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Conserved ‘Late’ Effector Genes From *Leptosphaeria maculans* Inducing Gene-For-Gene Quantitative Resistance in *Brassica napus* Semi-Winter Genotypes

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ABSTRACT

Leptosphaeria maculans is a phytopathogenic fungus responsible for stem canker on *Brassica napus*. Its infectious cycle goes through an early phase of leaf infection and a late phase of colonisation and infection of the stem. The disease is mainly controlled by plant genetic resistances targeting a limited set of early fungal effector genes overexpressed during leaf infection and located in dynamic repeat-rich genomic regions. Thus, these resistances can be rapidly overcome by the pathogen. To find new sources of resistance, we focused on late effector genes, expressed during stem infection and located in gene-rich regions. A previous study revealed a quantitative resistance in the stem, partly relying on a gene-for-gene interaction with a late effector gene. In this study, we deciphered whether all late effector genes shared the same genomic and evolutionary characteristics and if they could be more stable than early effector genes, rendering the resistance they trigger more durable. In addition, as previous studies highlighted new criteria for selecting late effectors and suggested *B. napus* semi-winter genotypes as an interesting genetic pool for uncovering resistance sources, we selected six new late effector gene candidates and screened an enlarged panel of semi-winter genotypes. We revealed that early and late effector genes diverged for most of their genomic characteristics, supporting the hypothesis of late effector genes being more conserved. Moreover, we revealed new resistance sources to late effector genes, almost all belonging to the semi-winter genetic pool, validating their importance to uncover new resistance sources.

1 | Introduction

During plant infection, fungal pathogens secrete effectors, mainly small secreted proteins, that modulate plant immunity and facilitate infection. Some of them are recognised by the plant immune system and termed avirulence effectors (Rocafort et al. 2020). Part of the plant pathogenic fungi display dual-compartmentalised genome structures divided into gene-dense/repeat-poor regions containing essential and highly conserved housekeeping genes, and gene-sparse/repeat-rich regions harbouring fast-evolving genes, including effector-encoding genes (Dong et al. 2015; Sánchez-Vallet et al. 2018). These repeat-rich

regions correspond to sub-telomeric regions, accessory chromosomes, or are dispersed along the chromosomes and are usually impacted by various mechanisms of rapid evolution: recombination, partial or complete gene deletion, mutation accumulation, and transposon insertion (Lo Presti et al. 2015). In ascomycetes, repeat-rich regions can also be submitted to the RIP (Repeat-Induced Point mutation) mechanism, a premeiotic process controlling repeated element invasion by introducing cytosine to thymine mutations generating stop codons (Cambareri et al. 1989). As RIP can extend beyond the duplicated sequences up to 4 kb (Irelan et al. 1994), it also plays an active role in rapid gene evolution.

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Genetic control, using plants displaying qualitative and/or quantitative resistance, is an efficient strategy to control fungal diseases (Jones and Dangl 2006). Quantitative resistance is a partial resistance taking place at the adult stage and under polygenic control, usually involving several quantitative trait loci (QTLs) with partial effects on the phenotype. These resistances are frequently dependent on the environment and do not prevent the colonisation of the plant but limit the development of the disease and reduce symptom severity (Delourme et al. 2006; Niks et al. 2015; St.Clair 2010). Conversely, qualitative resistance is usually conferred by race-specific resistance (*R*) genes recognising defined effector genes through a gene-for-gene interaction (Flor 1971). This recognition generally induces a localised cell death, called hypersensitive response (HR), preventing further colonisation by avirulent isolates. However, in the case of extensive use of a specific *R* gene, the resistance can be rapidly overcome by the pathogen that becomes virulent (Sprague et al. 2006). Several mechanisms allow pathogens to overcome *R* genes, including a partial or complete deletion of the avirulence gene, a point mutation allowing to escape recognition while maintaining effector function, a down-regulation of the avirulence gene, or the acquisition of new effectors that suppress recognition. Many of these mechanisms are favoured by the location of avirulence genes in repeat-rich regions of the fungal genomes, leading to a rapid bypass of specific resistant sources deployed in the fields. We hypothesise that *R* genes interacting with effector genes located in conserved regions of fungal genomes would be arduous to overcome.

Leptosphaeria maculans is an ascomycete responsible for one of the most damaging diseases on *Brassica napus* (rapeseed): phoma stem canker. This disease can cause yield losses reaching up to 50% and US\$900 million per year (Fitt et al. 2008). Phoma stem canker epidemics are monocyclic and usually initiated by ascospores, produced after sexual reproduction on stem residues, landing on the aerial organs of *B. napus*. After spore germination, hyphae penetrate leaves and cotyledons through stomata or wounding. Once inside the plant, the fungus colonises the apoplast during a short biotrophic stage (5–12 days) and then switches to a necrotrophic lifestyle, inducing leaf spots (Fitt et al. 2006; Rouxel and Balesdent 2005). After leaf infection, a lengthy endophytic systemic colonisation of leaf and stem tissues takes place and can last several months in Europe (West et al. 2001). At the end of the cultural season, the fungus suddenly switches to necrotrophy, causing crown canker responsible for lodging of the plant and associated yield losses. *L. maculans* exhibits a compartmentalised genome structure, with alternating GC-, gene-rich and AT-, repeat-rich regions (Rouxel et al. 2011). Furthermore, Soyer et al. (2021) revealed that this dual compartmentalisation was also visible through chromatin methylation enrichments during axenic growth, gene-rich regions being enriched in the trimethylation of lysine 27 of histone H3 (H3K27me3) associated with facultative heterochromatin and in the di-methylation of lysine 4 of histone H3 (H3K4me2) specific to euchromatin. In contrast, repeat-rich regions were enriched in the trimethylation of lysine 9 of the histone H3 (H3K9me3) associated with constitutive heterochromatin.

During the complex interaction between *L. maculans* and rapeseed, an arsenal of effectors is secreted, facilitating infection

of the host. In a first transcriptomic analysis comparing cotyledon and stem infection of *B. napus* by *L. maculans*, Gervais et al. (2017) differentiated two types of effector genes according to their expression kinetics: ‘early’ effector genes overexpressed during the cotyledon stage of infection, and ‘late’ effector genes or *LmSTEE* (*Leptosphaeria maculans* Stem Expressed Effectors; Jiquel et al. 2021) overexpressed during stem colonisation. Complementing these results, a larger-scale transcriptomics analysis covering all stages of interaction between *L. maculans* and its host identified clusters of effector gene expression (Gay et al. 2021). The first cluster grouped genes expressed during conidia germination and hyphae penetration into cotyledons, and the third cluster grouped genes expressed during necrotrophic stages of infection on cotyledons and petioles. Cluster 5 was specific to asymptomatic colonisation of the stem, and cluster 6 to the necrotrophic stage of stem infection. In contrast, clusters 2 and 4 contained genes overexpressed at both the early and late stages of infection, with genes of cluster 2 expressed during all the biotrophic/asymptomatic stages in cotyledons, petioles and stem, and cluster 4 containing genes highly expressed during the shifts from biotrophy to necrotrophy in cotyledons and the stem base. Thus, the combination of both studies refined the classification of early effector genes to genes grouped in clusters 1–3 and late effector genes in clusters 4–6.

Most of the resistant rapeseed cultivars currently deployed in the field harbour specific *R* genes targeting early effector genes (Vasquez-Teuber et al. 2024) located in AT-rich regions. *Rlm1*, a resistance gene targeting the early effector *AvrLm1*, was overcome in less than three cultural cycles (Rouxel et al. 2003). The main event explaining the switch to virulence was a deletion of the genomic region containing *AvrLm1* (Gout et al. 2007). To identify more durable resistance sources to *L. maculans*, Jiquel et al. (2021) focused on late effector genes, located in gene-rich regions. They hypothesised that gene-for-gene interactions with late effector genes may occur during stem colonisation and contribute to quantitative resistance in *B. napus*. To demonstrate this hypothesis, an innovative phenotyping method, consisting of overexpressing a selection of late effector genes at early stages of infection, allowed the identification of new resistance sources. Indeed, four semi-winter cultivars, Yudal, RG021, RG047 and RG072, presented an HR when inoculated with transformants overexpressing *AvrLmSTEE98*, a late effector gene from cluster 4, and one semi-winter cultivar, RG007, was similarly resistant to *LmSTEE6826*, a late effector gene from cluster 5 (Jiquel et al. 2021, 2022). CRISPR-Cas9 experiments inactivating *AvrLmSTEE98* demonstrated that the interaction at the cotyledon stage followed a classical gene-for-gene scheme. In addition, stem inoculation with a wild-type isolate and transformants inactivated for *AvrLmSTEE98* demonstrated that the presence of *RlmSTEE98* induced a reduction of symptom severity in the stem, indicating that the *AvrLmSTEE98/RlmSTEE98* interaction contributed to the quantitative resistance phenotype.

These pioneer studies highlighted the interest of using late effector genes to search for new resistance sources. However, the refined classification brought by Gay et al. (2021) raised the question of whether all late effector genes shared the same characteristics and if they would be more stable than early effector genes, rendering the resistance they triggered more durable. In addition, Jiquel et al. (2022) highlighted new criteria for

selecting late effectors and suggested that semi-winter rapeseed and rutabagas would be an interesting genetic pool to identify new resistance sources.

In this study, we tested these hypotheses by comparing the characteristics of a large set of early and late effector genes: genomic location, proximity of transposable elements (TE), enrichment in chromatin methylation marks and conservation in a worldwide collection of *L. maculans* isolates (Van de Wouw et al. 2024). We then selected six new late effector candidates using refined criteria of selection, and enriched the previous *B. napus* panel from Jiquel et al. (2022) with 100 additional semi-winter rapeseed and rutabaga genotypes. This study revealed that early and late effector genes diverged for most of their genomic characteristics, strengthening the hypothesis of late effector genes being more conserved. Finally, we identified two new resistance sources to two late effector genes and 14 new genotypes resistant to *AvrLmSTEE98*, all belonging to the semi-winter panel.

2 | Results

2.1 | Early and Late Effector Genes Harboured Highly Distinct Genomic Surroundings

Among the 1207 genes up-regulated *in planta* described by Gay et al. (2021), we selected 151 genes predicted to encode effectors to possess a signal peptide or to have an extracellular location and one or no predicted transmembrane domain (Table S1). This selection was divided into 75 early effector genes (8 from cluster 1, 59 from cluster 2 and 8 from cluster 3) and 76 late effector genes (41 from cluster 4, 26 from cluster 5 and 9 from cluster 6).

The genomic location of effector genes was assessed using isochore annotation on the JN3 genome (Dutreux et al. 2018). We categorised three genomic locations: GC-equilibrated, AT-rich isochores and borders corresponding to a 10kb overlapping region in between. We observed that late effector genes were distributed between GC-equilibrated isochores and borders. In contrast, early effector genes were equally present in the three types of genomic regions (χ^2 test, $p = 8.3e-10$; Figure 1a).

Soyer et al. (2021) previously characterised the chromatin landscape of the *L. maculans* genome during axenic growth. Using these data, we found that early and late effector genes were essentially enriched in heterochromatin methylation marks. Indeed, 53 late effector genes were enriched in H3K27me3, 5 in H3K9me3, and 7 in H3K4me2. Seven late effector genes were enriched with both H3K27me3 and H3K4me2 or H3K27me3 and H3K9me3, and four presented no enrichment. Early effector genes also showed enrichments in heterochromatin methylation marks but presented a more even distribution between H3K27me3 and H3K9me3 (χ^2 test; $p = 2.0e-05$; Figure 1b).

Using the TE annotation of Grandaubert et al. (2014), we assessed their presence, number, distance, and type up to 4kb around effector genes (Figure 2). There was no difference between early and late effector genes for the presence of TE in the interval (χ^2 test, $p = 0.09$; Figure 2a). However, both sets differed in the number of TE in their surroundings (Kruskal–Wallis test, $p = 7.7e-06$; Figure 2b) and the distance from the first TE

(Kruskal–Wallis test, $p = 1.0e-07$; Figure 2c). There was a mean of 4.9 TE close to early effector genes, with the first TE being, on average, at 209bp, compared to 1.5 TE close to late effector genes and the first TE being at 1123bp. Finally, we found no significant difference in the type of TE surrounding the two sets of effector genes, even if we noticed a higher frequency of the DNA transposon ‘DTx’ close to late effector genes (31% compared to 5%; Figure 2d).

2.2 | Late Effector Genes Were More Conserved Than Early Effector Genes in a Worldwide Collection of *L. maculans* Isolates

We used the genomic data of a worldwide collection gathering 205 isolates of *L. maculans* (de Wouw et al. 2024) to determine whether the 151 effector genes were conserved. We were able to assemble 201 isolate genomes and determined protein sequences for the 151 effectors using JN3 as a reference (Table S2). Four effector genes were not sufficiently well assembled to be analysed: two from cluster 2 (*Lmb_jn3_08794* and *Lmb_jn3_12242*), one from cluster 5 (*Lmb_jn3_07669*) and one from cluster 6 (*Lmb_jn3_09959*).

Nine early and four late effectors were absent in more than 10% of the isolates. Three effector genes were absent from a large majority of isolates: *Lmb_jn3_03238* and *AvrLm1* (*Lmb_jn3_13126*) (cluster 2) present in 70 and 34 isolates, respectively, and *Lmb_jn3_05550* (cluster 5) present in 11 isolates. We found that late effectors were significantly more present in the collection than early effectors, with a mean difference of 4 isolates (Kruskal–Wallis test, $p = 3.4e-04$). The number of protein isoforms varied from 0 to 26, with a mean of 2.9 for early effectors and 1.2 for late effectors (Kruskal–Wallis, $p = 0.004$; Figure 3a). The percentage of isolates carrying a protein isoform differing from JN3 ranged from 0% to 97% with a mean of 4% for early effectors and 0.32% for late effectors (Kruskal–Wallis, $p = 0.0014$; Figure 3b).

2.3 | New Selection Criteria Led to a Set of Six Promising Effector Candidates to Uncover New Resistance Sources

Jiquel et al. (2022) had displayed recommendations for the selection of late effectors candidate genes toward the identification of new plant resistance: (i) belonging to cluster 4 or 5, (ii) having no homologue in other fungal species and (iii) being located in a GC-equilibrated region or in a border.

Among the 67 late effector genes belonging to clusters 4 and 5, we first selected 54 genes not previously studied. Using Gay et al. (2021) gene expression analysis, we selected effectors having an expression profile similar to *AvrLmSTEE98* or *LmSTEE6826* in controlled and field conditions (i.e., genes overexpressed during stem infection, preferentially during early stages of stem colonisation, but with a low expression in cotyledons; Figure S1), leading to 32 candidates. Among these candidates, only 16 had no homologue in other fungal species. We selected candidates enriched in H3K27me3 chromatin methylation marks, leading to 11 potential candidates. Finally, when a reliable 3-D structure prediction was available using AlphaFold

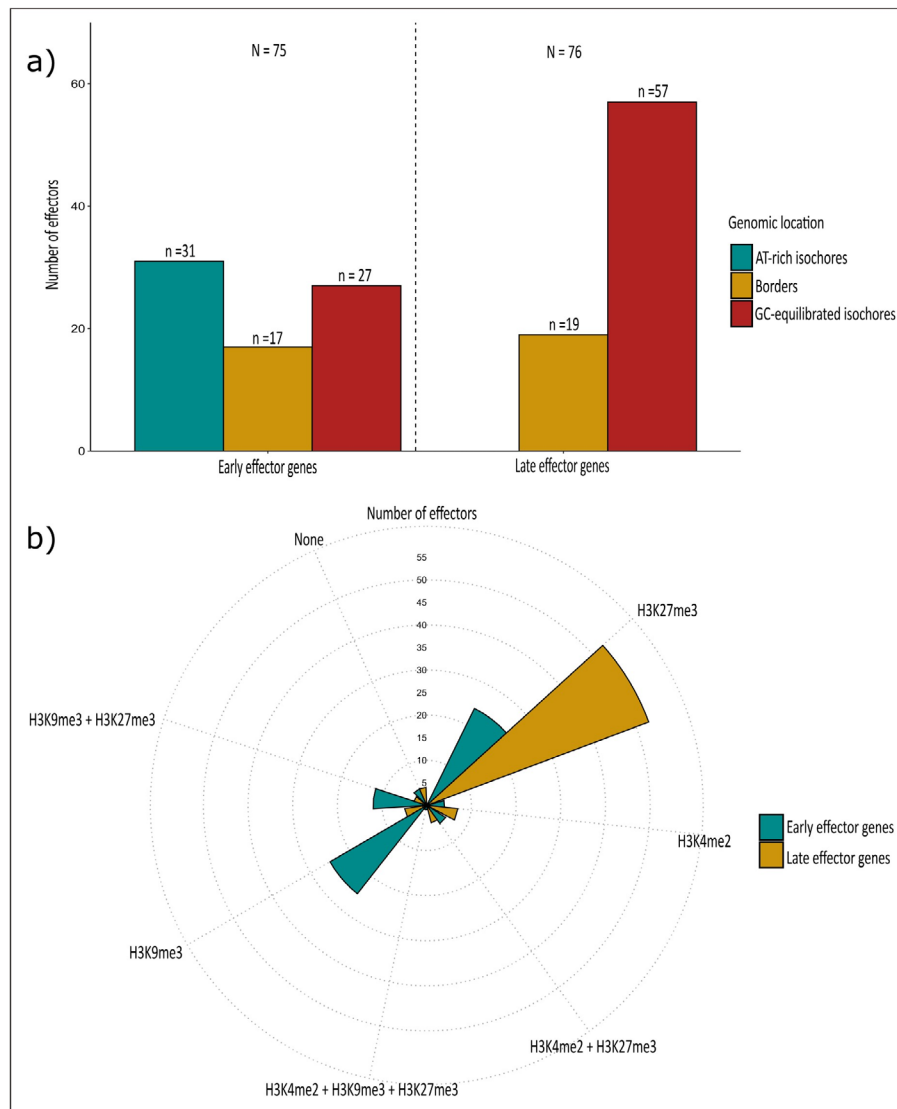


FIGURE 1 | Genomic location (a) and chromatin methylation enrichment during axenic growth (b) of early and late effector genes from *Leptosphaeria maculans*. Seventy-five early and 66 late effector genes overexpressed during rapeseed leaf infection and stem infection, respectively, were characterised. Genomic location of effector genes was assessed using the annotations on the JN3 genome (Dutreux et al. 2018). Enrichment in chromatin methylation marks was assessed using ChIP-seq data from Soyer et al. (2021).

(Mirdita et al. 2022), we favoured a diversity of structural analogs in our six final candidates (Table 1).

Five of these candidates were assigned to cluster 4 and one to cluster 5. They all encoded small secreted proteins ranging from 84 to 124 amino acids (aa). Two were enriched in cysteines, *Lmb_jn3_00833* with 9 cysteines for 99 aa (9%) and *Lmb_jn3_01853* with 10 cysteines for 110 aa (9%). Five candidate genes were located in GC-equilibrated regions, while *Lmb_jn3_01853* was located in a border. Two candidates, *Lmb_jn3_00833* and *Lmb_jn3_09385*, harboured a DNA transposon ‘Gimli’ respectively at 200bp and 1500bp. Two other candidates harboured a retrotransposon at less than 400bp. One candidate was not surrounded by TE and one harboured three DNA transposons ‘Gimli’, and two retrotransposons. Except for *Lmb_jn3_00833*, which was predicted to have structural analogy with KP6 Killer Toxin proteins, all candidates had a medium to low structural prediction.

These six candidate effector genes were renamed *LmSTEE833*, *LmSTEE1853*, *LmSTEE2187*, *LmSTEE4385*, *LmSTEE5765*, and *LmSTEE9385* and completed the 10 effector genes studied in Jiquel et al. studies: *LmSTEE1*, *LmSTEE1277*, *LmSTEE1852*, *LmSTEE35*, *LmSTEE5465*, *LmSTEE78*, *LmSTEE7919*, *LmSTEE10933*, *LmSTEE6826* and *AvrLmSTEE98*.

2.4 | Overexpression of New *LmSTEE* Genes at the Cotyledon Stage of Infection

We placed the six effector genes selected for this study, plus *AvrLmSTEE98* and *LmSTEE6826* (Jiquel et al. 2021, 2022) effector genes, under the control of the *AvrLm4-7* promoter. We introduced the eight constructs into the X83.51 isolate, virulent toward most known *Rlm* genes. We obtained from four up to 12 transformants for each construct. Transformants’ growth was tested on V8 agar medium, and pathogenicity was assessed by

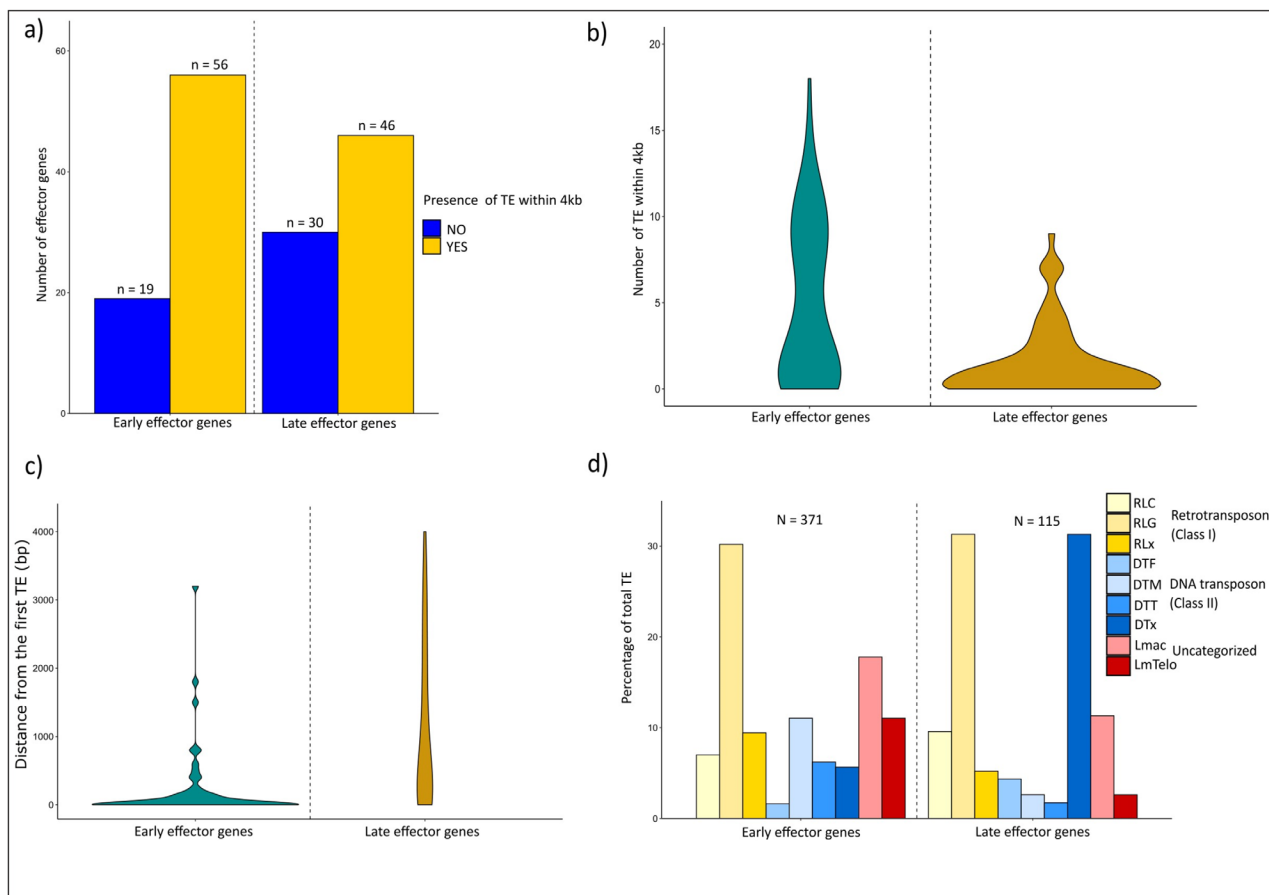


FIGURE 2 | Presence (a), number (b), distance (c) and type (d) of transposable elements (TEs) in the genomic surroundings of effector genes of *Leptosphaeria maculans*. The presence, number, and distance of TEs within 4 kb upstream and downstream of the effector genes were assessed using the annotations of Grandaubert et al. (2014). The type of TE (DNA transposon, retrotransposon or uncategorised) was calculated as a percentage of the total number of TEs in the surrounding genomic region.

inoculation on a susceptible genotype, Darmor. All transformants displayed no growth or pathogenicity defects except for pA4-7::*LmSTEE98.15* and pA4-7::*LmSTEE98.16*, which were not virulent on Darmor. All transformants were amplified and sequenced to confirm the correct insertion of the construct. We tested the level of *LmSTEE* gene expression at 7 days post-inoculation (dpi) in a maximum of four transformants per gene. Expression in the infected cotyledons varied between 1.1×10^2 and 1.1×10^4 compared to *Actin* expression, and all transformants overexpressed the *LmSTEE* gene when compared with the native gene in X83.51 (from a factor 100 to a factor 1000; Figure 4). For each *LmSTEE*, the two transformants having the highest level of expression at 7 dpi and a correct sequencing result were selected for the screening of 207 genotypes of *B. napus* (Table S3).

2.5 | New Resistance Responses Were Highlighted Using New *LmSTEE* Genes and an Enlarged *B. napus* Panel

OverExpressed in Cotyledons (OEC) transformants expressing *LmSTEE* genes described by Jiquel et al. (2021, 2022) were inoculated on 100 genotypes belonging to the semi-winter genetic

pool, including rapeseed and rutabaga (Table 2, Table S4). This screening revealed 14 genotypes, 13 rapeseed and one rutabaga displaying an HR when inoculated with *AvrLmSTEE98* OEC transformants in INV13.269 genetic background. All these 14 genotypes displayed the same resistance response when inoculated with *AvrLmSTEE98* OEC transformants obtained during the present study in the X83.51 background (Figure 5a).

In addition, an HR was induced by *LmSTEE1277* on the semi-winter rapeseed INN_GMLm_RG136. This genotype displayed a resistance response toward the two independent OEC transformants but not with the wild-type isolates INV13.269 (Kruskal-Wallis test, $p = 0.001$; Figure 5b).

The screening of the 207 genotypes with new *LmSTEE* genes also revealed promising interactions (Table 2, Table S4). The rutabaga INN_GMLm_RG200 was resistant to *LmSTEE833* (Kruskal-Wallis test, $p = 1.5 \times 10^{-8}$; Figure 5c), and the spring genotype INN_GMLm_RG078 displayed an HR when inoculated with *LmSTEE5765* OEC transformants. However, depending on the biological replicate, this genotype switched between susceptible and resistant responses when inoculated with X83.51, making the resistance phenotype induced by *LmSTEE5765* difficult to interpret.

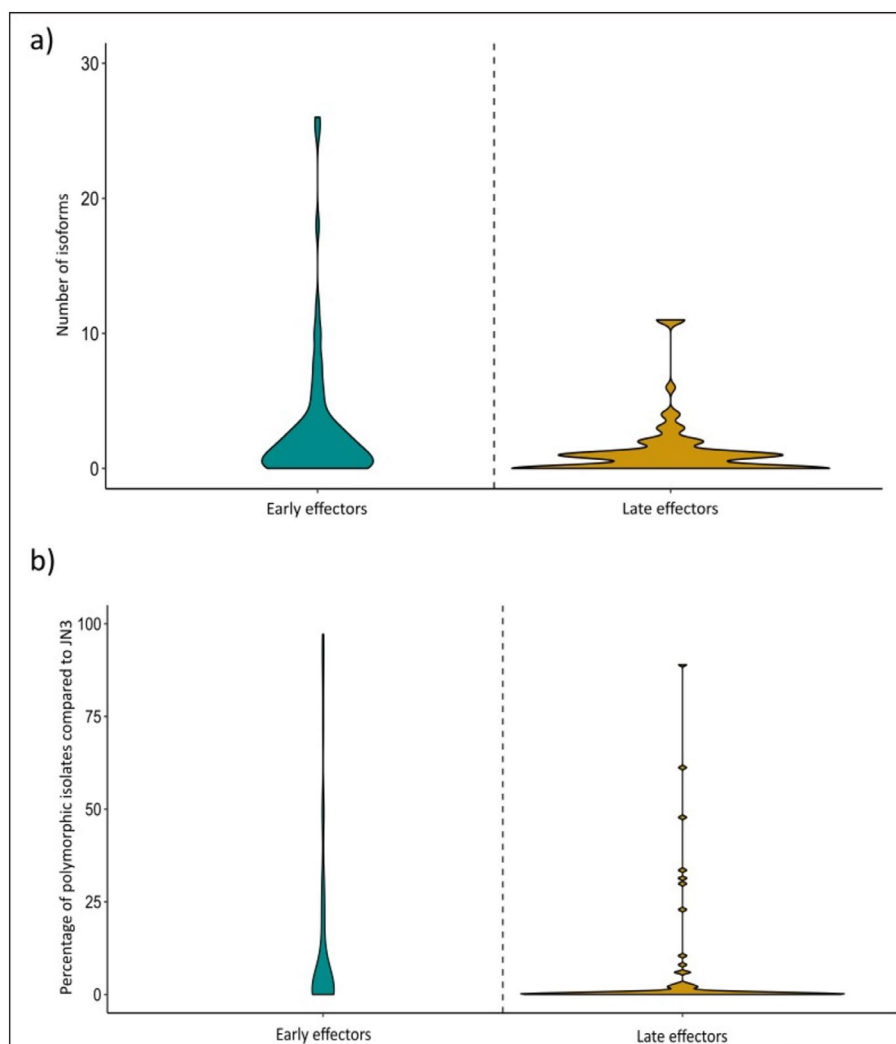


FIGURE 3 | Conservation of early and late effectors in a worldwide collection of *Leptosphaeria maculans* isolates (de Wouw et al. 2024). We compared the protein sequences of 201 *L. maculans* isolates for 73 early and 74 late effectors overexpressed during rapeseed leaf infection and stem infection, respectively, using JN3 as a reference. We assessed the number of isoforms identified for each effector (a) and the percentage of isolates carrying a polymorphic effector compared to the JN3 proteome (b).

One intermediate resistance corresponding to a delayed resistance phenotype was triggered by *LmSTEE5765* on the semi-winter rapeseed INN_GMLm_RG047. Several heterogeneous resistance phenotypes were revealed by *LmSTEE* transformants on various semi-winter genotypes, resulting in an alternance of susceptible and resistant phenotypes. Finally, 10 genotypes harboured resistance phenotypes when inoculated with X83.51.

Except for the spring genotype INN_GMLm_RG078, all genotypes displaying an HR to *LmSTEE* genes belonged to the semi-winter genetic pool, including rapeseed and rutabaga (Figure 6).

3 | Discussion

Genetic resistance is the most efficient strategy to control stem canker disease in rapeseed crops. However, as gene-for-gene resistance to *L. maculans* conferred by *Rlm* genes can rapidly be overcome in the fields, there is an urge to find new resistance sources with better durability combined with a coordinated and managed *R* gene deployment in the fields (Vasquez-Teuber

et al. 2024). In the present study, we revealed that late effector genes highly differed in their genomic and epigenomic context from early effector genes. We also showed that late effector genes were more conserved in a worldwide collection of *L. maculans* isolates, suggesting that the potential cognate resistances they could trigger would be more difficult to overcome. In addition, the screening assays confirmed the interest of late effector genes in uncovering new resistance sources, with the identification of three new potential gene-for-gene interactions. Except for one spring genotype, all resistance sources were found in the semi-winter genetic pool, emphasising the importance of this latter to identify resistance to late effector genes.

We studied a set of 151 effector genes (75 early and 76 late) and revealed that their genomic characteristics were significantly distinct. Late effector genes were specifically located in GC-equilibrated isochores and borders with AT-rich isochores, while early effector genes were well distributed between the three genomic locations, with all known *AvrLm* genes located in AT-rich isochores, in agreement with previous studies. Rouxel et al. (2011) described AT-rich isochores as a genomic

TABLE 1 | Description of the six *Leptosphaeria maculans* late effector genes selected to uncover new resistance sources in *Brassica napus*. The effector genes were predicted by Dutreux et al. (2018). The expression clusters were defined by Gay et al. (2021). Genomic location in AT-rich (AT), GC-equilibrated (GC) isochores or borders between these two locations were defined using Dutreux et al. (2018) annotation. Transposable elements present 4 kb upstream and downstream of the gene were annotated by Grandaubert et al. (2014). Enrichment in chromatin methylation marks during axenic growth was detected by Soyer et al. (2021) using ChIP-seq. Protein homologues were identified using the NCBI nr database, the quality of the structural prediction using AlphaFold (Mirdita et al. 2022), and close structural analogs according to the Dali database.

Gene ID ^a	Expression cluster ^b	Genomic location ^c	Transposable elements close to the gene ^d	Enrichment in chromatin methylation mark ^e	Protein size (amino acids)	Number of cysteines	First five protein homologues ^f	Structural prediction ^g	First five structural analogs ^h
<i>Lmb_jn3_00833</i>	4	GC	DTx_Gimli (1500pb)	H3K27me3	99	9	None	Good	KP6 Killer Toxin Subunit Alpha
<i>Lmb_jn3_01853</i>	4	Border	DTx_Gimli (100pb) DTx_Gimli (300pb) DTx_Gimli (1700pb) RLC_Zolly-1 (2500pb) RLG_Olly (2700pb)	H3K27me3	110	10	None	Low	NA
<i>Lmb_jn3_02187</i>	4	GC	RLG_Brawly (chevauchant)	H3K27me3	107	5	None	Low	NA
<i>Lmb_jn3_04385</i>	5	GC	RLG_Olly (400pb)	H3K27me3	90	6	None	Medium	NA
<i>Lmb_jn3_05765</i>	4	GC		H3K27me3	84	6	None	Low	NA
<i>Lmb_jn3_09385</i>	4	GC		H3K27me3	124	4	None	Medium	NA

^aFrom JN3 annotation (Dutreux et al. 2018).

^bData from Gay et al. (2021).

^cFrom JN3 annotation (Dutreux et al. 2018); Border = 10 kb interval between AT-rich and GC-equilibrated isochores.

^dFrom JN3 TE annotation (Grandaubert et al. 2014).

^eData from Soyer et al. (2021).

^fBlastp outside *L. maculans*, from NCBI nr database: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

^gPrediction by AlphaFold2 (ColabFold v1.5.5, Mirdita et al. 2022).

^hDali server, Z > 5, RMSD < 3: <http://ekhidna2.biocenter.helsinki.fi/dali/>.

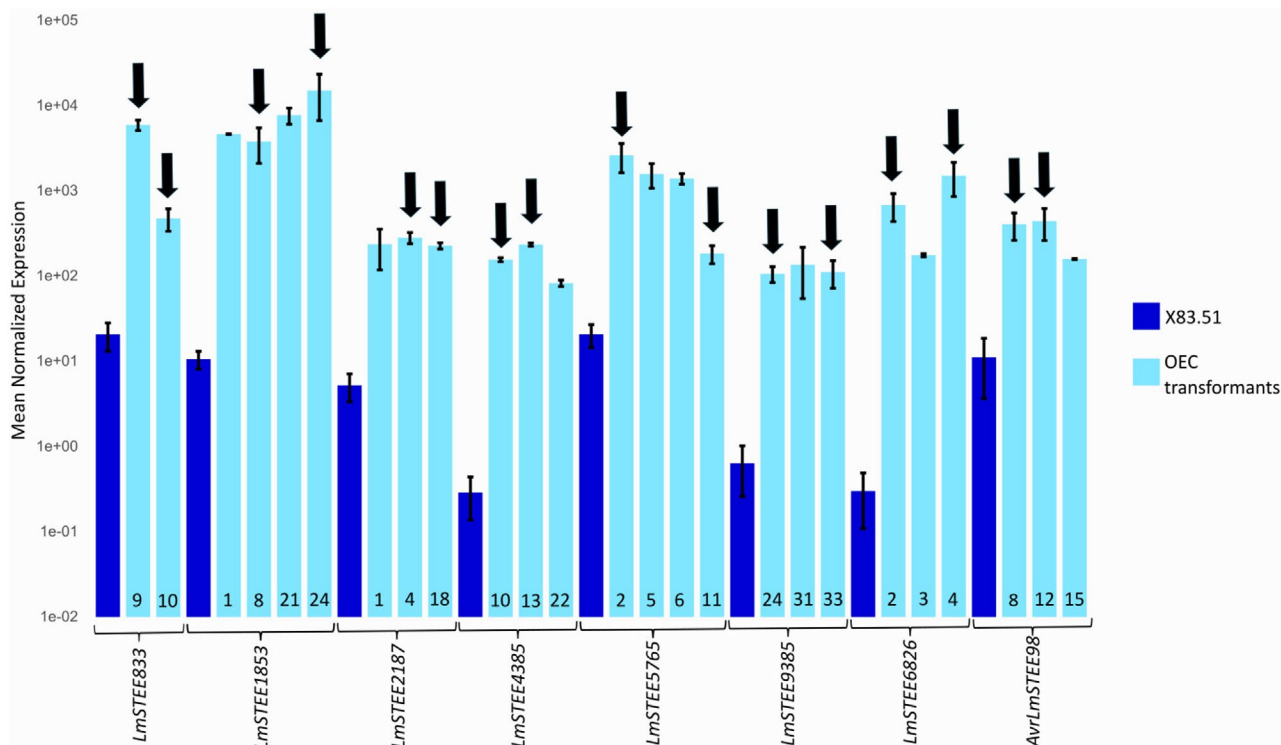


FIGURE 4 | Expression of six new *LmSTEE* genes in ‘overexpressed in cotyledons’ (OEC) transformants of *Leptosphaeria maculans* at an early stage of cotyledon colonisation. We obtained OEC transformants overexpressing the *LmSTEE* genes during rapeseed cotyledon infection by putting these genes under the control of the *AvrLm4-7* promoter. The expression of *LmSTEE* genes was assessed by reverse transcription-quantitative PCR in infected cotyledons of the cultivar Darmor, 7 days post-inoculation. Transformant identification numbers are mentioned at the bottom of the expression bars. Black arrows indicate OEC transformants selected for the screening of plant genotypes. Mean expression is normalised against *Actin*, with *EF1α* used as a control (Fudal et al. 2007). Error bars represent the standard error for two biological and two technical replicates.

environment promoting rapid sequence diversification and underpinning the evolutionary potential of the fungus to rapidly adapt to novel host-derived constraints. The absence of late effector genes from this dynamic genomic environment suggests that they are not affected by rapid evolutionary mechanisms and are conserved in *L. maculans* populations. TEs also have an important role in this rapid genomic evolution. Indeed, they can be inserted into gene sequences or promoters and are targeted by the RIP mechanism. RIP was detected in *L. maculans* by Idnurm and Howlett (2003) and is known to have an active role in effector gene rapid evolution as it can extend to 4 kb beyond the targeted duplicated sequence (Irelan et al. 1994). To determine if late effector genes could be affected by the presence of TEs, we investigated their presence, number, distance, and type 4 kb around the 151 effector genes. We demonstrated that both sets of effector genes are not different in terms of the presence of TEs in their close genomic surrounding. However, early effector genes harboured, on average, three times more TEs than late effector genes, and their first TE was six times closer. The lower number of TEs in the late effector gene environment also suggests that they could be less affected by RIP leaks, as RIP in *L. maculans* required large repeated regions (Van De Wouw et al. 2019). In addition, the higher distance between late effector genes and their first TE suggests that TEs are less susceptible to affect their promoter or coding sequences. These results support the hypothesis that late effector genes are better conserved in *L. maculans* populations, and that their associated resistances, if existing, may be more durable.

To test this hypothesis, we determined the presence and polymorphism of early and late effector genes in a worldwide collection. We demonstrated that late effector genes: (i) were significantly more present in the collection, (ii) presented half fewer isoforms and (iii) had 12 times fewer polymorphic isolates compared to JN3 than early effector genes. Except for *AvrLm10A*, all known *AvrLm* genes were absent or polymorphic in at least 20% of isolates, with a maximum observed for *AvrLm1* (absent in 167 isolates and polymorphic in three isolates). In contrast, *LmSTEE* genes, described in previous and present studies, presented some polymorphic isolates ranging from one for *LmSTEE6826* to four for *AvrLmSTEE98*, all isoforms differing by one amino acid substitution. These results confirmed the better conservation of late effector genes in *L. maculans* populations. However, these analyses are preliminary as Illumina Novaseq sequencing was used by Van de Wouw et al. (2024), making the assembly and annotation difficult. In the analysis for isoform number and polymorphic isolates ratio, we did not consider isolates lacking the start or the end of the protein, as it could be due to an error during sequencing or assembly. Long-read sequencing should be used to validate these first results.

The results of both genomic characteristics and conservation analyses in *L. maculans* populations highlighted the interest in late effector genes to uncover new resistance sources in *B. napus*. Using Jiquel et al. (2022) recommendations, we selected six new late effector genes added to *AvrLmSTEE98* and *LmSTEE6826*, previously characterised, and expressed them in X83.51 under

TABLE 2 | Incompatible interactions between *Brassica napus* genotypes and *LmSTEE* genes. 207 genotypes were screened using transformants overexpressing six new *LmSTEE* genes and 10 *LmSTEE* genes previously selected for the identification of new resistance sources (Jiquel et al. 2021, 2022).

Genotype	Diversity group	<i>LmSTEE</i> ^a	Type of resistance response ^b
INN_GMLm_RG123	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG125	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG129	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG145	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG149	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG160	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG168	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG170	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG171	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG172	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG177	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG194	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG198	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG199	Semi-winter	<i>LmSTEE98</i> (INV13.269 and X83.51 background)	HR
INN_GMLm_RG108	Semi-winter	<i>LmSTEE98</i> (INV13.269 background)	HR
INN_GMLm_RG110	Semi-winter	<i>LmSTEE98</i> (X83.51 background)	HR
INN_GMLm_RG136	Semi-winter	<i>LmSTEE1277</i> (INV13.269 background)	HR
INN_GMLm_RG078	Spring	<i>LmSTEE5765</i> (X83.51 background)	HR
INN_GMLm_RG200	Semi-winter	<i>LmSTEE833</i> (X83.51 background)	HR
INN_GMLm_RG047	Semi-winter	<i>LmSTEE5765</i> (X83.51 background)	Intermediate resistance
INN_GMLm_RG024	Semi-winter	<i>LmSTEE5765</i> (X83.51 background)	Heterogeneous resistance
INN_GMLm_RG056	Semi-winter	<i>LmSTEE5765</i> (X83.51 background)	Heterogeneous resistance
INN_GMLm_RG089	Semi-winter	<i>LmSTEE6826</i> (X83.51 background)	Heterogeneous resistance
INN_GMLm_RG136	Semi-winter	<i>LmSTEE10933</i> (INV13.269 background)	Heterogeneous resistance
INN_GMLm_RG139	Semi-winter	<i>LmSTEE35</i> (INV13.269 background)	Heterogeneous resistance
INN_GMLm_RG151	Semi-winter	<i>LmSTEE35</i> (INV13.269 background)	Heterogeneous resistance
INN_GMLm_RG151	Semi-winter	<i>LmSTEE5465</i> (INV13.269 background)	Heterogeneous resistance
INN_GMLm_RG151	Semi-winter	<i>LmSTEE10933</i> (INV13.269 background)	Heterogeneous resistance
INN_GMLm_RG163	Semi-winter	<i>LmSTEE1853</i> (X83.51 background)	Heterogeneous resistance
INN_GMLm_RG163	Semi-winter	<i>LmSTEE9385</i> (X83.51 background)	Heterogeneous resistance
INN_GMLm_RG198	Semi-winter	<i>LmSTEE5765</i> (X83.51 background)	Heterogeneous resistance
INN_GMLm_RG020	Unknown	X83.51	HR
INN_GMLm_RG072	Semi-winter	X83.51	HR
INN_GMLm_RG090	Semi-winter	X83.51	HR
INN_GMLm_RG091	Semi-winter	X83.51	Heterogeneous resistance
INN_GMLm_RG093	Unknown	X83.51	HR

(Continues)

TABLE 2 | (Continued)

Genotype	Diversity group	<i>LmSTEE</i> ^a	Type of resistance response ^b
INN_GMLm_RG147	Semi-winter	X83.51	Heterogeneous resistance
INN_GMLm_RG170	Semi-winter	X83.51	HR
INN_GMLm_RG172	Semi-winter	X83.51	HR
INN_GMLm_RG178	Semi-winter	X83.51	HR
INN_GMLm_RG179	Semi-winter	X83.51	HR
INN_GMLm_RG078	Spring	X83.51	Heterogeneous resistance

^a*LmSTEE* gene involved in the interaction with the genotype and background isolates in which the *LmSTEE* gene was introduced to obtain Over-Expressed in Cotyledons (OEC) transformants (INV13.269 or X83.51).

^bResistance phenotype observed during the interaction. HR = hypersensitive response, intermediate resistance corresponds to a delayed resistance phenotype, and heterogeneous resistance corresponds to an alternance of susceptible and resistant phenotype for a genotype × *LmSTEE* interaction.

the control of *AvrLm4-7* promoter. We confirmed the resistance phenotypes triggered by *AvrLmSTEE98* in the four genotypes Yudal, RG021, RG047 and RG072 and by *LmSTEE6826* in RG007.

We also revealed new resistance sources to *LmSTEE* genes through the combination of two strategies: the use of a new set of *LmSTEE* genes added to the 10 previously selected, and the enrichment of the *B. napus* panel in semi-winter rapeseed and rutabaga genotypes. Indeed, the screening of a semi-winter panel revealed 14 new genotypes resistant to *AvrLmSTEE98*. While the four already known genotypes resistant to *AvrLmSTEE98* were all from Asian origins (Korea and Japan), among the 14 genotypes uncovered in this study, six were from Japan and two from Korea, but four genotypes were from Australia, one from Germany, and one from the United States (Table S5). However, as Australian breeding programmes were initiated with Japanese spring varieties and French winter varieties (Salisbury et al. 1995), we can hypothesise that most resistance sources to *AvrLmSTEE98* are derived from Asian genotypes. The absence of Asian isolates in the IBCN collection (Van de Wouw et al. 2024) did not allow us to determine whether *AvrLmSTEE98* was conserved in Asian isolates or if the gene was subject to selection pressure in these populations.

Screening semi-winter and rutabaga genotypes also allowed the discovery of two new resistances induced by *LmSTEE* genes. The Japanese semi-winter rapeseed INN_GMLm_RG136 displayed HR to *LmSTEE1277*, and the Danish rutabaga INN_GMLm_RG200 was resistant to *LmSTEE833*. *LmSTEE1277* had already been studied by Jiquel et al. (2022), but no HR had been observed, confirming the interest in the semi-winter genetic pool. The absence of resistance sources to *LmSTEE* genes in the winter genetic pool compared to the semi-winter raises questions about their divergence. We can hypothesise a sampling bias, an independent evolution between both pools or a linkage disequilibrium between resistance to *LmSTEE* genes and another deleterious character, which would have been eliminated by selection from the elite varieties composing winter genotypes.

One last resistance phenotype was more difficult to interpret. Indeed, INN_GMLm_RG078 presented a resistance phenotype to *LmSTEE5765* for all experiments; however, depending on the experiment, the genotype alternated between susceptibility and resistance to X83.51, making it difficult to conclude on the sole

recognition of *LmSTEE5765* to trigger the HR. INN_GMLm_RG078 is an old (1963) German elite line with 13% of residual heterogeneity (Table S5) potentially acting on the resistance phenotype to X83.51. We can hypothesise that INN_GMLm_RG078 has a resistance gene recognising X83.51 (eventually *Rlm5*, *Rlm10* or *Rlm14*) but not fixed at the locus, explaining the heterogeneity of the phenotype. Additionally, even if performed in controlled conditions, the pathogenicity assays may have been subjected to temperature or humidity variations that could have influenced the resistance phenotype, as previously found for other *AvrLm*–*Rlm* interactions (Neik et al. 2022; Yang et al. 2021).

One intermediate resistance interaction was induced by *LmSTEE5765* in INN_GMLm_RG047, a Korean semi-winter rapeseed already known for its resistance to *AvrLmSTEE98*. First described in Blondeau et al. (2015), intermediate resistance phenotypes correspond to delayed recognition of an *AvrLm* protein by its cognate *Rlm* protein. As already postulated by Jiquel et al. (2022), this type of interaction could derive from a time lag between the peak of expression of *LmSTEE5765* in the OEC transformants and the expression profile of the corresponding *Rlm* gene. Another hypothesis is a partial recognition of the *AvrLm* protein due to an allelic variation in its encoding gene (Blondeau et al. 2015).

The two strategies developed in this study for uncovering new resistance sources were efficient. On one hand, the enlargement of the screening panel with genotypes from the semi-winter genetic pool allowed the identification of 14 new resistance sources to *AvrLmSTEE98* and one to *LmSTEE1277*, two late effector genes previously studied. On the other hand, the selection of six new late effector genes with improved selection criteria permitted the uncovering of two other resistance sources, one to *LmSTEE833* and one to *LmSTEE5765*. Resistance to *LmSTEE833* was found in a rutabaga, underlying the importance of combining the two strategies to maximise the chances of discovering new resistance.

Our study provides new perspectives for the identification of new resistant genotypes and their use for durable disease management strategies. However, resistance to *LmSTEE* genes has not yet been tested in the field. As semi-winter genotypes are not adapted to European growing conditions, resistance to *LmSTEE* genes should be tested by introgression in elite winter

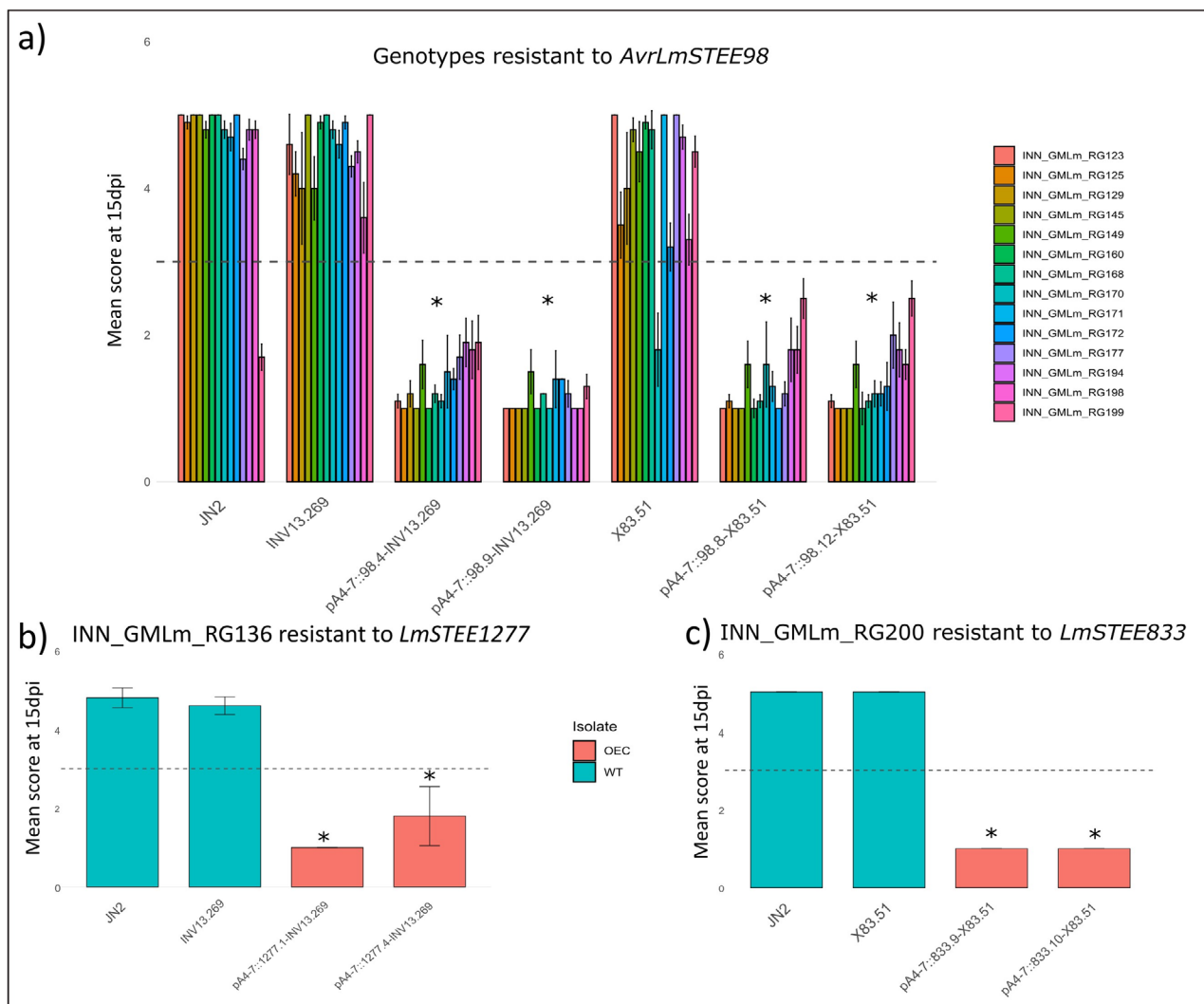


FIGURE 5 | Resistance of *Brassica napus* to *LmSTEE* genes revealed by pathogenicity assays using transformants overexpressing these genes at the cotyledon stage of infection. (a) 14 semi-winter genotypes displayed hypersensitive responses (HR) when inoculated with *AvrLmSTEE98* OverExpressed in Cotyledons (OEC) transformants (in INV13.269 and X83.51 backgrounds). (b) INN_GMLm_RG136 displayed an HR when inoculated with *LmSTEE1277* OEC transformants. (c) INN_GMLm_RG200 displayed an HR when inoculated with *LmSTEE833* OEC transformants. Scoring was performed 15 days post-inoculation (dpi). Scores from 1 to 3 correspond to a resistant phenotype and scores from 4 to 6 to a susceptible phenotype (IMAScore scale, Balesdent et al. 2001). Error bars represent the standard error for ≥ 6 biological replicates. The asterisks indicate a significant difference between the wild-type isolates and the OEC transformants (Kruskal-Wallis test: $*p < 0.001$).

cultivars. In addition, while we proved that late effector genes were more conserved in the *L. maculans* worldwide population, there is currently no proof that this conservation would be maintained in the case of extensive use of the corresponding resistance. Before deployment of resistance to late effectors, the *L. maculans* population should be monitored for *LmSTEE* gene polymorphism in the field in order to avoid rapid evolution of the pathogen against these new resistances.

4 | Experimental Procedures

4.1 | Fungal and Plant Materials

The JN2 isolate (v.23.1.2; Balesdent et al. 2002), closely related to JN3 (v.23.1.3; Balesdent et al. 2002), INV13.269 (Plissonneau et al. 2016), and X83.51 were used as controls in

inoculation tests. X83.51 results from a cross between IBCN14 (Balesdent et al. 2005) and X22.14, itself being an offspring of INV13.269 crossed with WT50-3 (Neik et al. 2020). OEC transformants described by Jiquel et al. (2021, 2022) were used for inoculation tests. All fungal isolates used in this study are described in Table S3. Fungal isolates were grown and sporulated on V8 juice agar medium as described by Ansan-Melayah et al. (1995).

The *B. napus* panel is composed of 207 genotypes. The first 107 genotypes were described by Jiquel et al. (2022) and are referred to as the 'EPHICAS panel'. This panel was set by genotyping *B. napus* genotypes with an internal array of 6331 SNPs covering the 19 chromosomes. A kinship matrix was created with the R package emma using the genotyping results. This Identity by State (IBS) matrix was used to compute a principal coordinate analysis (PCA) using the R packages FactoMineR and

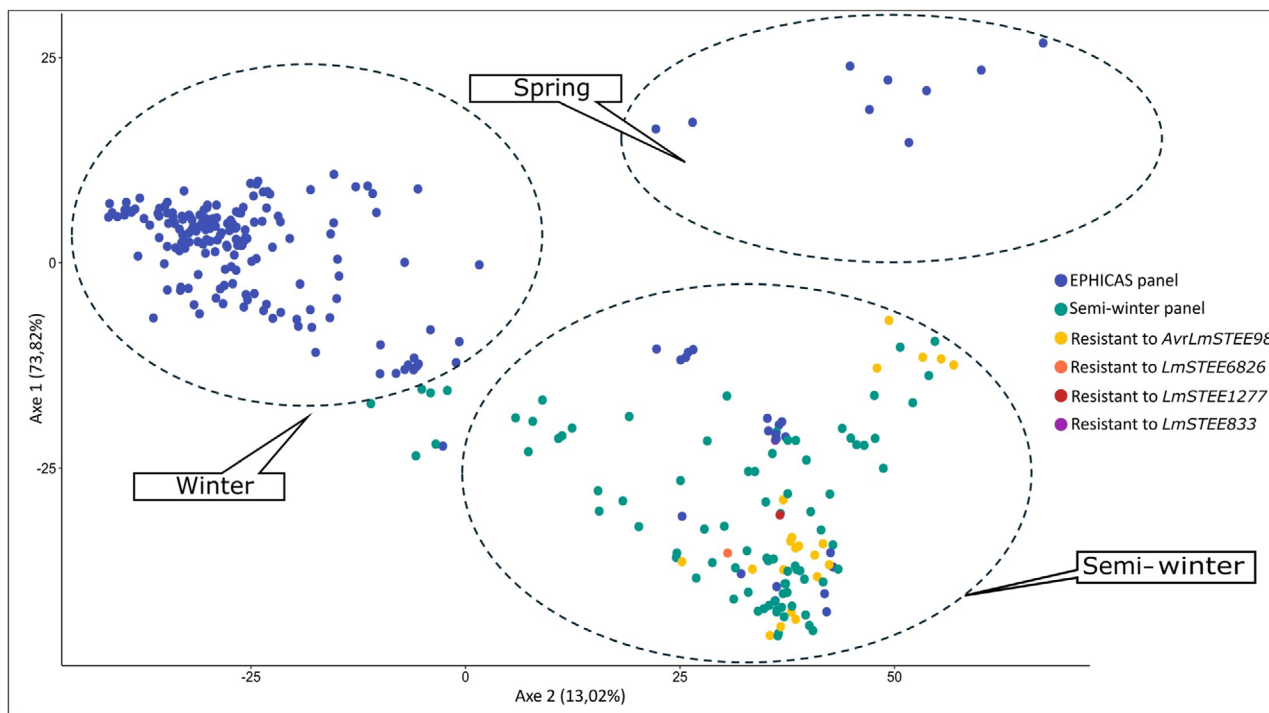


FIGURE 6 | Genotypic diversity of *Brassica napus* genotypes used for the screening experiments. 207 genotypes were screened, including 107 genotypes from the EPHICAS panel (Jiquel et al. 2022) and 100 from the semi-winter panel (present study). A principal coordinate analysis was performed based on an Identity by State matrix resulting from a genome-wide genotyping with 6631 single-nucleotide polymorphisms. Black frames distinguish the three main types of *B. napus*: winter, spring, and semi-winter. Genotypes resistant to *LmSTEE* genes are indicated in yellow, orange, red, and purple.

factoextra. Graphical representations were obtained using the R package ggplot2.

Using the same method, 100 genotypes were added to the 'EPHICAS panel' for their belonging to the semi-winter diversity group, which included rapeseed and rutabaga. This second panel is referred to as the 'semi-winter panel'. Genotypes from EPHICAS and semi-winter panels are described in Table S5.

4.2 | Plant Inoculation Tests

Plant inoculation tests were performed independently in two locations with different protocols.

The *LmSTEE* transformants described by Jiquel et al. (2021, 2022) were inoculated on the semi-winter panel at Innolea. Fifteen individuals per genotype were distributed in three repetitions of five plants following a longitudinal gradient in a growth chamber. The plants were inoculated with two control isolates, JN2 and INV13.269, and with one transformant per *LmSTEE* gene. One isolate was deposited on each half of the cotyledons. Inoculation was performed by puncturing 12-day-old seedlings and depositing 10 μ L of inoculum (10^7 pycnidiospores per mL) on each point. Plants were incubated for 48 h in the dark at 18°C at night and 22°C during the day, and then in the same temperature conditions with a 12-h photoperiod. Symptoms were scored at 13 and 15 dpi using the IMAScore scale, scores of 1–3 corresponding to resistance and 4–6 to susceptibility phenotypes (Balesdent et al. 2001).

The OEC transformants obtained in this study were inoculated on the 207 genotypes from EPHICAS and semi-winter panels. Six individuals per genotype were inoculated with two control strains, JN2 and X83.51, and one transformant per *LmSTEE* gene. Inoculations were done as described above, but on cotyledons of 10-day-old seedlings, with an incubation of 48 h at room temperature and growth chamber conditions set at 19°C (night)/24°C (day), 16-h photoperiod, 90% humidity. Symptoms were scored at 10 and 13 dpi using the IMAScore rating scale.

For promising interactions, a validation test was made. Inoculation was performed on 6–12 plants per genotype, but using two OEC transformants per *LmSTEE* gene. Symptoms were scored at 10, 13, and 15 dpi using the IMAScore rating scale.

4.3 | Vector Construction and Fungal Transformation

The expression of *LmSTEE* genes was induced during cotyledon colonisation using the promoter of *AvrLm4-7*, an early effector gene known to be up-regulated at 7 days post-infection of cotyledons (Parlange et al. 2009).

The promoter of the *AvrLm4-7* gene was cloned into the pPZ-PNat1 vector as described in Jiquel et al. (2021). The six new *LmSTEE* genes were amplified from their start codon to their terminator region using primers described in Table S6. The

amplicons were then digested by EcoRI and XhoI or SalI and XhoI and ligated into the pPZPNat1_AvrLm4-7 vector. Because of the presence of the XhoI restriction site in *LmSTEE5765*'s terminator, this gene was cloned with *LmSTEE833*'s terminator. In addition, *LmSTEE4385* was inserted into pPZPNat1_AvrLm4-7 vector using Gibson assembly (GeneArt Gibson Assembly HiFi Cloning Kit, Thermo Fisher Scientific) according to the manufacturer's recommendations.

The plasmids were introduced into *Agrobacterium tumefaciens* C58 pGV2260 by electroporation at 2.5 kV, 200 Ω and 25 μ F. *L. maculans* isolate X83.51 was transformed with the resulting *A. tumefaciens* following the transformation protocol described by Idnurm et al. (2017). X83.51 isolate is virulent against most known *Rlm* genes except *Rlm5*, *Rlm10*, and *Rlm14*, allowing the screening of a large panel of *B. napus* genotypes without interference with known *AvrLm*–*Rlm* interactions.

Selection of positive transformants was made using nourseothricin (50 μ g mL⁻¹) and cefotaxime (50 μ g mL⁻¹). Positive transformants were grown on V8 with antibiotics and sporulated for isolation of a single pycnidiospore.

X83.51 was also transformed with pPZPNat1_AvrLm4-7:LmSTEE98 and pPZPNat1_AvrLm4-7:LmSTEE6826, previously constructed by Jiquel et al. (2021, 2022), following the same protocol.

Fungal transformants were checked for their growth in vitro using V8 agar medium and for their pathogenicity by inoculation on a susceptible cultivar, Darmor. Finally, for each selected transformant, the correct insertion of pPZPNat1_AvrLm4-7 in frame with the corresponding *LmSTEE* gene was verified by PCR and sequencing on pycnidiospores harvested in sterile water.

4.4 | RNA Manipulation and RT-qPCR

LmSTEE gene expression at 7 dpi was quantified as described by Jiquel et al. (2021). Total RNA was extracted from inoculated cotyledons at 7 dpi on two biological replicates. We generated cDNA using oligo(dT)-primed reverse transcription with the PowerScript reverse transcriptase (Clontech), according to the manufacturer's protocol. RT-qPCR was performed on two technical replicates as described by Fudal et al. (2007) with the primers indicated in Table S6. *Actin* was used as a constitutively expressed reference gene, and levels of *EF1 α* expression relative to *Actin* expression were used as a control.

4.5 | Bioinformatics and Statistical Analyses

Leptosphaeria maculans genomic analyses were performed using JN3 genome assembly and annotations (Dutreux et al. 2018, https://bioinfo.bioger.inrae.fr/myGenomeBrowser?browse=1&portalname=Leptosphaeria_maculans_JN3&owner=bioinfobioger@inrae.fr&key=7d2D4XyQ, https://bioinfo.bioger.inrae.fr/myGenomeBrowser?browse=1&portalname=Leptosphaeria_maculans_JN3&owner=bioinfobioger@inrae.fr&key=7d2D4XyQ). Effectors were predicted using EffectorP

(Sperschneider et al. 2016). The presence of a signal peptide was predicted by SignalP (v. 4.1, Nielsen 2017) and the extracellular location by TargetP (v. 1.1, Almagro Armenteros et al. 2019). The number of transmembrane domains was assigned using the TMHMM tool (v. 2.0, Möller et al. 2001). Structural prediction of effectors was performed using AlphaFold2 (ColabFold v. 1.5.5) with standard parameters (Mirdita et al. 2022).

Effector genes were assigned to 'early' or 'late' categories using the expression clusters defined by Gay et al. (2021). Effectors belonging to clusters 1 to 3 were defined as early, and effectors from clusters 4 to 6 as late. The genomic location of effector genes was determined using Occultercut (Testa et al. 2016) on the JN3 genome. The location was assigned as 'border' when the gene was within a 10 kb distance of an overlap between AT-rich and GC-equilibrated isochores. TEs were annotated by Grandaubert et al. (2014) on the JN3 genome. The enrichment in chromatin methylation marks on effector genes was determined using ChIP-seq data generated by Soyer et al. (2021) during axenic growth of JN3.

The 205 previously published *L. maculans* genomes (de Wouw et al. 2024) were assembled and analysed for sequence variation content relative to the reference strain JN3 (<https://doi.org/10.57745/R7XVL3>). Raw reads were cleaned with Trimmomatic v. 0.32 (Bolger et al. 2014) and aligned with bwa-mem v. 0.7.7 (Li and Durbin 2009). Variant calling was performed with Freebayes v. 0.9 (Garrison and Marth 2012) before filtering with the same workflow previously established on other fungal species (Amezrou et al. 2024; Zhong et al. 2017). Raw reads were also assembled with a pipeline using a combination of Velvet (Zerbino and Birney 2008), SOAPdenovo and SOAP GapCloser (Luo et al. 2012) as follows: (1) reads were trimmed at the first N, (2) contigs were generated with several k-mer values using SOAPdenovo, (3) several Velvet assemblies were built using several k-mer values and as the input the trimmed reads and all SOAPdenovo contigs considered as 'long reads', (4) the assembly that maximises the criterion (N50*size of the assembly) was selected, (5) SOAP GapCloser was run on the selected assembly and (6) contigs completely enclosed in other longer contigs were removed. The gene annotation was transferred from the JN3 reference to other strains using LiftOff (Shumate and Salzberg 2021), enabling the variant content and detection of protein isoforms for each genetic locus to be analysed. The results are available on the web portal <https://bioinfo.bioger.inrae.fr/portal/variant-explorer/>. The protein sequence alignments were performed using the msa package (v. 1.34.0) with the ClustalW method.

Statistical analyses were performed using RStudio (R 4.3.2). χ^2 test, Kruskal–Wallis test, and ANOVA were done using the package Rstatix (v. 0.7.2).

Author Contributions

Camille Rabeau: methodology, investigation, formal analysis, visualization, writing – original draft, writing – review and editing. **Armand Wagner:** investigation, formal analysis, visualization, resources, writing – review and editing. **Nicolas Lapalu:** investigation, writing – review and editing, software, data curation, methodology. **Audren Jiquel:** conceptualization, methodology, validation, supervision, visualization, writing – review and editing. **Sébastien Faure:**

conceptualization, validation, methodology, supervision, resources, project administration, funding acquisition, writing – review and editing, visualization. **Isabelle Fudal**: conceptualization, funding acquisition, supervision, resources, project administration, writing – review and editing, methodology, validation, visualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Recherche Data Gouv at <https://doi.org/10.57745/R7XVL3>, reference number DOI: 10.57745/R7XVL3.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** *In planta* expression data of a selection of late effector genes of *Leptosphaeria maculans* in controlled conditions (a) and in field conditions during leaf infection (b) and stem infection (c). Left graphics present the expression of the 11 late effectors studied by Jiquel et al. (2021, 2022), and right graphics the expression of the candidate late effector genes selected in the present study. The expressions of *LmSTEE6826* (*Lmb_jn3_06826*) and *AvrLmSTEE98* (*Lmb_jn3_11364*) are framed in red, such as the effector genes presenting the same profile of expression in the same conditions. DPI, day post infection; MPI, month post infection. **Table S1:** Characteristics of the 151 effector genes of *Leptosphaeria maculans* analysed in this study. The effector genes were predicted by Dutreux et al. (2018), and the expression clusters were defined by Gay et al. (2021). Genomic location in AT-rich (AT), GC-equilibrated (GC) isochores or the border between these two locations was defined by Dutreux et al. (2018). Transposable elements present 4kbp upstream and downstream of the gene were annotated by Grandaubert et al. (2014). Enrichment in chromatin methylation marks during axenic growth was detected by Soyer et al. (2021) using ChIP-seq. Peptide signals were predicted by SignalP (Nielsen 2017), extracellular location by TargetP (Almagro Armenteros et al. 2019), and transmembrane domains by the TMHMM tool (Möller et al. 2001). Protein homologues were identified by BLASTp using the NCBI nr database, the quality of the structural prediction using AlphaFold (ColabFold v1.5.5, Mirdita et al. 2022), and close structural analogs according to the Dali database. **Table S2:** Description of 147 effector gene conservation in the *Leptosphaeria maculans* IBCN collection described by Van de Wouw et al. (2024). Genomes of 205 isolates from the new IBCN collection were analysed for sequence variation content relative to the reference strain JN3 using a variant calling method. **Table S3:** *Leptosphaeria maculans* isolates used in the present study. Transformants used for the first screening are indicated with an asterisk. The validations were done by inoculating with two transformants per *LmSTEE* gene. **Table S4:** Interactions between 207 genotypes of *Brassica napus* and 16 *LmSTEE* genes from *Leptosphaeria maculans*. Transformants used for the first screening are indicated with an asterisk. The validations were done by inoculating with two transformants per *LmSTEE* gene. Type of resistance response: hypersensitive response (HR), intermediate resistance (RI) corresponding to a delayed resistance phenotype and heterogeneous resistance (RH) corresponding to an alternance of susceptible and resistant phenotypes for a genotype x *LmSTEE* interaction. **Table S5:** 207 *Brassica napus* genotypes used for the pathogenicity assays. **Table S6:** Primers used in the present study.