A gene-for-gene interaction involving a ‘late’ effector contributes to quantitative resistance to the stem canker disease in *Brassica napus*  

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Received: 22 December 2020  
Accepted: 15 February 2021  

New Phytologist (2021) 231: 1510–1524  
doi: 10.1111/nph.17292  

Key words: *Brassica napus*, effector, gene-for-gene interaction, *Leptosphaeria maculans*, quantitative disease resistance.  

Summary  
• The control of stem canker disease of *Brassica napus* (rapeseed), caused by the fungus *Leptosphaeria maculans* is based largely on plant genetic resistance: single-gene specific resistance (*Rlm* genes) or quantitative, polygenic, adult-stage resistance. Our working hypothesis was that quantitative resistance partly obeys the gene-for-gene model, with resistance genes ‘recognizing’ fungal effectors expressed during late systemic colonization.  
• Five *LmSTEE* (stem-expressed effector) genes were selected and placed under the control of the *AvrLm4-7* promoter, an effector gene highly expressed at the cotyledon stage of infection, for miniaturized cotyledon inoculation test screening of a gene pool of 204 rapeseed genotypes.  
• We identified a rapeseed genotype, ‘Yudal’, expressing hypersensitive response to *LmSTEE98*. The *LmSTEE98*-*RlmSTEE98* interaction was further validated by inactivation of the *LmSTEE98* gene with a CRISPR-Cas9 approach. Isolates with mutated versions of *LmSTEE98* induced more severe stem symptoms than the wild-type isolate in ‘Yudal’. This single-gene resistance was mapped in a 0.6 cM interval of the ‘Darmor_bzh’ × ‘Yudal’ genetic map.  
• One typical gene-for-gene interaction contributes partly to quantitative resistance when *L. maculans* colonizes the stems of rapeseed. With numerous other effectors specific to stem colonization, our study provides a new route for resistance gene discovery, elucidation of quantitative resistance mechanisms and selection for durable resistance.

Introduction  
Genetic resistance to plant diseases is a highly desirable trait for plant breeding, to ensure global food security and avoid the adverse effects of pesticides. Furthermore, genetic resistance is the only means of disease control available in some crop species lacking other efficient or sustainable control methods. Two types of resistance are deployed in the field for efficient plant disease control: qualitative and quantitative resistance. Qualitative resistance usually is controlled by a single resistance gene and confers complete resistance to pathogen populations harboring the corresponding avirulence gene. It is often associated with a hypersensitive response (HR) and localized cell death, preventing the pathogen from colonizing its host (Greenberg & Yao, 2004). By contrast, quantitative resistance does not prevent the pathogen from infecting the plant but instead limits symptom severity, thereby reducing the impact on yield. It is based on the combined effects of several genes, associated with genomic regions or QTL (quantitative trait loci), each making a quantitative contribution to plant defense (Delourme et al., 2006; St Clair, 2010; Niks et al., 2015). Many disease resistance QTL have been identified in plants over the last two decades. For example, Wilfert & Schmid-Hempel (2008) analyzed 194 publications describing 445 QTL involved in resistance to various bioaggressors in diverse plants, such as wheat, barley, maize and melon. Despite the importance of quantitative resistance to the control of plant diseases, the mechanisms underlying such resistance remain largely unknown (Poland et al., 2009) and are probably highly diverse (Kushalappa et al., 2016; Corwin & Kliebenstein, 2017). Several studies have suggested that some quantitative resistance may be isolate-specific (Arru et al., 2003; Calenge et al., 2004; Rocherieux et al., 2004; Zenbayashi-Sawata et al., 2005; Niks et al., 2015), and that partial resistance may be at least partly due
to gene-for-gene interactions, as hypothesized by Parlevliet & Zadoks (1977).

The Dothideomycete *Leptosphaeria maculans* is responsible for one of the most damaging diseases of rapeseed (*Brassica napus*), phoma stem canker disease, also known as blackleg. This disease, which has been known to cause losses exceeding US$900 million per year, is controlled principally by genetic resistance in most parts of the world (Fitt et al., 2008). The ascospores of *L. maculans* produced on stem residues land on aerial organs of the plant, where they germinate, and the hyphae penetrate the leaves and cotyledons through stomata. After entering the plant, the fungus goes through a short biotrophic stage (5–15 d), during which it colonizes the apoplast, subsequently switching to a necrotrophic lifestyle, in which it causes leaf spots (Fitt et al., 2006; Rouxel & Balesdent, 2005). After this first stage of colonization, the fungus migrates toward the stem tissues, from the leaves to the crown, during an asymptomatic phase that may last several months, before switching to a necrotrophic lifestyle in which necrosis is induced at the stem base (Hammond et al., 1985; West et al., 2001; Huang et al., 2014; Rouxel & Balesdent, 2005).

Qualitative and quantitative resistances to *L. maculans* have been described in *B. napus* (Delourme et al., 2006). Qualitative resistance typically is conferred by race-specific resistance genes, the Rlmx or LepRx genes, conferring complete resistance to avirulent isolates and preventing such isolates from colonizing cotyledons or leaves (Balesdent et al., 2001; Huang et al., 2006; Larkan et al., 2013). Qualitative resistance can be assessed easily in controlled conditions using cotyledon inoculation assays, which are reproducible and used widely, and provide a reliable method for the high-throughput screening of large collections of *B. napus* or populations of *L. maculans* (Balesdent et al., 2006; Rouxel et al., 2003; Ghanbarnia et al., 2012). Quantitative resistance, which operates during colonization of the petiole and stem, is a partial resistance under polygenic control mediated by a large number of QTL; its efficacy for limiting necrosis can be highly dependent on environmental conditions (Pilet et al., 2001; Fopa Fomeju et al., 2015; Jestin et al., 2015; Huang et al., 2016; Raman et al., 2016; Kumar et al., 2018). Quantitative resistance limits the onset and severity of stem necrosis, but its mechanistic determinants are unknown. However, effects on the growth rate of the pathogen in tissues, the ability of the pathogen to move from the petiole to the stem and stem necrosis development have been described (Huang et al., 2009, 2014). By contrast to qualitative resistance, reproducible methods for assessing quantitative resistance in controlled conditions have yet to be developed. This type of resistance currently is evaluated in field assays, by scoring disease severity on mature plants at the end of the growing season, which can last from six to 10 months, depending on geographical location. Furthermore, owing to the influence of the environment on quantitative resistance, the identification of stable resistance QTL requires replicated field-plot experiments at different sites and in different years (Jestin et al., 2015; Huang et al., 2016; Kumar et al., 2018).

As already reported for other pathogens, the infection and colonization of rapeseed by *L. maculans* is dependent on a cocktail of effectors, molecules mainly corresponding to small secreted proteins which modulate plant immunity and facilitate infection (Sánchez-Vallet et al., 2018). Two waves of *L. maculans* effector gene expression were identified initially, the molecules involved in the first wave being described by Gervais et al. (2017) as ‘early effectors’ and those of the second wave, ‘late effectors’ (aka ‘LmSTEE genes’ for ‘*L. maculans* stem expressed effector’). More extensive studies have since discriminated eight waves of genes specifically expressed during the interaction with the plant, all being enriched in genes encoding effectors (Gay et al., 2021).

‘Early effector’ genes, including all known avirulence genes (*AvrLm*) ‘recognized’ by the plant and leading to qualitative resistance, are upregulated during cotyledon/leaf colonization. Genetic manipulations or crosses of the fungus to generate isolates harboring the smallest possible number of *AvrLm* genes and isogenic isolates differing by only a single *AvrLm* gene, are now routinely used as tools for identifying the corresponding *Rlm/ LepR* genes in *Brassica* genotypes, for screening genetic resources, or for use in plant breeding (Balesdent et al., 2002; Rouxel et al., 2003; Van de Wouw et al., 2014; Larkan et al., 2015). An easy-to-use, medium- to high-throughput cotyledon inoculation test is used widely worldwide for such purposes (Larkan et al., 2016b). Quantitative resistance operates during the systemic colonization of rapeseed, and, during this phase, *L. maculans* expresses several genes encoding effectors thought to interfere with plant defenses (Gervais et al., 2017). These *LmSTEE* genes are expressed only very weakly, if at all, during axenic growth and cotyledon/leaf colonization, but are strongly expressed during stem colonization, many months after the initial leaf infection. Our working hypothesis is that at least some *LmSTEE* genes encode products recognized by specific plant resistance gene products during systemic colonization, in a manner similar to that described for *AerLm–Rlm* interactions, accounting for at least part of the quantitative resistance. However, it is not possible to detect gene-for-gene interactions between *LmSTEE* genes and the resistance genes of *B. napus* in the usual cotyledon inoculation assay, because the corresponding avirulence proteins are not produced at this stage. In addition, stem inoculation assays (Gervais et al., 2017; Gay et al., 2021) for the identification of such resistance are not amenable to medium-/high-throughput screening, due to the length of the process, low reproducibility, and the time and space required to screen a large number of genotypes of fully grown plants. We therefore designed a new strategy, allowing the expression of ‘late’ effectors during cotyledon infection, for the identification of putative new resistance genes in rapeseed interacting with the late effectors from *L. maculans* in the cotyledon inoculation test.

With this approach we identified a *B. napus* genotype expressing hypersensitive resistance to one late effector: LmSTEE98. The LmSTEE98–RlmSTEE98 interaction was further validated by inactivation of the *LmSTEE98* gene with a CRISPR-Cas9 approach. The genetic control of this response was investigated, and a double-haploid (DH) population was used to map the associated region on the *B. napus* genome. These results provide proof-of-principle for this approach to the identification of new, but difficult-to-detect, sources of resistance for the effective control of *L. maculans* and show for the first time in this model that
gene-for-gene interactions contribute to quantitative resistance in grown-up plants.

Materials and Methods

Fungal and plant materials

The sequenced isolate JN3 (v.23.1.3; Rouxel et al., 2011) and the closely related isolate JN2 (v.23.1.2) correspond to two sister progenies from an in vitro cross between European field isolates (Balesdent et al., 2001). They were used as controls in inoculation tests. A representative selection of isolates from worldwide collections and reference isolates from Australia, Canada, the USA, Chile, France, Mexico and New Zealand, also was analyzed (Supporting Information Table S1; Mendes-Pereira et al., 2003; Dilmaghani et al., 2009, 2012; this study). INV13.269 is a single pycnidiospore isolate recovered from a French field in 2013.

Fungal cultures were maintained on V8 juice agar medium, and sporulating cultures were obtained on V8 medium, as described previously (Ansan-Melayah et al., 1995).

A collection of 204 genotypes of Brassica napus (Table S2) was screened for gene-for-gene interactions with LmSTEE genes. This panel mostly comprised varieties previously used for association studies of quantitative resistance to Leptosphaeria maculans (Fopa Fomeju et al., 2015). The segregating doubled-haploid population BnaDYDH was derived from a ‘Darmor-bzh’ × ‘Yudal’ cross (Foisset et al., 1996). The ‘Darmor-bzh’ parent is a French winter rapeseed cultivar and the ‘Yudal’ parent is a Korean spring rapeseed cultivar.

Plant inoculations

The cotyledons of 10-d-old seedlings were inoculated, by puncture, with 10 µl of inoculum (107 pycnidiospores ml–1), as described by Balesdent et al. (2001). Four different isolates, one per half-cotyledon, were used to inoculate each plant. At least six plants were inoculated with each isolate. Plants were incubated for two days at room temperature in the dark, and then in a growth chamber at 19°C (night) and 24°C (day) with a 16 h:8 h, light: dark photoperiod and a relative humidity of 90%. Symptoms were scored 10, 14 and 18 d post inoculation (dpi), with the IMASCORE rating scale (Balesdent et al., 2001). Mean scores for symptoms and the percentage of virulent and avirulent phenotypes induced by the LmSTEE-over-expressed in cotyledons’ (OEC) transformants were determined for each cultivar and compared with the symptoms induced by the wild-type (WT) isolate on the same cultivar, on the same date (Kruskal–Wallis test).

Adult plants were inoculated as described by Gervais et al., 2017. For the analysis of stem colonization by L. maculans, we cut the petiole of the second leaf horizontally, 1 cm from the insertion point of the leaf. Inoculum (10 µl, containing 107 pycnidiospores ml–1) was applied to the wounds. Inoculated plants were incubated as described previously. The necrosis of infected stems was assessed by cutting the whole stem section between the insertion point of the inoculated petiole on the stem and the ground into successive 3-mm slices. The sections were scanned and the percentage of the stem section showing internal necrosis was measured with ImageJ.

Vector construction and fungal transformation

The promoter of the AvrLm4-7 gene was amplified with the primers indicated in Table S3. It was then digested with NheI and BamHI, and ligated into a SpeI-BamHI-digested pPZPNat1 vector. The LmSTEE genes (LmSTEE1, LmSTEE30, LmSTEE35, LmSTEE98 and LmSTEE78) were amplified from their Start codon to their terminator regions and the amplicons were digested with EcoRI and XhoI or SalI and XhoI (Table S3). The resulting fragments were ligated into the pPZPNat1 vector containing the AvrLm4-7 promoter, digested with the same enzymes.

For CRISPR-Cas9 gene inactivation, the pLAU2 (hygromycin resistance), containing the Cas9 gene, and pLAU53 (geneticin resistance), in which the guide RNA was inserted, were used, as described by Idnurm et al. (2017). The CRISPOR prediction tool was used, with L. maculans as the reference genome (Dutreux et al., 2018), to design the guide RNA (gRNA) targeting the gene for inactivation (http://crispor.tefor.net; Table S3). The guide RNA was selected as the sequence with the fewest off-target predictions. The corresponding DNA fragment then was amplified with the universal primers MAI0309 and MAI0310 (Table S3). Gibson assembly (Silayeva & Barnes, 2018) was used to insert the guide RNA into the XhoI site of pLAU53, generating pLAU53-gRNA plasmids.

The four mutated versions of LmSTEE98 (see below) were amplified from the Start codon to the terminator region, as for the WT version of LmSTEE98 (Table S3). The ΔlmSTEE98_06 and ΔlmSTEE98_10 variants were cloned by Gibson assembly (Silayeva & Barnes, 2018), with the primers Gip_pA4-7: Lm98_F and Gip_pA4-7: Lm98_R. The ΔlmSTEE98_20 and ΔlmSTEE98_30 variants were amplified with the LmSTEE98_SalI_Up and LmSTEE98_XhoI_Low primers and the resulting amplicons were digested with SalI and XhoI (Table S3). For both cloning techniques, the pPZPNat1 vector containing the AvrLm4-7 promoter was digested with the same enzymes and the fragments were inserted, by Gibson assembly or enzyme-mediated ligation. Both cloning methods produced constructs identical to those used for genotype screening (pA4-7:: LmSTEE98), except for the mutated sites.

The various plasmids were introduced into Agrobacterium tumefaciens C58 by electroporation at 2.5 kV, 200 µF and 25 µF. Leptosphaeria maculans was transformed with the resulting A. tumefaciens strains as described in a previous study (Gout et al., 2006). For CRISPR-Cas9-mediated gene inactivation, pLAU2-Cas9 was introduced into the JN2 isolate. Transformants then were transformed with pLAU53-gRNA plasmids. Fungal transformants were selected on nourseothricin (50 µg ml–1) for pPZPNat1, hygromycin (50 µg ml–1) for pLAU2-Cas9 and geneticin (50 µg ml–1) for pLAU53-gRNA.

We checked that the fungal transformants with LmSTEE98 inactivation displayed no growth defects in vitro. A plug of

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DNA manipulation and high-resolution melting (HRM) experiments

Genomic DNA was extracted from suspensions of conidia with the DNeasy 96 or DNeasy Plant Mini Kit (Qiagen), in accordance with the manufacturer’s recommendations. LmSTEE genes were amplified by PCR with the primers in Table S3. We performed HRM as described by Plissonneau et al. (2016), to analyze SNPs in the natural population of L. maculans for the various LmSTEE genes considered, with the primers described in Table S3. If variant HRM profiles were detected, the LmSTEE genes were sequenced by Eurofins Genomics (Eurofins, Ebersberg, Germany).

RNA manipulation and quantitative reverse transcription (qRT)-PCR

Total RNA was extracted from the inoculated cotyledons 7 dpi, the time point corresponding to the peak of AvrLm4-7 expression (Parlange et al., 2009), or from inoculated stems 30 dpi. The content of all RNA samples was adjusted to 1 µg of RNA and single-strand cDNA was generated by oligo-dT-primed reverse transcription with the PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA), according to the manufacturer’s protocol. Plants inoculated with water were used as negative controls. For each condition, two to three technical replicates were performed on one (stem samples) or two (cotyledon samples) biological replicates. The qRT-PCR experiments were performed as described by Fudal et al. (2007), with the primers indicated in Table S3. Ct values were analyzed as described elsewhere (Muller et al., 2002). Actin was used as a constitutively expressed reference gene and levels of tubulin expression relative to actin expression were used as a control.

Genetic mapping

Genotyping data for the BnDYDH population included an analysis of SNPs from the Brassica 60K Illumina Infinium SNP array (Clarke et al., 2016), the 20K Illumina Infinium SNP array (Chalhoub et al., 2014) and the 8K Illumina Infinium SNP array (Delourme et al., 2013). The genetic map contained 28,000 loci that represented 3592 unique loci, and covered 2128.2 cM (Laperche et al., 2017). The phenotypic data were mapped as quantitative traits with the R/qtl package (Broman et al., 2003) or as a qualitative traits where scores of 1 to 3 were considered to indicate plant resistance and scores of 4 to 6 were considered to indicate susceptibility.

Bioinformatics and statistical analyses

BLAST analyses were performed by aligning the five LmSTEE protein sequences against the non-redundant protein sequences from the GenPept, Swissprot, PIR, PDF, PDB and NCBI RefSeq databases with default parameters. Hits with an e-value lower than 1.10^{-8} were selected. The PSI-BLAST method with default parameters was used to align the LmSTEE protein sequences against all proteins of Leptosphaeria biglobosa (Dutreux et al., 2018). Hits with an e-value lower than 5 x 10^{-2} were selected.

Statistical analyses were performed with R (R Core Team, 2015). The effect of the mutants used for inoculation on the area affected by stem necrosis was assessed by applying a generalized linear model (GLM), assuming a quasipoisson distribution of the data.

Results

Selection of five late effector candidates

We selected five LmSTEE genes from the 307 effector candidates upregulated in the stem (Gervais et al., 2017). These genes were selected to cover the range of characteristics of the effector gene repertoire (Table 1) and were thus representative of the diversity present in this gene pool. The five LmSTEE genes encoded for small predicted secreted proteins with size ranging from 55 to 291 amino acids (Table 1). Some of them were particularly enriched in cysteins, such as LmSTEE1 with 10 cysteins for 80 amino acids (12.5%) compared to only two for LmSTEE78 (0.7%). The five genes belonged to three of the eight clusters of gene expression identified by Gay et al. (2021); LmSTEE35 and LmSTEE98 belonged to cluster 4 (‘biotrophy to necrotrophy transition’), LmSTEE1 and LmSTEE30 to cluster 5 (‘stem biotrophy’) and LmSTEE78 to cluster 6 (‘stem necrotrophy’; Table 1).

All genes were much less expressed in cotyledons than ‘early’ effector genes and were all highly expressed in stems after inoculation in controlled conditions (Fig. S1). LmSTEE1 and LmSTEE30 were particularly highly expressed during stem colonization. Under field conditions, each gene had a specific expression pattern, with distinct time of peak expression (March for LmSTEE35, April for LmSTEE1, LmSTEE78 and LmSTEE98, and May for LmSTEE30; Fig. S1). Following its peak of expression in April, LmStee98 expression level remained intermediate at all stages except at the last time point, corresponding to stem necrosis (Fig. S1; Gay et al., 2021).

With the exception of LmSTEE98, we were able to find homologs for all the LmSTEE proteins in databases, but all the closest hits corresponded to hypothetical proteins (Table 1). Three proteins paralogous to LmSTEE35 were also found in L. maculans (54% identity for the best hit; Table S4), but not in the related species from the same species complex, L. biglobosa, another pathogen of oilseed rape (Dilmaghani et al, 2009). For each of the other LmSTEE proteins, we identified one to four homologs in L. biglobosa (Table S5).

Conservation of late effector candidates in field populations of L. maculans

We used a collection of 186 isolates from around the world (Dilmaghani et al., 2009, 2012), to study the conservation of the...
Leptosphaeria maculans

Table 1

| Characteristic                        | L. biglobosa | L. maculans |
|---------------------------------------|--------------|-------------|
| **Number of hits on**                 |              |             |
| **Pfam**                              |              |             |
| **CAZymes**                           |              |             |
| **Domain hit**                        |              |             |
| **E-value**                           |              |             |
| **BLAST hit**                         |              |             |
| **(amino acids)**                     |              |             |
| **Expression**                        |              |             |
| **Domain hit**                        |              |             |
| **E-value**                           |              |             |
| **BLAST hit**                         |              |             |
| **(amino acids)**                     |              |             |
| **Expression**                        |              |             |
| **Domain hit**                        |              |             |
| **E-value**                           |              |             |
| **BLAST hit**                         |              |             |
| **(amino acids)**                     |              |             |
| **Expression**                        |              |             |
| **Domain hit**                        |              |             |
| **E-value**                           |              |             |
| **BLAST hit**                         |              |             |
| **(amino acids)**                     |              |             |
| **Expression**                        |              |             |
| **Domain hit**                        |              |             |
| **E-value**                           |              |             |
| **BLAST hit**                         |              |             |
| **(amino acids)**                     |              |             |
| **Expression**                        |              |             |
| **Domain hit**                        |              |             |
| **E-value**                           |              |             |
| **BLAST hit**                         |              |             |
| **(amino acids)**                     |              |             |
| **Expression**                        |              |             |

The five LmSTEE genes were amplified by PCR in almost all isolates (Table 2). Only two French isolates, from two different regions, lacked LmSTEE35, and another two unrelated French isolates yielded no amplicon for LmSTEE98. Sequence polymorphism also was rare, and the sequences of LmSTEE1, LmSTEE35 and LmSTEE78 obtained were invariant. One silent mutation was detected in LmSTEE30, in 18% of the isolates tested, mostly from Western or Central Canada. Only LmSTEE98 displayed sequence polymorphism at the protein level, with one allele containing two nonsynonymous mutations leading to two substituitions in the protein sequence. This allele was found at only one site in Mexico, where it was present in 70% of the local population (17% of the Mexican isolates analyzed here; Tables 2, S1).

The use of an avirulence gene promoter makes it possible to overexpress LmSTEE genes during cotyledon colonization

We induced the expression of LmSTEE genes during cotyledon colonization, by creating mutant strains expressing the genes under the control of the promoter of the avirulence gene AvrLm4-7 (pA4-7), which is known to be upregulated by 7 dpi in cotyledons (Parlange et al., 2009; Fig. S1). Constructs were introduced into the INV13.269 isolate, which is virulent against all Rlm genes potentially present in current B. napus resources (Rlm1, Rlm2, Rlm3, Rlm4, Rlm7 and Rlm9), for the subsequent screening of a large panel of genotypes without interference with known AvrLm–Rlm interactions. The resulting OEC transformants (pA4-7::LmSTEE) strongly expressed LmSTEE genes during cotyledon infection (Fig. 1). We obtained such transformants for four genes, but not for LmSTEE30, which we were unable to overexpress with this strategy. Expression in the cotyledons varied between transformants and genes, but was similar to that of the native AvrLm4-7 gene. The gene most highly expressed in OEC transformants was LmSTEE1, for which the level of expression was upregulated by a factor of 1000 in the transformants at 7 dpi; by contrast, transformants obtained with a construct containing the native promoter had expression levels similar to that of the WT isolate (Fig. 1). These findings demonstrate the usefulness of the AvrLm4-7 promoter for modifying the expression profile of ‘late’ effector genes. The two transformants per construct displaying the strongest overexpression (Fig. 1) were selected for the screening of rapeseed genotypes in the cotyledon inoculation assay.

Two LmSTEE genes expressed in cotyledons induce resistance response in two different cultivars

The OEC transformants were used to inoculate a panel of 204 B. napus genotypes (Table S2). This panel comprised mostly European winter-type rapeseed genotypes, and included only one spring genotype of Asian origin. With this approach, we
identified two cultivars displaying specific resistance to two late effectors: ‘Dariot’, which was resistant to LmSTEE1 and ‘Yudal’, which was resistant to LmSTEE98 (Figs 2, S2). These genotypes displayed resistance responses with the two independent OEC transformants of each construct, but not with the other OEC transformants or the WT isolate. Heterogeneous resistance to other effectors was observed in ‘Dariot’ (Fig. 2), justifying the focusing of our analysis on the specific HR-type resistance to LmSTEE98 observed in ‘Yudal’. We hypothesized that there must be a resistance gene, RlmSTEE98, interacting with LmSTEE98, at least at the cotyledon stage.

**LmSTEE98 inactivation suppresses its recognition in ‘Yudal’ at the cotyledon stage**

For validation of the interaction between LmSTEE98 and RlmSTEE98, we used the CRISPR-Cas9 technology (Idnurm et al., 2017) to inactivate LmSTEE98 in L. maculans. Using the transformant strain JN2 expressing the Cas9 protein (JN2_Cas9), we generated L. maculans LmSTEE98 mutants. Four independent mutants with four different mutations resulting in different truncations or modifications to the protein were selected: A\textsubscript{LmSTEE98}_06 (+1 bp), A\textsubscript{LmSTEE98}_10 (−2 bp), A\textsubscript{LmSTEE98}_20 (−9 bp) and A\textsubscript{LmSTEE98}_30 (−17 bp; Fig. 3a).

In order to test whether these altered versions of the protein were still able to elicit the RlmSTEE98-mediated resistance, these four mutated versions of LmSTEE98 were placed under the control of the \textit{AvrLm4-7} promoter and introduced into INV13.269, to generate OEC mutants. All transformants displayed high levels of expression of the mutated versions of LmSTEE98 on cotyledons at 7 dpi (Fig. S3). All of the OEC mutants were virulent on the susceptible genotype ‘Darmor’, as were the control isolates with the WT version of LmSTEE98 (Fig. 3b). Three OEC mutants with major defects of LmSTEE98 – A\textsubscript{LmSTEE98}_06, A\textsubscript{LmSTEE98}_10 and A\textsubscript{LmSTEE98}_30 – were virulent on ‘Yudal’, suggesting that the corresponding altered versions of the protein were not recognized by the plant (Fig. 3c). By contrast, the pA4-7::A\textsubscript{LmSTEE98}_20 OEC mutant, lacking only three amino acids, was avirulent (Fig. 3c), as were the control isolates pA4-7::LmSTEE98\_4 and pA4-7::LmSTEE98\_9 (Fig. 2), suggesting that this mutant version of the protein was still recognized by the plant. These results indicate that the recognition of LmSTEE98 at the cotyledon stage is impaired by major mutations or truncations of LmSTEE98.

**LmSTEE98 inactivation leads to stronger symptoms during stem colonization**

We investigated the role of LmSTEE98 in stem colonization, using the four mutants inactivated with CRISPR-Cas9 (Fig. 3a). These mutants displayed no \textit{in vitro} growth or sporulation defects and were as virulent as the control isolate JN2_Cas9 when used to inoculate the cotyledons of a susceptible cultivar (Fig. S4). Moreover, they displayed no defect of LmSTEE98 gene expression during stem colonization (Fig. S5). We then tested the
ability of these four mutants to cause stem necrosis on 'Yudal', and on 'Darmor' as a control.

The three mutants with LmSTEE98 gene impairments – DLMSTEE98_06, DLMSTEE98_10 and DLMSTEE98_30 – generated significantly higher percentage areas of stem necrosis on 'Yudal' than the control isolate JN2_Cas9 (Fig. 4a). By contrast, the effects of the DLMSTEE98_20 mutant, which lacked only three amino acids, did not differ significantly from those of JN2_Cas9. On 'Darmor'; no significant effect of LmSTEE98 gene inactivation was observed (Fig. 4b).

Genetic control and mapping of resistance to LmSTEE98

A doubled-haploid population derived from the ‘Darmor-bzh’ × ‘Yudal’ cross (BnDYDH; Foisset et al., 1996) was used to investigate the segregation of the phenotype of resistance to LmSTEE98 in ‘Yudal’. We found that 87 of the 258 offspring tested were resistant to the LmSTEE98 OEC transformants, whereas 171 were susceptible (Table S6). This segregation does not correspond to the expected 50 : 50 (resistant : susceptible) ratio for monogenic resistance in a DH population. However, genetic mapping of the resistant phenotype indicated that the resistance was controlled by a single locus mapping to a 0.6 cM interval on oilseed rape chromosome A09, flanked on one side by the marker Bn-A09-p33777987 and on the other side by Bn-A09-p33971771 (Table S6; Fig. 5). This genetic interval corresponded to a 266.33 kb sequence of chromosome A09 and an unanchored 84 kb sequence (potentially located on chromosome A09). In the reference genome of ‘Darmor-bzh’, this interval was predicted to contain 70 genes (Chalhoub et al., 2014), but a comparison with this genetic interval in ‘Yudal’ was not possible, as a consequence of a lack of sequence data for ‘Yudal’. The control of the interaction by single genes in both the plant and the fungus is consistent with a typical gene-for-gene interaction, and LmSTEE98 was therefore renamed AvrLmSTEE98.

Discussion

The use of resistance genes is the most efficient strategy for controlling stem canker disease in rapeseed crops. However, the resistance to avirulent populations of Leptosphaeria maculans conferred by Rlm/LepR genes is rapidly broken down in the field after the release of resistant varieties (Rouxel & Balesdent, 2017). New sources of resistance to the pathogen are, therefore, urgently required. High-throughput screening for resistance at the cotyledon stage, with genetically improved isolates, is now well developed. However, screening for adult-stage resistance is much more difficult, time-consuming, and less reproducible, with high levels of variation between experiments and between plants. We hypothesized that adult-stage resistance was at least partly dependent on gene-for-gene interactions involving fungal effectors expressed only during stem colonization. We tested this...
hypothesis by selecting a small number of effectors belonging to stem-specific waves of expression, and designing an innovative strategy in which the genes were placed under the control of an early effector gene promoter, making it possible to screen a collection of *Brassica napus* genotypes for resistance genes operating in the stem in a miniaturized cotyledon test. Not only did this efficient strategy identify and map a new resistance source, but, following CRISPR-Cas9 inactivation of the late effector gene identified, we were able to demonstrate its involvement in stem canker severity and recognition by the cognate resistance gene.

**Fig. 2** Resistance response of the rapeseed cultivars ‘Yudal’ and ‘Dariot’ to two ‘late’ effector genes. *Leptosphaeria maculans* transformants expressing *LmSTEE35, LmSTEE1, LmSTEE98* or *LmSTEE78* under the control of the promoter of the avirulence gene *AvrLm4-7* were used to inoculate the ‘Yudal’, ‘Dariot’ and susceptible ‘Eurol’ cultivars. Two independent transformants were used per construct. The bars represent the percentage of plants displaying susceptible (in red) or resistant (in green) phenotypes, based on the IMASCORE rating scale (Balesdent et al., 2001), 14 d post inoculation. INV13.269 is the wild-type isolate and the JN2 and JN3 isolates were used as additional controls. The resistance of ‘Dariot’ to JN3 and JN2 is linked to the presence of *Rlm7*, which recognizes *AvrLm7* in these isolates.
Fig. 3. Generation of CRISPR-Cas9-mutated versions of LmSTE98 and effect of the mutations on their recognition by RlmSTE98 in cultivar ‘Yudal’. Four CRISPR-Cas9-mutated versions of LmSTE98 were generated and their deduced protein sequences were aligned with that of the wild-type (WT) protein in (a). The mutated alleles were placed under the control of the promoter of the avirulence gene AvrLm4-7, and transformants of Leptosphaeria maculans were used to inoculate the susceptible cultivar ‘Darmor’ (b) and the resistant cultivar ‘Yudal’, containing the RlmSTE98 gene (c). Three independent transformants were used per construct. Scoring was based on the IMASCORE rating scale, in which scores of 1–3 indicate resistance and scores of 4–6 indicate susceptibility (Balesdent et al., 2001), 14 d post inoculation. INV13.269 is the WT isolate. Error bars represent the SE for ≥10 biological replicates.
We selected five late effector candidates from three different waves of expression that had previously been shown to be specifically upregulated in rapeseed stems during systemic colonization (Gay et al., 2021). We recently showed that all ‘early’ effectors (including all known avirulence effectors) are actually specifically upregulated during all of the biotrophic stages of plant colonization (leaf/cotyledon, petiole and stems; Gay et al., 2021). The decrease in expression of early effectors is relayed, at least during stem colonization, by a stem-specific biotrophic wave of gene expression including \textit{LmSTEE1} and \textit{LmSTEE30}, followed by another wave involved in the biotrophy-to-necrotrophy transition, which includes \textit{LmSTEE78}, \textit{LmSTEE35} and \textit{LmSTEE98} belong to a less well-defined wave, illustrating a series of biotrophy-to-necrotrophy transitions. In field samples, the expression of \textit{LmSTEE} genes other than \textit{LmSTEE98} and \textit{LmSTEE78}, decreases with the development of stem necrosis (Gay et al., 2021). Some AvrLm effectors, produced during the initial stages of cotyledon colonization, may decrease the magnitude of symptom expression on \textit{B. napus} cotyledons (Petit-Houdenot et al., 2019). Likewise, the \textit{LmSTEE} genes associated with the long systemic colonization of the tissues and involved in biotrophic stem colonization have been suggested to manipulate plant innate immunity, to enable the fungus to colonize its host efficiently, ultimately leading to the development of stem necrosis (Gay et al., 2021).

Both ‘early’ and ‘late’ candidate effectors are structurally similar, as they are small secreted proteins (SSPs) generally enriched in cysteine residues, and most have no predicted function or recognizable domain. However, they differ by their location within the genome. ‘Early’ effector genes are located in dispensable regions of the genome enriched in transposable elements acting as drivers of evolution by favoring deletions and translocations, and diversifying or inactivating point mutations (Rouxel & Balesdent, 2017; Gay et al., 2021; Soyer et al., 2020). By contrast, ‘late’ effector genes are located in GC-equilibrated regions of the genome, an unusual feature for most effector genes in fungi, and a genome environment much less conducive in generating deletions or inactivation by Repeat-Induced Point mutation, as frequently found for \textit{AvrLm} genes (Rouxel et al., 2011; Rouxel & Balesdent, 2017). As a consequence, \textit{LmSTEE} genes also differ from ‘early’ effector genes by their lack of polymorphism or presence/absence variation in field populations of \textit{L. maculans}, with only one nonsynonymous mutation identified in \textit{LmSTEE98}. This suggests that, unlike effector genes expressed at the onset of plant colonization, \textit{LmSTEE} genes, which are not expressed in early stages of infection, are not readily subjected to selection pressures exerted by the

![Fig 4](https://www.newphytologist.com/)

**Fig. 4** The inactivation of \textit{LmSTEE98} by CRISPR-Cas9 method increases stem necrosis in ‘Yudal’ rapeseed stems. \textit{LmSTEE98} mutants of \textit{Leptosphaeria maculans} (Fig. 3a) were used to inoculate the petiole of the second true leaf of \textit{Brassica napus} ‘Darmon’ and ‘Yudal’, cut 1 cm from the point of insertion into the stem. Symptoms were evaluated on stem sections, 30 d post inoculation. (a) Percentage of the stem section displaying necrosis for cultivar ‘Yudal’ \((n \geq 18)\). (b) Percentage of the stem section displaying necrosis for cultivar ‘Darmon’ \((n \geq 18)\). The asterisks indicate a significant difference in the area under the curve between the control isolate JN2_Cas9 and the \textit{LmSTEE98} mutants, for the two biological replicates of the experiment (GLM: **, \(P < 0.05\); ***, \(P < 0.01\)).
plant, such as those generated by Rlm genes. As the corresponding RlmSTEE genes also may have only a partial effect, contributing to adult-stage resistance, they may contribute to more stable and durable resistance in B. napus.

We designed a new approach based on the widely used cotyledon inoculation test, for identifying new sources of resistance to L. maculans in B. napus that could contribute to quantitative resistance. This type of resistance has not, to date, been amenable to miniaturized, medium-throughput screening in growth chambers. We modified the expression of the LmSTEE genes such that they are expressed, under the control of the AvrLm4-7 promoter, during cotyledon infection. This approach was particularly efficient, with all strains transformed with the constructs expressing the target genes to at least the same level as avirulence genes on cotyledons at 7 dpi. We screened a large collection of rapeseed genotypes such that they are expressed, under the control of the AvrLm4-7 promoter, during cotyledon infection. This approach was particularly efficient, with all strains transformed with the constructs expressing the target genes to at least the same level as avirulence genes on cotyledons at 7 dpi. We screened a large collection of rapeseed genotypes to identify easy-to-record plant resistance responses to late effectors. We identified at least one such interaction between LmSTEE98 and a resistance gene in the ‘Yudal’ cultivar. This interaction would not have been identified with the usual screening approaches.

Following inoculation with LmSTEE98 ‘over-expressed in cotyledons’ (OEC) transformants, we observed a clear hypersensitive response (HR) phenotype, which segregated in the BnDYDH population. The segregation pattern in this population was not consistent with the 1:1 ratio expected for monogenic control of the resistance. However, markers closely linked to RlmSTEE98 on A09 also displayed distorted segregation patterns. Such distortions often have been observed in segregating doubled-haploid (DH) populations in B. napus, even in other regions of the genome, and are thought to be due to in vitro androgenesis (Foisset et al., 1996; Delourme et al., 2013). To confirm that our approach revealed a genuine gene-for-gene interaction contributing to quantitative resistance, we used the CRISPR-Cas9 method to introduce a range of mutations into AvrLmSTEE98. Heavily altered versions of the protein did not induce a HR on cotyledons, suggesting a lack of recognition by RlmSTEE98. In addition, the mutant isolates induced larger areas of stem necrosis in ‘Yudal’, also suggesting that the RlmSTEE98-AvrLmSTEE98 interaction contributes to stem resistance to wild-type (WT) isolates.

Our results thus highlighted a role for a single genomic region in ‘Yudal’, and functional analyses strongly suggest that the interaction was controlled by a single resistance gene. The participation of single major genes in adult-stage resistance through gene-for-gene interactions already has been reported in other models, such as the Brassica oleracea Plasmaphthora brassicae (Rocherieux et al., 2004), apple tree/Venturia inaequalis (Calenge et al., 2004) and barley/Pyrenophora graminea (Arru et al., 2003) pathosystems, but has never before been reported for the B. napus-L. maculans interaction. Gene-for-gene interactions underlying partial resistance also have been identified in a few models, such as the wheat/Zymoseptoria tritici system (Meile et al., 2018), or the rice/Magnaporthe oryzae pathosystem, in which an avirulence gene (AvrPi34) corresponding to the single dominant resistance gene Pi34 was shown to be responsible for the partial resistance phenotype (Zenbayashi-Sawata et al., 2005).

The BnDYDH population has been used extensively to search for quantitative trait loci (QTL) for resistance to L. maculans following the scoring of stem necrosis severity in the field (Pilet et al., 1998, 2001; Jestin et al., 2011; Huang et al., 2016). Using the same BnDYDH population we mapped RlmSTEE98 to chromosome A09, but, surprisingly, this locus originated from ‘Yudal’, the parent of the cross considered susceptible under field conditions. QTL for resistance may originate from the susceptible parent, and resistance QTL have been identified in the vicinity of the genomic region containing RlmSTEE98, but no such QTL for resistance was identified in ‘Yudal’ in previous studies, the closest QTL associated with stem canker resistance located on A09 originating from ‘Darmor-bzh’ (Jestin et al., 2011). Stem canker resistance QTL or associated markers on A09 from other cultivars also were detected by Jestin et al. (2015), Larkan et al. (2016a) and Raman et al. (2016). However, all these regions mapped to areas upstream from the physical position of RlmSTEE98.

A09 (Darmor-bzh x Yudal)

| Marker | Location |
|--------|----------|
| Bn-A09-p33456655 | Bn-A09-p33459299 |
| Bn-A09-p33464355 | Bn-A09-p33271433 |
| Bn-A09-p33415187 | Bn-A09-p33473254 |
| Bn-A09-p33485784 | Bn-A09-p33487356 |
| Bn-A09-p33496888 | Bn-A09-p33499505 |
| Bn-A09-p33582715 | BSN008606 |
| Bn-A09-p33544191 | BS011027 |
| Bn-A09-p33535118 | Bn-A09-p33595174 |
| Bn-A09-p33567432 | scaffoldv4_6574_3030 |
| Bn-A09-p33574107 | Bn-A09-p33542334 |
| Bn-A09-p33641390 | scaffoldv4_1659_34722 |
| BSN01723 | BSN012011 |
| Bn-A09-p33694154 | Bn-A09-p33694194a |
| Bn-A09-p33706030 | | 
| scaffoldv4_1975_26115 | Bn-A09-p33841506 |
| scaffoldv4_1426_32643 | Bn-A09-p3385531 |
| scaffoldv4_3931_30023 | Bn-A09-p33892344 |
| Bn-A09-p33777546 | Bn-A09-p33777987 |
| Bn-A09-p33821269 | Bn-A09-p33925635 |
| Bn-A09-p33926185 | | 

**Fig. 5** Genetic mapping of the resistance gene RlmSTEE98 in the ‘Darmor-bzh’ × ‘Yudal’ cross. RlmSTEE98 was mapped to chromosome A09 of Brassica napus. The genetic distances (in cM), are shown on the left, with marker order shown on the right.
There are four possible reasons for the identification of a resistance QTL originating from the susceptible genotype and not identified previously at this genomic location: (1) *Rlm*STEE98 is differentially expressed at different stages of plant growth, being expressed in the cotyledons and leaves, for example, but not in stems, preventing the recognition of *AvrLm*STEE98 during stem colonization, when this gene is expressed. Stage-specific expression patterns have been observed for other resistance genes, such as the wheat *Lr34* gene, which is weakly expressed at the cotyledon stage but strongly induced in adult plants, conferring quantitative resistance to several pathogens (Krattinger et al., 2009). This hypothesis also is supported by the identification of differential expression profiles between cotyledons and stems for the genes present in the *Rlm*STEE98 region in ‘Darmor–bz’ (data not shown). However, our results showing that *AvrLm*STEE98 inactivation increases the size of stem lesions in ‘Yudal’, but not in ‘Darmor–bz’, in controlled conditions strongly suggests that recognition takes place in the stem, rendering this hypothesis highly unlikely. Genomic and transcriptomic data for different developmental stages are now required for ‘Yudal’, to resolve this issue. (2) Alternatively, *AvrLm*STEE98 may be expressed too late or at too low a level during systemic colonization, leading to late or insufficient recognition by the plant, and an inability to prevent the development of stem canker at this stage. Indeed, transcriptomic analyses in ‘Darmor–bz’ have shown that *AvrLm*STEE98 is not expressed in field conditions from November to March, and that its expression peaks in April, but at a lower level than for two of the other *Lm*STEE genes analyzed here, *Lm*STEE1 and *Lm*STEE30 (Gay et al., 2021; Fig. S1). According to this hypothesis, the choice of *Lm*STEE genes for resistance screening in the approach developed here, should be based on a precise characterization of their expression profiles under field conditions, with particular attention paid to effectors expressed at the earliest stages of stem colonization. (3) Another possible explanation for the absence of resistance QTL associated with this region is that the *Rlm*STEE98 region is effectively associated with a resistance QTL conferring partial resistance to the fungus but not identified as such under field conditions. Several studies have shown that the detection of resistance QTL can be strongly influenced by environmental conditions, particularly for the *B. napus–L. maculans* system under European cropping conditions (Kaur et al., 2009; Raman et al., 2012). The effect of the *Rlm*STEE98 gene may be subject to such influences, preventing its detection. (4) Finally, the partial resistance conferred by *Rlm*STEE98 may have remained undetected owing to a combination of insufficient sensitivity and accuracy of the disease severity scoring in the field and a relatively low contribution to quantitative resistance relative to other QTL in this cross.

This study provides a new approach and tools for enriching the *Rlm* gene pool for a system in which the scarcity of *Rlm* genes has proved a critical limitation to crop sustainability. Our study also highlights the importance of the diversity of the plant material screened. Our screening was of limited diversity, with only European winter rapeseed varieties plus ‘Yudal’. More distantly related genotypes also might display new specific resistances to *Lm*STEE effectors. Also, choice of *Lm*STEE genes expressed early during stem infection could allow the identification of new *Rlm*STEE resistance in winter-type European cultivars known to display a high level of adult-stage resistance. Many other late effector candidates have been described, with 40 effector candidates belonging to the stem biotrophy wave of expression (Gay et al., 2021). These genes will be targets of choice for this strategy, now that proof-of-principle has been obtained for its validity.

With time, the *B. napus–L. maculans* system has become a model of choice to conceptualize mechanisms of plant–fungal pathogen coevolution, with a range of adaptive mechanisms set up by the fungus to break-down qualitative resistance while maintaining effector function (Rouxel & Balesdent, 2017). The finding that *Lm*STEE genes may behave as avirulence effectors operating very late in the disease cycle and not prone to accelerated mutation rate make the picture more complex and suggests the plant surveillance machinery has been able to adapt to all tissue-specific waves of effector gene expression to recognize the fungal presence.

The data obtained thus provide new tools and strategies for knowledge-driven breeding for quantitative resistance in *B. napus*. They also cast new light on the mechanisms of quantitative resistance and question on the durable quantitative resistance paradigm in this model.

Acknowledgements

We wish to thank Bertrand Auclair and Yanick Monet (INRAE BIOGER) for plant management. We thank Alexander Idrum (Melbourne University, Australia) for providing us with the vector and protocols for the CRISPR-Cas9 approach. We thank Pascal Glory and the CRB BrACySol, INRAE-IGEPP, France, for providing the BnDHYD population. JG was funded by a joint grant from the Santé des Plantes et Environnement (SPE) department of INRAE and Terres Inovia. AJ was funded by ANRT and Euralis semences (Cifre project no. 2017/1374). The authors acknowledge financial support from SOFIPROTEOL as part of the FASO EPICHAS Project. The UMR Bioger benefits from the support of Saclay Plant Sciences-SPS (ANR-17-EUR-0007).

Author contributions

M-HB, TR, RD designed the research; AJ, JG, AGK, BO, IF, M-HB and TR performed the research; AJ, EJG, RD and M-HB analyzed, collected and interpreted the data; M-HB, TR and SF supervised the work; and AJ, JG, TR, M-HB, RD and SF wrote the manuscript. AJ and JG contributed equally to this work.

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Data availability

Data are available in the main document or supplementary files. Other data (DarmorX/Yudal map, RNAs sequences accession no. for LmSTEE effectors, etc.) are available in the literature.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Expression profiles of *LmSTE* genes during rapeseed colonization.
Fig. S2 Mean disease ratings of three rapeseed cultivars post inoculation with *L. maculans* transformants expressing ‘late’ effector genes in cotyledons.

Fig. S3 Expression of the mutated *LmSTEE98* gene in OEC transformants during early stages of cotyledon colonization.

Fig. S4 *In vitro* growth and pathogenicity phenotypes of *L. maculans* *LmSTEE98* mutants.

Fig. S5 Expression of *LmSTEE98* in mutants during stem infection.

Table S1 Isolates used for polymorphism studies in *L. maculans* populations.

Table S2 List of *B. napus* genotypes screened for resistance.

Table S3 List of primers used in this study.

Table S4 The top 20 BLAST results for each ‘late’ effector.

Table S5 PSI-BLAST results for each late effector against *L. biglobosa* proteins.

Table S6 Cosegregation of resistance to LmSTEE98 and two molecular markers in the ‘Darmor-bzh’ × ‘Yudal’ doubled haploid population.

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