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SUMMARY

- The control of stem canker disease of *Brassica napus* (rapeseed), caused by the fungus *Leptosphaeria maculans* is largely based on plant genetic resistance: single-gene specific resistance (*Rlm* genes) or quantitative, polygenic, adult-stage resistance. Our working hypothesis was that quantitative resistance partly obeys the gene-for-gene model, with resistance genes "recognizing" fungal effectors expressed during late systemic colonization.

- Five *LmSTEE* (stem-expressed effector) genes were selected and placed under the control of the *AvrLm4-7* promoter, an effector gene highly expressed at the cotyledon stage of infection, for miniaturized cotyledon inoculation test screening of a gene pool of 204 rapeseed genotypes.

- We identified a rapeseed genotype, 'Yudal', expressing hypersensitive response to LmSTEE98. The LmSTEE98-RlmSTEE98 interaction was further validated by inactivation of the *LmSTEE98* gene with a CRISPR-Cas9 approach. Isolates with mutated versions of *LmSTEE98* induced more severe stem symptoms than the wild-type isolate in 'Yudal'. This single-gene resistance was mapped in a 0.6 cM interval of the 'Darmor_*bzh*' x 'Yudal' genetic map.

- One typical gene-for-gene interaction contributes partly to quantitative resistance when *L. maculans* colonizes the stems of rapeseed. With numerous other effectors specific to stem colonization, our study provides a new route for resistance gene discovery, elucidation of quantitative resistance mechanisms, and selection for durable resistance.

KEY WORDS: *Brassica napus*, effector, gene-for-gene interaction, *Leptosphaeria maculans*, quantitative disease resistance

INTRODUCTION

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

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

asymptomatic phase that may last several months, before switching to a necrotrophic lifestyle in which necrosis is induced at the stem base (Hammond *et al.*, 1985; West *et al.*, 2001; Rouxel & Balesdent, 2005; Huang *et al.*, 2014).

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

As already reported for other pathogens, the infection and colonization of rapeseed by *L. maculans* is dependent on a cocktail of effectors, molecules mainly corresponding to small secreted proteins which modulate plant immunity and facilitate infection (Sánchez-Vallet *et al.*, 2018). Two waves of *L. maculans* effector gene expression were initially identified, the molecules involved in the first wave being described by Gervais *et al.* (2017) as “early effectors” and those of the second wave, “late effectors” (aka “LmSTEE genes” for “*L. maculans* stem expressed effector”). More extensive studies have since discriminated eight waves of genes specifically expressed during the interaction with the plant, all being enriched in genes encoding effectors (Gay *et al.*, 2020). "Early effector" genes, including all known avirulence genes (*AvrLm*) “recognized” by the plant and leading to qualitative resistance, are up-regulated during cotyledon/leaf

colonization. Genetic manipulations or crosses of the fungus to generate isolates harboring the smallest possible number of *AvrLm* genes and isogenic isolates differing by only a single *AvrLm* gene, are now routinely used as tools for identifying the corresponding *Rlm/LepR* genes in *Brassica* genotypes, for screening genetic resources, or for use in plant breeding (Balesdent *et al.*, 2002; Rouxel *et al.*, 2003; Van de Wouw *et al.*, 2014; Larkan *et al.*, 2015). An easy-to-use, medium- to high-throughput cotyledon inoculation test is widely used worldwide for such purposes (Larkan *et al.*, 2016a). Quantitative resistance operates during the systemic colonization of rapeseed, and, during this phase, *L. maculans* expresses several genes encoding effectors thought to interfere with plant defenses (Gervais *et al.*, 2017). These *LmSTEE* genes are expressed only very weakly, if at all, during axenic growth and cotyledon/leaf colonization, but are strongly expressed during stem colonization, many months after the initial leaf infection. Our working hypothesis is that at least some *LmSTEE* genes encode products recognized by specific plant resistance gene products during systemic colonization, in a manner similar to that described for *AvrLm-Rlm* interactions, accounting for at least part of the quantitative resistance. However, it is not possible to detect gene-for-gene interactions between *LmSTEE* genes and the resistance genes of *B. napus* in the usual cotyledon inoculation assay, because the corresponding avirulence proteins are not produced at this stage. In addition, stem inoculation assays (Gervais *et al.*, 2017; Gay *et al.*, 2020) for the identification of such resistance are not amenable to medium/high-throughput screening, due to length of the process, low reproducibility and the time and space required to screen a large number of genotypes of fully grown plants. We therefore designed a new strategy, allowing the expression of “late” effectors during cotyledon infection, for the identification of putative new resistance genes in rapeseed interacting with the late effectors from *L. maculans* in the cotyledon inoculation test.

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

MATERIALS AND METHODS

Fungal and plant materials

The sequenced isolate JN3 (v23.1.3) (Rouxel *et al.*, 2011) and the closely related isolate JN2 (v23.1.2) correspond to two sister progenies from an *in vitro* cross between European field isolates (Balesdent *et al.*, 2001). They were used as controls in inoculation tests. A representative selection of isolates from worldwide collections and reference isolates from Australia, Canada, the USA, Chile, France, Mexico, and New Zealand, was also analyzed (Table S1) (Mendes-Pereira *et al.*, 2003; Dilmaghani *et al.*, 2009, 2012; this study). INV13.269 is a single pycnidiospore isolate recovered from a French field in 2013.

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

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

Plant inoculations

The cotyledons of 10-day-old seedlings were inoculated, by puncture, with 10 µl of inoculum (10^7 pycnidiospores ml^{-1}), as described by Balesdent *et al.* (2001). Four different isolates, one per half-cotyledon, were used to inoculate each plant. At least six plants were inoculated with each isolate. Plants were incubated for two days at room temperature in the dark, and then in a growth chamber at 19°C (night) and 24°C (day) with a 16 h photoperiod and a relative humidity of 90%. Symptoms were scored 10, 14 and 18 dpi, with the IMASCORE rating scale (Balesdent *et al.*, 2001). Mean scores for symptoms and the percentage of virulent and avirulent phenotypes induced by the *LmSTEE* "over-expressed in cotyledons" (OEC) transformants were determined for each cultivar and compared with the symptoms induced by the wild-type isolate on the same cultivar, on the same date (Kruskal-Wallis test).

Adult plants were inoculated as described by Gervais *et al.*, 2017. For the analysis of stem colonization by *L. maculans*, we cut the petiole of the second leaf horizontally, 1 cm from the insertion point of the leaf. Inoculum (10 µl, containing 10^7 pycnidiospores ml^{-1}) was applied to the wounds. Inoculated plants were incubated as previously described. The necrosis of infected stems was assessed by cutting the whole stem section between the insertion point of the inoculated petiole on the stem and the ground into successive 3 mm slices. The sections were scanned and the percentage of the stem section showing internal necrosis was measured with ImageJ.

Vector construction and fungal transformation

The promoter of the *AvrLm4-7* gene was amplified with the primers indicated in Table S3. It was then digested with *Nhe*I *and Bam*HI and ligated into a *Spe*I-*Bam*HI-digested pPZPNat1 vector. The *LmSTEE* genes (*LmSTEE1*, *LmSTEE30*, *LmSTEE35*, *LmSTEE98*, *and LmSTEE78*) were amplified from their Start codon to their terminator regions and the amplicons were digested with *Eco*RI and *Xho*I or *Sal*I and *Xho*I (Table S3). The resulting fragments were ligated into the pPZPNat1 vector containing the *AvrLm4-7* promoter, digested with the same enzymes.

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

The four mutated versions of *LmSTEE98* (see below) were amplified from the Start codon to the terminator region, as for the wild-type version of *LmSTEE98* (Table S3). The *ΔlmSTEE98_06* and *ΔlmSTEE98_10* variants were cloned by Gibson assembly (Silayeva and Barnes, 2018), with the primers Gib_pA4-7:Lm98_F and Gib_pA4-7:Lm98_R. The *ΔlmSTEE98_20* and *ΔlmSTEE98_30* variants were amplified with the LmSTEE98_SalI_Up and LmSTEE98_XhoI_Low primers and the resulting amplicons were digested with *Sal*I and *Xho*I (Table S3). For both cloning techniques, the pPZPNat1 vector containing the *AvrLm4-7* promoter was digested with the same enzymes and the fragments were inserted, by Gibson assembly or enzyme-mediated ligation. Both cloning methods produced constructs identical to those used for genotype screening (pA4-7::LmSTEE98), except for the mutated sites.

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

We checked that the fungal transformants with *LmSTEE98* inactivation displayed no growth defects *in vitro*. A plug of mycelium was deposited in the center of a Petri dish containing V8 agar media, and mycelial growth was measured every two days for six days (nine replicates).

DNA manipulation and HRM experiments

Genomic DNA was extracted from suspensions of conidia with the DNeasy 96 or DNeasy Plant Mini Kit (Qiagen), in accordance with the manufacturer's recommendations. LmSTEE genes were amplified by PCR with the primers in Table S3.

High-resolution melting (HRM) was performed as described by Plissonneau *et al.* (2016), to analyze SNPs in the natural population of *L. maculans* for the various *LmSTEE* genes considered, with the primers described in Table S3. If variant HRM profiles were detected, the *LmSTEE* genes were sequenced by Eurofins Genomics (Eurofins, Ebersberg, Germany).

RNA manipulation and RT-qPCR

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

Genetic mapping

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

Bioinformatics and statistical analyses

BLAST analyses were performed by aligning the five LmSTEE protein sequences against the non-redundant protein sequences from the GenPept, Swissprot, PIR, PDF, PDB, and NCBI RefSeq databases with default parameters. Hits with an e-value lower than 1.10^{-5} were selected. The Psi-BLAST method with default parameters was used to align the LmSTEE protein sequences against all proteins of *Leptosphaeria biglobosa* (Dutreux *et al*.; 2018). Hits with an e-value lower than 5.10^{-2} were selected.

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

RESULTS

Selection of five late effector candidates

We selected five *LmSTEE* genes from the 307 effector candidates up-regulated in the stem (Gervais *et al.*, 2017). These genes were selected to cover the range of characteristics of the effector gene repertoire (Table 1) and were thus representative of the diversity present in this gene pool. The five *LmSTEE* genes encoded for small predicted secreted proteins with size ranging from 55 to 291 amino acids (Table 1). Some of them were particularly enriched in cysteins, such as LmSTEE1 with 10 cysteins for 80 amino acids (12.5%) compared to only 2 for LmSTEE78 (0.7%). The five genes belonged to three of the eight clusters of gene expression identified by Gay *et al*. (2020); *LmSTEE35* and *LmSTEE98* belonged to cluster 4 ('biotrophy to necrotrophy transition'), *LmSTEE1* and *LmSTEE30* to cluster 5 ('stem biotrophy') and *LmSTEE78* to cluster 6 ('stem necrotrophy') (Table 1). All genes were much less expressed in cotyledons than 'early' effector genes and were all highly expressed in stems after inoculation in controlled conditions (Figure S1). *LmSTEE1* and *LmSTEE30* were particularly highly expressed during stem colonization. Under field conditions, each gene had a specific expression pattern, with distinct time of peak expression (March for *LmSTEE35*, April for *LmSTEE1*, *LmSTEE78* and *LmSTEE98* and May for *LmSTEE30*; Figure S1). Following its peak of expression in April, *LmStee98* expression level remained intermediate at all stages except at the last time point, corresponding to stem necrosis (Figure S1; Gay *et al*., 2020).

With the exception of LmSTEE98, we were able to find homologs for all the LmSTEE proteins in databases, but all the closest hits corresponded to hypothetical proteins (Table 1). Three proteins paralogous to LmSTEE35 were also found in *L. maculans* (54% identity for the best hit, Table S4), but not in the related species from the same species complex, *Leptosphaeria biglobosa*, another pathogen of oilseed rape

(Dilmaghani et al, 2009). For each of the other LmSTEE proteins, we identified one to four homologs in *L. biglobosa* (Table S5).

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

We used a collection of 186 isolates from around the world (Dilmaghani *et al.*, 2009, 2012), to study the conservation of the *LmSTEE* genes in populations of *L. maculans* (Table S1). All isolates were obtained from *B. napus,* with the exception of the Mexican isolates, which were obtained from broccoli (*Brassica oleracea*).

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

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

We induced the expression of *LmSTEE* genes during cotyledon colonization, by creating mutant strains expressing the genes under the control of the promoter of the avirulence gene *AvrLm4-7* (pA4-7)*,* which is known to be up-regulated seven days post inoculation (dpi) in cotyledons (Parlange *et al.*, 2009) (Figure S1). Constructs were introduced into the INV13.269 isolate, which is virulent against all *Rlm* genes potentially present in current *B. napus* resources (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9*), for the subsequent screening of a large panel of genotypes without interference with known *AvrLm-Rlm* interactions. The resulting OEC transformants (*pA4-7::LmSTEEi*) strongly expressed *LmSTEE* genes during cotyledon infection (Figure 1). We obtained such transformants for four genes, but not for *LmSTEE30*, which we were unable to overexpress with this strategy. Expression in the cotyledons varied between transformants and genes, but was similar to that of the native *AvrLm4*-7 gene. The gene most highly expressed in OEC transformants was *LmSTEE1,* for which the level of expression was upregulated by a factor of 1000 in the transformants at 7 dpi; by contrast, transformants obtained with a construct containing the native promoter had expression levels similar to that of the wild-type isolate (Figure 1).

These findings demonstrate the usefulness of the *AvrLm4-7* promoter for modifying the expression profile of "late" effector genes. The two transformants per construct displaying the strongest overexpression (Figure 1) were selected for the screening of rapeseed genotypes in the cotyledon inoculation assay.

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

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

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

For validation of the interaction between LmSTEE98 and RlmSTEE98, we used the CRISPR-Cas9 technology (Idnurm *et al.*, 2017) to inactivate *LmSTEE98* in *L. maculans*. Using the transformant strain JN2 expressing the Cas9 protein (JN2_Cas9), we generated *L. maculans LmSTEE98* mutants. Four independent mutants with four different mutations resulting in different truncations or modifications to the protein were selected: *ΔLmSTEE98_06* (+1 bp), *ΔLmSTEE98_10* (-2 bp), *ΔLmSTEE98_20* (-9 bp) and *ΔLmSTEE98_30* (-17 bp) (Figure 3A).

To test whether these altered versions of the protein were still able to elicit the RlmStee98-mediated resistance, these four mutated versions of *LmSTEE98* were placed under the control of the *AvrLm4-7* promoter and introduced into INV13.269, to generate OEC mutants. All transformants displayed high levels of expression of the mutated versions of *LmSTEE98* on cotyledons at 7 dpi (Figure S3). All of the OEC mutants were virulent on the susceptible genotype 'Darmor', as were the control isolates with the wild-type version of *LmSTEE98* (Figure 3B). Three OEC mutants with major defects of *LmSTEE98* — *ΔLmSTEE98_06*, *ΔLmSTEE98_10* and *ΔLmSTEE98_30* — were virulent on 'Yudal', suggesting that the corresponding altered versions of the protein were not recognized by the plant (Figure 3C). By contrast, the *pA4-7::ΔLmSTEE98_20* OEC mutant, lacking only three amino acids, was avirulent (Figure 3C), as were the control isolates *pA4-7::LmSTEE98_4* and *pA4-7::LmSTEE98_9* (Figure 2), suggesting that this mutant

version of the protein was still recognized by the plant. These results indicate that the recognition of LmSTEE98 at the cotyledon stage is impaired by major mutations or truncations of *LmSTEE98.*

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

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

The three mutants with *LmSTEE98* gene impairments — *ΔLmSTEE98_06*, *ΔLmSTEE98_10* and *ΔLmSTEE98_30* — generated significantly higher percentage areas of stem necrosis on 'Yudal' than the control isolate JN2_Cas9 (Figure 4A). By contrast, the effects of the *ΔLmSTEE98_20* mutant, which lacked only three amino acids, did not differ significantly from those of JN2_Cas9. On 'Darmor'; no significant effect of *LmSTEE98* gene inactivation was observed (Figure 4B).

Genetic control and mapping of resistance to LmSTEE98

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

DISCUSSION

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

We selected five late effector candidates from three different waves of expression that had previously been shown to be specifically up-regulated in rapeseed stems during systemic colonization (Gay *et al.*, 2020). We recently showed that all "early" effectors (including all known avirulence effectors) are actually specifically up-regulated during all the biotrophic stages of plant colonization (leaf/cotyledon, petiole and stems) (Gay *et al.*, 2020). The decrease in expression of early effectors is relayed, at least during stem colonization, by a stem-specific biotrophic wave of gene expression including *LmSTEE1* and *LmSTEE30*, followed by another wave involved in the biotrophy-to-necrotrophy transition, which includes *LmSTEE78. LmSTEE35* and *LmSTEE98* belong to a less well-defined wave, illustrating a series of biotrophy-to-necrotrophy transitions. In field samples, the expression of *LmSTEE* genes other than *LmSTEE98* and *LmSTEE78*, decreases with the development of stem necrosis (Gay *et al.*, 2020). Some AvrLm effectors, produced during the initial stages of cotyledon colonization, may decrease the magnitude of symptom expression on *B. napus* cotyledons (Petit-Houdenot *et al.*, 2019). Similarly, the *LmSTEE* genes associated with the long systemic colonization of the tissues and involved in biotrophic stem colonization have been suggested to manipulate plant innate immunity, to enable the fungus to colonize its host efficiently, ultimately leading to the development of stem necrosis (Gay *et al.*, 2020).

Both "early" and "late" candidate effectors are structurally similar, as they are small secreted proteins (SSPs) generally enriched in cysteine residues, and most have no predicted function or recognizable

domain. However, they differ by their location within the genome. ”Early” effector genes are located in dispensable regions of the genome enriched in transposable elements acting as drivers of evolution by favoring deletions, translocations and diversifying or inactivating point mutations (Rouxel & Balesdent, 2017; Gay *et al.*, 2020; Soyer *et al.*, 2020). By contrast, “late” effector genes are located in GC-equilibrated regions of the genome, an unusual feature for most effector genes in fungi, and a genome environment much less conducive in generating deletions or inactivation by Repeat-Induced Point mutation, as frequently found for *AvrLm* genes (Rouxel et al., 2011; Rouxel & Balesdent, 2017). As a consequence, *LmSTEE* genes also differ from “early” effector genes by their lack of polymorphism or presence/absence variation in field populations of *L. maculans*, with only one non-synonymous mutation identified in *LmSTEE98*. This suggests that, unlike effector genes expressed at the onset of plant colonization, *LmSTEE* genes, which are not expressed in early stages of infection, are not readily subjected to selection pressures exerted by the plant, such as those generated by *Rlm* genes. As the corresponding *RlmSTEE* genes may also have only a partial effect, contributing to adult-stage resistance, they may contribute to more stable and durable resistance in *B. napus*.

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

Following inoculation with *LmSTEE98* OEC transformants, we observed a clear HR phenotype, which segregated in the BnDYDH population. The segregation pattern in this population was not consistent with the 1:1 ratio expected for monogenic control of the resistance. However, markers closely linked to *RlmSTEE98* on A09 also displayed distorted segregation patterns. Such distortions have often been observed in segregating DH populations in *B. napus,* even in other regions of the genome, and are thought to be due to *in vitro* androgenesis (Foisset *et al.*, 1996; Delourme *et al.*, 2013). To confirm that our approach revealed a genuine gene-for-gene interaction contributing to quantitative resistance, we used CRISPR-Cas9 method to introduce a range of mutations into *AvrLmSTEE98*. Heavily altered versions of the

protein did not induce a HR on cotyledons, suggesting a lack of recognition by RlmSTEE98. In addition, the mutant isolates induced larger areas of stem necrosis in 'Yudal', also suggesting that the RlmSTEE98-AvrLmSTEE98 interaction contributes to stem resistance to wild-type isolates.

Our results thus highlighted a role for a single genomic region in 'Yudal', and functional analyses strongly suggest that the interaction was controlled by a single resistance gene. The participation of single major genes in adult-stage resistance through gene-for-gene interactions has already been reported in other models, such as the *Brassica oleracea*/*Plasmodiophora brassicae* (Rocherieux *et al.*, 2004), apple tree/*Venturia inaequalis* (Calenge *et al.*, 2004) and barley/*Pyrenophora graminea* (Arru *et al.*, 2003) pathosystems, but has never before been reported for the *B. napus*-*L. maculans* interaction. Gene-for-gene interactions underlying partial resistance have also been identified in a few models, such as the wheat/*Zymoseptoria tritici* system (Meile et al, 2019), or the rice/*Magnaporthe oryzae* pathosystem, in which an avirulence gene (*AvrPi34*) corresponding to the single dominant resistance gene *Pi34* was shown to be responsible for the partial resistance phenotype (Zenbayashi-Sawata *et al.*, 2005).

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

There are four possible reasons for the identification of a resistance QTL originating from the susceptible genotype and not previously identified at this genomic location: (i) *RlmSTEE98* is differentially expressed at different stages of plant growth, being expressed in the cotyledons and leaves, for example, but not in stems, preventing the recognition of AvrLmSTEE98 during stem colonization, when this gene is expressed. Stage-specific expression patterns have been observed for other resistance genes, such as the wheat *Lr34* gene, which is weakly expressed at the cotyledon stage but strongly induced in adult plants, conferring quantitative resistance to several pathogens (Krattinger *et al.*, 2009). This hypothesis is also supported by the identification of differential expression profiles between cotyledons and stems for the genes present

in the *RlmSTEE98* region in 'Darmor-*bzh*' (data not shown). However, our results showing that *AvrLmSTEE98* inactivation increases the size of stem lesions in 'Yudal', but not in 'Darmor-*bzh*' in controlled conditions strongly suggests that recognition takes place in the stem, rendering this hypothesis highly unlikely. Genomic and transcriptomic data for different developmental stages are now required for 'Yudal', to resolve this issue. (ii) Alternatively, *AvrLmSTEE98* may be expressed too late or at a too low level during systemic colonization, leading to late or insufficient recognition by the plant, and an inability to prevent the development of stem canker at this stage. Indeed, transcriptomic analyses in 'Darmor-*bzh*' have shown that *AvrLmSTEE98* is not expressed in field conditions from November to March, and that its expression peaks in April, but at a lower level than for two of the other *LmSTEE* genes analysed here, *LmSTEE1* and *LmSTEE30* (Gay *et al.*, 2020; Figure S1). According to this hypothesis, the choice of *LmSTEE* genes for resistance screening in the approach developed here, should be based on a precise characterization of their expression profiles under field conditions, with particular attention paid to effectors expressed at the earliest stages of stem colonization. (iii) Another possible explanation for the absence of resistance QTL associated with this region is that the RlmSTEE98 region is effectively associated with a resistance QTL conferring partial resistance to the fungus but not identified as such under field conditions. Several studies have shown that the detection of resistance QTL can be strongly influenced by environmental conditions, particularly for the *B. napus*-*L. maculans* system under European cropping conditions (Kaur *et al.*, 2009; Raman *et al.*, 2012). The effect of the *RlmSTEE98* gene may be subject to such influences, preventing its detection. (iv) Finally, the partial resistance conferred by *RlmSTEE98* may have remained undetected due to a combination of insufficient sensitivity and accuracy of the disease severity scoring in the field and a relatively low contribution to quantitative resistance relative to other QTL in this cross.

This study provides a new approach and tools for enriching the *Rlm* gene pool for a system in which the scarcity of *Rlm* genes has proved a critical limitation to crop sustainability. Our study also highlights the importance of the diversity of the plant material screened. Our screening was of limited diversity, with only European winter rapeseed varieties plus 'Yudal'. More distantly related genotypes might also display new specific resistances to LmSTEE effectors. Also, choice of *LmSTEE* genes expressed early during stem infection could allow to identify new RlmSTEE resistance in winter-type European cultivars known to display a high level of adult-stage resistance. Many other late effector candidates have been described, with 40 effector candidates belonging to the stem biotrophy wave of expression (Gay *et al.*, 2020). These genes will be targets of choice for this strategy, now that proof-of-principle has been obtained for its validity.

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

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

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DATA AVAILABILITY : Data are available in the main document or supplementary files. Other data (DarmorxYudal map, RNASeq, accession no. for LmSTEE effectors, etc.) are available in the literature.

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Supporting information

Additional supporting information may be found ine the online version of this article.

Fig S1 Expression profiles of *LmSTEE* genes during rapeseed colonization

Fig. S2 Mean disease ratings of three rapeseed cultivars following inoculation with *L. maculans* transformants expressing “late” effector genes in cotyledons

Fig. S3 Expression of the mutated *LmSTEE98* gene in “over-expressed in cotyledons” (OEC) transformants during early stages of cotyledon colonization

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

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

Table S1 Isolates used for polymorphism studies in *Leptosphaeria maculans* populations

Table S2 List of *Brassica napus* genotypes screened for resistance

Table S3 List of primers used in this study

Table S4 The top 20 BLAST results for each "late" effector

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

Table S6 Cosegregation of resistance to LmSTEE98 and two molecular markers in the 'Darmor-*bzh*' x 'Yudal' doubled haploid population

Figure 1: Expression of *LmSTEE* genes in "over-expressed in cotyledons" (OEC) transformants of *Leptosphaeria maculans* during early stages of cotyledon colonization

We constructed OEC transformants specifically overexpressing the *LmSTEE1*, *LmSTEE35*, *LmSTEE78* and *LmSTEE98* genes under the control of the promoter of the *AvrLm4-7* gene during the early colonization of cotyledons. The expression of *LmSTEE* genes in the various OEC transformants was assessed by RT-qPCR in infected cotyledons of the cultivar 'Eurol', 7 days post-inoculation. Gray bars correspond to expression data from three to four different transformants; black bars represent expression in the wild-type isolate (INV13.269) and white bars indicate the expression in control transformants with the endogenous promoter (LmSTEE1 only). Red arrows indicate the OEC transformants selected for the screening of plant genotypes. Mean expression is normalized against actin, with tubulin used as a control (Fudal *et al*., 2007). Error bars represent the standard error for two biological and three technical replicates.

Figure 2: Resistance response of the rapeseed cultivars 'Yudal' and 'Dariot' to two "late" effector genes

Leptosphaeria maculans transformants expressing *LmSTEE35, LmSTEE1, LmSTEE98 or LmSTEE78* under the control of the promoter of the avirulence gene *AvrLm4-7* were used to inoculate the 'Yudal', 'Dariot' and susceptible 'Eurol' cultivars. Two independent transformants were used per construct. The bars represent the percentage of plants displaying susceptible (in red) or resistant (in green) phenotypes, based on the IMASCORE rating scale (Balesdent *et al.*, 2001), 14 days after inoculation. INV13.269 is the wild-type isolate and the JN2 and JN3 isolates were used as additional controls. The resistance of 'Dariot' to JN3 and JN2 is linked to the presence of *Rlm7,* which recognizes *AvrLm7* in these isolates.

Figure 3: Generation of CRISPR-Cas9-mutated versions of LmSTEE98 and effect of the mutations on their recognition by RlmSTEE98 in cultivar 'Yudal'

Four CRISPR-Cas9-mutated versions of *LmSTE98* were generated and their deduced protein sequences were aligned with that of the WT protein in (A). The mutated alleles were placed under the control of the promoter of the avirulence gene *AvrLm4-7,* and transformants of Leptosphaeria maculans were used to inoculate the susceptible cultivar 'Darmor' (B) and the resistant cultivar 'Yudal', containing the *RlmSTEE98* gene (C). Three independent transformants were used per construct. Scoring was based on the IMASCORE rating scale, in which scores of 1-3 indicate resistance and scores of 4-6 indicate susceptibility (Balesdent

et al., 2001), 14 days after inoculation. INV13.269 is the wild-type isolate. Error bars represent the standard error for at least ten biological replicates.

Figure 4: The inactivation of *LmSTEE98* by CRISPR-Cas9 method increases stem necrosis in 'Yudal' rapeseed stems

LmSTEE98 mutants of *Leptosphaeria maculans* (Fig. 3A) were used to inoculate the petiole of the second true leaf of *B. napus* 'Darmor' and 'Yudal', cut 1 cm from the point of insertion into the stem. Symptoms were evaluated on stem sections, 30 days after inoculation. (A) Percentage of the stem section displaying necrosis for cultivar 'Yudal' ($n \geq 18$). (B) Percentage of the stem section displaying necrosis for cultivar 'Darmor' ($n \geq 18$). The asterisks indicate a significant difference in the area under the curve between the control isolate JN2_Cas9 and the *LmSTEE98* mutants, for the two biological replicates of the experiment (GLM: **: p-value < 0.05 and ***: p-value < 0.01).

Figure 5: Genetic mapping of the resistance gene *RlmSTEE98* in the 'Darmor-*bzh*' x 'Yudal' cross

RlmSTEE98 was mapped to chromosome A09 of *Brassica napus*. The genetic distances, indicated in centiMorgans, are shown on the left, with marker order shown on the right.

Table 1: Characteristics of the "late" effector candidates of *Leptosphaeria maculans* studied here

Table 2: PCR amplification and HRM analysis of *LmSTEE* genes in *Leptosphaeria maculans* isolates

Table 1: Characteristics of the "late" effector candidates studied here

Name	Accession Nb	Size (aa)	Cysteines	Expression rank[a]	Expression wave[b]	Number of BLAST hits[c]	Closest BLAST hit	E-value (% Identities)	BLAST hit coverage	Pfam domain (E-value)	CAZymes hit (E-value)	Number of hits on *L. biglobosa 'brassicae'* proteins[d]
LmSTEE1	*Lmb_jn3_03177*	80	10 (12.5%)	2	Stem biotrophy (cluster 5)	24	*Aspergillus bombycis* hypothetical protein	3.00E-19 (56%)	99%	none	none	1
LmSTEE30	*Lmb_jn3_04778*	107	10 (9.3%)	12	Stem biotrophy (cluster 5)	230	*Epicoccum nigrum* hypothetical protein	8E-35 (68%)	98%	CFEM (2.7E-10)	none	4
LmSTEE35	*Lmb_jn3_08094*	123	7 (5.7%)	93	Biotrophy-to-necrotrophy transition (cluster 4)	26	*Colletotrichum salicis* hypothetical protein	3E-17 (39%)	94%	none	none	0
LmSTEE78	*Lmb_jn3_00617*	291	2 (0.7%)	114	Stem necrotrophy (cluster 6)	458	*Stagonospora sp. SRC1lsM3a* hypothetical protein	4.00E-156 (74%)	99%	none	GH131 (7.8E-64)	3

LmSTEE98	*Lmb_jn3_11364*	55	6 (10.9%)	101	Biotrophy-to-necrotrophy transition (cluster 4)	0	none	none	none	none	none	2

[a]Fold-change in rank between stems and cotyledons of *L. maculans* effectors from RNA-seq data (Gervais *et al.*, 2017).

[b]Names of expression waves and corresponding genes cluster, as defined by Gay *et al*., 2020

[c]Number of hits with an E-value < 1E-5

[d]Results from PSI-BLAST against all *L. biglobosa* proteins with E-values < 5E-2 (Table S5)

Table 2: PCR amplification and HRM analysis of *LmSTEE* genes in *Leptosphaeria maculans* isolates

Origin of isolates[a]	*LmSTEE1*			*LmSTEE30*			*LmSTEE35*			*LmSTEE78*			*LmSTEE98*		
	Presence	Absence [b]	Mutation [c] (Nb isolates)	Presence	Absence [b]	Mutation [c] (Nb isolates)	Presence	Absence [b]	Mutation [c] (Nb isolates)	Presence	Absence [b]	Mutation [c] (Nb isolates)	Presence	Absence [b]	Mutation [c] (Nb isolates)
France	93	0	nd	93	0	nd	91	2	nd	93	0	nd	91	2	nd
Oceania	20	0	0/18	20	0	1/20[d]	20	0	0/20	20	0	0/20	20	0	0/18
Canada	17	0	0/14	17	0	11/17 [d]	17	0	0/17	17	0	0/16	17	0	0/17
USA	10	0	0/9	10	0	1/10 [d]	10	0	0/9	10	0	0/10	10	0	0/10

Mexico	41	0	0/37	41	0	0/41	41	0	0/40	41	0	0/41	41	0	7/41 [e]
Chile	5	0	0/5	5	0	3/5 [d]	5	0	0/5	5	0	0/5	5	0	0/5
Total	186	0	0/83	186	0	16/88 [d]	184	2	0/91	186	0	0/88	184	2	7/90 [e]

[a] Isolates are described in Table S3.

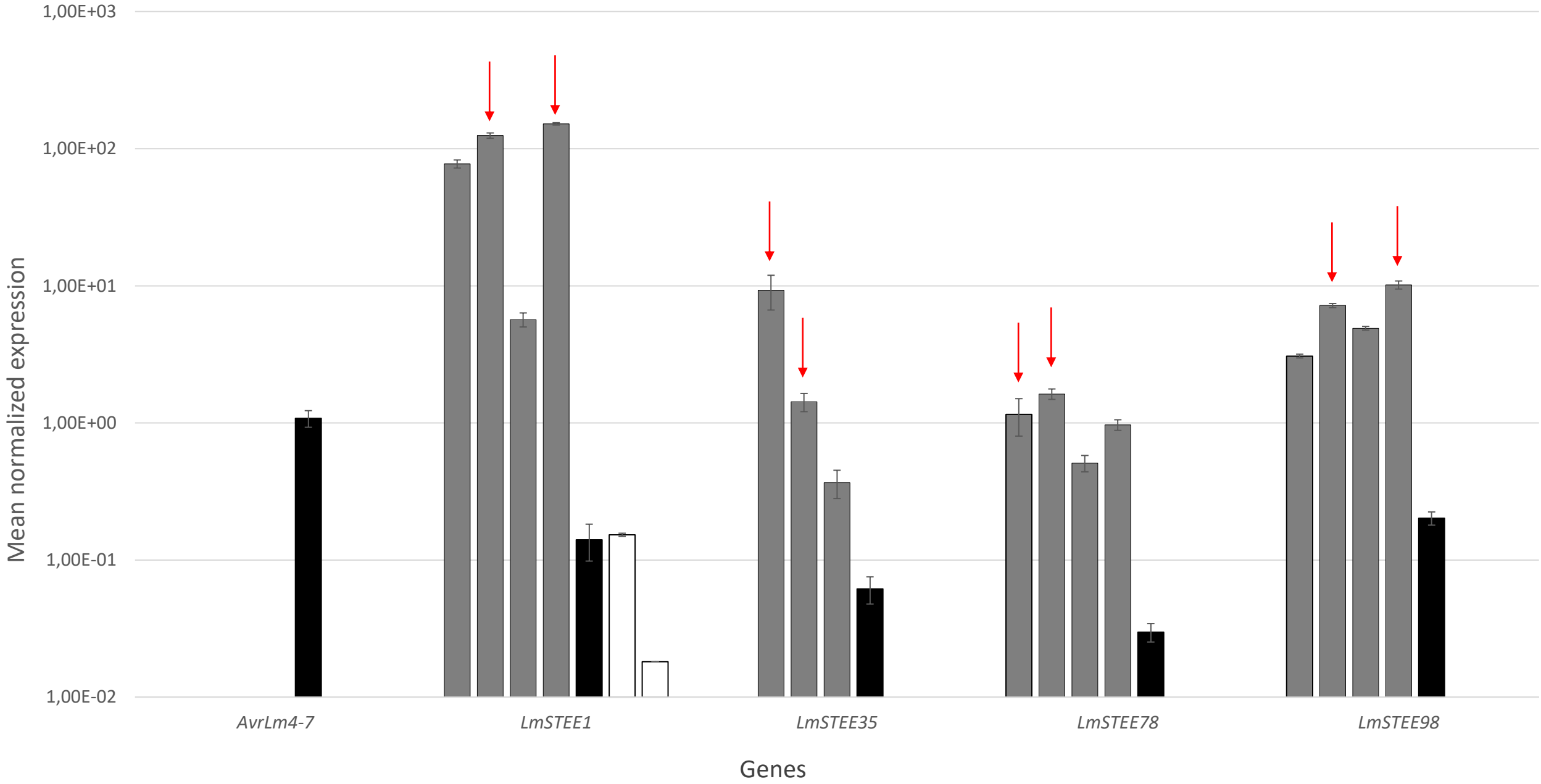
[b] Lack of PCR amplification.

[c] Number of isolates with SNP compared to the reference sequence JN3, based on HRM results coupled with sequencing.

[d] All mutations for *LmSTEE30* corresponded to one single silent mutation.

[e] Observed mutations for *LmSTEE98* corresponded to two SNPs modifying the protein sequence: A>I(17)and I>P(27).

nd: not done


1,00E+03
1,00E+02
1,00E+01
1,00E+00
1,00E-01
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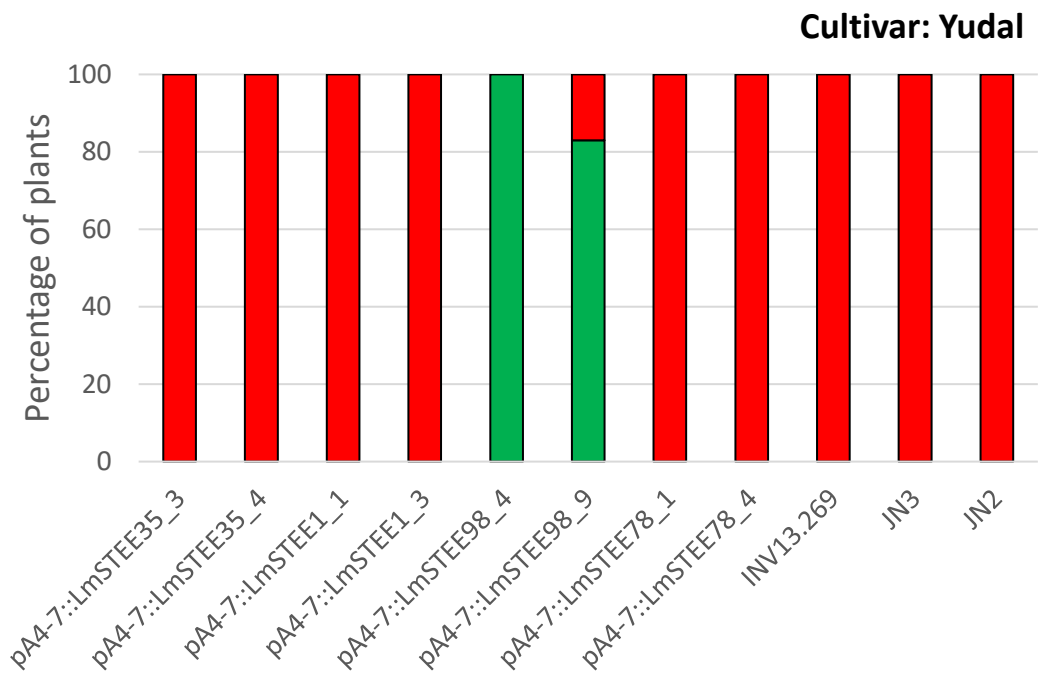
Mean normalized expression
AvrLm4-7
LmSTEE1
LmSTEE35
LmSTEE78
LmSTEE98
Genes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Percentage of plants
100
80
60
40
20
0
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pA4-7::LmSTEE35_4
pA4-7::LmSTEE1_1
pA4-7::LmSTEE1_3
pA4-7::LmSTEE98_4
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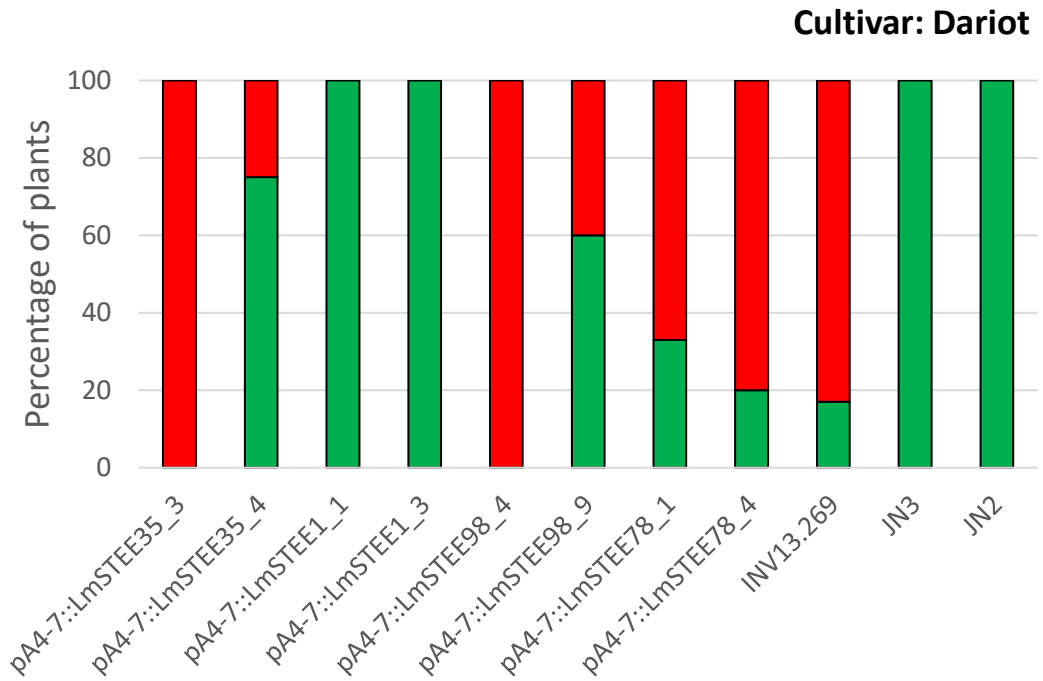
INV13.269
JN3
JN2


Cultivar: Dariot
Percentage of plants
100
80
60
40
20
0
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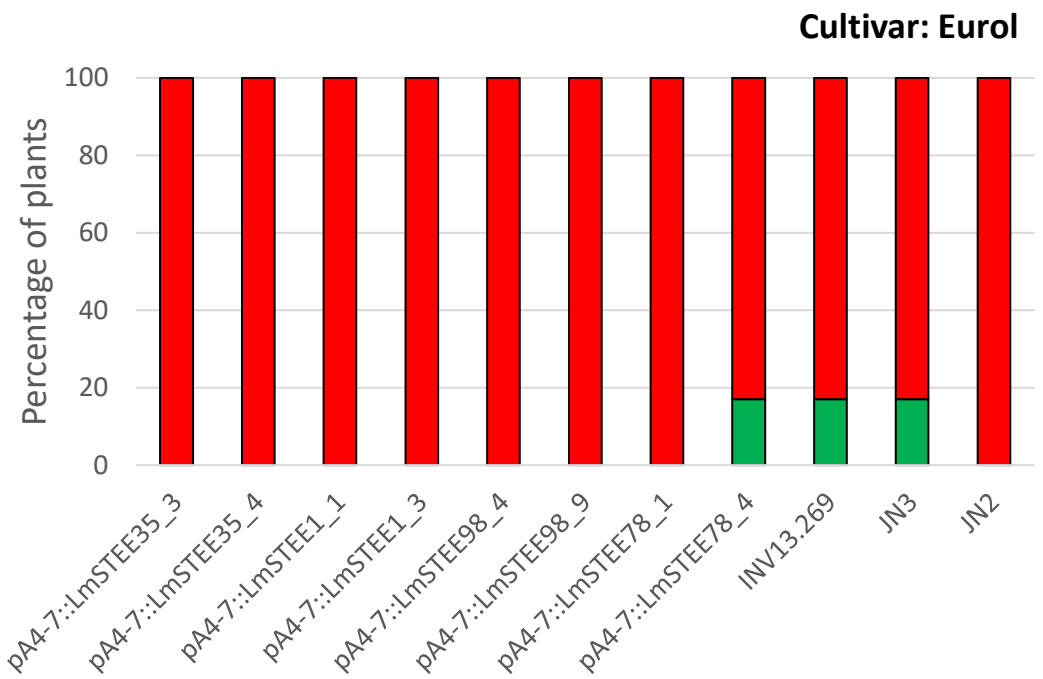
pA4-7::LmSTEE35_4
pA4-7::LmSTEE1_1
pA4-7::LmSTEE1_3
pA4-7::LmSTEE98_4
pA4-7::LmSTEE98_9
pA4-7::LmSTEE78_1
pA4-7::LmSTEE78_4
INV13.269
JN3
JN2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Percentage of plants
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80
60
40
20
0
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pA4-7::LmSTEE35_4
pA4-7::LmSTEE1_1
pA4-7::LmSTEE1_3
pA4-7::LmSTEE98_4
pA4-7::LmSTEE98_9
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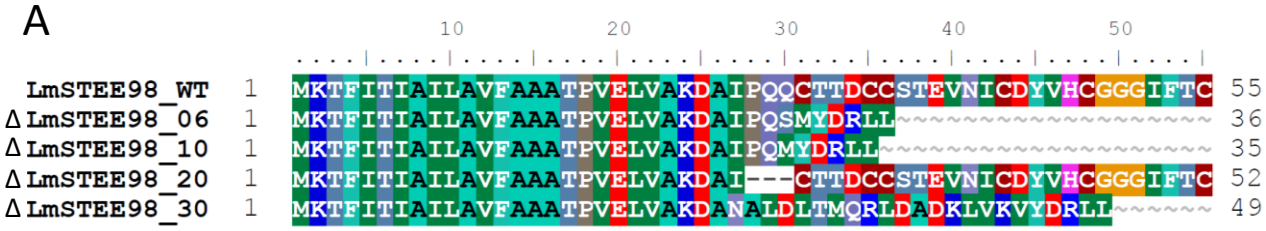
INV13.269
JN3
JN2

A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20
30
40
50
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ΔLmSTEE98_10 1 MKTFITIAILAVFAAATPVELVAKDAIPQMYDRLL~~~~~~~~~~~~~~~~~~~~ 35
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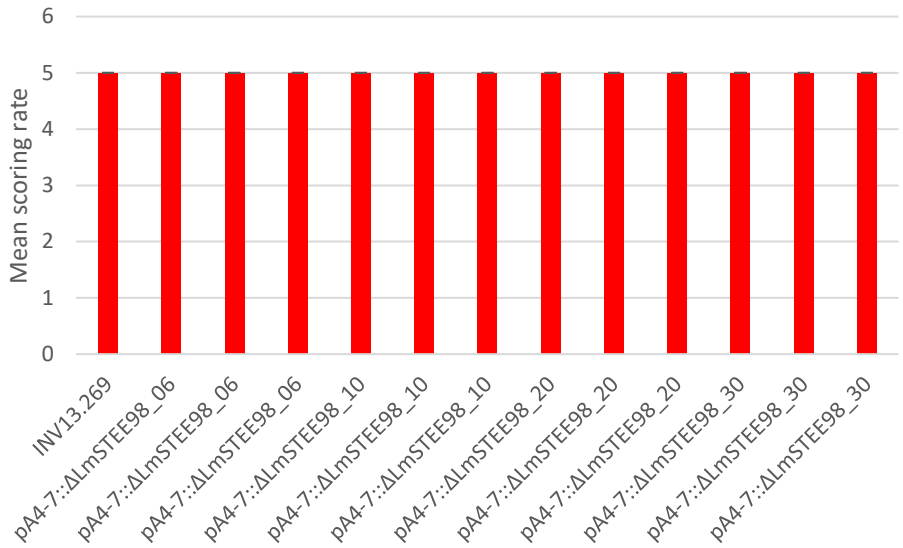
B


Mean scoring rate
0
1
2
3
4
5
6
INV13.269
pA4-7::ΔLmSTEE98_06
pA4-7::ΔLmSTEE98_06
pA4-7::ΔLmSTEE98_06
pA4-7::ΔLmSTEE98_10
pA4-7::ΔLmSTEE98_10
pA4-7::ΔLmSTEE98_10
pA4-7::ΔLmSTEE98_20
pA4-7::ΔLmSTEE98_20
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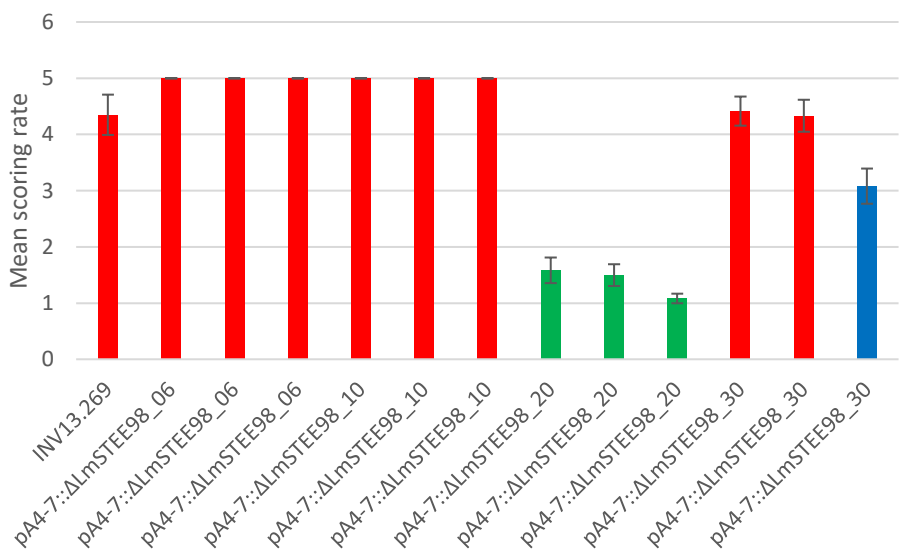
pA4-7::ΔLmSTEE98_30

C


Mean scoring rate
0
1
2
3
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5
6
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pA4-7::ΔLmSTEE98_06
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pA4-7::ΔLmSTEE98_10
pA4-7::ΔLmSTEE98_10
pA4-7::ΔLmSTEE98_20
pA4-7::ΔLmSTEE98_20
pA4-7::ΔLmSTEE98_20
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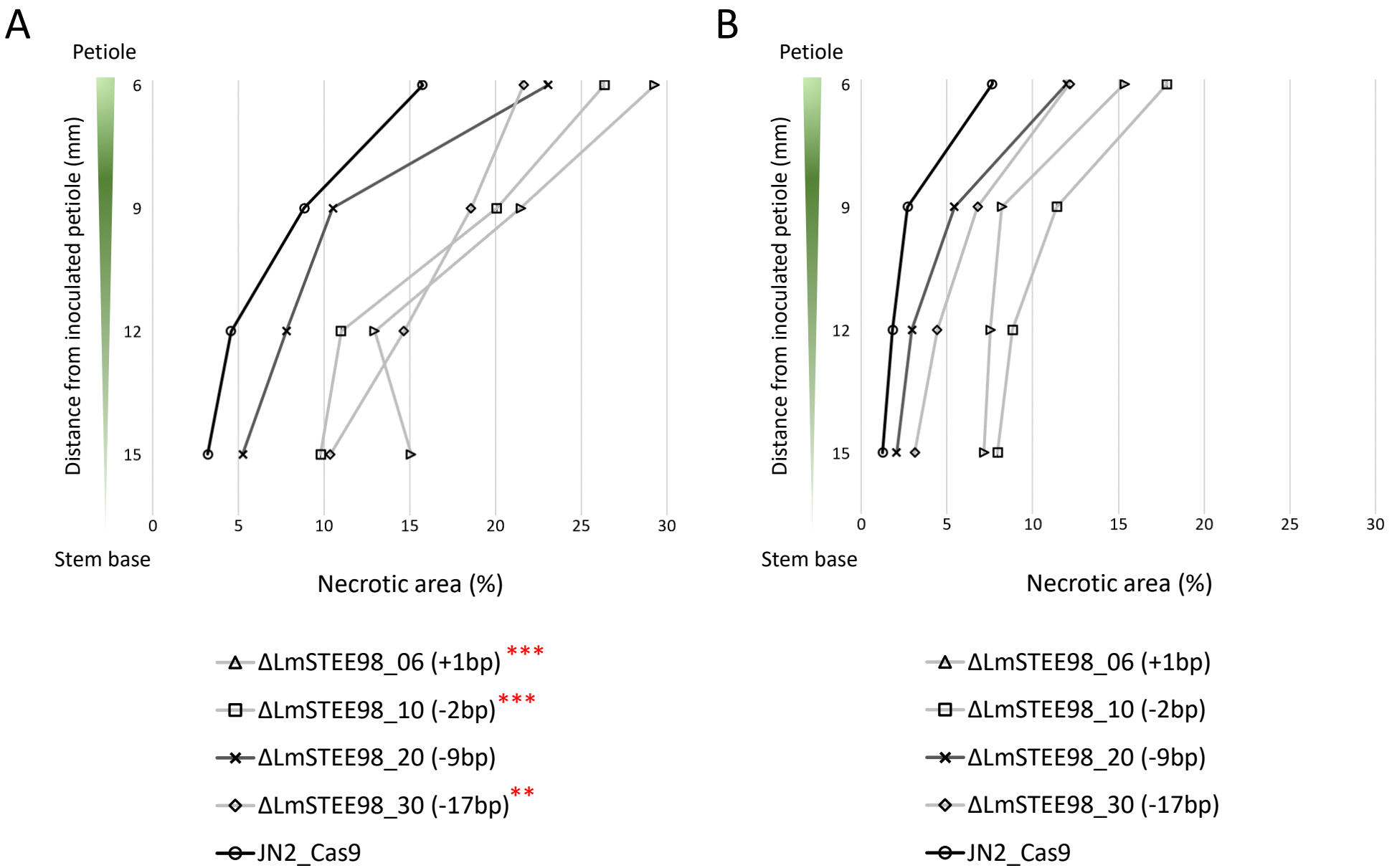
A
Petiole
Distance from inoculated petiole (mm)
6
9
12
15
0
5
10
15
20
25
30
Stem base
Necrotic area (%)
ΔLmSTEE98_06 (+1bp) ***
ΔLmSTEE98_10 (-2bp) ***
ΔLmSTEE98_20 (-9bp)
ΔLmSTEE98_30 (-17bp) **
JN2_Cas9
B
Petiole
Distance from inoculated petiole (mm)
6
9
12
15
0
5
10
15
20
25
30
Stem base
Necrotic area (%)
ΔLmSTEE98_06 (+1bp)
ΔLmSTEE98_10 (-2bp)
ΔLmSTEE98_20 (-9bp)
ΔLmSTEE98_30 (-17bp)
JN2_Cas9

A09 (Darmor-*bzh* x Yudal)