Leptosphaeria maculans* effector AvrLm4-7 affects salicylic acid (SA) and ethylene (ET) signalling and hydrogen peroxide (H₂O₂) accumulation in *Brassica napus

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SUMMARY

To achieve host colonization, successful pathogens need to overcome plant basal defences. For this, (hemi)biotrophic pathogens secrete effectors that interfere with a range of physiological processes of the host plant. AvrLm4-7 is one of the cloned effectors from the hemibiotrophic fungus *Leptosphaeria maculans* 'brassicaceae' infecting mainly oilseed rape (*Brassica napus*). Although its mode of action is still unknown, AvrLm4-7 is strongly involved in *L. maculans* virulence. Here, we investigated the effect of AvrLm4-7 on plant defence responses in a susceptible cultivar of *B. napus*. Using two isogenic *L. maculans* isolates differing in the presence of a functional AvrLm4-7 allele [absence ('a4a7') and presence ('A4A7') of the allele], the plant hormone concentrations, defence-related gene transcription and reactive oxygen species (ROS) accumulation were analysed in infected *B. napus* cotyledons. Various components of the plant immune system were affected. Infection with the 'A4A7' isolate caused suppression of salicylic acid- and ethylene-dependent signalling, the pathways regulating an effective defence against *L. maculans* infection. Furthermore, ROS accumulation was decreased in cotyledons infected with the 'A4A7' isolate. Treatment with an antioxidant agent, ascorbic acid, increased the aggressiveness of the 'a4a7' *L. maculans* isolate, but not that of the 'A4A7' isolate. Together, our results suggest that the increased aggressiveness of the 'A4A7' *L. maculans* isolate could be caused by defects in ROS-dependent defence and/or linked to suppressed SA and ET signalling. This is the first study to provide insights into the manipulation of *B. napus* defence responses by an effector of *L. maculans*.

Keywords: AvrLm4-7, *Brassica napus*, effector, ethylene, *Leptosphaeria*, ROS, salicylic acid.

INTRODUCTION

Plant immunity consists of multi-layered defence responses (Chisholm *et al.*, 2006) as represented by the 'zig-zag' model designed by Jones and Dangl (2006). The first layer is based on the recognition of pathogen- (or microbe-) associated molecular patterns (PAMPs or MAMPs), components common to whole classes of microbes, by surface transmembrane pattern recognition receptors (PRRs). The perception activates PAMP-triggered immunity (PTI), also referred to as basal innate immunity. However, adapted pathogens have acquired effector proteins that overcome PTI. This phase in the plant–pathogen interaction is called effector-triggered susceptibility (ETS) and leads to a compatible interaction (i.e. susceptibility of the plant). In the co-evolutionary arms race, plants have struck back by the recognition of these effectors through additional intracellular receptors. This second layer of plant immunity is called effector-triggered immunity (ETI) (Dodds and Rathjen, 2010) and usually provides an incompatible interaction (i.e. resistance of the plant and avirulence of the pathogen). As the effectors are recognized by plant immunity on ETI, they are termed avirulence (*Avr*) effectors encoded by *Avr* genes. A plethora of cellular events follows the activation of both PTI and ETI: a rapid influx of calcium ions, an oxidative burst characterized by the production of reactive oxygen species (ROS), hormonal changes and transcriptional reprogramming. Often, ETI is followed by localized cell death in the form of a hypersensitive response (HR) (Dodds and Rathjen, 2010; Tsuda and Katagiri, 2010).

The key components of the plant immune system are the plant hormones that operate signal transduction after the perception of a pathogen (Pieterse *et al.*, 2009). Although salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the principal defence hormones in plants, other phytohormones, such as abscisic acid (ABA), auxin (indole-3-acetic acid, IAA), cytokinins (CKs), brassinosteroids (BRs) and gibberellins (GAs), also intervene in plant defences. Hormonal homeostasis and crosstalk between signalling pathways are crucial for the fine regulation of plant immunity (Pieterse *et al.*, 2012; Robert-Seilaniantz *et al.*, 2011). The changes in phytohormone

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levels (or sensing) lead to transcriptional reprogramming to favour defence over other cellular processes, such as growth and development (Buscaill and Rivas, 2014; Denancé *et al.*, 2013; Robert-Seilanianz *et al.*, 2011). SA, JA and ET signalling can all be activated during some cases of PTI and ETI (Tsuda and Katagiri, 2010). Although there are exceptions, SA signalling is generally implicated in defence against biotrophs, the types of pathogen that grow and retrieve nutrients from living plant tissue, whereas JA/ET signalling mediates defence against necrotrophic pathogens that kill plant cells and feed on dead tissue (Glazebrook, 2005). Naturally, plant pathogens have evolved a variety of strategies to overcome plant hormone-mediated immunity or to induce host susceptibility by interfering with various hormonal processes (Dou and Zhou, 2012).

Typically, effectors are defined as small secreted proteins (c. 300 amino acids) that are highly expressed during host infection, do not include known conserved domains or motifs and are often enriched in cysteine residues engaged in disulphide bridges for protein stability after secretion (Doehlemann and Hemetsberger, 2013). A more general definition describes effectors as 'small secreted proteins or molecules that alter host-cell structure or function' (Hogenhout *et al.*, 2009). The recent advances in fungal genomics have allowed the prediction of hundreds of effector genes in the genomes of different fungal pathogens (Schmidt and Panstruga, 2011).

Although more is known about the interference of bacterial effectors with the plant immune system (Block and Alfano, 2011; Deslandes and Rivas, 2012), notably in the *Pseudomonas-Arabidopsis* pathosystem, recent progress has also been made in deciphering the roles of fungal effectors (Giraldo and Valent, 2013; Rafiqi *et al.*, 2012). Despite the diversity of fungal effectors and the differences between fungal and bacterial effectors, it seems that they impair the host plant immune system at similar key steps, such as the perception of PAMPs and downstream signalling, hormonal homeostasis and crosstalk, plant cell death or the production of antimicrobial compounds (Doehlemann and Hemetsberger, 2013; Dou and Zhou, 2012).

Leptosphaeria maculans is a fungal plant extracellular pathogen belonging to the *Dothideomycetes*. The fungus infects mainly *Brassica* crops. In oilseed rape (*Brassica napus*), *L. maculans* causes blackleg (or phoma stem canker), the most damaging disease of this crop in Australia, Canada and Europe (Howlett, 2004; West *et al.*, 2001). The infection cycle begins with the germination of ascospores on the leaf surface, which penetrate cotyledons and younger leaves via stomata or wounds. The fungus grows in the extracellular space (apoplast) and initially colonizes the tissue as a biotroph, but, behind the hyphal front, the fungus becomes necrotrophic and kills plant cells. Concomitantly, hyphae spread down the petiole in an endophytic manner, eventually reaching the stem cortex and causing black/brown blackleg necrotic lesions (Hammond and Lewis, 1987; West *et al.*, 2001). In *L. maculans*, the genomic location of 11 *Avr* genes, designated *AvrLm1*, 2, 3, 4, 5, 6, 7, 8, 9, 11, *AvrLepR1* and *AvrLmJ1*, has been identified to

date (Balesdent *et al.*, 2002, 2005; Ghanbarnia *et al.*, 2012; Van de Wouw *et al.*, 2014). Among these, six have been cloned: *AvrLm1* (Gout *et al.*, 2006), *AvrLm2* (Ghanbarnia *et al.*, 2015), *AvrLm4-7* (Parlange *et al.*, 2009), *AvrLm6* (Fudal *et al.*, 2007), *AvrLm11* (Balesdent *et al.*, 2013) and *AvrLmJ1* (Van de Wouw *et al.*, 2014). These are involved in PTI with a series of *Brassica* hosts, such as *B. napus*, *B. rapa* and/or *B. juncea*. Although some of the *L. maculans* effectors have been shown to be involved in fungal aggressiveness (Huang *et al.*, 2006, 2010), we currently have no mechanistic understanding of their function and how they may interfere with plant defences or other metabolic pathways.

One of the most interesting candidates for such an interaction is the *Avr* protein *AvrLm4-7*, recognized by two resistance proteins coded by *Rlm4* and *Rlm7*. During adaptation to resistance, a single non-synonymous base mutation in *AvrLm4-7* led to the escape of effector recognition by *Rlm4*, whereas the recognition by *Rlm7* was conserved (Parlange *et al.*, 2009). Moreover, comparison of two near-isogenic *L. maculans* isolates differing in the presence of an *AvrLm4-7* allele showed that the virulent *avrLm4* allele was associated with a decrease in fungal aggressiveness (Huang *et al.*, 2006, 2010). The importance of the effector is underlined by the fact that, in nature and (before the use of *Rlm7*) in agronomic practice, all the examined strains virulent on susceptible genotypes of *B. napus* (*rlm4 rlm7*) possessed *AvrLm4-7* (Balesdent *et al.*, 2006). The *AvrLm4-7* effector was thus postulated to be implicated as a virulence factor of *L. maculans* (Rouxel and de Wit, 2012). Although *AvrLm4-7* has been studied mainly as an *Avr* protein, we focus here on its activity as an effector and investigate how the *AvrLm4-7* effector alters the *B. napus* immune system during ETS. For this objective, we investigated the effect of two isogenic isolates of *L. maculans*, harbouring or not the *AvrLm4-7* allele, following inoculation on a susceptible *B. napus* cultivar (*rlm4 rlm7*). Different defence responses were monitored, including the production of plant hormones, expression of defence-related genes and ROS accumulation. Our additional scope was to characterize the defence responses triggered after the recognition of *AvrLm4-7* by *Rlm4* in *B. napus* in the incompatible interaction and compare them with previously reported *AvrLm1*-triggered defence responses (Šašek *et al.*, 2012).

RESULTS

The presence of a functional allele of *AvrLm4-7* promotes the aggressiveness of *L. maculans* during a compatible interaction

It has been reported previously that the *AvrLm4-7* effector contributes to *L. maculans* fitness and increases its virulence, and that even differences in alleles (i.e. *AvrLm4-AvrLm7* versus *avrLm4-AvrLm7* alleles) could lead to fitness differences, with the virulent allele being responsible for a fitness deficit (Huang *et al.*, 2006, 2010). Here, we expanded the experiment by comparing 26

isogenic *L. maculans* isolates differing at the *AvrLm4-7* locus for aggressiveness on two susceptible *B. napus* cultivars, Eurol and ES Astrid, both lacking the corresponding resistance genes (*rlm4 rlm7*) (Fig. 1A). The wild-type (WT) isolate Nzt-4 (*a4a7*) contains a highly

Fig. 1 Introduction of a functional allele of *AvrLm4-7* increases *Leptosphaeria maculans* aggressiveness on susceptible *Brassica napus* cultivars lacking corresponding resistance genes (*rlm4* and *rlm7*). (A) Isogenic isolates of Nzt-4, with the *a4a7*, *a4A7* or *A4A7* allele of *AvrLm4-7*, were puncture inoculated on cv. 'Eurol' and cv. 'ES Astrid'. At 12 days after inoculation (dai), the cotyledons were scanned. The box-plots represent the median lesion area obtained for each isolate/plant genotype combination. For a given plant genotype, groups with the same letter do not induce significantly different median leaf lesions (red trait) (Kruskal–Wallis test, 5% threshold). (B) Disease symptoms at 13 dai caused by two isogenic β -glucuronidase (GUS)-tagged Nzt-4 isolates lacking (*a4a7*) or complemented (*A4A7*) with the *AvrLm4-7* gene. (C) Quantification of disease symptoms caused by the two isogenic GUS-tagged Nzt-4 isolates. Lesion areas were measured using image analysis. Values represent means $\pm$ standard error (SE) ($n > 22$). Asterisks indicate statistically significant differences between 'a4a7' and 'A4A7' isolates (** $P < 0.01$, Student's *t*-test). The experiment is a representative from three independent biological experiments.

degenerated non-functional allele of *AvrLm4-7*. A series of Nzt-4 isolates complemented with either the *AvrLm4-7* (*A4A7*, 11 isolates) or *avrLm4-7* (*a4A7*, eight isolates) allele of the gene (Parlange *et al.*, 2009) was compared with control WT Nzt-4 isolates that underwent the transformation process but did not integrate the construct (*a4a7*, seven isolates). In both *B. napus* cultivars, the group of *L. maculans* isolates complemented with the *A4A7* allele caused significantly larger cotyledon lesions than the control group of isolates lacking *AvrLm4-7* (Fig. 1A). Cotyledons of cv. ES Astrid infected with isolates complemented with the *a4A7* allele exhibited more severe symptoms in comparison with the control group, whereas, on cv. Eurol, the *L. maculans* infection with isolates carrying the *a4A7* allele increased the lesion size only slightly, but without statistical significance (Fig. 1A).

In further experiments, a pair of isogenic *L. maculans* Nzt-4 isolates differing only in the presence of the *AvrLm4-7* gene (Parlange *et al.*, 2009) were chosen, transformed by the β -glucuronidase (GUS) gene from *Escherichia coli* using *Agrobacterium tumefaciens* (Gardiner and Howlett, 2004), and referred to as 'a4a7' and 'A4A7' hereafter. Although the GUS transformation increased slightly the aggressiveness of the 'a4a7' isolate compared with the WT (Fig. S2, see Supporting Information), the difference between the transformed isolates 'a4a7' and 'A4A7' was conserved. On susceptible *B. napus* cv. Columbus, the 'A4A7' isolate exhibited about 40% larger lesions than the 'a4a7' isolate at 13 days after inoculation (dai) (Fig. 1B,C).

In vitro and in planta growth of isogenic *L. maculans* Nzt-4 isolates and expression of *AvrLm4-7*

We first monitored the *in vitro* growth of the 'a4a7' and 'A4A7' isolates to check whether any growth difference could be observed that might have an impact on symptom severity. Both *L. maculans* isolates differing in *AvrLm4-7* grew similarly on V8 juice agar *in vitro* (Fig. S1 and Table S1, see Supporting Information).

Fig. 2 *In planta* growth of *Leptosphaeria maculans* isolates differing in the presence of the AvrLm4-7 gene on susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*), visualized and quantified using β -glucuronidase (GUS) activity. Plants (14 days old) were puncture inoculated by either water (mock) or a spore suspension of GUS-tagged *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented (*A4A7*) with the AvrLm4-7 effector. (A) Disease symptoms and histochemical visualization of fungal mycelia by GUS staining at 6 or 7, 8 and 10 days after inoculation (dai). (B) GUS activity in inoculated cotyledons measured by a 4-methylumbelliferone glucuronide (MUG) assay at 4, 6, 8 and 10 dai. Relative fluorescence is reported to fungal mass at the infection site. Values represent means $\pm$ standard error (SE) from three biological replicates. Asterisks indicate statistically significant differences between '*a4a7*' and '*A4A7*' isolates (* $P < 0.05$ and ** $P < 0.01$; Student's *t*-test). The experiment was repeated three times with similar results.

The spread of infection *in planta* was then monitored in susceptible *B. napus* Columbus (*rlm4 rlm7*) cotyledons infected by puncture with either an *L. maculans* '*a4a7*' or '*A4A7*' isolate starting from 4 dai. None of the isolates developed macroscopically visible symptoms until 8 dai. To precisely monitor the growth of *L. maculans* isolates in infected plant tissue, we adopted a fluorescence measurement method of GUS activity using 4-methylumbelliferone glucuronide (MUG). As prerequisite, the GUS activity in *in vitro*-grown mycelia was shown to be similar (Fig. S3, see Supporting Information). Both isolates colonized the plant tissue similarly until 8 dai (Fig. 2A,B). Later, at 10 dai, the colonization by the '*A4A7*' isolate was more intense and its mycelium quantity reached three times that of the '*a4a7*' isolate (Fig. 2B).

Typically, the expression of *L. maculans* effectors is linked to the biotrophic stage of infection. To confirm this, we analysed the expression of AvrLm4-7 in *L. maculans* isolate Nzt-4 complemented with an AvrLm4-7 allele originating from JN3 ('*A4A7*' isolate) during infection of susceptible *B. napus* cv. Columbus and after *in vitro* growth. The expression of AvrLm4-7 in *L. maculans* isolate JN3 was also determined *in vitro*. Consistent with previous observations (Parlange *et al.* 2009; Soyer *et al.*, 2014), the expression of AvrLm4-7 was strongly repressed *in vitro* (Fig. 3). During infection, almost 10 000 times more AvrLm4-7 transcripts were detected in the complemented '*A4A7*' isolate compared with *in vitro* growth (Fig. 3). The peak occurred during the asymptomatic stage of infection until 8 dai.

AvrLm4-7 decreases SA and ABA levels during compatible interactions

We hypothesized that the presence of AvrLm4-7 could subvert plant immunity. First, in infected *B. napus* cv. Columbus cotyledons, we checked the levels of the principal plant hormones by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

The SA concentration increased strongly with infection. Differences between isolates were observed at 6 dai, but reached a maximum at 8 dai. Being surprisingly low, the level of SA in '*A4A7*'-infected cotyledons then increased strongly, reaching a comparable level to that of cotyledons infected with the '*a4a7*' isolate at 10 dai (Fig. 4).

Fig. 3 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) relative expression of AvrLm4-7 during the growth of *Leptosphaeria maculans* isolates JN3 and Nzt-4 '*A4A7*' *in vitro* and during infection on susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*) infected with isolate Nzt-4 '*A4A7*'. *In vitro* spore suspensions of JN3 and Nzt-4 were grown in Gamborg B5 medium for 10 days. Plants (14 days old) were inoculated by puncture inoculation with a spore suspension of *L. maculans* Nzt-4 isolate complemented with (*A4A7*) the AvrLm4-7 gene. Expression of AvrLm4-7 is normalized to *LmITS1* and relative to AvrLm4-7 expression in JN3 *in vitro*. Values represent means $\pm$ standard error from three biological replicates.

Fig. 4 Liquid chromatography–mass spectrometry analysis of plant hormone levels in susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*) infected with *Leptosphaeria maculans* isolates differing in the presence of the *AvrLm4-7* gene. Salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and its catabolic product phaseic acid (PA), indole-3-acetic acid (IAA) and its oxidized product 2-oxo-IAA (OxIAA), gibberellin 1 (GA1) and gibberellin 4 (GA4) were determined in extracts from cotyledons. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. Values represent means $\pm$ standard error (SE) from three independent experiments. Asterisks indicate statistically significant differences between cotyledons infected with '*a4a7*' and '*A4A7*' isolates (* $P < 0.05$ and ** $P < 0.01$; one-tailed Student's *t*-test).

Interestingly, the ABA content was significantly reduced in cotyledons infected with the '*A4A7*' isolate compared with '*a4a7*'-infected cotyledons at 8 dai (Fig. 4). The level of the ABA degradation product, phaseic acid (PA), increased more slowly in '*A4A7*'-infected

plants and, at 10 dai, the content of PA was significantly reduced in comparison with plants infected with the '*a4a7*' isolate.

The contents of gibberellins, auxin and its degradation product 2-oxo-IAA (OxIAA) exhibited no change in comparison with

either the mock-treated cotyledon or between the two isolates (Fig. 4).

The JA concentration increased similarly in the mock-treated and infected cotyledons (Fig. 4). We explain the slight JA increase in mock-treated and infected cotyledons between 6 and 10 dai as being the effect of puncture inoculation, as JA responds to wounding (Bell *et al.*, 1995).

SA- and ET-dependent signalling is lowered in susceptible *B. napus* cotyledons infected with the 'A4A7' isolate

To confirm our findings, we examined whether decreased concentrations of plant hormones are also reflected at the transcript level. For this, we used a set of *B. napus* defence signalling marker genes that were identified in our previous study (Šašek *et al.*, 2012).

The expression induction of a gene coding for the key enzyme in the SA biosynthetic pathway, *ISOCHORISMATE SYNTHASE 1* (*ICS1*), was lower in cotyledons infected with the 'A4A7' isolate compared with the virulent recipient at 8 dai (Fig. 5). Moreover, we observed a delayed expression induction of the widely used SA marker *PATHOGENESIS-RELATED GENE 1* (*PR1*) in cotyledons infected with the 'A4A7' isolate. In cotyledons infected with the 'a4a7' isolate, the transcription of marker genes increased almost linearly from 6 to 10 dai, whereas, in cotyledons infected with the 'A4A7' isolate, the increase in expression was only seen from 8 dai (Fig. 5).

In addition, we also analysed the transcription of genes related to ET signalling. The transcription rate of a gene involved in the biosynthesis of 1-aminocyclopropane (ET precursor), *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2* (*ACS2*), was always lower in cotyledons infected with the 'A4A7' isolate compared with those infected with 'a4a7'. Similarly, *hevein-like protein* (*HEL*; also classified as *PR4*), responding to ET and JA signalling pathways concomitantly, was induced to a lesser degree in cotyledons infected with the 'A4A7' isolate (Fig. 5). The transcription of the ABA-related marker genes *NCED3*, a gene coding for the key enzyme of ABA biosynthesis, and *RD26*, another ABA-inducible gene, was not significantly affected (Fig. 5).

Hydrogen peroxide (H₂O₂) accumulation is decreased in cotyledons infected with the 'A4A7' isolate at late stages of infection

Reactive oxygen species (ROS) are components of importance to the plant immune system, acting either as signalling molecules or exerting a direct toxic effect on invading microbes (Apel and Hirt, 2004; Foyer and Noctor, 2013). As several plant pathogens subvert ROS production (Doehlemann and Hemetsberger, 2013), we examined the possibility that AvrLm4-7 also affects ROS levels. The production of H₂O₂ was monitored from 6 to 10 dai by

diaminobenzidine (DAB) staining. At 6 dai, no cotyledon showed brown staining (Fig. 6A). At this stage of the infection, only a little *L. maculans* mycelium is detected at the site of infection (Fig. 2B). In infected cotyledons, H₂O₂ stained by DAB appeared from 8 dai, showing more intense accumulation at 10 dai. We detected significantly less DAB-stained area in cotyledons infected with the 'A4A7' isolate than in those infected with the 'a4a7' isolate at both time points (Fig. 6). Furthermore, we examined the expression of NADPH oxidase genes during the response of cv. Columbus to 'a4a7' and 'A4A7' *L. maculans* isolates. NADPH oxidases are involved in the production of superoxide, an important precursor of several ROS. Amongst these, the transcription of *rbohF* was slightly induced in cotyledons when infected with the 'a4a7' isolate only (Fig. S4, see Supporting Information).

Treatment with an antioxidant agent increases 'a4a7' aggressiveness

Ascorbic acid belongs to the natural antioxidants in plants and, together with other antioxidants, determines the lifetime of ROS (Foyer and Noctor, 2013). Infected cotyledons were treated with water or 5 mM ascorbic acid at 3 and 6 dai, and disease symptoms were quantified at 13 dai. Although the necrotic lesion area caused by the 'A4A7' isolate was unaffected by treatment, the lesions produced by the 'a4a7' isolate increased after ascorbic acid treatment to reach the level obtained following 'A4A7' infection (Fig. 7). This result indicates that H₂O₂ is involved in the defence response of *B. napus* to *L. maculans* at late stages of the primary infection, and that its accumulation is affected by the action of AvrLm4-7.

Recognition of AvrLm4-7 by Rlm4 induces SA and ET signalling

In addition to the effect of AvrLm4-7 during a compatible interaction, we also aimed to characterize the defence pathways triggered by the recognition of AvrLm4-7 in *B. napus*. For this purpose, the same two isogenic isolates were also inoculated onto an *Rlm4 B. napus* genotype (cv. Pixel). In the incompatible interaction, the recognition of AvrLm4-7 by Rlm4 led to the inhibition of infection spread. Indeed, only small greyish lesions with sharp margins developed on cotyledons and were restricted to a small area surrounding the site of inoculation. In contrast, the 'a4a7' isolate lacking the AvrLm4-7 effector (compatible interaction) caused large greyish necrotic lesions that spread up to the cotyledon margin at 10 dai (Fig. 8A).

Compared with the isolate lacking AvrLm4-7, the 'A4A7' isolate induced higher transcription of the SA marker genes *ICS1* and *PR1*. The transcription of *WRKY 70*, an SA-responsive transcription factor, was also induced on AvrLm4-7 recognition, but without statistical significance (Fig. 8B). Similar to *PR1* and *ICS1*, the

Fig. 5 Relative expression of salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) marker genes in susceptible *Brassica napus* 'Columbus' (rlm4 and rlm7) infected with *Leptosphaeria maculans* isolates differing in the presence of the *AvrLm4-7* gene. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. The expression of *B. napus* signalling marker genes was determined by reverse transcription-quantitative polymerase chain reaction and normalized to *B. napus* *Actin*. Values represent means $\pm$ standard error (SE) from three independent experiments. Asterisks indicate statistically significant differences between cotyledons infected with '*a4a7*' and '*A4A7*' isolates (* $P < 0.05$; one-tailed Student's *t*-test).

ET-related marker gene *ACS2* was also strongly induced in cotyledons infected with the '*A4A7*' isolate. In addition, *HEL* and chitinase (*CHI*), which respond concomitantly to ET and JA, were

induced. Here, we attribute the increase to the effect of ET, as the JA-dependent marker genes *AOS* and *LOX3* revealed no difference in transcription (Fig. 8B). Interestingly, the recognition of *AvrLm4-*

Fig. 6 Detection of hydrogen peroxide in susceptible *Brassica napus* 'Columbus' cotyledons infected with *Leptosphaeria maculans* isolates differing in the presence of the AvrLm4-7 effector using diaminobenzidine (DAB) staining. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the AvrLm4-7 gene. (A) DAB-stained cotyledons at 6, 8 and 10 days after inoculation (dai). (B) DAB-stained area was measured using image analysis at 8 and 10 dai. Values represent means $\pm$ standard error from 22 cotyledons. Asterisks indicate statistically significant differences between *L. maculans* isolates (* $P < 0.05$; Student's *t*-test). The experiment was performed twice with similar results.

Fig. 7 Treatment with ascorbic acid increases the aggressiveness of *Leptosphaeria maculans* isolate Nzt-4 'a4a7' on susceptible *Brassica napus* 'Columbus'. Plants (14 days old) were inoculated by puncture inoculation by water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the AvrLm4-7 gene. At 3 and 6 days after inoculation (dai), inoculation sites were treated with either water (mock) or 5 mM ascorbic acid (Asc). (A) Quantification of disease symptoms at 13 dai. The necrotic lesion area was measured using image analysis. Values represent means $\pm$ standard error (SE) ($n \geq 40$). Different letters above the columns indicate statistically significant differences according to Student's *t*-test ($P < 0.05$). (B) Representative cotyledons at 13 dai.

7 induced a slight but statistically significant increase in the transcription of the ABA-dependent genes *NCED3* and *RD26* (Fig. 8B).

In summary, the recognition of *L. maculans* AvrLm4-7 by Rlm4 triggered strong activation of SA- and ET-dependent signalling pathways, whereas ABA signalling was only slightly induced and

Fig. 8 Compatible and incompatible interaction of *Leptosphaeria maculans* isolates differing in the presence of the *AvrLm4-7* effector with *Brassica napus* 'Pixel' carrying the resistance gene (*Rlm4*). Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or complemented with (*A4A7*) the *AvrLm4-7* gene. (A) Disease symptoms developed at 10 days after inoculation (dai). (B) Expression of salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) marker genes relative to mock at 6 dai determined by reverse transcription-quantitative polymerase chain reaction. Values represent means $\pm$ standard error (SE) from three biological replicates. Asterisks indicate statistically significant differences between *L. maculans* isolates (* $P < 0.01$; Student's *t*-test).

JA-dependent signalling exhibited no activation. The successful elimination of *L. maculans* growth by the defence triggered by the SA and ET signalling pathways also illustrated the expression decrease of activated marker genes at 10 dai (Fig. S5, see Supporting Information). In 'A4A7'-infected cotyledons, SA and ET signalling marker genes exhibited a lower transcription rate at 10 dai in comparison with 6 or 8 dai, whereas the expression increased steadily from 6 to 10 dai in cotyledons infected with the virulent isolate 'a4a7' (Fig. S5).

DISCUSSION

This study aimed to confirm and find a cause for the increased aggressiveness of *L. maculans* isolates harbouring the *AvrLm4-7* effector by investigating its effect on *B. napus* defence responses. This work is the first attempt so far to investigate the role of *AvrLm4-7* as an effector during compatible interactions with *B. napus*, with the working hypothesis that it may interfere with plant defence signalling.

First, using a set of *L. maculans* isolates differing at the *AvrLm4-7* locus, we showed that the absence of a functional allele of *AvrLm4-7* was responsible for the reduced aggressiveness on cotyledons, as measured by the size of the lesions in control conditions. In contrast with *AvrLm1*, which has also been shown to contribute to pathogen aggressiveness to some extent (Huang *et al.*, 2006, 2010), the increase in aggressiveness caused by the presence of the *AvrLm4-7* gene was much higher and cultivar independent, as three different susceptible cultivars of *B. napus*, namely Euro1, Columbus and ES Astrid, revealed greater symptom development during primary infection when infected with isolates complemented with the *AvrLm4-7* gene. Huang *et al.* (2006) have reported previously decreased aggressiveness associated with the loss of *AvrLm4* specificity as a result of a single base mutation in the *AvrLm4-7* gene, but did not analyse the effect of the loss of the gene. In our study, we found decreased aggressiveness following the loss of the gene, but no significant effect of the single amino acid change. The reasons for these different findings are unclear, but we stress the very different starting material and inoculation protocols between the two studies. In particular, Huang *et al.* (2006) departed from two field isolates that showed, or not, *AvrLm4* specificity, and used the progeny of isolates obtained following a series of backcrosses to generate batches of progeny harbouring, or not, the *AvrLm4* allele in a related, but not isogenic, genetic background. In this study, genetic complementation allowed us to compare the different alleles of *AvrLm4-7* (including the virulent degenerated allele) in the same genetic background. Moreover, Huang *et al.* (2006) used inoculations with ascospores without wounding the cotyledons; this is a tedious procedure, more closely resembling what happens in the field than the usual cotyledon inoculation test involving the inoculation of conidia on wounded cotyledons. It is possible that the inoculation with ascospores is more sensitive and may reveal fitness differences as a result of a single amino acid change that cannot be seen using the cotyledon inoculation test; for example, the ascospore inoculation procedure may reveal a fitness deficit linked to the penetration step that is not accessible when wounding the tissues prior to inoculation. All in all, the importance of the *a4a7* allele in fungal aggressiveness strongly suggests that the single mutation in the *AvrLm4-7* gene, allowing it to escape *Rlm4* resistance only slightly (if at all), compromises the effector function of the gene.

Further, we demonstrated that the two selected isogenic isolates 'a4a7' and 'A4A7', differing by the presence of *AvrLm4-7*, grew similarly *in vitro*, in accordance with the findings of Huang *et al.* (2006), substantiating the fact that *AvrLm4-7*, as a regular effector, does not have an intrinsic function for the fungus, but rather exerts its effect on the plant during the interaction. However, it must be remembered that the expression of *AvrLm4-7*, similar to that of other effector genes located in AT isochores of *L. maculans*, is strongly repressed during vegetative growth (Soyer *et al.*, 2014).

Once in the plant, the repression of effector gene expression was relieved, and a sharp increase in expression was observed between 3 and 6–8 dai, followed by reduced levels of expression at later stages (Parlange *et al.*, 2009; this study). Both isolates 'a4a7' and 'A4A7' colonized the plant tissue comparably and grew asymptotically on susceptible *B. napus* cotyledons until 8 dai. We assume that the effect of AvrLm4-7 is linked to the biotrophic stage of the primary infection, as the wave of effector expression to which that of AvrLm4-7 belongs peaked during the asymptomatic stage of leaf colonization prior to the development of leaf lesions. This is a commonly found feature for many biotrophic and hemibiotrophic filamentous phytopathogens for which waves of expression of effectors associated with the biotrophic stage of infection are common (Gan *et al.*, 2013; Lee and Rose, 2010). In other cases, however, such as that of *Zymoseptoria tritici*, waves of expression of putative effectors seem to be linked rather to the necrotic stage of hemibiotrophic behaviour (Rudd *et al.*, 2015). In later stages of infection, AvrLm4-7 had a significant impact on the development of necrotic lesions. Similarly, the presence of the AvrLm4-AvrLm7 allele of AvrLm4-7 increased the hyphal growth towards the petiole to reach the stem (Huang *et al.*, 2006).

Analogy in signalling after perception of AvrLm1 and AvrLm4-7

The recognition of AvrLm4-7 by Rlm4 examined in this study revealed a strong induction of SA- and ET-dependent signalling pathways early in the incompatible interaction. The same signalling pathways mediated defence in our previous study (Šašek *et al.*, 2012), in which the incompatible interaction based on the recognition of AvrLm1 by Rlm1 was examined. AvrLm1 is recognized by the *LepR3* resistance gene, which is an RLP (Larkan *et al.*, 2013), and by *Rlm1* (not cloned to date), but there is still debate on whether or not the two R genes may be the same (Rouxel and Balesdent, 2013). Nevertheless, one difference between the defence signalling mediated by AvrLm1 and AvrLm4-7 recognition still exists. The perception of the AvrLm4-7 effector by Rlm4 in *B. napus* cv. Pixel also slightly induced marker genes related to ABA signalling. Moreover, all compatible interactions in the two studies revealed elevated ABA levels, but we were unable to detect the up-regulation of the ABA-dependent marker genes *NCED3* and *RD26* (Šašek *et al.*, 2012). Considering our previous finding that the induction of ABA signalling prior to infection decreased symptoms caused by a virulent isolate (Šašek *et al.*, 2012), the role of ABA in the *L. maculans*–*B. napus* interaction remains obscure and requires further investigation.

Increased aggressiveness is linked to the suppression of signalling pathways by AvrLm4-7

In *B. napus*, SA and ET signalling pathways mount an effective defence response to *L. maculans* (Šašek *et al.*, 2012), as was also

shown here during the course of the AvrLm4–Rlm4 interaction. Our study suggests that, in a compatible interaction, these signalling pathways may be the primary targets of the AvrLm4-7 effector. Our results indicate that the presence of AvrLm4-7 in infected cotyledons causes suppression of SA signalling as a whole, affecting biosynthesis, the SA level and the response of the SA-related marker gene *PR1*. Moreover, ET signalling seems to be lowered in infected cotyledons when AvrLm4-7 is present: the transcription of the ET-responsive genes *ACS2* and *HEL* was repressed from 6 to 10 dai.

To our knowledge, this is the first study to indicate the manipulation of SA pathways by an effector from hemibiotrophic fungi. A strategy of other (hemi)biotrophic fungi involves effectors targeting JA signalling to promote susceptibility in a host (Kazan and Lyons, 2014). For example, *Fusarium oxysporum* secretes the Fo-SIX4 effector that induces JA signalling in *Arabidopsis thaliana* and contributes to disease development (Thatcher *et al.*, 2009). The SA pathway is also manipulated by bacterial effectors (Kazan and Lyons, 2014). The type III effector XopD from *Xanthomonas campestris* pv *vesicatori* represses SA-dependent gene expression and SA production in tomato plants (Kim *et al.*, 2008). Recently, Kim *et al.* (2013) have reported that XopD interacts directly with the tomato ET response factor ERF4 in subnuclear foci and also suppresses ET-stimulated immunity late in infection. In this aspect, the effect of AvrLm4-7 on *B. napus* defence signalling resembles that exerted by XopD in tomato.

H₂O₂ as the key player in the playground?

Previously, Jindřichová *et al.* (2011) have shown that H₂O₂ accumulates during *L. maculans* infection. In this study, we demonstrated that the removal of H₂O₂ by ascorbic acid during the biotrophic stage of the infection increased the lesion development of the less aggressive isolate 'a4a7' lacking the AvrLm4-7 effector. This illustrates the importance of H₂O₂ in the defence response to *L. maculans* infection. Interestingly, the presence of AvrLm4-7 in the interaction led to a decreased accumulation of H₂O₂ during the later stages of the primary infection. Moreover, the transcription of *rbohF*, a gene coding for one of the NADPH oxidases, which are a source of ROS in the apoplast, was reduced when cotyledons were infected with the 'A4A7' isolate harbouring the AvrLm4-7 effector. These results suggest a role for AvrLm4-7 in affecting the accumulation of H₂O₂. Among fungal effectors known to interfere with ROS production in plants, the pep1 effector of the biotrophic pathogen *Ustilago maydis* has been shown to inhibit the maize peroxidase POX12 in the apoplast (Hemetsberger *et al.*, 2012). Restricted colonization of the plant tissue by *U. maydis* is associated with the accumulation of ROS when pep1 is absent in the interaction. Until the effect of purified AvrLm4-7 protein on ROS-producing enzymes is examined, the possibility that AvrLm4-7 also acts in the apoplast by direct inhibition of ROS

production in a pep1-like manner cannot be excluded. However, alternative explanations of the H₂O₂ decrease are possible. ROS have been proposed to act synergistically in a signal amplification loop with SA to drive the HR and the establishment of systemic defences (Draper, 1997; Herrera-Vásquez *et al.*, 2015). Possibly, the modest ROS accumulation at the infection site of cotyledons infected by isolates harbouring AvrLm4-7 could reflect the decreased SA level and SA signalling. Nevertheless, the difference in SA level between the isolates disappeared at 10 dai, whereas the difference in ROS accumulation increased. A noticeable role in the regulation of H₂O₂ accumulation could also be played by ET signalling. ET can induce programmed cell death and senescence, processes that are also associated with the action of ROS (de Jong *et al.*, 2002). Therefore, in the absence of AvrLm4-7, the accumulation of ROS at 10 dai could be stimulated by increased ET signalling.

Taken together, we speculate that the increased aggressiveness of *L. maculans* isolates harbouring the functional AvrLm4-7 allele could be caused by defects in ROS accumulation or by the complex effects (involving ROS accumulation) exerted by AvrLm4-7 on the *B. napus* defence system through the suppression of SA and ET signalling. Recent findings of Blondeau *et al.* (2015), showing that AvrLm4-7 is translocated into the plant cell, rather suggest that SA and/or ET signalling is the primary target of AvrLm4-7. The identification of the interacting partner of AvrLm4-7 upstream of these processes represents the next step in our understanding of the molecular mechanisms of this unique *L. maculans* effector.

EXPERIMENTAL PROCEDURES

Plant and pathogen cultivation

Brassica napus plants were grown in soil mixture Atami BioGrowmix (GROWMAN PLAINS, Prague, Czech Republic) in a cycle of 14 h of daylight (150 µE/m/s, 24°C) and 10 h of night (19°C) at 70% relative humidity in a cultivation chamber (Snijders Labs, Tilburg, the Netherlands). *Leptosphaeria maculans* isolates Nzt-4 AvrLm4_6 and Nzt-4 AvrLm4_3 (Parlange *et al.*, 2009), here referred to as 'a4a7' and 'A4A7', respectively, were cultivated on V8 juice agar medium at 26°C in the dark. Sporulation was obtained as described by Ansan-Melayah *et al.* (1995). Spores were washed once with distilled water after harvesting, diluted to 10⁸ spores/mL and stored at -20°C. For aggressiveness studies following inoculation on compatible *B. napus* varieties, additional isogenic isolates were used: (i) a total of seven single-conidia isolates of Nzt-4, lacking AvrLm4-7; (ii) a total of eight Nzt-4 isogenic isolates having integrated the *avrLm4-AvrLm7* allele of AvrLm4-7 (a4A7); and (iii) a total of 11 isogenic isolates having integrated the *AvrLm4-AvrLm7* allele of AvrLm4-7 (A4A7) (Parlange *et al.*, 2009).

Plant inoculation and treatments

Plants were inoculated by puncture inoculation, i.e. by placing 10 µL of spore suspension (10⁶ spores/mL) on a cotyledon that was punctured by a sterile needle.

For experiments with antioxidant agent, 10 µL of water (mock) or 5 mM ascorbic acid (Farmakon, Olomouc, Czech Republic) were placed on the puncture at 3 and 6 dai.

For aggressiveness studies, isolates were inoculated with the puncture method on the two compatible varieties, i.e. devoid of *Rlm4* and *Rlm7*, Eurol and ES-Astrid, using 10–12 inoculation points per isolate per plant variety. At 12 dai, cotyledons were scanned to measure lesion areas. The experiment was repeated twice. Median lesion areas were calculated and the data were subjected to non-parametric tests (Kruskal–Wallis) to test the significance of the differences observed between the three groups of isolates.

Transformation of *L. maculans*

Leptosphaeria maculans isolates Nzt-4 AvrLm4_6 and Nzt-4 AvrLm4_3 were transformed with a pS01 construct (Persson *et al.*, 2009) carrying the gene for GUS from *E. coli*. The transformation was performed using *A. tumefaciens* LBA4404 according to Gardiner and Howlett (2004). GUS-transformed isolates were assessed for GUS activity *in vitro* and compared with WT (recipient) isolates for phenotype changes and virulence to exclude possible alterations caused by transformation. Unless stated otherwise, all inoculations were performed using GUS-tagged isolates.

GUS staining

Discs (diameter, 12 mm) surrounding the inoculation sites were cut from *B. napus* cotyledons and stained as described previously (Šašek *et al.*, 2012). In addition, cotyledon discs were submerged in 10% (w/w) NaOH (Lachema, Brno, Czech Republic) and incubated for 1 h at 37°C with shaking at 130 rpm. Rehydrated cotyledon discs were scanned in a thin layer of water under transmission light.

MUG assay

Fluorescence measurements of GUS activity were performed according to a method described by Jefferson *et al.* (1987) with several modifications.

For the determination of GUS activity in mycelia of GUS-tagged *L. maculans* isolates, 15 mg of mycelia filtered from the cultivation medium Gamborg B5-MES were homogenized in a 2-mL screw cap filled with 0.5 g of 1.3-mm-diameter silica beads with 500 µL of GUS extraction buffer (Jefferson *et al.*, 1987) using a FastPrep®-24 Instrument (MP Bio-medicals, Santa Ana, CA, USA), centrifuged and 50 µL of supernatants were pipetted to a 96-well plate maintained at 37°C. To each well, 200 µL of 1 mM MUG (Duchefa Biochemie, Haarlem, the Netherlands), pre-heated to 37°C, was added. Immediately, 50 µL were removed to 300 µL of STOP buffer (0.2 M Na₂CO₃) in a black Nunc™ F96 Microwell Polystyrene plate (Thermo Fisher Scientific, Waltham, MA, USA) for blank measurement. The mixture was incubated for 15 min at 37°C and stopped as described above. The fluorescence of 4-methylumbelliferone (MU) formed by GUS activity was measured in a Tecan Infinite F200 plate reader (Tecan, Männedorf, Switzerland) equipped with a 360/20-nm excitation filter and 465/25-nm emission filter.

For the determination of GUS activity in *B. napus* cotyledons infected with GUS-tagged *L. maculans* isolates, four discs (approximately 50 mg fresh weight) surrounding the inoculation sites were cut by Harris Uni-Core® (diameter, 6 mm) from four plants. The extraction of GUS, the

reaction with MUG and the detection of fluorescence proceeded similarly to that with *L. maculans* mycelium, except that the reaction time was 30 min.

Analysis of plant hormones

Plant hormones were extracted from approximately 200 mg of fresh tissue and determined as described previously (Dobrev and Kamínek, 2002; Dobrev and Vankova, 2012). The addition of appropriate internal standards to the samples preceded the analyses. Quantification was performed on an Ultimate 3000 high-performance liquid chromatograph (Dionex, Bannockburn, IL, USA) coupled to a 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA).

Gene expression analysis

Discs surrounding the inoculation sites were cut by Harris Uni-Core® (diameter, 6 mm) from at least six plants. Homogenization, total RNA extraction, treatment with DNA-Free kit (Ambion, Austin, TX, USA) and reverse transcription of RNA to cDNA were performed as described previously (Šašek *et al.*, 2014). The reverse transcription-quantitative polymerase chain reactions (RT-qPCRs) were performed as described previously (Janda *et al.*, 2015). For *ACS2a*, the annealing conditions were modified to 55°C for 20 s in all reactions. The relative expression of *B. napus* and *L. maculans* genes was calculated with efficiency correction and normalization to *B. napus Actin* and *L. maculans ITS1* (Persson *et al.*, 2009), respectively. Primer sets for *B. napus* signalling marker genes were characterized, designed and verified in our previous study, where a complete list is given (Šašek *et al.*, 2012). Primers for *LmITS1* (FJ172239) and *AvrLm4-7* were designed by Persson *et al.* (2009) and Parlange *et al.* (2009), respectively.

H₂O₂ detection using DAB

Detection of H₂O₂ in *B. napus* cotyledons using staining with DAB (Sigma-Aldrich) was performed as described previously (Šašek *et al.*, 2012).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 Phenotypes of aerial colonies of *Leptosphaeria maculans* wild-type Nzt-4 and β -glucuronidase (GUS)-tagged Nzt-4 isolates harbouring (A4A7) or lacking (a4a7) the *AvrLm4-7* effector. The transformed and wild-type (WT) isolates were grown on V8 juice agar medium and the photographs were taken 7 days after subculture.

Table S1 *In vitro* growth rate (mm/day) of wild-type *Leptosphaeria maculans* and β -glucuronidase (GUS)-tagged isolates

Figure S2 Aggressiveness of *Leptosphaeria maculans* wild-type (recipient) Nzt-4 and β -glucuronidase (GUS)-tagged Nzt-4 isolates harbouring (A4A7) or lacking (a4a7) the *AvrLm4-7* effector following inoculation on the susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*). Plants (14 days old) were inoculated by infiltration of a spore suspension of wild-type *L. maculans* (WT) or GUS-tagged (GUS) Nzt-4 isolates (a4a7 and

A4A7). The area of the necrotic lesion relative to the total cotyledon area was measured using image analysis at 10 days after inoculation. Values represent means $\pm$ standard error (SE) ($n > 21$). Different letters above the columns indicate statistically significant differences according to Student's *t*-test ($P < 0.05$).

Figure S3 β -Glucuronidase (GUS) activity in mycelia of GUS-tagged *Leptosphaeria maculans* isolates Nzt-4 differing in the presence of the AvrLm4-7 effector. *Leptosphaeria maculans* mycelium was harvested from axenic culture in Gamborg B5 by filtration at 10 days after inoculation of the medium. GUS activity was measured by a 4-methylumbelliferone glucuronide (MUG) assay. Relative fluorescence indicates the fluorescence of 4-methylumbelliferone formed by GUS activity. Values represent means $\pm$ standard errors from three biological replicates. No statistically significant difference was observed between 'a4a7' and 'A4A7' isolates ($P > 0.05$, Student's *t*-test).

Figure S4 Relative expression of *rboh* genes in susceptible *Brassica napus* 'Columbus' (*rlm4* and *rlm7*) infected with *Leptosphaeria maculans* isolates differing in the AvrLm4-7 effector, determined by reverse transcription-quantitative polymerase

chain reaction. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or harbouring (A4A7) the AvrLm4-7 effector. Expression was normalized to *B. napus Actin*. Values represent means $\pm$ standard error (SE) from three independent experiments. Asterisks indicate statistically significant differences between cotyledons infected with 'a4a7' and 'A4A7' isolates ($*P < 0.05$; one-tailed Student's *t*-test).

Figure S5 Relative expression of salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) marker genes in resistant *Brassica napus* 'Pixel' (*Rlm4*) infected with *Leptosphaeria maculans* virulent (*a4a7*) and avirulent (A4A7) Nzt-4 isolates, determined by reverse transcription-quantitative polymerase chain reaction. Plants (14 days old) were inoculated by puncture inoculation with either water (mock) or a spore suspension of *L. maculans* Nzt-4 isolates lacking (*a4a7*) or harbouring (A4A7) the AvrLm4-7 effector. Values represent means $\pm$ standard error from three biological replicates. Asterisks indicate statistically significant differences between *L. maculans* isolates ($*P < 0.05$ and $**P < 0.01$; Student's *t*-test).