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ORIGINAL RESEARCH

"Late" effectors from *Leptosphaeria maculans* as tools for identifying novel sources of resistance in *Brassica napus*

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Abstract

The Dothideomycete *Leptosphaeria maculans*, causing stem canker (blackleg) of *Brassica napus*, secretes different cocktails of effectors at specific infection stages. Some effectors ("Late" effectors) are specifically produced during the long asymptomatic phase of stem colonization. By manipulating their expression so that they are overexpressed during cotyledon infection (OEC transformants of the fungus), we previously postulated that resistance genes operating in the stem may be involved in gene-for-gene relationship and thus contribute to quantitative disease resistance (QDR). Here, we selected 10 relevant new effector genes, and we generated OEC transformants to screen a collection of 130 *B. napus* genotypes, representative of the available diversity in the species. Five *B. napus* accessions showed a typical hypersensitive response when challenged with effectors LmSTEE98 or LmSTEE6826 at the cotyledon stage, and all belong to the semi-winter type of the diversity panel. In addition, five winter-type genotypes displayed an intermediate response to another late effector, LmSTEE7919. These new interactions now have to be genetically validated to check that they also correspond to gene-for-gene interactions. In all cases, they potentially provide novel resources, easy to breed for, and accounting for part of the quantitative resistance in a species for which we are currently facing limited resistance sources.

Highlight

Overexpression of "late" effector genes from *Leptosphaeria maculans* was used to screen a diversity panel of *Brassica napus* genotypes and allowed to identify novel resistances potentially contributing to quantitative resistance.

KEYWORDS
late effectors, *Leptosphaeria maculans*, manipulation of effector expression, qualitative resistance, quantitative resistance, rapeseed

1 | INTRODUCTION

Quantitative (also termed horizontal, incomplete, minor-gene, broad-spectrum, adult-stage, …) disease resistance (QDR) in plants is a complex and somewhat blurred notion that encompasses many different realities depending of the plant life cycle and its duration (from annual to perennial), the pathogen life cycle (e.g., differentiating monocyclic vs. polycyclic diseases), and the duration of the interaction between the plant and its pathogen (Poland et al., 2009). Thus, depending on the model, QDR may increase latency period, reduce number or size of symptoms, reduce sporulation for polycyclic diseases, and eventually reduce damages to crops without suppressing the expression of the disease (symptoms) and without suppressing the next-cycle inoculum. For breeders, QDR is of paramount importance due to its famed durability compared with major single gene resistance. However, being polygenic in nature, with each locus contributing only to a portion of the QDR, markers for marker-assisted selection (MAS) are difficult to develop and genes underlying the phenomenon remain elusive.

Stem canker disease of rapeseed (*Brassica napus*), due to the ascomycete *Leptosphaeria maculans*, is controlled mostly by qualitative resistance and QDR. QDR to *L. maculans*, also termed "adult-stage" resistance involves several QTLs with minor effects (Kumar et al., 2018). In Western Europe, the pathogenic cycle of *L. maculans* is long and complex, and the outcome of the disease, the stem canker, is assessed at the end of the growing season (May–July), whereas the first leaf symptoms develop in autumn (October–November), 7–10 months before. Thus, differences in QDR between genotypes are difficult to evaluate in the field, as the robustness and efficiency of QDR (and the stability of the corresponding QTLs) can depend on environmental conditions summed up during this long growing period in the fields (Fopa Fomeju et al., 2015; Huang et al., 2016; Jestin et al., 2015; Kumar et al., 2018; Pilet et al., 2001; Raman et al., 2016). It is even more difficult to accurately phenotype QDR under controlled conditions because it would ideally necessitate to reproduce the whole of the pathogenic cycle in the greenhouse (Jiquel et al., 2021).

In Western Europe, the lifecycle of *L. maculans* alternates several times between saprotrophic, necrotrophic, and biotrophic lifestyles. At the end of the growing season, *L. maculans* develops onto crop residues and performs its sexual reproduction (Fitt et al., 2006; Rouxel & Balesdent, 2005). Produced ascospores, which are transported by wind, infect cotyledons and leaves of rapeseed through stomata. After a short phase of biotrophic colonization of leaves, the fungus switches to a necrotrophic lifestyle and induces the formation of leaf spots. Following the appearance of leaf symptoms, fungal hyphae develop from the leaves to the stem crown, during an asymptomatic and endophytic phase that lasts several months. At the end of the growing season, the fungus switches again to a necrotrophic phase, by inducing necrotic lesions at the stem crown (Hammond et al., 1985; Huang et al., 2014; Rouxel & Balesdent, 2005). While qualitative resistance expressed at the cotyledon/leaf stage is well-known and a couple of major genes for resistance have been cloned (*LepR3*, Larkan et al., 2013; Rouxel & Balesdent, 2013; *Rlm2*, Larkan et al., 2015; *Rlm9*, Larkan et al., 2020), the QDRs supposed to operate during petiole and stem colonization associated to the long asymptomatic phase are not well characterized, in terms of mechanisms and molecules involved and in terms of variability between genotypes displaying QDR.

To colonize their hosts, pathogenic, endophytic, and symbiotic fungi secrete an arsenal of effectors, used to promote infection and interfere with plant defenses (Lo Presti et al., 2015; Sánchez-Vallet et al., 2018). Eight waves of expression of genes enriched in effector genes, showing infection stage specificity or trophic specificities, have been described during the long interaction between *L. maculans* and its host (Gay et al., 2021). Four of them may be of specific relevance for QDR (Figure 1). Effector genes of Wave 2 ("biotrophy," described by Gervais et al., 2017 as "early" effectors) are expressed at all stages of biotrophic colonization of tissues (cotyledons, leaves, petioles, stems), and their expression is switched on/off multiple times during the plant colonization. *Rlm* and *LepR* qualitative resistance genes operate by "recognizing" effectors of Wave 2, encompassing all currently known avirulence (*AvrLm*) genes produced during infection and colonization of cotyledons and leaves (Gay et al., 2021; Rouxel & Balesdent, 2017). The Wave 4 corresponds to the transition from biotrophy to necrotrophy, with genes expressed in cotyledons, petioles, and stems, whose expression is repressed before necrotic symptoms begin to develop. The Wave 5 corresponds to stem biotrophy with genes exclusively expressed during many months of asymptomatic stem colonization. Lastly, the Wave 6 corresponds to stem necrotrophy with genes exclusively expressed in the stem at the end of the growing season. These three latter waves include effectors named "late" effectors by Gervais et al. (2017) or LmSTEE (*L. maculans* STem Expressed Effectors) by Jiquel et al. (2021). At these stages of colonization, quantitative resistance operates, and, in view of the existence of fungal effectors exclusively produced during stem colonization (Figure 1), Jiquel et al. (2021) postulated that resistance genes operating in the stem may be involved in gene-for-gene relationship and thus contribute to QDR.

Screening for resistance is routinely done at the cotyledon stage, preferably using isogenic isolates differing by only one *AvrLm* gene. However, as *LmSTEE* genes are weakly or not expressed during cotyledon and leaf infection, Jiquel et al. (2021) used an innovative approach by placing *LmSTEE* genes under the control of the promoter of the avirulence gene *AvrLm4-7*, so that they are highly expressed at the cotyledon stage of the infection. This original approach allowed the authors to screen a 204-plant genotype collection and to identify one resistance gene recognizing one late effector, by using the high-throughput test at cotyledon stage.

With the use of this approach, Jiquel et al. (2021) showed that at least part of the quantitative resistance to *L. maculans* is due to a canonical gene-for-gene interaction, thus opening avenues for easy generation of markers for MAS and knowledge-driven construction of genotypes summing up diverse types of qualitative resistance and QDR, previously shown to increase the durability of major genes (Brun et al., 2010).

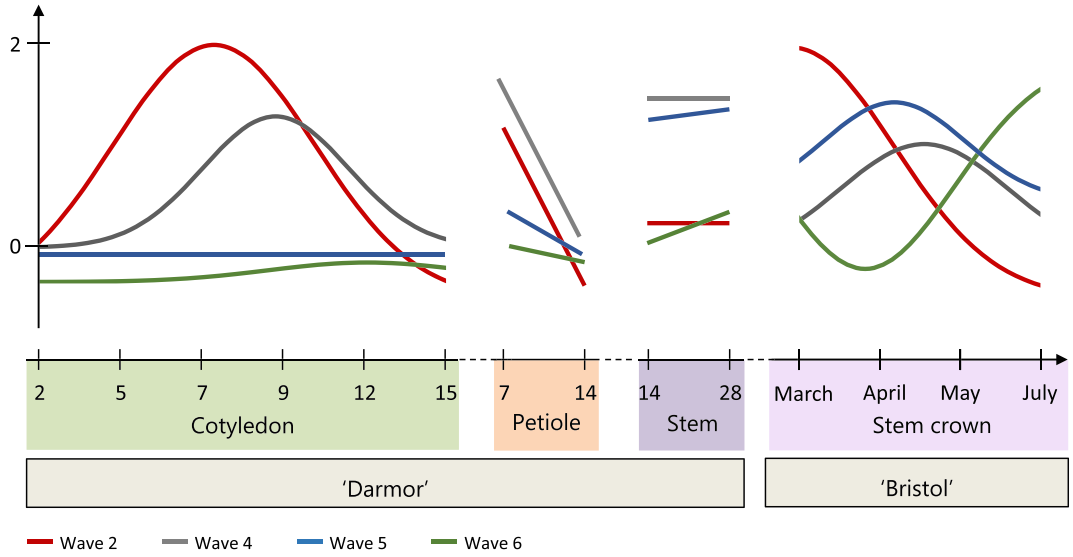

FIGURE 1 Schematic representation of waves of expressions of genes highly expressed during colonization of *Brassica napus* by *Leptosphaeria maculans* (adapted from Gay et al., 2021). The timing of infection is indicated in days post-inoculation for inoculations performed in controlled conditions (cotyledons, petioles, and stems) and according to the month of sampling (March to July) when the samples originate from the field under natural inoculum infection. Expression levels are indicated along the *y* axis, in $Log_2(FPKM + 1)$. Color code as follows: red, wave 2 "Biotrophy"; gray, Wave 4 "Transition from biotrophy to necrotrophy"; blue, wave 5 "Stem biotrophy"; green, Wave 6 "Stem necrotrophy"

However, this pioneer study also highlighted several limitations about criteria of choice of effector genes and diversity of the collection of *B. napus* genotype screened, preventing the authors to conclude whether the new gene-for-gene interaction identified was an exception or if other such interactions putatively involved in QDR exist. First, with >100 effectors produced during stem colonization, the authors selected five that were scattered in Waves 4–6. We thus wondered whether focusing on effectors exclusively produced in stems and not in cotyledons (Wave 5; Figure 1) could increase the chance to find matching *Rlm* genes expressed in stems. Second, the only oilseed rape genotype that carried a new source of resistance, now identified as *RlmSTEE98*, was also the only "exotic" one (semi-winter type of Asian origin) among the screened collection composed of a vast majority of European, winter-type genotypes. Therefore, we postulated that the diversity in the screened genotype collection should be as wide as possible, to increase the probability to identify new resistance genes.

In the present study, we partially addressed these limitations, by diversifying the screened genotype collection and by selecting new effectors for screening, using a wide diversity of transcriptomic data, and choosing those showing no expression in cotyledons and specifically expressed during petiole and stem colonization. We selected six new *LmSTEE* genes to screen a panel of 130 genotypes of *B. napus*, showing a wide diversity of origins. We confirmed that the over expression of "late" effectors in cotyledons allows us to identify new sources of resistance to different late effectors. This study and that of Jiquel et al. (2021) thus confirm the postulate that gene-for-gene interactions operate during stem colonization and provide us with additional criteria to choose effector genes that may be the best avirulence candidates and genetic resources to focus on for uncovering new resistance genes.

2 | MATERIALS AND METHODS

2.1 | Fungal and plant materials

The isolate JN2 (v23.1.2), a progeny from an *in vitro* cross between European field isolates (Balesdent et al., 2001), was used as a control in inoculation tests. Isolate INV13.269, isolated from a single pycnidiospore in a French field in 2013, was used as background for fungal transformation to screen for resistance. A worldwide collection of isolates (Table S1), from Australia, New Zealand, Canada, the United States, Mexico, and Chile, was used for diversity analysis (Dilmaghani et al., 2009, 2012; Mendes-Pereira et al., 2003; this study). This collection was enriched with a recent population collected in France in 2017 from four locations in France as single-pycnidium isolates recovered from individual leaf lesions as described previously (Balesdent et al., 2006; Table S1). V8 juice agar medium was used for fungal growth and sporulation, as described by Ansan-Melayah et al. (1995).

To search for gene-for-gene interactions with late effectors, we screened two types of oilseed rape materials (Table S2A): (1) a diversity panel including 99 accessions selected based on materials available at Innolea and representing different crop morphotypes (winter, spring, semi-winter, or rutabagas), geographical origins (mostly European or Asian), and seed qualities (either high or low in erucic acid or glucosinolates or both); and (2) a selection of 31 varieties based on their known field behavior as described in Terres Inovia's

crop guide (https://www.terresinovia.fr/p/guide-culture-colza) or according to Innolea's knowledge with an emphasis on genotypes devoid of known efficient major *Rlm* genes (Table S2B).

2.2 | Vector construction and fungal transformation

The vector pPZPNat1 containing the promoter of the *AvrLm4-7* gene was obtained as described in Jiquel et al. (2021). The six late effector genes were amplified from their Start codon to their terminator regions, with primers described in Table S3. The gene *LmSTEE6826* was cloned by Gibson assembly (Silayeva & Barnes, 2018), after digestion of pPZPNat1 by *Sal*I and *Xho*I. Amplicons of *LmSTEE1277*, *LmSTEE1852*, *LmSTEE5465*, *LmSTEE7919*, and *LmSTEE10933* were digested with *Eco*RI and *Xho*I or *Eco*RI and *Sal*I (Table S3). By digestion with the same enzymes, fragments were ligated into the pPZPNat1 vector containing the *AvrLm4-7* promoter.

Each construct was introduced by electroporation (2.5 kV, 200 Ω, and 25 μF) into *Agrobacterium tumefaciens* C58. Transformations of *L. maculans* with *A. tumefaciens* strains were performed as described by Gout et al. (2006). Selection of fungal transformants was conducted on nourseothricin (50 μg ml^{-1}).

2.3 | Fungal DNA manipulation and polymorphism analysis

To extract fungal genomic DNA, the DNeasy 96 or DNeasy Plant Mini Kits (Qiagen) were used on conidia suspensions, in accordance with the manufacturer's recommendations. *LmSTEE* genes were amplified by PCR, using the primers in Table S3, and visualized on 1% agarose gels to detect presence/absence polymorphism. Amplicons were sequenced for six late effector candidates by Eurofins Genomics (Eurofins, Ebersberg, Germany), using the primers in bold in Table S3.

2.4 | Fungal RNA manipulation and RT-qPCR

Total RNA was extracted from a disk of inoculated cotyledons, centered on the inoculation point and sampled with a 5-mm cork borer, at 7 dpi corresponding to the peak of *AvrLm4-7* expression (Parlange et al., 2009). For the reverse transcription reaction, RNA samples were adjusted to 1 μg of RNA. Single-strand cDNAs were generated by oligo-dT-primed reverse transcription with the PowerScript reverse transcriptase according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). Two technical replicates were performed on two biological replicates obtained from two independent experiments. The RT-qPCR experiments were performed with the primers indicated in Table S3 as described by Fudal et al. (2007). The method from Muller et al. (2002) was used to analyze Ct values. Actin was used as a constitutively expressed reference gene, and levels of β-tubulin expression relative to actin expression were analyzed as a control.

2.5 | Transcriptomic analysis

Biological samples from in vitro conditions, *in planta* controlled conditions and field conditions (varieties "Darmor-*bzh*" and "Bristol"), and RNA-Seq sequencing were described in Gay et al. (2021). In this study, we used RNA-Seq data from cotyledon (6 time-points), petiole (2 time-points), and stem (2 time-points) infection by *L. maculans* JN2 in controlled conditions, from naturally infected plants in the field (3 time-points) and from JN2 growth on V8 medium as an *in vitro* condition. Mapping of RNA-Seq data has been done in the study of Gay et al. (2021), using the reference genomes of *B. napus* (Chalhoub et al., 2014) and of *L. maculans* (Dutreux et al., 2018). Only genes with at least 15 reads in one condition were analyzed, and RNA-Seq data were normalized using « Trimmed Mean of M-values » method, with the EdgeR package (Robinson et al., 2010). The glmQLFit function was used to fit a negative binomial generalized linear model to the data. To compare gene expression between conditions, we used the glmLRT function. Differentially expressed genes were selected with a Log_2(FC) > 1.5 and a p value < .05. Clustering was done on genes with an FPKM > 1, which were Log2(FPKM + 1)-transformed and scaled. Then effectors specifically overexpressed in stem or petiole conditions compared with the six cotyledon conditions were extracted, and the Kohonen R package was used to cluster these genes.

2.6 | Structuration of the *B. napus* genotype collection

Both sets of *B. napus* materials (Table S2) were genotyped with an internal array of 6331 SNP covering the 19 chromosomes. Briefly, DNA was extracted from eight punches of young leaves per genotype using the DNeasy plant 96 kit (Qiagen), and 1.5 μg were used on an Affymetrix Axiom MyDesign chip array according to manufacturer's protocol (Thermo Fisher Scientific Inc.). The resulting allelic matrix was used to compute kinship matrix using the R package emma represented by the Identity By State (IBS) between each accession. A principal coordinate analysis (PCA) was also performed based on the IBS matrix using the R packages FactoMineR and factoextra, and graphical representations were obtained using the R package ggplot2, allowing a visualization of the overall diversity present in the panel.

2.7 | Plant inoculations and resistance assessment

Plant genotypes were tested independently in two locations with slightly different protocols. The first step of the screening was performed on five plants at Innolea and on 10 plants at INRAE, with four transformants/control isolates deposited on four inoculation points (one per half cotyledon) per plants. The experiment was repeated for promising interactions, allowing us to have at least 20 plants, in two biological replicates, still with four transformants/control isolates on each plant. Inoculations performed at INRAE Bioger were done as described by Balesdent et al. (2001), on cotyledons of 10-day-old seedlings.

Punctures were inoculated with 10 µl of inoculum (10^7 pycnidiospores ml^{-1}). Inoculated plants were incubated 48 h at room temperature and then placed in a growth chamber (19°C (night) /24°C (day), 16-h photoperiod, 90% humidity). Symptoms were assessed at 10, 14, and 16 dpi using the IMASCORE rating scale, with scores of 1 and 2 corresponding to resistance, 3 to intermediate phenotype, and 4–6 to susceptibility (Balesdent et al., 2001). Inoculations at Innolea were slightly different, starting by inoculations on 16-day-old seedlings. After inoculations, plants were incubated 48 h in the dark at 18°C (night) and 22°C (day) and then, in the same temperature conditions, with a photoperiod of 16 h. Symptoms were scored at 12, 13, and 14 dpi, using the rating scale of Williams (1985), with scores of 1 and 3 corresponding to resistance, 5 to intermediate phenotype, 7 and 9 to susceptibility.

For both experiments, mean scores for symptoms and the percentage of virulent and avirulent phenotypes induced by OEC transformants were determined for each genotype and compared with the symptoms induced by the wild-type isolate INV13.269 on the same genotype, at the same date (Kruskal–Wallis test, Chi^2 test and general linear model [GLM] test, assuming a quasi-Poisson distribution of the data). Statistical analyses were performed with R (R Core Team, 2015).

3 | RESULTS

3.1 | Transcriptomic analysis to select relevant "late" effector candidates

To identify relevant "late" effector candidates, we exploited RNA-Seq data from the LEPTOLIFE project, some of which, but not all of them, having been described in Gay et al. (2021). The samples were collected at different time points in controlled conditions, and we focused on six sampling time-points during cotyledon infection on variety "Darmor-*bzh*," two sampling time-points during petiole infection on "Darmor-*bzh*" and one on "Bristol," and two sampling time-points during stem infection on each variety "Darmor-*bzh*" and "Bristol" (Tables S4 and S5).

To identify genes specifically expressed during petiole or stem infection, we then discriminated them from genes also expressed during cotyledon infection. We thus found a total of 267 differentially expressed genes (DEGs). A total of 134 genes were overexpressed during stem infection of "Darmor-*bzh*" compared with cotyledon infection, and during petiole colonization, 19 genes were overexpressed compared with cotyledon infection, all in common with the stem infection (Figure S1A). During stem and petiole colonization of "Bristol," we found 151 and 153 genes, respectively, overexpressed compared with cotyledon infection, with 54 in common between stem and petiole expression (Figure S1A).

The repertoire of *L. maculans* candidate effectors has recently been updated by Gay et al. (2021), allowing us to determine the set of effector genes present in our set of DEG genes (Figure S1B). Thereby, 35 effector candidate genes were overexpressed during stem and/or petiole colonization of "Darmor-*bzh*" and/or "Bristol" (excluding those exclusively expressed in petioles of "Bristol"), compared with cotyledon infection (Figure 2), including three previously identified "late" effector candidates, *LmSTEE1* (renamed here *LmSTEE3177*), *LmSTEE30* (now *LmSTEE4778*), and *LmSTEE35* (now *LmSTEE8094*) (Jiquel et al., 2021). These could be discriminated into four groups according to their level of expression during *in vitro* culture, overexpression in petioles and differential expression in petioles 7 dpi versus 14 dpi (Figure 2). Gay et al. (2021) performed a precise analysis of gene expression during all steps of the interaction between *L. maculans* and *B. napus*, from the penetration in cotyledons up to the saprophytic stage on plant residues, using a wider set of samples from the LEPTOLIFE project, including field samples. By comparing the gene list of our analysis to the eight expression waves defined by Gay et al. (2021), most of these (23 out of 35, including *LmSTEE3177* and *LmSTEE4778*) were included in the "Stem biotrophy" wave (Wave 5) of genes exclusively expressed during the biotrophic colonization of stems (Figure 1). Five genes (including *LmSTEE8094*) were included in the "Biotrophy-to-necrotrophy transition" wave (Wave 4), and one effector gene belonged to the "Stem necrotrophy" wave (Wave 6) (Figure 1). Finally, six "late" effector genes were not included in the expression waves described in Gay et al. (2021), likely due to a non-negligible level of expression during in vitro growth and/or to a less drastic threshold of differential expression used here compared with what was used by Gay et al. (2021), (Log_2(FC) > 1.5 in this study versus Log_2(FC) > 4.0 in the Gay et al. study) (Figure 2).

3.2 | *LmSTEE* genes are conserved in field populations of *L. maculans*

Fifteen *LmSTEE* genes among the 35 were further selected for a deeper characterization. These were selected based on their localization in GC-isochores (Gervais et al., 2017; Rouxel et al., 2011), their size (less than 300 amino acids), and their lack of a predicted function (Figure S2). Their presence/absence polymorphism was first analyzed in a collection of *L. maculans* isolates consisting of 90 French isolates and 93 world-wide isolates (Table S1) (Dilmaghani et al., 2009, 2012). Consistent with what was previously observed for *LmSTEE* genes (Gervais, 2017; Jiquel et al., 2021), these 15 genes were present in all studied isolates following PCR amplification.

For screening the *B. napus* diversity panel, we then selected six out of the 15 based on their belonging to the "Stem biotrophy" wave described by Gay et al. (2021) and with the lowest expression level during cotyledon infection (Figures 1 and 3). This subset of six effectors, LmSTEE1277, LmSTEE1852, LmSTEE5465, LmSTEE6826, LmSTEE7919, and LmSTEE10933, had common characteristic typical of effectors; they displayed a predicted signal peptide (SignalP), they were predicted as effectors according to EffectorP, and, except for LmSTEE7919, had a high cysteine rate (Table 1). Four of them displayed high homologies with proteins from other related fungal species (Dothideomycetes/Pleosporales), including in the most closely related species *Leptosphaeria biglobosa* (Tables 1, S6, and S7). However, all closest blast hits corresponded to hypothetical proteins (Table S6). LmSTEE1852 and LmSTEE6826 had no homologs in other fungal species, including in *L. biglobosa*, a feature that was previously observed for LmSTEE98 (Jiquel et al., 2021).

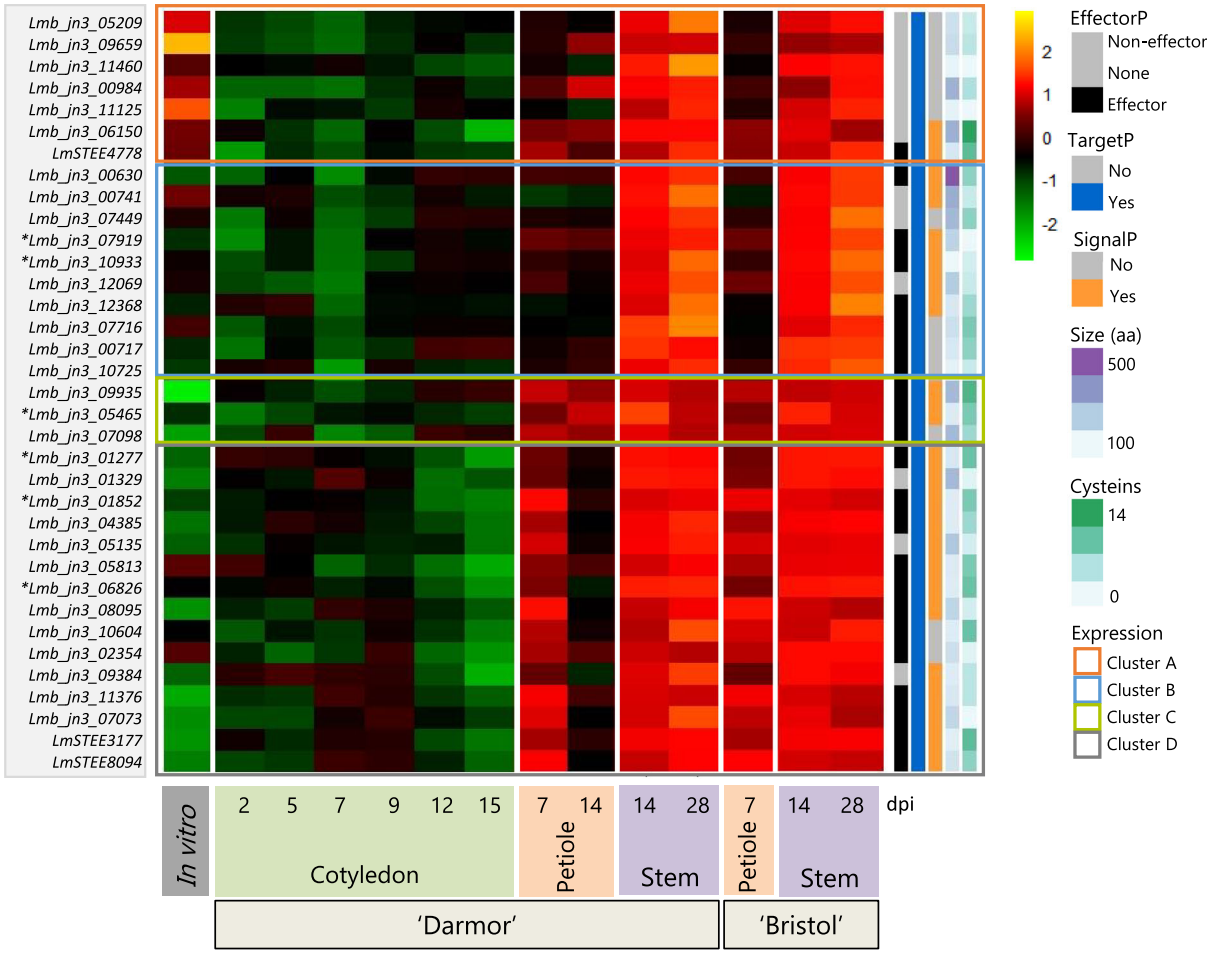

FIGURE 2 Heatmap representing the clustering of 35 late effector candidates of *Leptosphaeria maculans*, overexpressed during stem colonization. The mean FPKM value of the biological replicates was calculated for each condition and then Log_2 transformed and scaled. Differentially expressed genes were selected with a Log_2(FC) > 1.5 and a *p* value < .05. To define expression clusters, the method of self-organizing was used on Log_2(FPKM + 1) values. Predictions by EffectorP, TargetP, SignalP, the size, and the number of cysteines are indicated by different colors in legend. The experimental conditions are along the *x* axis, and the number indicates sampling time-points in days post-inoculation (dpi). The stars indicate the six candidates selected for screening of the *Brassica napus* diversity panel. *LmSTEE3177*, *LmSTEE4778*, and *LmSTEE8094* were formerly named *LmSTEE1*, *LmSTEE30*, and *LmSTEE35*, respectively (Jiquel et al., 2021).

The six genes were sequenced in the French and worldwide populations, and only a very limited number of sequence polymorphisms were observed (Tables 2 and S1). *LmSTEE6826* and *LmSTEE10933* had no mutation in their coding sequences, whereas *LmSTEE1852* had only two silent mutations, in one Canadian isolate. *LmSTEE1277* and *LmSTEE5465* had rare non-synonymous mutations. *LmSTEE1277* presented one V > M(13) substitution found in two French isolates and a L > M(100) substitution found in another French isolate. *LmSTEE5465* presented one silent mutation in three French and one Canadian isolates and one non-synonymous L > F(41) substitution in one French isolate. Finally, one non-synonymous V > I (38) substitution was found in *LmSTEE7919* and was present at a low frequency in all populations, except in Mexican isolates obtained from *B. oleracea* in which it was the most frequent isoform of the protein (65% of the Mexican isolates analyzed here). Allelic variants identified for the six late effectors are always present in distinct isolates.

3.3 | Genomic location of *LmSTEE* genes

Genomic location was investigated manually using the *L. maculans* genome browser and pointed to four different genome environments for 10 *LmSTEE* genes, that is, the six selected here along with four previously used for screening (Jiquel et al., 2021) (Figure S3). (i) Three of them, *LmSTEE617* (aka *LmSTEE78*), *LmSTEE5465*, and *LmSTEE10933*, were located in the middle of gene-rich GC-isochores; (ii) three of them, *LmSTEE1277*, *LmSTEE6826*, and *LmSTEE98* (aka *LmSTEE11364*), were also located in the middle of GC-isochores, but neighbored by degenerated DNA transposons (DTx_Gimli or DTM_Sahana) inserted as solo transposable elements (TEs) in the middle of GC-isochores; (iii) two genes, *LmSTEE1852* and *LmSTEE7919*, were located at the border between GC- and TE-rich AT isochores, and *LmSTEE1852* was also closely associated to DTx_Gimli; (iv) and the last two, *LmSTEE3177* (aka *LmSTEE1*) and *LmSTEE8094* (aka *LmSTEE35*), were

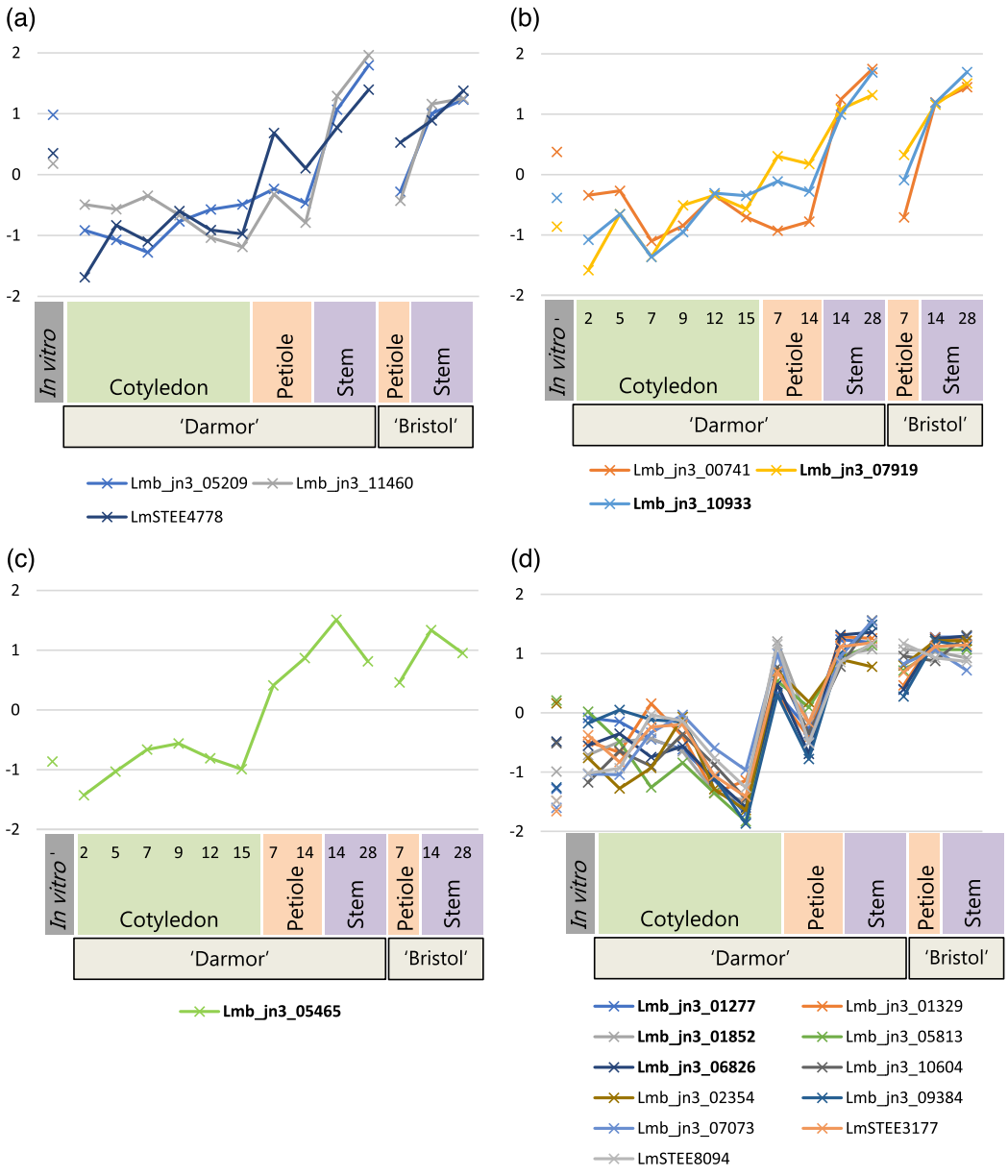

FIGURE 3 Expression profiles of 15 late effector genes during cotyledon and stem colonization in controlled conditions. Experimental conditions are displayed on the *x* axis. Expression values (CPM—count per millions of mapped reads) were Log_2-transformed and then mean-centered. Two or three independent biological replicates were used per sampling time-point. Expression profiles are clustered as in Figure 1, corresponding to the cluster A in panel (a), cluster B in panel (b), cluster C in panel (c), and cluster D in panel (d). Raw data and statistics of differential expressions between the dates are indicated in Tables S4 and S5. The six late effector candidates selected for further characterization are indicated in bold.

in GC islands comprising 2–6 genes in the middle of large AT-isochores.

3.4 | Diversity in the collection of *B. napus* genotypes

To increase the probability of identifying new sources of resistance corresponding to late effectors, it was necessary to screen a large panel of diversity of *B. napus* genotypes. For this purpose, we explored a selection of 99 accessions from a worldwide collection available at Innolea (Table S2A). The diversity present in this collection was investigated following genotyping with 6631 SNP well-distributed on the 19 chromosomes. This grouped genotypes into three main subgroups roughly corresponding to morphotypes, that is, winter types (approximately 69 genotypes), spring types (nine genotypes), and rutabagas (*B. napus* var. *rapifera*, three genotypes) grouped with semi-winter types, mainly of Asian origin (approximately 16 genotypes) (Figure 4). The grouping and representative diversity was consistent with other collection studies (Rahman et al., 2021; Wu et al., 2019). Additionally, a set of 31 "modern" winter-type varieties grown in France, known for their behavior in the field with regard to

TABLE 1 Characteristics of the "late" effector candidates studied here

Name	Accession no	Size (aa)[a]	Number of cysteins	Expression waves[b]	Number of BLAST hits[c]	Closest BLAST hit	*E* value (% identities)	BLAST hit coverage	Pfam domain (*E* value)	Number of hits on *L. biglobosa* "*brassicae*" proteins[d]
LmSTEE1277	*Lmb_jn3_01277*	109	9 (8.3%)	Stem biotrophy (cluster 5)	48	*Plenodomus tracheiphilus*	5E-43 (68%)	94%	CFEM (1.0E-09)	1
LmSTEE1852	*Lmb_jn3_01852*	89	8 (9.0%)	Stem biotrophy (cluster 5)	0	-	-	-	None	0
LmSTEE5465	*Lmb_jn3_05465*	96	8 (8.3%)	Stem biotrophy (cluster 5)	214	*Plenodomus tracheiphilus*	2E-61 (82%)	98%	None	1
LmSTEE6826	*Lmb_jn3_06826*	87	9 (10.3%)	Stem biotrophy (cluster 5)	0	-	-	-	None	0
LmSTEE7919	*Lmb_jn3_07919*	176	0 (0.0%)	Stem biotrophy (cluster 5)	629	*Alternaria gaisen*	7E-94 (79.44%)	99%	None	1
LmSTEE10933	*Lmb_jn3_10933*	50	2 (4.0%)	Stem biotrophy (cluster 5)	37	*Setomelanomma holmii*	5E-13 (64.15%)	98%	None	1

[a]Amino acids.
[b]Names of expression waves and corresponding genes cluster, as defined by Gay et al. (2021).
[c]Number of hits with an *E* value < 1E-5.
[d]Results from BLASTp against all *L. biglobosa* proteins with *E* value < 1E-5 (Table S5, *L. biglobosa* genome available at http://www.genoscope.cns.fr/leptolife, Dutreux et al., 2018).

TABLE 2 PCR amplification and sequencing analysis of six selected *LmSTEE* genes in *Leptosphaeria maculans* isolates

Origin of isolates[a]	*LmSTEE1277*			*LmSTEE1852*			*LmSTEE5465*			*LmSTEE6826*			*LmSTEE7919*			*LmSTEE10933*		
	Presence	Absence[b]	Mutation[c]	Presence	Absence[b]	Mutation[c]	Presence	Absence[b]	Mutation[c]	Presence	Absence[b]	Mutation	Presence	Absence[b]	Mutation[c]	Presence	Absence[b]	Mutation[c]
France	90	0	**3/90**[d]	90	0	0/90	90	0	**4/90**[e]	90	0	0/90	90	0	**4/90**[f]	90	0	0/90
Oceania	23	0	0/23	23	0	0/23	23	0	0/23	23	0	0/23	23	0	**2/23**[f]	23	0	0/23
Canada	21	0	0/21	21	0	0/21	21	0	**1/21**[e]	21	0	0/21	21	0	**1/21**[f]	21	0	0/21
USA	10	0	0/10	10	0	**1/10**[g]	10	0	0/10	10	0	0/10	10	0	**1/10**[f]	10	0	0/10
Mexico	34	0	0/34	34	0	0/34	34	0	0/34	34	0	0/34	34	0	**22/34**[f]	34	0	0/34
Chile	5	0	0/5	5	0	0/5	5	0	0/5	5	0	0/5	5	0	**0/5**	5	0	0/5
Total	183	0	**3/183**	183	0	**1/183**	183	0	**5/183**	183	0	0/183	183	0	**30/183**	183	0	0/183

[a]Isolates are described in Table S1.
[b]Lack of PCR amplification.
[c]Number of isolates with SNP compared with the reference sequence JN3, based on sequencing results.
[d]Observed mutations for LmSTEE1277 correspond to two SNPs impacting the protein sequences: V > M(13) and L > M(100).
[e]Observed mutations for LmSTEE5465 correspond to one silent mutation for 4 isolates and to one SNP: L > F(41) for one French isolate.
[f]Observed mutations for LmSTEE7919 correspond to one single mutation for 29 isolates: V > I(38) and one silent mutation T > T(20) for one isolate from Australia.
[g]The only isolate with mutations for LmSTEE1852 has two silent mutations.

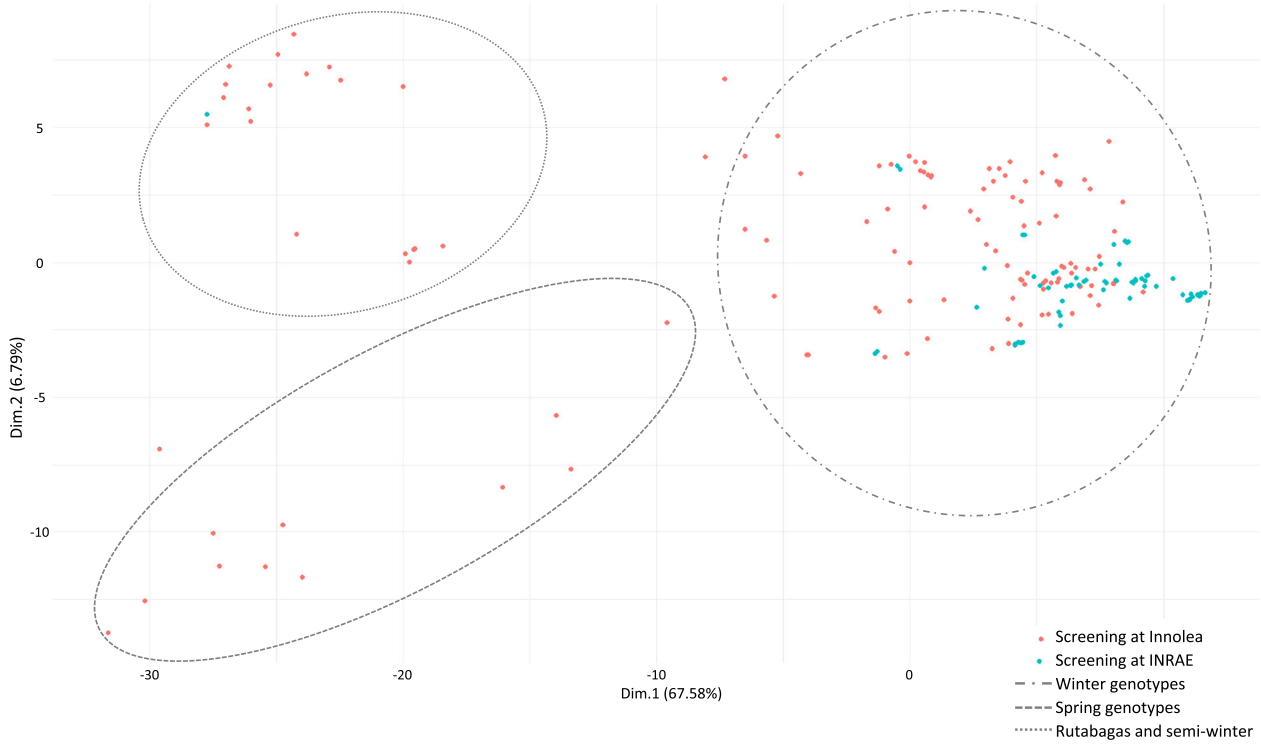

FIGURE 4 Principal coordinates analysis of *Brassica napus* genotype diversity; 130 genotypes are represented, including 99 genotypes (in red) screened at Innolea and 31 genotypes at INRAE Bioger (in blue). Genotyping data were obtained with 6631 SNP well-distributed on the 19 chromosomes. The resulting allelic matrix was used to compute an Identity by State (IBS) matrix. Then a principal coordinate analysis was performed based on the IBS matrix.

stem canker resistance, was also genotyped (Figure 4; Table S2B). Most of these are elite varieties with quantitative resistance and cluster along with the winter type "00" varieties.

3.5 | Identifying sources of resistance to selected "late" effectors in the collection of 130 genotypes

The six *LmSTEE* genes selected here were placed under the control of *AvrLm4-7* promoter to generate "over-expressed in cotyledons" (OECs) transformants, and their expression level in cotyledons was checked in all transformants (Figure S4). Lack of pathogenicity defects of transformants was also controlled on a susceptible genotype (Figure S5). Two OEC transformants were selected for each late effector gene, and the subset of 130 plant genotypes (Table S2) was screened with the two independent OEC transformants for each of the six new late effectors along with four late effector genes previously used (*LmSTEE3177*, *LmSTEE8094*, *LmSTEE617*, and *LmSTEE98*; Jiquel et al., 2021). This screening was done in three steps.

A first screening was done with a small number of seedlings, to eliminate genotypes with no resistance phenotype (Tables S2 and S8). This first step allowed us to identify 36 promising interactions involving 22 different *B. napus* genotypes and 8 different "late" effectors. Four additional genotypes, "RG009," "RG034," "RG074," and "RG099," displayed a general response to the inoculation with OEC transformants and with control isolates INV13.269 and JN2 (Table S9).

These potential interactions were challenged with an increased number of plants and in two locations with slightly different inoculation conditions, except for RG099, for which we did not have enough seeds. Three genotypes ("RG021," "RG047," and "RG072") consistently displayed resistance responses to the *LmSTEE98* late effector (Figures 5 and S6), as previously found with the "Yudal" genotype (Jiquel et al., 2021) and replicated here using "Yudal" as a control. The resistance response of "RG021" or "RG047" to *LmSTEE98* corresponded to a typical hypersensitive response (HR), as previously observed for "Yudal," while a slightly different, more intermediate interaction phenotype was observed with "RG072" (Figure 5). OEC transformants expressing another late effector candidate, *LmSTEE6826*, also induced a clear HR-type resistance phenotype when inoculated on genotype "RG007" (Figures 5 and S6).

While these genotypes displayed a clear-cut resistance response, some other genotypes consistently responded to the late effector *LmSTEE7919* along with one or the other *LmSTEE8094* or *LmSTEE6826*. However, they did not induce typical resistance response phenotypes, but rather late resistance responses expressed as large patches of dark tissue (Figures 6, S7, and S8). INN_VAR04 and INN_VAR14 reacted to both *LmSTEE7919* and *LmSTEE8094*, whereas INN_VAR11 and INN_VAR17 reacted to *LmSTEE6826* and *LmSTEE7919* (Figures 6 and S7). RG074 and RG009, while displaying

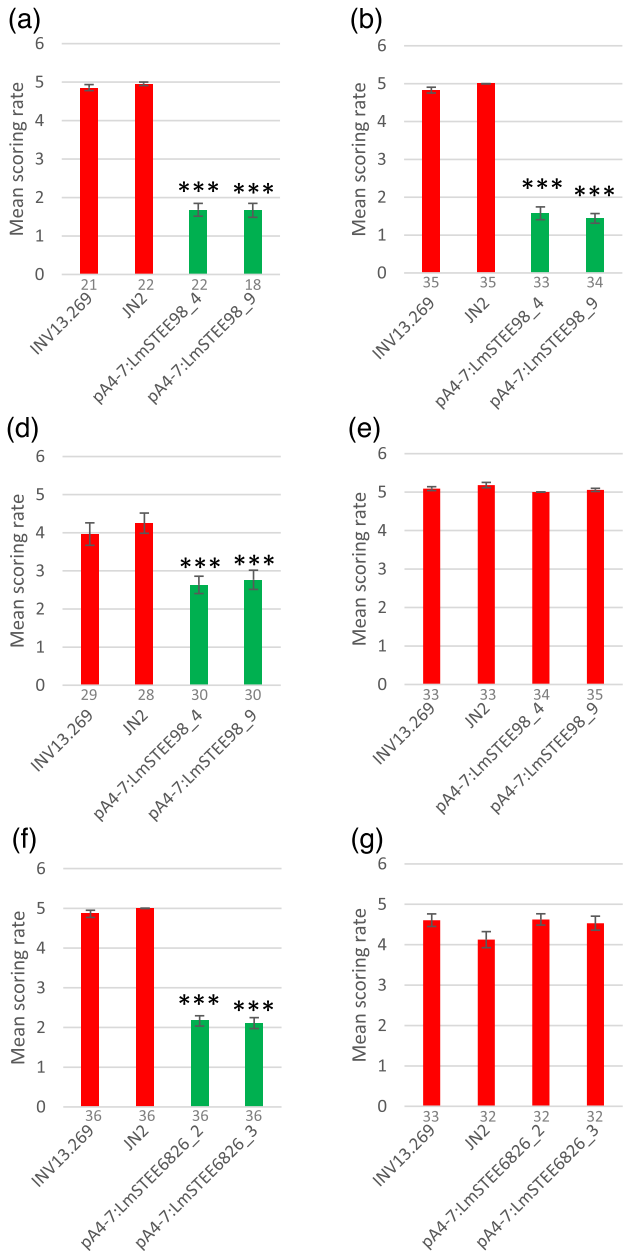

FIGURE 5 Resistance response of five oilseed rape varieties towards *Leptosphaeria maculans* late effector genes, observed at INRAE. *L. maculans* transformants expressing *LmSTEE98* (aka *LmSTEE11364*) and *LmSTEE6826* under the promoter of the avirulence gene *AvrLm4-7* were inoculated onto the varieties (a) "Yudal," (b) RG021, (c) RG047, (d) RG072, (e) RG030, (f) RG007, or (g) RG088; RG030 and RG088 correspond to susceptible controls. Two independent transformants were used per construction. The bars represent the mean score of symptoms (±standard error), based on the IMASCORE rating scale, with 1 and 2 corresponding to resistance (green bars for resistance responses), 3 to intermediate phenotype, 4–6 to susceptibility (red bars) (Balesdent et al., 2001), 14 days after inoculation. The number of plants is indicated below the *x* axis. Isolate INV13.269 is the wild-type isolate, and isolate JN2 is used as an additional control. The asterisks indicate a significant difference between the control isolate INV13.269 and the *L. maculans* transformants (Kruskal–Wallis test: ***p value < .001)

a general level of resistance to OEC transformants and control isolates at the cotyledon stage, also reacted differentially when challenged with *LmSTEE98* (RG009) or *LmSTEE7919* and *LmSTEE8094* (RG074), displaying again late responses different from typical HR (Figures 6, S7, and S8). Other promising interactions were either not reproducible or were apparently dependent on the environmental conditions used during inoculation (Tables S8 and S9).

3.6 | Relatedness of genotypes displaying resistance responses to "late" effectors

Among the 130 genotypes tested, a total of 11 were found to react to OEC transformants expressing five different late effectors, of which three were identified in this study. The genotypes displayed consistent resistance phenotypes, but the phenotypes varied from one genotype to the other, ranging from a strong HR to a delayed resistance response (Figure 7a). Interestingly, the four genotypes recognizing LmSTEE98, and reacting to it either with a typical HR ("RG021" and "RG047") or a delayed resistance response ("RG009" and "RG072"), are genetically close to the genotype "Yudal" (Minimum IBS value between "Yudal" and the four other genotypes: 0.72). They belong to the "rutabaga and semi-winter" group and are all of Asian origin (Japan or Korea) (Figure 7b). The genotype "RG007", which displayed an HR after infection by *LmSTEE6826* OEC transformants, also belonged to this "semi-winter group" of Asian genotypes (IBS value: 0.71). In summary, five genotypes among the 12 genotypes tested from this "rutabaga and semi-winter" group displayed a resistance response to the selected late effectors expressed at cotyledon stage.

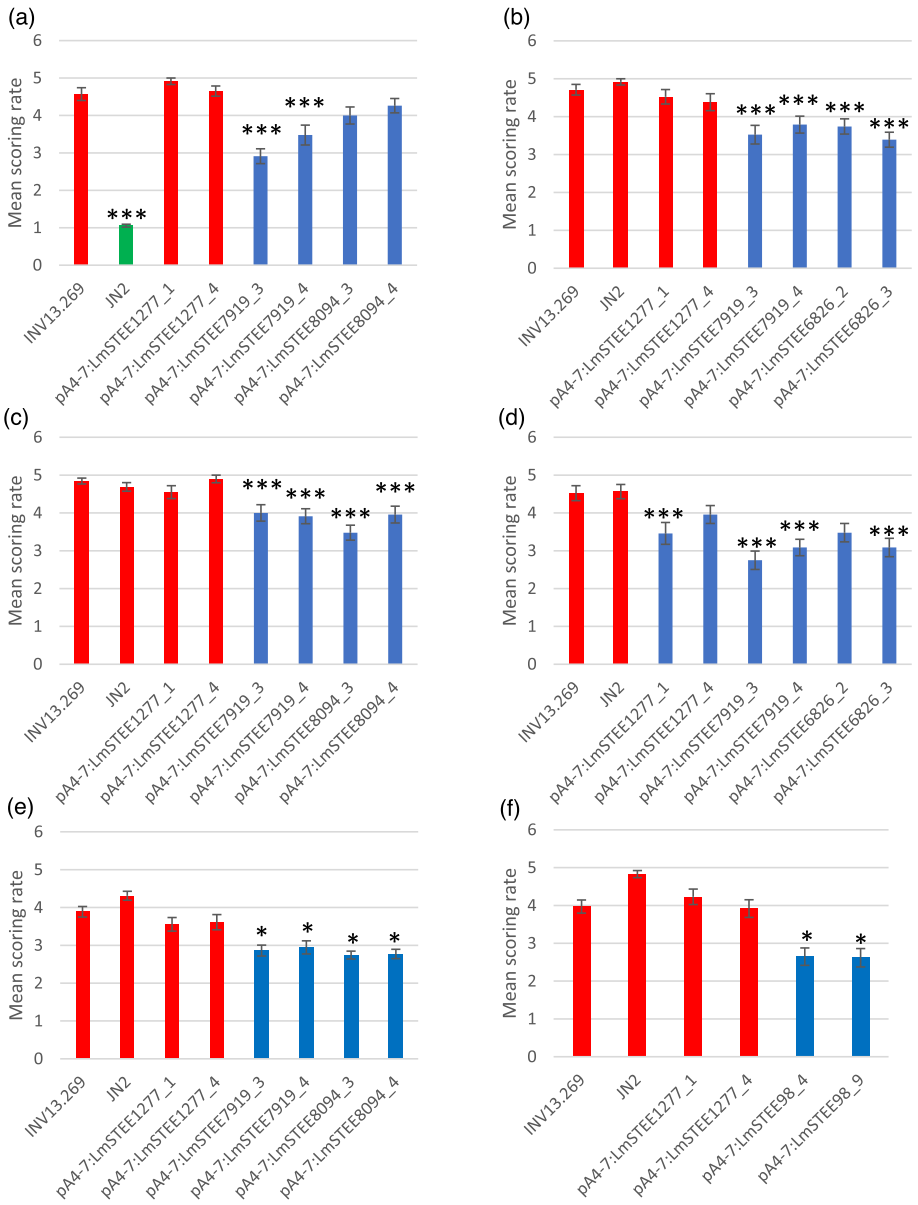

FIGURE 6 Reduced susceptibility of six oilseed rape varieties towards "late" effector genes, observed at INRAE. *L. maculans* transformants expressing *LmSTEE1277*, *LmSTEE98*, *LmSTEE8094*, *LmSTEE6826*, or *LmSTEE7919* under the promoter of the avirulence gene *AvrLm4-7* were inoculated onto the varieties (a) INN_VAR04, (b) INN_VAR11, (c) INN_VAR14, (d) INN_VAR17, (e) RG074, and (f) RG009. Two independent transformants were used per construction. The bars represent the mean score of symptoms (±standard error, $n > 20$), based on the Imascore rating scale, with 1 and 2 corresponding to resistance (green bars for resistance), 3 to intermediate phenotype, 4–6 to susceptibility (red bars) (Balesdent et al., 2001), 14 days after inoculation. Blue bars indicate delayed resistance, with scoring generally lower than that of susceptible interactions. Isolate INV13.269 is the wild-type isolate, and isolate JN2 is used as an additional control. Resistance phenotype of INN_VAR04 to JN2 is linked to the presence of Rlm7 recognizing AvrLm4-7. The asterisks indicate a significant difference between the control isolate INV13.269 and the *L. maculans* transformants (general linear model [GLM] test: *p value < .05; ***p value < .001), combining two biological replicates.

The four genotypes displaying intermediate responses to *LmSTEE7919* and either *LmSTEE8094* or *LmSTEE6826* were all modern varieties of European origin and grouped together in the dense area of the PCA, corresponding mainly to winter type oilseed rape (Figure 7c). RG074, also reacting to *LmSTEE7919* and *LmSTEE8094*, was distant from all of them (Maximum IBS value: 0.62) but was also of European origin.

4 | DISCUSSION

In a previous paper (Jiquel et al., 2021), we investigated the feasibility to genetically engineer "late" effectors so that they are expressed during cotyledon infection and then to use them to uncover putative matching resistance genes in collections of *B. napus* genotypes to identify new sources of resistance to *L. maculans*. As a proof-of-principle, we found one late effector, LmSTEE98 (aka LmSTEE11364), that allowed us to identify one new, single-locus source of resistance in cv. "Yudal," operating when the fungus colonizes the stem of the plant. This finding suggested that at least part of the QDR was attributable to gene-for gene interactions. If such a finding could be generalized, it would have important practical consequences for breeders, allowing them to diversify the genepool of *Rlm* genes, to generate easy-to-use markers of QDR and eventually to provide them with knowledge-driven tools to improve QDR to *L. maculans* in rapeseed. However, as mentioned by Jiquel et al. (2021), this work, as a first shot, suffered flaws preventing the authors from reaching general conclusions on the possibility to find other such resistances with such an approach.

To address these flaws, we firstly duplicated the phenotyping experiments on the diversity panel in two locations, with slightly different operating protocols, to only keep sources of resistance

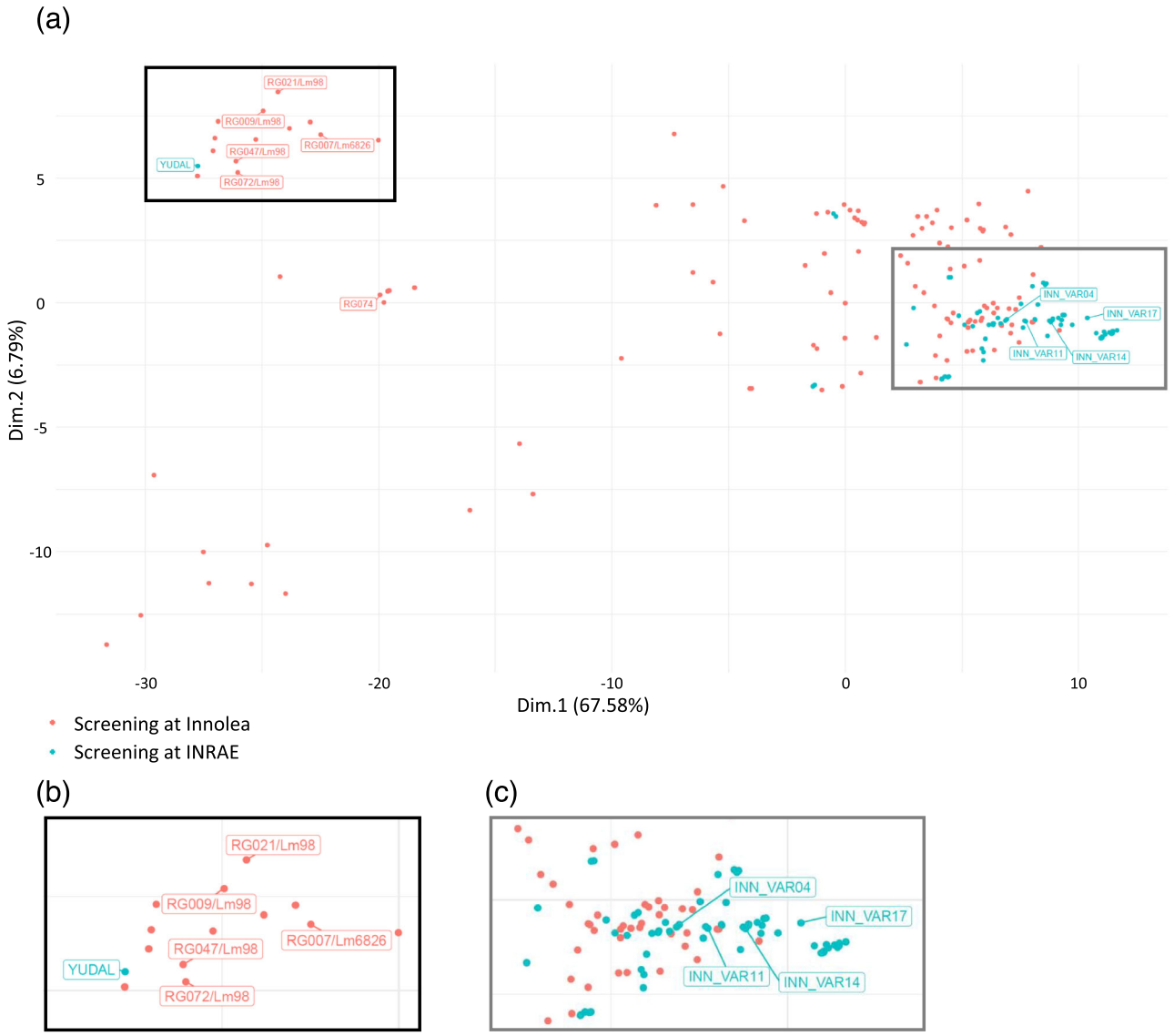

FIGURE 7 Genotypic diversity of Brassica napus resistant genotypes; 130 genotypes are represented, including 99 genotypes (in red) screened at Innolea and 31 genotypes at INRAE Bioger (in blue). (a) Genotyping data were obtained with 6631 SNP well-distributed on the 19 chromosomes. The resulting allelic matrix was used to compute an Identity by State (IBS) matrix. Then a principal coordinate analysis was performed based on the IBS matrix of the 130 genotypes. (b) Enlargement of the black framed area in (a), including four genotypes and "Yudal," displaying hypersensitive responses to the late effectors LmSTEE98 or LmSTEE6826. (c) Enlargement of gray framed area in (a), displaying genotypes partially responding to one or two *LmSTEE* genes

expressed similarly in the two locations. We then decided to focus our choice of effectors to be investigated to those that are exclusively expressed during the stem/petiole colonization of rapeseed by *L. maculans*, that is, which show no expression at the cotyledon stage. Last, we strongly enhanced the diversity of the *B. napus* material screened, using a subset of a large world-wide collection of *B. napus* genotypes available at Innolea. Eventually, our objective was to get an evaluation grid to rank the most important criteria to privilege to identify diversified resistances.

With these improvements, we found that (i) *LmSTEE98*, that induces HR in cv. "Yudal," also displays similar interaction phenotypes in three other *B. napus* genotypes, likely to also contain the putative *RlmSTEE98* gene; (ii) one other late effector, *LmSTEE6826*, induces a clear resistance response in one genotype; (iii) three "late" effectors, including *LmSTEE6826*, induce intermediate resistance responses in six *B. napus* genotypes when OECs; (iv) a series of genotypes display resistance responses to the same effector (four genotypes reacted to LmSTEE98; five to LmSTEE7919) and are genetically related, suggesting the genetic background is shared by these different genotypes. In this study, we did not genetically identify the resistance loci corresponding to the new *LmSTEE* genes when expressed at the cotyledon stage, but we can postulate that, on the model of the AvrLmSTEE98–RlmSTEE98 interaction, major resistance genes also are present that recognize *LmSTEE6828*, *LmSTEE7919*, and *LmSTEE8094*, suggesting at

least three new gene-for-gene interactions may operate. We also noticed phenotypic differences between the interactions involving *LmSTEE98* and *LmSTEE6828* compared with those involving *LmSTEE7919* and *LmSTEE8094*. These later were always expressed as intermediate phenotypes of resistance that may evolve towards susceptibility. However, we also observed genotype-dependent interaction phenotypes as illustrated by interaction phenotypes with OEC transformants expressing *LmSTEE98*, with three genotypes expressing typical HR, whereas two others, RG009 and RG072, express delayed resistance responses. This may be due to expression levels of R gene during infection (Cook et al., 2012), insufficient matching between the avirulence effector and the resistance protein leading to incomplete or delayed expression of resistance, sometimes related to "weak" alleles of resistance genes that could lead to QDR (Roux et al., 2014). We can also imagine that the expression peak of late effectors in this OEC system incompletely matches with the expression profile of the cognate R gene that could be expressed at a low level, or too late during the infection in cotyledons, leading to an intermediate response in these genotypes. We can thus postulate that our approach will identify only those resistance genes that are expressed constitutively throughout the life of the plant, resistance genes whose expression is induced upon pathogen attack in both cotyledon (like *Rlm9*; Larkan et al., 2020) and stem tissues, and resistance genes that are expressed late during cotyledon infection and whose expression is then maintained in petioles and stems. We thus will miss all resistance genes that are only expressed in the stem or induced during petiole or stem colonization (if any).

Jiquel et al. (2021) screened a collection of 204 rapeseed genotypes, quasi-exclusively comprising winter-types. The only genotype identified as expressing an HR-type resistance to one late effector, "Yudal," was genetically divergent from the winter-type varieties, and the authors stressed that the limited diversity in the genepool screened may be a limitation in novel resistance discovery. Here, we overpassed this limitation by screening a wide diversity of genetic material comprising three main *B. napus* types: winter types, spring types, and semi-winter types/rutabagas. By analyzing the diversity in the screened collection of 99 genotypes, we firstly found no resistance in the 13 spring-type genotypes, which is consistent with the scarcity of native major resistance genes in these genotypes (Rouxel et al., 2003), and the usually famed low level of QDR in spring types/canola (Raman et al., 2016). Genotypes recognizing late effectors are all grouped into the "rutabagas and semi-winter" group of genotypes, to which "Yudal" also belongs. Among the 12 genotypes screened in this group, five displayed an HR-type response to two distinct late effectors, LmSTEE98 and LmSTEE6826. The identification of genotypes recognizing late effectors mainly in the semi-winter group could be explained by the recent domestication of *B. napus*, occurring about 400 years ago. The first oilseed rape is hypothesized to be a semi-winter type (Rahman et al., 2021). After this, a selection of genotypes adapted to more diverse environmental conditions was conducted, leading to the development of winter and spring genotypes, accompanied by a reduced diversity in both these groups and possible loss of interesting agronomic traits. This recent history of *B. napus* could explain the greater diversity and the higher number of genotypes recognizing late effectors in the semi-winter type, compared with spring and winter type. The semi winter-type/rutabaga genepool, with a prevalence of Asian genotypes, is thus a peculiarly promising source of novel resistances. Different studies have recently obtained deep molecular data on large collections (Rahman et al., 2021; Wu et al., 2019), showing that the genebank collections hold 75–145 accessions that could be related to this semi-winter-type/rutabaga genepool and thus representing promising routes for more resistance gene discovery.

While the diversity panel of 99 genotypes was screened at both Innolea and Bioger, with a focus on clear-cut, easy to reproduce HR-type responses, and absence of selection of intermediate responses, the 31 modern winter-type genotypes were screened at Bioger only and resulted in the identification of more diffuse resistance responses. We often observed inconsistent responses to late effectors in these genotypes, including irreproducible results between repeats or different behavior between two transformants with the same late effector gene (Table S8). This is similar to what has been described in our previous screening mainly comprising winter-type genotypes (Jiquel et al., 2021) preventing us to conclude on their use as a source of novel resistance. By contrast, our study indicates that a limited amount of modern winter type genotypes (4 out of 31 analyzed) consistently reacted to *LmSTEE7919* expressed at the cotyledon stage (and to either one of *LmSTEE8094* or *LmSTEE6826*) but with intermediate phenotypes rather than typical HR. The link between the recognition of a late effector in our somewhat artificial system and actual QDR in the field remains difficult to establish, as we currently have no information on the field behavior of the semi-winter genotypes, other than "Yudal." Also, while the panel of 31 modern winter-types was firstly selected on their famed QDR in the field, we only have partial information on how the four winter type varieties recognizing LmSTEE7919 behave, with three of them displaying a good behavior in field experiments assessing the quantitative resistance at the end of the growing season and a fourth genotype being variable between years (data not shown).

For setting up the OEC strategy with late effectors, Jiquel et al. (2021) selected five "late" effectors produced in the stem during systemic colonization but belonging to diverse waves of expression: *LmSTEE3177* and *LmSTEE4778* belong to the stem biotrophy wave (Wave 5) displaying an exclusive and early expression in stems, before the development of the stem canker symptoms; *LmSTEE8094* and *LmSTEE98* belong to the "biotrophy-to necrotrophy transition" wave (Wave 4), expressed later than Wave 5 in stems, and also displaying a limited level of expression in cotyledons; and *LmSTEE617* belongs to the stem necrotrophy wave (Wave 6) and is expressed exclusively in stems at later stages of stem infection (Gay et al., 2021) (Figure 1). For a reasoned choice of most relevant effectors for screening, we chose here to enrich the LmSTEE genepool with genes exclusively expressed in petioles and stems of *B. napus*, the places where QDR is postulated to be operating, and therefore on effector genes associated to the "stem biotrophy" wave. If pooling information from Jiquel et al. (2021) and those of this study, we can notice that two out of the six

new effectors chosen here, *LmSTEE6826* and *LmSTEE7919*, identified potential new resistance sources following screening. However, *LmSTEE98* (Jiquel et al., 2021; this study) and *LmSTEE8094* (this study) belong to Wave 4 and identified new sources of resistance. Another criterium for the choice of the most promising candidates for screening was conservation between species or polymorphism in populations, and we found that the two late effectors that lead to typical HR responses, *LmSTEE98* and *LmSTEE6826*, are two out of three of those that have no homologs in other fungal species. This characteristic is reminiscent of "classical" avirulence genes, often with no homolog also in other species (for example, *AvrLm1*, Gout et al., 2006; *AvrLm3*, Plissonneau et al., 2016; *AvrLmS*, Neik et al., 2022). *LmSTEE7919* and *LmSTEE8094*, in contrast, have homologs in other fungal species and even paralogs in *L. maculans* for *LmSTEE8094* (Jiquel et al., 2021). Another criterion of choice that may be considered is conservation in *L. maculans* populations. For these four effectors, contrasted data were obtained, with *LmSTEE6826* being always present in field populations with no sequence variation, *LmSTEE7919* being always present in field populations with one rare non-silent mutation, and *LmSTEE98* and *LmSTEE8094* may be absent in a very limited number of field isolates, and only *LmSTEE98* displays sequence variation in a very special population obtained from cabbages in Mexico (Jiquel et al., 2021). Lastly, we can consider genome location as an additional criterium for decision making, as we found that *LmSTEE* genes identifying new resistance sources were located in AT-GC borders, GC islands in AT-isochores or associated to DNA transposons in GC regions, but that none of the genes that are in a canonical GC-equilibrated environment induced resistance responses.

The strategy to choose the best effector candidates for screening may thus be a combination of three non-exclusive criteria: (i) belonging to Waves 4 or 5 of expression; (ii) having low or no homologs in other species; and (iii) being located in a transition region of the genome between AT-rich and GC-isochores. If only focusing on the first two criteria, 13 effectors among the 40 effectors clustered in the Wave 5 have no homologs, including *LmSTEE1852* and *LmSTEE6826* studied here. Among the 56 effectors clustered in the Wave 4, 21 genes have no homologs either, including *LmSTEE98*. Last, among the six late effectors identified in our transcriptomic analysis, but absent from the Wave 5 described by Gay et al. (2021), four also have no homologs in other fungal species. There is thus a pool of at least 35 new "late" effectors that could be candidates of choice to generate OEC transformants for identifying genotypes specifically recognizing these effectors.

Our study and the previous one by Jiquel et al. (2021) thus provide new perspectives for knowledge informed building of resistant genotypes and their use for durable disease management strategies. While major genes for resistance expressed at the leaf stage are extremely efficient and useful, their scarcity in current genetic resources limits their reasoned use in the field. In contrast, if genes reacting to "late" effectors indeed are involved in QDR, our study provides both prospects for numerous diversified genes and easy to design markers for QDR. With this diversification of genetic resources and markers (or cloned genes) at hand, it will be easier to build new genotypes including relevant genes of "semi-winter" in winter-types or spring-types and to generate diversified combinations of genes involved in qualitative and quantitative resistance that may display an additive effect in the field.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

SF, MHB, and TR did the conceptualization. AJ and EJG performed the formal analysis. SF, TR, and MHB did the funding acquisition. AJ, EJG, JM, PG, and MHB performed the investigation. AJ, SF, MHB, and TR did the methodology. SF, MHB, and TR performed the project administration. AJ, JM, PG, and MHB were responsible for the resources. SF, TR, and MHB were responsible for the supervision. TR, MHB, and SF did the validation. AJ, AW, and AF did the visualization. AJ, TR, MHB, and SF wrote the draft. AJ, EJG, SF, JM, PG, AW, AF, MHB, and TR did the writing—review.

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SUPPORTING INFORMATION

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