INTRODUCTION

Sclerotinia sclerotiorum is a plant-parasitic fungus that causes white mould disease. It is known for its aggressive necrotrophic lifestyle, which means that the fungus actively kills the plant host cells and thrives by feeding on the dead plant material, and for exhibiting a broad host range. S. sclerotiorum can infect more than 600 host plants including economically important species such as tomato.

Abstract

Sclerotinia sclerotiorum is a notorious generalist plant pathogen that threatens more than 600 host plants, including wild and cultivated species. The molecular bases underlying the broad compatibility of S. sclerotiorum with its hosts is not fully elucidated. In contrast to higher plants and animals, alternative splicing (AS) is not well studied in plant-pathogenic fungi. AS is a common regulated cellular process that increases cell protein and RNA diversity. In this study, we annotated spliceosome genes in the genome of S. sclerotiorum and characterized their expression in vitro and during the colonization of six host species. Several spliceosome genes were differentially expressed in planta, suggesting that AS was altered during infection. Using stringent parameters, we identified 1,487 S. sclerotiorum genes differentially expressed in planta and exhibiting alternative transcripts. The most common AS events during the colonization of all plants were retained introns and the alternative 3′ receiver site. We identified S. sclerotiorum genes expressed in planta for which (a) the relative accumulation of alternative transcripts varies according to the host being colonized and (b) alternative transcripts harbour distinct protein domains. This notably included 42 genes encoding predicted secreted proteins showing high-confidence AS events. This study indicates that AS events are taking place in the plant pathogenic fungus S. sclerotiorum during the colonization of host plants and could generate functional diversity in the repertoire of proteins secreted by S. sclerotiorum during infection.

KEYWORDS
alternative splicing, computational analysis, host adaptation, isoforms, RNA sequencing (RNA-seq), Sclerotinia sclerotiorum
studies of the systematic searches for candidates (Guyon et al., 2014). Large-scale sequence (Amselem et al., 2011; Derbyshire et al., 2017) enabled in addition, suggested to interfere with the transcription of host defence genes (Derbyshire et al., 2019). In addition, S. sclerotiorum employs small secreted proteins to alter host cell physiology. A number of these have been characterized through mutant studies (Mbengue et al., 2016; Xia et al., 2019) and the release of the S. sclerotiorum genome sequence (Amselem et al., 2011; Derbyshire et al., 2017) enabled systematic searches for candidates (Guyon et al., 2014). Large-scale studies of the S. sclerotiorum genome, proteome, and transcriptome also identified a large repertoire of secreted hydrolytic enzymes (Seilbarghi et al., 2017) produced during infection. Furthermore, S. sclerotiorum produces secondary metabolites such as sclerin and botcinic acid acting as toxins to facilitate infection (Graham-Taylor et al., 2020; Pedras and Ahiahonu, 2004). Finally, S. sclerotiorum is able to detoxify plant defence compounds, enabling the colonization of certain hosts (Pedras and Ahiahonu, 2005), but the underlying molecular mechanisms are known in rare cases only (Chen et al., 2020). Whether the requirement for these diverse virulence mechanisms varies from one host to another remains largely elusive.

The coevolution of pathogen-secreted proteins with their host targets (Dong et al., 2015) suggests that the ability to infect very diverse host species would associate with expanded repertoires of secreted proteins. However, the repertoire of secreted protein-coding genes in S. sclerotiorum is within the average for ascomycete fungal pathogens (Derbyshire et al., 2017). To support infection of very diverse hosts, S. sclerotiorum exhibits codon usage optimization for secreted proteins, increasing the efficiency of protein translation with the potential to confer fitness benefits on multiple hosts (Badet et al., 2017). In addition, S. sclerotiorum hyphae organize in cooperating units, sharing the metabolic cost of virulence and growth during the colonization of resistant plants (Peyraud et al., 2019). Posttranscriptional regulation has been proposed as a mechanism to diversify effector proteins produced by a single gene (Betz et al., 2016), but support for this hypothesis remains scarce. Here, we investigated the extent to which posttranscriptional regulation could generate diversity in virulence factor candidates produced by S. sclerotiorum during the colonization of plants from diverse botanical families.

Alternative splicing (AS) is a process in eukaryotic cells that increases the cellular capacity to shape their transcriptome diversity and proteome complexity. Splicing is an important mechanism that regulates the maturation of the precursor messenger RNAs (pre-mRNA) by subjecting it to the removal of noncoding sequences (introns). AS occurs in many eukaryotes under certain conditions, resulting in multiple isoforms of transcripts that retain specific intronic sequences or lack specific exonic sequences. The transcripts with retained introns (RI) then have a prolonged lifetime compared to the completely mature mRNA transcript (Braunschweig et al., 2014; Naro et al., 2017; Schmitz et al., 2017).

The efficiency and accuracy of the splicing mechanisms play a critical role in gene transcription and subsequent protein function. Imprecise splicing may result in abnormal and nonfunctional transcripts that may lead to the production of defective proteins, thus disturbing cellular processes. Previous studies showed that inaccurate splicing may cause diseases in humans, for example Parkinson’s disease and leukaemia (David and Manley, 2010; Fu et al., 2013), and increases plant sensitivity to abiotic or biotic stresses (Cui et al., 2014). In line with this, the importance of AS in plant immunity against pathogen attacks is well established (Rigo et al., 2019). AS regulation and the factors that control it, the prediction of their cis-regulatory sequences, and trans-acting elements have been intensively studied in plants and in animals (Blanco and Bernabeu, 2011; Eckardt, 2013; Zhang et al., 2017), while only few reports are available from fungal phytopathogens. Therefore, the extent to which AS is regulated and functional during host colonization in fungal phytopathogens remains elusive.

Recently, Jin et al. (2017) found that transcripts of the plant fungal pathogen Verticillium dahliae undergo splicing of retained introns, producing different isoforms of transcripts. These isoforms have predicted roles in controlling many conserved biological functions, such as ATP synthesis and signal transduction. The involvement and regulation of the retained intron isoforms and splicing during the infection of host plants are still unexplored. Moreover, AS is detected during V. dahliae microsclerotia development (Xiong et al., 2014). Interestingly, 90% of the detected alternative transcripts exhibit retained introns. However, there is no further evidence to support the contribution of AS in microsclerotia development. In the same fashion, alternative transcripts are annotated in the genomes of the plant-pathogenic fungi Colletotrichum graminicola and Fusarium graminearum (Schliebner et al., 2014; Zhao et al., 2013).

AS is pivotal in regulating gene expression and in diversification of the protein repertoire in the plant-pathogenic oomycete Pseudoperonospora cubensis during pathogen development and transition from sporangia to zoospores (Burkhardt et al., 2015). In this study 4,205 out of 17,558 genes with c.10,000 potential AS events were identified, of which c.83% had evidence of retained introns. Interestingly, no exon skipping events were detected. Intriguingly, two genes encoding putative secreted RXLR and QXLR effectors showed evidence for a retained intron specifically at the sporangium stage, while the spliced version was abundant during the host-associated stage. The retained intron may, therefore, regulate gene expression instead of affecting the function of the protein. Similarly, AS of the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3-phosphoglycerate kinase (PGK) modulates their localization in the smut fungus Ustilago maydis. In particular, AS gives rise to GAPDH carrying a peroxisome-targeting signal. Importantly, U. maydis mutants lacking the specific isoforms with peroxisomal localization have reduced virulence (Freitag et al., 2012). These examples highlight the crucial role of AS in the pathogenicity of plant-pathogenic fungi.
A predicted splicing factor 8 corresponding to the U5-associated component Prp8 (GenBank accession number SS1G_03208) was reported recently from S. sclerotiorum (McLoughlin et al., 2018). This prompted us to test for AS in S. sclerotiorum during the infection of diverse host plants. To this end, we exploited RNA-seq data of S. sclerotiorum infecting host plants from six botanical families, that is, Arabidopsis thaliana (Brassicales), tomato (Solanales), sunflower (Asterales), beetroot (Caryophyllales), castor bean (Ricinus communis, Malphigiales), and common bean (Fabales), in addition to the RNA-seq of S. sclerotiorum cultivated in vitro as control (Peyraud et al., 2019; Sucher et al., 2020). We found that S. sclerotiorum has a functional splicing machinery and that at least 4% of the S. sclerotiorum secretome undergoes AS regulation, resulting in multiple differentially expressed isoforms that may have modified or altered functions. Some of the novel transcripts exhibit different predicted function or localization. Based on our analysis, we suggest that AS has the potential to give rise to transcriptional flexibility, thus contributing to the broad host spectrum of the plant-pathogenic fungus S. sclerotiorum.

2 | RESULTS

2.1 | S. sclerotiorum spliceosome is differentially regulated during host colonization

To study AS in the fungal plant pathogen S. sclerotiorum, we first searched the predicted proteome of S. sclerotiorum for components associated with splicing (spliceosome) using BLASTp and UniProtKB. We identified all the main components encompassing the entire pre-mRNA splicing cycle, that is, U1/U2/U4/U5/U6-associated components, PRP19/NTC complex proteins, the proteins catalysing the splicing of the intron (exon junction complex; EJC), the mRNA export complex TRES, and the mRNA and intron release components PRP43 and PRP22 (Figure 1).

We documented the transcriptional regulation of S. sclerotiorum spliceosome components during plant infection by exploiting RNA-seq reads of S. sclerotiorum 1980 cultivated in vitro on potato dextrose agar (PDA) (Peyraud et al., 2019) and during the infection of host plants from six botanical families (Sucher et al., 2020): A. thaliana (At), tomato (Solanum lycopersicum, Sl), sunflower (Helianthus annuus, Ha), common bean (Phaseolus vulgaris, Pv), castor bean (Ricinus communis, Rc), and beetroot (Beta vulgaris, Bv) (Figure 1). We found 116 proteins probably associated with (alternative) splicing, all but one of which were expressed at >10 fragments per kilobase of transcript per million mapped reads (FPKM) across all conditions (Figure 1 and Table S1). Sscl02g018420, encoding a U2AF, was not expressed at detectable levels (FPKM < 1). By performing BLASTp searches we identified 81 of these 116 spliceosome-associated genes to be conserved in related ascomycetes, such as Botrytis species (Table S2). Interestingly, many components exhibited the strongest expression in vitro, but appeared to be down-regulated on some or all of the hosts. Eighty of the 116 genes were significantly down-regulated (p < .01) on at least one host plant, and one gene (Sscl03g031850, encoding a U2AF) was up-regulated on all hosts except sunflower (Table S3). For example, 63 components were down-regulated on B. vulgaris, while the U2AF-encoding gene Sscl03g031850 displayed 4.7-fold up-regulation during infection of B. vulgaris (Figure 1). Overall, 81 of the 116 components appeared to be differentially modulated dependent on the host plant, suggesting host plant-specific regulation of the spliceosome in S. sclerotiorum.

2.2 | Alternatively spliced genes are differentially expressed during host infection

To search for AS events in the S. sclerotiorum transcriptome in planta and to reduce false discovery rate due to pipeline-dependent bias, we applied a stringent strategy based on two pipelines employing either transcriptome alignment or de novo transcriptome assembly (Figure 2a). Transcriptome alignment is a robust and effective method of characterizing transcripts that are mapped to a provided reference transcriptome (including isoforms with skipped exons) while de novo transcriptome assembly mainly focuses on recovering transcripts with segments of the genome that are missing from the transcriptome alignment method, including retained introns (Martin and Wang, 2011).

In the transcriptome alignment pipeline (Figure 2a), the trimmed reads (steps I and II) were aligned to the S. sclerotiorum 1980 reference genome (Derbyshire et al., 2017) (step III). Expression of transcripts (transcripts per million, TPM) was determined in Salmon with the QUASI mapping algorithm (Patro et al., 2017) and Kallisto (Bray et al., 2016). Next, we used SUPPA2 to identify differentially expressed (DE) transcripts (step IV), with a cut-off TPM > 30 and p < .05 (Trincado et al., 2018). We found 6,399 DE transcripts in total with this approach among all samples (lesion edge on six plant species) and compared to the control (edge S. sclerotiorum cultivated in vitro on PDA). Then, SUPPA2 was applied to DE genes to identify the different AS events and to measure the percentage spliced in index (PSI; ψ), which represents the ratio between reads excluding or including exons (step V). These PSI values indicate the inclusion of sequences into transcripts (Alamancos et al., 2015; Wang et al., 2008) using the normalized transcript abundance values (TPM) of the isoforms from Salmon. The differential splicing analysis of the events (dpsi values) at p < .05 identified 1,369 DE genes with significant splicing events producing 3,409 DE transcripts (Figure 2a,b).

In the de novo assembly pipeline, transcripts were assembled from fungal reads using StringTie. To identify fungal reads in our samples, the trimmed reads (step I and II) were aligned to the S. sclerotiorum 1980 reference genome (Derbyshire et al., 2017) using HISAT2 (Kim et al., 2015), yielding between 10,258,270 and 26,314,353 mapped reads per sample (Table S4) (step III). S. sclerotiorum reads were then used for de novo transcriptome assembly in a modified Tuxedo differential expression analysis pipeline (Trapnell et al., 2010, 2012). Because StringTie was proven to be a more accurate and improved transcript assembler and quantifier (Pertea et al., 2015, 2016), we used StringTie instead of cufflinks for the de novo assembly step (step III’ and III’').
This resulted in 3,393 transcripts, including 2,633 transcripts from genes present in the reference transcriptome of *S. sclerotiorum* isolate 1980 (Derbyshire et al., 2017), 410 gene fusions and 337 novel genes encoding 350 transcripts. We compared FPKM expression values of original and novel transcripts and found similar distributions for the two sets. We therefore expect the rate of spurious transcripts to be
FIGURE 2  Pipeline for genome-wide detection of alternative splicing (AS) in *Sclerotinia sclerotiorum*. (a) Raw RNA-seq data was first inspected with FastQC (I) and quality-trimmed using Trimmomatic (II). We then applied two pipelines for detection and analysis of novel transcripts, the de novo assembly pipeline (yellow box) and the transcriptome alignment pipeline (blue box). For detection of novel transcripts, we mapped reads with HISAT (III) to the *S. sclerotiorum* reference genome; this data was used in a modified StringTie de novo assembly (III’). Using Cuffmerge, gffcompare, and Transdecoder, we identified novel transcripts compared with the reference gene annotation (III’’) and generated a new reference annotation. Using the new annotation and the reference genome, we performed differential expression analysis (IV) and filtered the differentially expressed (DE) genes for those encoding at least two DE transcripts (DET; V). In the transcriptome alignment pipeline, mapping was done with Kallisto or Salmon (III), DE analysis and AS detection with SUPPA2 (IV and V). (b) A Venn diagram summarizing the results from DE analysis in (a) for both pipelines; numbers are given only for genes encoding multiple transcripts. AS, alternative splicing; DE, differentially expressed; DET, differentially expressed transcript; incl., including; ref., reference; TPM, transcripts per million.
limited and similar in the two sets of transcripts (Figure S1 and FPKM values in Table S5). Differential expression analysis on the complete transcriptome including both reference and novel transcripts with cuffdiff (step IV) identified 4,111 DE genes accounting for 4,735 DE transcripts on any of the six host species compared to the control. Out of those, there were 368 genes that encoded several DE transcripts each, producing 772 transcripts in total. These represent candidate genes harbouring AS in planta (step V).

Finally, we compared transcripts identified with the two pipelines and found a total number of 3,671 transcripts differentially expressed in planta in total, originating from 1,487 genes ("full set" of candidates). Among those, the two pipelines identified a common set of 250 genes of S. sclerotiorum encoding more than one transcript and expressed differentially in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2). This common set of genes produced 510 transcripts differentially expressed in planta ("common set" of candidates, Figures 2b and S2).

To document the extent to which host plant species associated with AS events in S. sclerotiorum, we performed hierarchical clustering and principal component analysis (PCA) for S. sclerotiorum alternatively spliced transcript accumulation in six host species (Figure 4a). The distribution of the plant variable according to the two principal components displayed host-specific clustering in which the AS transcripts produced on each host could be clearly separated, except for AS transcripts produced on A. thaliana and S. lycopersicum. This analysis suggested that the relative accumulation of alternative transcripts produced by a given gene could vary according to the host being colonized.

We tested whether this was the case for the gene Sscle03g026100, encoding a predicted phosphoenolpyruvate kinase-like protein. The Sscle03g026100 locus harboured RNA-seq reads that aligned in the 3′ region of intron 1, indicative of alternative 3′ receiver sites in exon 2 of the reference transcripts (TCONS_00008522, Figure 4b). This splicing event is predicted to cause an extension of the alternatively spliced exon in transcript variant TCONS_00008521. Thanks to its N-terminal extension, the protein isoform TCONS_00008522 but not TCONS_00008521 is recognized as a member of the PIRSF034452 family (TIM-barrel signal transduction protein). According to Cuffdiff transcript quantification, the ratio between alternative and reference transcript varied from 0.32 in A. thaliana to 0.73 in B. vulgaris (Figure 4c). To confirm AS of the Sscle03g026100 transcript, we performed RT-PCR with primers spanning the variant exon 2 on RNAs collected from five host species (Figure 4d). We retrieved a 418 bp amplicon corresponding to the reference transcript (TCONS_00008522), a 535 bp amplicon corresponding to the TCONS_00008521 alternative transcript, as well as a third c.750 bp amplicon. In agreement with the RNA-seq read coverage, bands corresponding to the alternative transcript TCONS_00008521 were much weaker than bands corresponding to the reference transcript TCONS_00008522 in A. thaliana, H. annuus, and in vitro.

2.4 AS is host-regulated in S. sclerotiorum

To document the extent to which host plant species associated with AS events in S. sclerotiorum, we performed hierarchical clustering and principal component analysis (PCA) for S. sclerotiorum alternatively spliced transcript accumulation in six host species (Figure 4a). The distribution of the plant variable according to the two principal components displayed host-specific clustering in which the AS transcripts produced on each host could be clearly separated, except for AS transcripts produced on A. thaliana and S. lycopersicum. This analysis suggested that the relative accumulation of alternative transcripts produced by a given gene could vary according to the host being colonized.

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2.5 AS is predicted to generate protein isoforms with modified functions

To study the functional consequences of AS in S. sclerotiorum, we first analysed gene ontology (GO) terms enriched in our list of high-confidence DE genes with AS. GO enrichment was determined using BiNGO, a tool package within the complex network visualizing platform Cytoscape (Shannon et al., 2003; Maere et al., 2005) (Table S6).
The most significantly enriched terms included "oxidoreductase activity" and "carbohydrate metabolic process", suggesting that genes involved in the degradation of carbohydrates and organic molecules were subject to AS during infection of host plants. According to BLASTP searches ($E < 10^{-25}$) 175 of the 250 genes in our common set of AS candidates are conserved in related ascomycetes, including Botrytis species (Table S7).

Second, to test if AS could alter the domain content of protein isoforms in our full set of AS candidates, we assigned PFAM domains to all isoforms and identified AS events leading to a change...
FIGURE 4  Alternative 3′ receiver splice site variation according to host in Sscl03g026100. (a) Principal component analysis map of the sample variable for the accumulation of reference and alternative transcripts produced by 250 high-confidence S. sclerotiorum genes showing alternative splicing (AS). Sample types are colour-coded according to infected host plant species. (b) Example of alternative 3′ receiver splice site in the reference transcript of Sscl03g026100. In the transcript diagrams, exons are shown as boxes, introns as lines. Read mappings are shown in grey for one RNA-seq sample of each treatment. (c) Ratio between the abundance of alternative over reference transcript for Sscl03g026100 determined by Cuffdiff. Error bars show 90% confidence interval. (d) Reverse transcription (RT)-PCR analysis of Sscl03g026100 transcripts produced during the colonization of five plant species and in vitro. The position of oligonucleotide primers used for RT-PCR is shown as arrows in (b). At, Arabidopsis thaliana; Bv, Beta vulgaris; Ha, Helianthus annuus; Pv, Phaseolus vulgaris; Rc, Ricinus communis; Sl, Solanum lycopersicum
in PFAM domain content. In total, 158 genes expressed alternative transcripts with changes in PFAM annotation profiles. Of these, 53 isoforms exhibited loss of PFAM domains, 85 isoforms displayed gain of PFAM domains, and 20 isoforms showed more complex changes of PFAM profiles (File S1). Only eight of the 158 genes with alterations in their PFAM annotation profiles were from the 250 common AS genes (Table 1). Four isoforms gained PFAM domains, for example the putative cutinase Sscle11g080920 where the alternative isoform TCONS_00002255 gained two PFAM domains, ETS_PEA3_N (PF04621.12) and CBM_1 (PF00734.17).

Furthermore, we explored the isoforms from alternatively spliced genes for signal peptides for secretion (Figure 5a). Of the 250 genes in our common set of AS candidates, 42 are predicted to encode a secreted protein, corresponding to 4% of the S. sclerotiorum secretome (Juan et al., 2019; Figure S3). Among those, five genes (Sscle02g014060, Sscle09g075820, Sscle09g070580, Sscle12g091110, and Sscle15g103140) showed possible gains of secretion peptide by AS and two cases of loss of secretion peptides in alternative isoforms (Sscle10g075480 and Sscle15g102380). In the set of 3,393 AS candidates detected in total, we found 26 possible gains of secretion peptides and 16 losses of secretion peptides in alternative isoforms.

Similarly, AS caused the gain and loss of transmembrane domains in novel isoforms (Figure 5b). In total, 20 novel isoforms exhibited gain of one and 25 isoforms gained more than one predicted transmembrane domains. Thirty-two of these did not harbour a putative transmembrane domain in the reference isoform, which suggests relocation to the plasma membrane or an intracellular membrane. Conversely, we observed the loss of one transmembrane domain in 30 isoforms and of more than one in 24 isoforms, including 32 isoforms that lost all transmembrane domains, suggesting subcellular relocalization of the respective novel isoform. Two genes that gained (Sscle05g040780 and Sscle16g109930) and five genes that lost (Sscle02g014060, Sscle02g019060, Sscle03g031900, Sscle05g043820, and Sscle08g066940) transmembrane domains are found in the 250 alternatively spliced and differentially expressed genes. Intriguingly, the novel isoform of Sscle02g014060 is predicted to be secreted as well as to have lost its transmembrane domain.

### 2.6 AS is predicted to modify the activity of S. sclerotiorum secreted proteins

Of the 250 genes with evidence for AS, 42 are predicted to encode a secreted protein, corresponding to 4% of the S. sclerotiorum secretome (Juan et al., 2019; Figure S3). For example, the alternatively spliced gene Sscle11g080920 was predicted to encode two secreted protein isoforms derived from the reference transcript TCONS_00002255.

### Table 1 Changes in PFAM profiles of differentially expressed and alternatively spliced genes of Sclerotinia sclerotiorum

| Gene         | Isoform      | PFAM accessions          | PFAM descriptors                          |
|--------------|--------------|--------------------------|-------------------------------------------|
| Sscle02g012330 | TCONS_00006935 | PF05277                  | DUF726                                    |
|              | TCONS_00006936 | PF04900; PF05277         | Fcf1; DUF726                              |
|              | TCONS_00006937 | PF04900; PF09388         | Fcf1; SpoOE-like                          |
| Sscle03g026280 | TCONS_00008535 | PF00172; PF05393; PF07690; PF08006; PF14960 | Zn_clus; Hum_adeno_E3A; MFS_1; DUF1700; ATP_synth_reg |
|              | TCONS_00008536 | PF00172; PF05393; PF07690; PF08006; PF14960; PF04082 | Zn_clus; Hum_adeno_E3A; MFS_1; DUF1700; ATP_synth_reg; Fungal_trans |
| Sscle03g030170 | TCONS_00008807 | PF06999                  | Suc_Fer-like                              |
| Sscle03g031900 | TCONS_00008259 | PF01601                  | Corona_S2                                 |
| Sscle03g031900 | TCONS_00008260 | PF01601                  | Corona_S2                                 |
| Sscle05g043820 | TCONS_0010732 | PF06172                  | Cupin_5                                   |
| Sscle05g043820 | TCONS_0010733 | PF06172                  | Cupin_5                                   |
| Sscle11g080920 | TCONS_00002254 | PF01083; PF08237         | Cutinase; PE-PPE                          |
| Sscle15g103140 | TCONS_00005366 | PF00169; PF01442; PF11932 | PH; Apolipoprotein; DUF3450               |
|              | TCONS_00005367 | PF00169; PF01442; PF11932; PF05592; PF17389; PF17390 | PH; Apolipoprotein; DUF3450; Bac_rhamnosid; Bac_rhamnosidC |
| Sscle16g109930 | TCONS_00006105 | PF00032; PF00083; PF03137; PF07690; PF12670 | Cytochrom_B_C; Sugar_tr; OATP; MFS_1; DUF3792 |
|              | TCONS_00006106 | PF00032; PF00083; PF03137; PF07690; PF12670; PF05977 | Cytochrom_B_C; Sugar_tr; OATP; MFS_1; DUF3793; MFS_3 |
alternatively spliced genes of *S. sclerotiorum*, where a score of 0.45 is the threshold for a putative secretion peptide. Orange data points indicate novel isoforms that may have gained a secretion peptide, blue data points indicate loss of the secretion peptide. (b) We predicted the number of transmembrane domains for reference (isoform 1) and novel isoforms (isoform 2) using TMHMM. Orange data points indicate isoforms that gained transmembrane domains, blue data points indicate novel isoforms that may have lost one or more transmembrane domains.

To gain insights into the functional consequence of AS in *S. sclerotiorum*, we analysed PFAM domains and performed structure modelling for the proteins encoded by the reference transcript TCONS_00002255 and the alternative transcript TCONS_00002254. The alternative transcript TCONS_00002254 encoded a 235 amino acid protein featuring a secretion signal and a cutinase (PF01083) domain (Figure 6d). Homology modelling and fold recognition in I-TASSER identified the acetylxylan esterase AXEII from *Penicillium purpureogenum* (PDB identifier 1B59) as the closest structural analog to TCONS_00002254 (RMSD 0.31Å). AXEII is a close structural analog of *Fusarium solani* cutinase, an esterase that hydrolyses cutin in the plant’s cuticle (Ghosh et al., 2001). The reference transcript TCONS_00002255 encoded a 296 amino acid protein featuring a secretion signal, a cutinase domain, and a short fungal cellulose-binding domain (PF00734) (Figure 6e). Its closest structural analog identified by I-TASSER was model 1G66 of AXEII. The superimposition of TCONS_00002254 and TCONS_00002255 protein models revealed that the C-terminal extension in TCONS_00002255 corresponds to a surface-exposed unstructured loop reaching the neighbourhood of the catalytic site cleft (Figure 6e). This additional exposed loop could modify protein–protein interactions in TCONS_00002255 or modify access to its catalytic site. These results suggest that AS is a mechanism to generate functional diversity in the repertoire of proteins secreted by *S. sclerotiorum* during the colonization of host plants.

### DISCUSSION

There are several approaches to study AS from RNA-seq data (Thakur et al., 2019), such as analysing splice junctions (Hu et al., 2013) or exonic regions (Anders et al., 2012), which largely rely on mapping strategies only. The pipeline we used in this study combines two fundamentally different strategies (de novo assembly based and reference mapping-based) to detect true novel splicing events and reduce algorithm bias. This approach, however, does not completely exclude false-positive or false-negative AS events, and also does not
allow a distinction between an AS event and correction of an incorrect reference gene model. Manual inspection or curation of gene models, as, for example, performed in *F. graminearum* (Zhao et al., 2013), is required to distinguish between these possibilities. In the current study, we limited our analysis to one stage of infection per host species, selected to represent similar infection stages on each host (Sucher et al., 2020). A time-course experiment as, for example, performed for *Brassica napus* (Seifbarghi et al., 2017) would be likely
AS in the plant-pathogenic fungus *S. sclerotiorum*. In this study, we present a comparative genome-wide survey of mechanism affecting the activity of a majority of genes in plant and secretion of effector proteins. A number of genes are spliced alternatively on different hosts. We have inspected AS predictions and experimentally validated alternative transcripts for a small subset of the AS events predicted here, supporting the accuracy of our analysis pipeline. Nevertheless, further efforts will be needed to improve the gene annotations of *S. sclerotiorum*, confirm alternative transcripts across all stages of infection, and identify further alternatively spliced transcripts missed by our pipeline at the genome scale.

### 3.1 A number of *S. sclerotiorum* genes are spliced alternatively on different hosts

Colonization of a host plant by a pathogen requires global changes in the gene expression of the pathogen and secretion of effector proteins and enzymes (van der Does and Rep, 2017). AS is a regulatory mechanism affecting the activity of a majority of genes in plant and animal cells at the posttranscriptional level. Whether AS contributes to the regulation of virulence in plant-pathogenic fungi remains elusive. In this study, we present a comparative genome-wide survey of AS in the plant-pathogenic fungus *S. sclerotiorum* during the infection of six different host plants compared to growth in vitro as a control. Using stringent criteria for the detection of alternatively spliced isoforms, and considering genes identified consistently with our two pipelines, we found 250 genes that expressed more than one isoform (Figure 2). These represent about 2.3% of the genome, which is consistent with estimates for the AS rate of 2.7% in the closely related fungal species *B. cinerea* (Grützmann et al., 2014). In *F. graminearum*, which causes head blight disease in cereal and stalk rot in maize, AS represents 1.7% of the total number of genes in mycelia grown in vitro (Zhao et al., 2013), while in *C. graminicola*, which causes anthracnose disease in maize, only 0.57% of all genes are predicted to exhibit AS during maize infection (Schlebner et al., 2014). Yet this percentage is strikingly low compared to the AS rate of the intron or multiexon-containing genes in plants such as *A. thaliana* or mammals such as *Homo sapiens*, which are reported to be 42% and 95%, respectively (Pan et al., 2008; Filichkin et al., 2010). AS is not well characterized in plant-pathogenic fungi and needs to be investigated in more detail.

(Grützmann et al., 2014). A previous study reported evidence for AS in the plant-pathogenic oomycete *Pseudoperonospora cubensis* that causes downy mildew in the Cucurbitaceae family (Burkhardt et al., 2015). In this work, 24% of the expressed genes showed novel isoforms with new AS events over the course of infection of cucumber at 1–8 days after infection. Moreover, recently Jin et al. (2017) found that the transcripts of two different isolates of the plant fungal pathogen *V. dahliae* undergo splicing of retained introns, producing different isoforms of transcripts that differ between the two isolates during the fungal development. These isoforms have predicted roles in controlling many conserved biological functions, such as ATP synthesis and signal transduction. Interestingly, *P. cubensis* exhibited 10-fold higher AS rates than what we observed in *S. sclerotiorum* and in contrast to *S. sclerotiorum* is an obligate biotrophic pathogen. It is tempting to speculate that the intricate interaction of a biotrophic pathogen with its host plant requires an even more flexible and refined transcriptome than in nonpathogenic, hemibiotrophic, or necrotrophic fungi. Unfortunately, systematic studies addressing the question of the role of AS in the lifestyle of plant-pathogenic fungi are lacking. Current research is limited to necrotrophic and hemibiotrophic fungi with a range of 2.3% (S. sclerotiorum) and 7.9% (the hemibiotrophic pathogen *Magnaporthe oryzae*) AS rate (Grützmann et al., 2014). Further studies should be conducted to uncover the contribution of AS to fungal lifestyles.

In our analysis, most of the AS events were retained introns (RI; 39.8%), which is consistent with previous studies where intron retention showed higher preference in the newly identified isoforms (Grützmann et al., 2014). Moreover, skipped exons represented a small frequency in our analysis (4.4%) but could be considered higher than usual compared to other fungi such as *V. dahliae* (2-fold higher; 2.2%). Interestingly, SE is the most common AS event in mammals (Sammeth et al., 2008).

### 3.2 Do these AS variants contribute to virulence on the respective hosts?

AS is a natural phenomenon in eukaryotes that is genetically tightly regulated, and proper spliceosome activity ensures adequate splicing...
(Chen et al., 2012). The operating mechanisms of splicing regulation and the extent to which components of the splicing machinery regulate splice site decisions remain poorly understood, however (Saltzman et al., 2011). The spliceosome activity is modulated by cis- and trans-acting regulatory factors. The trans-acting elements include the SR (serine/arginine-rich) and hnRNP (heterogeneous ribonucleoprotein) families (Chen and Manley, 2009; Nilsen and Graveley, 2010), and generally regulate AS by enhancing or inhibiting the assembly of the spliceosome at adjacent splice sites after perceiving cis-acting elements in exon or intron regions of pre-mRNAs.

Because spliceosome components strictly regulate splicing, any changes in spliceosome component abundance may result in inaccurate splicing and/or generation of alternative transcripts in accordance with the environmental condition that causes the changes. Although tightly regulated, AS is influenced by external stimuli in eu-karyotes such as biotic and abiotic stresses. For instance, the LSM2–8 complex and SmE, which are regulatory components of the spliceosome, differentially modulate adaptation in response to abiotic stress conditions in Arabidopsis (Carrasco-López et al., 2017; Huertas et al., 2019). Similarly, U1A is essential in adapting Arabidopsis plants to salt stress. Mutation in AtU1A renders Arabidopsis plants hypersensitive to salt stress and results in reactive oxygen species (ROS) accumulation (Gu et al., 2018).

In our analysis we found that many of the spliceosome components are down-regulated in S. sclerotiorum during infection, in particular on hosts where we detected a high number of AS events. For instance, during infection of B. vulgaris, S. sclerotiorum exhibited the highest abundance of alternative transcripts and showed down-regulation of the majority of the spliceosome components (Figures 1 and 3). This suggests that AS in S. sclerotiorum could result from the down-regulation of spliceosome genes and raises the question of whether host plant defences actively interfere with the regulation of S. sclerotiorum spliceosomal machinery to trigger the observed down-regulation. A related goal for future research will be determining whether AS confers fitness benefits to S. sclerotiorum during host colonization. Dedicated functional analyses will be required to clarify the role of AS in S. sclerotiorum adaptation to the host.

In some cases, more than one isoform is present in a host plant. The reason could be that the new isoforms have new functions that assist the establishment of pathogenesis, while the dominant isoform(s) has/have substantial biological functions that are needed for Sclerotinia under any condition. The different isoforms produced by AS in S. sclerotiorum during infection of the different host plants could be a way to increase pathogen virulence. For instance, the alternatively spliced gene Sscl03g026100 encodes a putative phosphonopyruvate hydrolyase. Phosphonopyruvate hydrolyses hydrolyse phosphonopyruvate (P-pyr) into pyruvate and phosphate (Liu et al., 2004). In plants, phosphonopyruvate plays an important intermediate role in the formation of organophosphonates, which function as antibiotics and play a role in pathogenesis or signalling. Therefore, the fungus may use these two different isoforms to detoxify one of the plant defence molecules to facilitate the infection process. In a previous study, Ochrobactrum anthropi and Achromobacter bacterial strains were found to degrade the organophosphates from surrounding environments and to use the degraded product as a source of carbon and nitrogen (Ermakova et al., 2017). Interestingly, the newly identified isoform of Sscl03g026100 (TCONS_00008521) showed the highest expression during infection of beans (B. vulgaris). Because B. vulgaris is well known for its production of antifungal secondary metabolites such as C-glycosyl flavonoids and betalains (Citores et al., 2016; Ninfali et al., 2017), this suggests that the new isoform may be required for S. sclerotiorum to overcome the plant resistance by degrading some of these metabolites. In addition, AS of Sscl11g080920, predicted to encode a secreted cutinase, could exhibit specificity for differently branched cellulose molecules. Taken together, our study revealed that S. sclerotiorum uses AS that gives rise to functionally divergent proteins. We further show that a number of these isoforms have differential expression on diverse host plants.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant inoculations and RNA sequencing

Raw RNA-seq data used in this work are available from the NCBI Gene Expression Omnibus under accession numbers GSE106811, GSE116194, and GSE138039. Samples and RNAs were prepared as described in Sucher et al. (2020). Briefly, the edge of 25 mm-wide developed necrotic lesions were isolated with a scalpel blade and immediately frozen in liquid nitrogen. Samples were harvested before lesions reached 25 mm width, at 24 hr (H. annuus), 47–50 hr (A. thaliana, P. vulgaris, R. communis, and S. lycopersicum) or 72 hr postinoculation (B. vulgaris). Material obtained from leaves of three plants were pooled together for each sample, all samples were collected in triplicates. RNA extractions were performed using NucleoSpin RNA extraction kits (Macherey-Nagel) following the manufacturer’s instructions. RNA sequencing was outsourced to Fasteris SA to produce Illumina single-end reads (A. thaliana, S. lycopersicum, in vitro control) or paired reads (other infected plants) using a HiSeq 2500 instrument.

4.2 | Quantification of isoform and transcript abundance

Quality control for the RNA-seq data was performed using FastQC (Babraham Bioinformatics). The quality-checked data were processed for trimming with the Java-based tool Trimomatic-0.36 (Bolger et al., 2014). Transcript abundances were quantified using a set of tools as follows. In the alignment pipeline, reads were first mapped to the S. sclerotiorum reference genome (Derbyshire et al., 2017) using HISAT2 (Kim et al., 2015). Annotation of reference genes and transcripts were provided in the input. The aligned reads were assembled and the transcripts were quantified in each sample using StringTie (Pertea et al., 2015, 2016). The assemblies produced by StringTie were merged with the reference annotation file in one GTF
file to incorporate the novel isoforms with the original ones using cuffmerge (Goff et al., 2019). The accuracy of the merged assembly was estimated by reciprocal comparison to the S. sclerotiorum reference annotation. In the de novo assembly pipeline, transcript abundances were quantified using gffcompare (Pertea et al., 2016) and cuffcompare (Trapnell et al., 2012). All the expressed transcripts, including novel genes and alternatively spliced transcripts, were merged into one annotation file using the Tuxedo pipeline merging tool, cuffmerge. The accuracy of the assembled annotation file was assessed by comparing it to the reference genome using gffcompare (Pertea et al., 2016).

### 4.3 Differential expression analysis of RNA-seq

The differential expression analyses of genes and isoforms were calculated using cuffdiff from the Tuxedo pipeline (Trapnell et al., 2012). We then used CummeRbund to visualize the cuffdiff results of the genes whose expression were marked as significant and at log2 fold change of ±2 across all samples, leaving 4,111 genes that had differentially expressed isoforms (Figure 2). quasi-mapping was applied on the same RNA-seq data for expression quantification of transcripts using Kallisto (Bray et al., 2016) and Salmon-0.7.0 (Patro et al., 2017). Differential expression analysis of the quantified transcript and isoform abundance of the RNA-seq data resulting from StringTie and Salmon were used in cuffdiff (Trapnell et al., 2012) and SUPPA2 (Trincado et al., 2018), respectively, according to the default parameters as referred to by the software manuals. The R Studio software package CummeRbund (Goff et al., 2019) was employed to determine the significant change in the transcript abundance across the different samples. All samples were compared with the PDA in vitro cultivation control. Default settings were used. Genes with a false discovery rate (FDR)-adjusted P(q) < .05 with a fold change of ±2 were considered differentially expressed.

### 4.4 RNA-seq data visualization and transcripts annotation

The Integrative Genomics Viewer (IGV) (Robinson et al., 2017) and WebApollo annotator (Lee et al., 2013) were used for visualizing the RNA-seq data. Heatmaps were generated with the heatmap.2 function of R (R Core Team 2018). Spliceosome genes were identified using several approaches. A first set of genes were identified based on map 03040 (Spliceosome) for S. sclerotiorum (organism code "ssl") in the Kyoto Encyclopedia of Genes and Genomes (KEGG). The annotation of these genes was verified using BLASTP searches against Saccharomyces cerevisiae and H. sapiens in the NCBI ReSeq database followed by searches in the UniprotKB database for detailed annotation. Second, we searched for all spliceosome components annotated in ascomycete genomes in the UniprotKB database and identified their orthologs in S. sclerotiorum using BLASTP searches. The gene ontology classification database with the Blast2GO package was used to perform the functional clustering of the differentially expressed or spliced genes. The method was performed using Fisher’s exact test with robust FDR correction to obtain an adjusted p value between certain tested gene groups and the whole annotation. SignalP v. 4.1 (Nielsen, 2017) was used to predict N-terminal secretion signals of reference and novel isoforms. Transmembrane domains were predicted with TMHMM v. 2.0 (Krogh et al., 2001).

### 4.5 RT-PCR

RNA was collected as for the RNA-seq experiment. Reverse transcription was performed using 0.5 µl of SuperScript II reverse transcriptase (Invitrogen), 1 µg of oligo(dT), 10 nmol of deoxynucleotide triphosphate (dNTP), and 1 µg of total RNA in a 20 µl reaction. RNA samples collected from three plants of each species were pooled together for cDNA synthesis. RT-PCR was performed using gene-specific primers (Table S8) on an Eppendorf G-storm GS2 Mastercycler with PCR conditions 4 min at 94°C, followed by 32 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by 10 min at 72°C.

### 4.6 Protein 3D structure modelling and visualization

Protein structure models were determined using the I-TASSER online server (Yang et al., 2015). Top protein models retrieved from I-TASSER searches were rendered using the UCSF Chimera v. 1.11.2 software. Models were superimposed using the MatchMaker function in Chimera, best-aligning pairs of chains with the Needleman–Wunsch algorithm with BLOSUM–62 matrix and iterating by pruning atom pairs until no pair exceeds 2.0 Å.

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### AUTHOR CONTRIBUTIONS

H.M.M.I. suggested the idea, designed the experiment, performed the bioinformatics analyses, and drafted the manuscript. S.K. contributed to the bioinformatics analyses and drafted the manuscript. M.D. performed the RT-PCR. S.R. monitored the RT-PCR experiment.
contributed to bioinformatics analyses, revised the manuscript, and provided feedback. All authors edited, proofread, and approved the final version of this manuscript.

**DATA AVAILABILITY STATEMENT**

All data sets generated for this study are included in the manuscript and the supplementary files. RNA-Seq data are deposited in the NCBI Gene Expression Omnibus (GEO) repository at https://www.ncbi.nlm.nih.gov/geo/ under accessions GSE106811, GSE116194, and GSE138039.

**ORCID**

Heba M.M. Ibrahim https://orcid.org/0000-0003-0603-7755
Stefan Kusch https://orcid.org/0000-0002-2472-5255
Marie Didelon https://orcid.org/0000-0002-8458-8117
Sylvain Raffaele https://orcid.org/0000-0002-2442-9632

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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