene (t test; $P = 0.05$).

and molecular disease parameters and significant negative correlations ($r = -0.64$ to -0.96) between disease severity and biometric traits.

These results are in agreement with previous reports, which showed no impact of *V. longisporum* on A and E in *B. napus* (Floerl et al. 2008). Similar findings were obtained in a study on *Arabidopsis*, where no negative impact of *V. longisporum* on plant water and nutrient relations occurred (Floerl et al. 2010). Such observations

have also been reported in several crops infected with various other *Verticillium* spp. In early developmental stages of potato, *V. dahliae* alone and in interaction with drought had no significant impact on E and gs (Haverkort et al. 1990). In sunflower, gs was unaffected by infection with *V. dahliae* (Sadras et al. 2000). Infection of alfalfa with *V. albo-atrum* caused no significant reduction in gs and A (Pennypacker et al. 1991). Similarly, Reusche et al. (2014) reported no effect of the wilt-inducing vascular pathogen *V. dahliae* on LWC of *Arabidopsis*. Reusche et al. (2012), however, demonstrated improved LWC in infected *Arabidopsis thaliana* plants caused by a de novo formation of xylem.

In contrast to these findings, a significant reduction of gas exchange rates due to *V. dahliae*-induced drought stress was reported in several crop species, including potato (Bowden et al. 1990), pepper (Goicoechea et al. 2000), and cotton (Hampton et al. 1990). Such contrasts may indicate that secretion of effective wilting toxins plays a crucial role in wilt induction by vascular fungal pathogens such as *V. albo-atrum* causing stomatal closure and inhibition of A in infected tomato (Lorenzini et al. 1997).

Responses of drought-inducible genes to drought stress and infection with *V. longisporum*. RT-PCR analysis of six drought-inducible marker genes showed up to sixfold increases of gene expression under severe drought stress, regardless of infection with *V. longisporum* (Fig. 6). This further confirms our physiological data that showed a similar and significant physiological impact by drought on both mock- and *V. longisporum*-inoculated plants. The transcription factor encoding *DREB2-23* and *CIPK1* genes that control expression of stress-responsive genes via an ABA-independent pathway (Yang et al. 2010) and *P5SC1*, which regulates proline biosynthesis in the cytosol and chloroplast (Szabados and Savaure 2010), showed the strongest relative increase in expression. Similarly, studies in *B. napus* have shown dehydration-induced increase of expression of *DREB2-23* (Kagale et al. 2007) and *P5SC1* (Xue et al. 2009). In *Arabidopsis*, expression of *DREB2A* under drought conditions was unaffected by infection with *V. dahliae* but showed a reduced expression upon infection with *V. longisporum* (Reusche et al. 2014). Chen et al. (2012) reported increased expression of *CIPK* genes in *B. napus* due to salt stress, and the overexpression of these genes in *Arabidopsis* led to enhanced salt tolerance. On the other hand, the relative expression of *P5SC2*, *CBF17*, and *HB6* was very low. *P5SC2* is one of the key regulators of proline biosynthesis in the mitochondria (Szabados and Savaure 2010). *CBF17* is a family of *DREB1* transcription factors which is known to be mainly induced by cold stress (Gao et al. 2002). The expression of *HB6* was only slightly increased by drought stress, whereas it was unaffected

TABLE 5. Pearson product-moment correlation (*r*) coefficients and significant levels of relationships among disease parameters and between disease and physiological or biometric responses of *Brassica napus* Falcon subjected to *Verticillium longisporum* or different levels of drought stress 7 and 28 days after initiation of drought treatments (28 and 49 days postinoculation [DPI], respectively)^a

Parameters ^b	28 DPI				49 DPI			
	AUDPC	DNA	RS	MSI	AUDPC	DNA	RS	MSI
Disease								
DNA	0.92***				0.97***			
RS	0.99***	0.95***			0.98***	0.95***		
MSI	0.89***	0.95***	0.91***		0.97***	0.96***	0.97***	
Physiological								
E	NS	NS	NS	NS	NS	NS	NS	NS
gs	NS	NS	NS	NS	NS	NS	NS	NS
A	NS	NS	NS	NS	NS	NS	NS	NS
WUE	NS	NS	NS	NS	NS	NS	NS	NS
RWC	NS	NS	NS	NS	NS	NS	NS	NS
LPC	NS	NS	NS	NS	NS	NS	NS	NS
Biometric								
PH	-0.96***	-0.92***	-0.97***	-0.88***	-0.94***	-0.93***	-0.96***	-0.92***
HD	ND	ND	ND	ND	-0.65**	-0.70**	-0.66**	-0.64**
SDM	-0.96***	-0.91***	-0.96***	-0.89***	-0.84***	-0.86***	-0.86***	-0.82***
RDM	-0.86***	-0.83***	-0.87***	-0.80***	-0.88***	-0.87***	-0.90***	-0.86***

^a AUDPC = net area under disease progress curve value, DNA = *V. longisporum* DNA in hypocotyl, and RS = relative stunting. NS = not significant; ND = not determined; and *, **, and *** indicate significant at *P* = 0.05, 0.01, and 0.001, respectively. Analysis was performed using the PROC CORR procedure (SAS 9.4; SAS Institute, Inc.).

^b MSI = multiple shoot induction, E = transpiration rate, gs = stomatal conductance of CO₂, A = photosynthesis rate, WUE = water use efficiency, RWC = relative leaf water content, LPC = leaf proline content, PH = plant height, HD = hypocotyl diameter, SDM = shoot dry matter, and RDM = root dry matter.

by drought stress when plants were infected with *V. longisporum*. This may explain findings in a previous report where infection with *V. longisporum* induced improved drought tolerance in *Arabidopsis* drought-stress reporter lines harboring the *HB* gene (Reusche et al. 2012). In *B. napus*, *BnHB6* is a late-responsive single-copy gene whose expression varies in a time-dependent manner (Yu et al. 2005). *HB* genes are members of the large family of homeodomain leucine-zipper transcription factors, which are unique to the plant kingdom. These genes regulate development and maintenance of outer cell layers of plant organs, thus playing an important role in plant defense against abiotic stress, including drought and salinity (Chew et al. 2013). Overall, gene expression data are consistent with phenotypic and physiological data, indicating no induction by infection with *V. longisporum* and no interaction between drought and *V. longisporum*-induced stress, thus implying a pathogenic impact of *V. longisporum* infection on *B. napus* independent from impairment of plant water relations.

This study provides physiological and molecular genetic explanations for why *V. longisporum* fails to induce wilt on *B. napus*, which distinguishes it from most other vascular pathogens. *V. longisporum* did not affect physiological markers of drought stress and, thus, plant damage induced by *V. longisporum* is not related to impairment of plant water relations. An important conclusion for oilseed rape cultivation is that severity of *Verticillium* stem striping is unaffected by drought-stress conditions and sensitivity to drought stress is not aggravated by *V. longisporum*. Although strong evidence is presented that drought stress and vascular infection with *V. longisporum* impair plant growth by different mechanisms, it remains to be determined by which other factors *V. longisporum* may induce crop loss.

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