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Short communication

The APSES transcription factor LmStuA is required for sporulation, pathogenic development and effector gene expression in *Leptosphaeria maculans*

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SUMMARY

Leptosphaeria maculans causes stem canker of oilseed rape (*Brassica napus*). The APSES transcription factor StuA is a key developmental regulator of fungi, involved in morphogenesis, conidia production and also more recently described as required for secondary metabolite production and for effector gene expression in phytopathogenic fungi. We investigated the involvement of the orthologue of *StuA* in *L. maculans*, *LmStuA*, in morphogenesis, pathogenicity and effector gene expression. *LmStuA* is induced during mycelial growth and at 14 days after infection, corresponding to the development of pycnidia on oilseed rape leaves, consistent with the function of StuA described so far. We set up the functional characterization of LmStuA using an RNA interference approach. Silenced *LmStuA* transformants showed typical phenotypic defects of *StuA* mutants with altered growth in axenic culture and impaired conidia production and perithecia formation. Silencing of *LmStuA* abolished the pathogenicity of *L. maculans* on oilseed rape leaves and also resulted in a drastic decrease in expression of at least three effector genes during *in planta* infection, suggesting either that *LmStuA* regulates, directly or indirectly, the expression of several effector genes in *L. maculans* or that the infection stage in which effectors are expressed is not reached when *LmStuA* expression is silenced.

Keywords: conidiation, effectors, *Leptosphaeria maculans*, morphogenesis, oilseed rape, regulation.

APSES transcription factors (TFs) are specific to fungi and are characterized by highly conserved basic helix–loop–helix (bHLH) DNA-binding domains (Ramirez-Zavala and Dominguez, 2008) that bind to a specific StuAp response element with the consensus sequence (A/T)CGCG(T/A)N(A/C) (Dutton *et al*., 1997). APSES TFs are known to regulate morphological changes during asexual and sexual reproduction (Doedt *et al*., 2004; Miller *et al*., 1992; Ohara and Tsuge, 2004; Scherer *et al*., 2002; Sheppard *et al*., 2005). In addition to regulating the genes involved in morphogenesis, APSES TFs also control the expression of genes encoding metabolic enzymes (Doedt *et al*., 2004; Sheppard *et al*., 2005), secreted enzymes (Korting *et al*., 2003) and cell wall proteins (Sohn *et al*., 2003). StuA from *Aspergillus nidulans* was the first APSES TF to be described (Miller *et al*., 1992), and is required for conidiation and perithecia formation. StuA has been characterized in several phytopathogenic fungi and has been demonstrated to be required for the formation of aerial hyphae, efficient conidiation and the formation of perithecia (IpCho *et al*., 2010; Nishimura *et al*., 2009; Ohara and Tsuge, 2004; Tong *et al*., 2007). Its involvement in pathogenicity has also been investigated. In *Fusarium oxysporum*, deletion of *FoStuA* has a major effect on sporulation processes, but does not result in decreased virulence (Ohara and Tsuge, 2004), whereas, in *Fusarium graminearum*, deletion of *FgStuA* greatly reduces pathogenicity on wheat heads and the expression of secondary metabolites (Lysøe *et al*., 2011), and, in *Fusarium culmorum*, deletion of *FcStuA* leads to a complete loss of pathogenicity towards wheat stem base and root tissues (Pasquali *et al*., 2013). In *Glomerella cingulata*, *StuA* is required for the generation of appressorial turgor pressure and full pathogenicity on intact apple fruit (Tong *et al*., 2007). In *Magnaporthe oryzae*, *StuA* is involved in the mobilization of lipid droplets and glycogen from conidia to appressoria, and is therefore required for appressorium-mediated infection (Nishimura *et al*., 2009). In *Stagonospora nodorum*, StuA has been reported to be required for pathogenicity, for the production of proteins involved in central carbon metabolism and of a metabolite mycotoxin (alternariol), and for the expression of *SnTox3* encoding a host-selective proteinaceous toxin (IpCho *et al*., 2010).

Leptosphaeria maculans is the hemibiotrophic fungus responsible for stem canker of oilseed rape (*Brassica napus*). *Leptosphaeria maculans* is present all over *Brassica*-growing areas,

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except China (Dilmaghani *et al*., 2009; Fitt *et al*., 2006; West *et al*., 2001). Its complex life cycle includes phases of saprophytism, endophytism and necrotrophy, demonstrating remarkable plasticity. Following sexual reproduction on stem debris, leaf infections by ascospores cause phoma leaf spots, supporting asexual multiplication. The life cycle of the pathogen is completed by a lengthy symptomless colonization phase during which the pathogen grows from the leaf lesions along the petiole to the stem, where cankers develop at the end of the growing season (Rouxel and Balesdent, 2005). The *L. maculans* genome has an isochore-like structure with GC-equilibrated isochores (average of 51% GC) which are gene rich, whereas AT-rich isochores (33.9% GC) are mostly devoid of active sequences and are made up of mosaics of intermingled and degenerated repeated elements (Rouxel *et al*., 2011). One hundred and twenty-two genes encoding putative effectors have been predicted in AT-rich isochores (Rouxel *et al*., 2011), including the five avirulence genes identified so far in *L. maculans*: *AvrLm1*, *AvrLm6*, *AvrLm4-7*, *AvrLm11* and *AvrLmJ1* (Balesdent *et al*., 2013; Fudal *et al*., 2007; Gout *et al*., 2006; Parlange *et al*., 2009; Van de Wouw *et al*., 2013). Seventy-three per cent of these effector genes share the same expression profile: a very low expression in axenic culture and a drastic increase in expression during primary leaf infection, with a peak of expression at 7 days post-inoculation (dpi). In Soyer *et al*. (2014), we showed that local loosening of the chromatin structure in the environment of effector genes is a prerequisite for the induction of their expression during infection of oilseed rape by *L. maculans*. We therefore hypothesized that the relaxation of the chromatin structure allows the action of one or several TFs, not yet identified, to efficiently coordinate effector gene expression during infection. Here, we report the identification and functional characterization of LmStuA, the *L. maculans* orthologue of StuA. We aimed to obtain an understanding of the role of LmStuA in fungal development, primary leaf infection of oilseed rape and expression of effector genes. We generated *L. maculans* transformants in which the expression of *LmStuA* was decreased, and analysed hyphal morphogenesis and growth, conidiation, perithecia formation and pathogenicity. The effect of *LmStuA* silencing on the expression of effectors was also investigated during plant infection.

Four genes encoding TFs with an APSES DNA-binding domain were retrieved from the genome of *L. maculans*. Among them, a StuA orthologue (GenBank accession number CBY0240) was identified by a BiDirectional Best Hit using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al*., 1990) with the amino acid sequence of StuA from *S. nodorum* as a reference (IpCho *et al*., 2010). Functional domains have been identified using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan). *LmStuA* encodes a protein of 645 amino acids that shares 82% identity with StuA of *S. nodorum* and contains the typical APSES domain (IPR003163) within its sequence. To validate the automated annotation of *LmStuA*, rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) analysis was performed. Untranslated regions (UTRs), transcriptional start and stop sites, and intron positions were annotated following PCR amplification and sequencing of the 3' and 5' ends of cDNA using a Creator SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA), according to the manufacturer's recommendations, and using *LmStuA*-5UTRL1, *LmStuA*-5UTRL2, *LmStuA*-3UTRU1 and *LmStuA*-3UTRU2 as specific primers (Table S1, see Supporting Information). Three introns of 51, 54 and 49 bp were identified in the coding sequence of *LmStuA* (Fig. S1, see Supporting Information). Amplification of the 3'UTR region of the gene produced a unique band of about 1000 bp that was cloned and sequenced. The 3'UTR showed a polyadenylation site 994 bp downstream of the stop codon. Amplification of the 5'UTR region of the gene produced two bands of about 1000 and 1300 bp that were cloned and sequenced. The sequencing of these two bands showed the presence of two lengthy introns in the 5'UTR sequence and that the 5' end was located at either 2479 or 1258 bp upstream of the start codon (Fig. S1). This is consistent with *StuA* transcripts of *A. nidulans* (Miller *et al*., 1992) and *G. cingulata* (Tong *et al*., 2007), which correspond to two different mRNAs including long 5' leaders, and suggests a post-transcriptional control of *LmStuA* expression. Post-transcriptional controls have been identified in fungi, in particular for genes involved in nutrition (Hinnebusch, 1997; Sachs, 1998). Yeast *GCN4* expression was one of the first examples of translational control in eukaryotes. This gene encodes a TF involved in the activation of the genes required for amino acid availability (Hinnebusch, 1997). Another example is the *CreA* gene involved in catabolic repression regulation. This gene is regulated at both the transcriptional and post-transcriptional level. Protein degradation is suspected to occur in the presence of derepressing carbohydrates (Strauss *et al*., 1999). More recently, environmental pH has been reported to be involved in transcription and post-transcriptional processing of the protease BcACP1 of *Botrytis cinerea* (Rolland *et al*., 2009).

LmStuA expression was investigated using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in the sequenced isolate v23.1.3 (Rouxel *et al*., 2011). Total RNA was extracted from mycelium grown for one week in Fries liquid medium, and from infected leaf tissues as described previously (Fudal *et al*., 2007). Three time points important for host invasion during primary infection were investigated: 3 dpi, when conidia have germinated and the fungus has just penetrated the leaves; 7 dpi, when the fungus is still growing asymptomatically and effector genes reach their peak of expression; 14 dpi, when the necrotrophic symptoms appear and pycnidia differentiate. qRT-PCR was performed as described previously (Fudal *et al*., 2007). For each condition tested, three different RNA extractions from three different biological samples and two reverse transcriptions for each biological replicate were performed. The primers used for qRT-PCR are described in Table S1. C_t values were analysed as

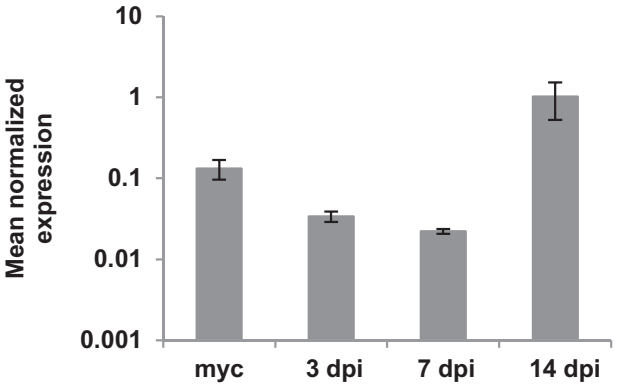

Fig. 1 Expression of *LmStuA* during *in vitro* growth of *Leptosphaeria maculans* and oilseed rape infection. The expression of *LmStuA* was analysed in the strain v23.1.3 by quantitative reverse transcription-polymerase chain reaction (qRT-PCR); 3, 7 and 14 dpi, RT-PCR products obtained from RNA isolated from oilseed rape cotyledons (Westar), 3, 7 and 14 days post-infection; myc, RT-PCR product obtained from RNA isolated from mycelial culture. RNA extracted from uninfected cotyledons and water was used as a negative control. Gene expression levels are relative to *elongation factor 1α* (*EF1α*). Each data point is the average of three biological replicates (extractions from different biological material) and two technical replicates (two RT-PCRs). Standard error of the mean normalized expression level is indicated by error bars.

described by Muller *et al*. (2002) for expression profile analysis. *Elongation factor 1α* (*EF1α*) was used as a constitutive reference gene. The expression of *actin* relative to *EF1α* was used as a control. The gene expression level of actin relative to *EF1α* showed no significant variations among the different *in planta* and *in vitro* conditions (data not shown). The expression of *LmStuA* relative to *EF1α* peaked at 14 dpi, correlating with the development of symptoms and conidia production, and was also high during mycelial growth in axenic culture (Fig. 1). This expression profile is consistent with that of *StuA* from *S. nodorum* and *F. oxysporum*. In these species, *StuA* is expressed during vegetative growth and conidiation, and, in *S. nodorum*, *StuA* expression increases during the late stages of infection correlating with the production of pycnidia (IpCho *et al*., 2010; Ohara and Tsuge, 2004).

LmStuA was silenced by RNA interference (RNAi). Indeed, in *L. maculans*, knock-out strategies were found to be particularly inefficient because of the need for large flanking homologous regions and rare homologous recombination events (Gardiner and Howlett, 2004), and RNAi was demonstrated to be an efficient alternative strategy (Fudal *et al*., 2007). Vector pPZPnat1-*LmStuA* for RNAi-mediated silencing of the *LmStuA* gene was constructed as described by Fudal *et al*. (2007). The pJK11 vector contains a *G. cingulata gpd*A promoter fragment and an *A. nidulans trp*C terminator fragment, separated by a multiple locus site in which inverted repeats of coding sequence of *LmStuA* have been cloned using the primers described in Table S1. The expression cassette was excised by digestion with *Spe*I and *Xho*I, and inserted into the binary vector pPZPnat1 digested with *Spe*I and *Xho*I, creating the vector pPZPnat1-*LmStuA*. *Agrobacterium tumefaciens*-mediated transformation of v23.1.3 from *L. maculans* was performed as described previously (Gout *et al*., 2006). Transformants were then plated onto minimal medium complemented with nourseothricin (50 mg/L) and cefotaxime (250 mg/L). After transformation of *L. maculans* with the pPZPnat1-*LmStuA* vector, 19 transformants resistant to nourseothricin were recovered. Residual expression of *LmStuA* was assessed by qRT-PCR during axenic culture. *LmStuA* expression in the transformants was never lower than 21% compared with the wild-type (WT) strain. Three transformants, s.*LmStuA*-21, s.*LmStuA*-49 and s.*LmStuA*-64, with reduced expression of *StuA* of 21%, 49% and 64%, respectively, compared with the WT strain (Table 1), were selected for further analysis.

We investigated axenic growth, conidiation and perithecia formation of the transformants s.*LmStuA*-21, s.*LmStuA*-49 and s.*LmStuA*-64 compared with the WT strain. Growth assays were performed by the deposition of a 5-mm plug at the centre of a 90-mm Petri dish containing 25 mL of V8 juice agar. Radial growth was measured at 8 days after incubation in a growth chamber at 25 °C, with three replicates. s.*LmStuA*-21 and s.*LmStuA*-49, but not s.*LmStuA*-64, grew significantly more slowly than the WT strain on V8 medium (Table 1). Compared with the WT strain, s.*LmStuA* mycelium appeared to be stunted, less aerial and embedded in the solid medium (Fig. 2A). We observed hyphal morphology on minimal MMII medium and noticed that the hyphae were less branched in the two transformants with the highest level of silencing (Fig. 2B). The hydrophobicity of s.*LmStuA* and WT isolates was assessed by depositing a drop of water at the surface of the mycelium. In the s.*LmStuA* transformants and the WT strain, the drop of water persisted at the surface of the mycelium and did not penetrate the hyphae, even after 10 min. Highly sporulating cultures were obtained on V8 juice agar medium, as described previously (Ansan-Melayah *et al*., 1995). Conidiation was affected in the three transformants compared with the WT strain (Table 1), with an average concentration of 3×10^7 conidia/mL for the transformants compared with more than 1×10^8 conidia/mL for the WT strain. The formation of perithecia was assessed in the three transformants and the WT strain. *In vitro* crosses were performed as established previously (Gall *et al*., 1994). Using v23.1.2 as a compatible strain, five crosses were performed per transformant, and crosses were considered to be fertile when more than five perithecia ejecting ascospores were produced per plate. Transformants s.*LmStuA*-21 and s.*LmStuA*-49 were affected in their fertility, whereas s.*LmStuA*-64 was as fertile as the WT strain v23.1.3 (Table 1). The APSES family of TFs controls a wide range of biological processes, and the deletion or mutation of *StuA* leads to pleiotropic defects in fungi. Accordingly, the silencing of *LmStuA* triggered multiple defects in *L. maculans*. Silenced *LmStuA* mycelia showed morphological defects consistent with the defects usually associated with *StuA* mutations or

Table 1 Effect of *LmStuA* silencing on growth, conidia production, fertility and pathogenicity of *Leptosphaeria maculans*.

Isolate	Relative expression level (%)*	Radial growth (mm)†	Conidia/mL‡	Sexual fertility (number of perithecia)§	Pathogenicity on cotyledons¶
Wild-type					
v23.1.3	100	51.6 ± 0.7	1.97×10^8	Yes (>5)	3.54 ± 0.72
Transformants					
s.*LmStuA*-21	21	43 ± 1.8**	2.17×10^7	No (0)	1 ± 0.00**
s.*LmStuA*-49	49	31 ± 0.75**	4.33×10^7	No (<5)	1 ± 0.00**
s.*LmStuA*-64	64	51.3 ± 0.6	2.83×10^7	Yes (>5)	1 ± 0.00**

**LmStuA* expression was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with RNA isolated from mycelial culture. Expression is relative to the elongation factor 1α (EF1α) expression level and to the expression of *LmStuA* in the wild-type isolate v23.1.3 ($2^{-\Delta\Delta Ct}$ method).

†Radial growth (mm) at 8 days post-inoculation on V8 agar plates.

‡Conidiation (number of conidia/mL). Conidia were collected from five plates per transformant.

§Fertility was measured by crossing v23.1.3 and the selected silenced *LmStuA* (s.*LmStuA*) transformants with the compatible isolate v23.1.2. Crosses were considered as fertile if more than five perithecia ejecting ascospores were produced per plate.

¶Pathogenicity was assayed 14 days after inoculation on the susceptible cultivar Westar of *Brassica napus*. Results are expressed as the mean score using the IMASCORE rating scale, comprising six infection classes (ICs), where IC1–IC3 correspond to resistance and IC4–IC6 to susceptibility (Balesdent *et al*., 2006).

***P* < 0.05. Disease scores and radial growth of each isolate were analysed using analysis of variance (ANOVA). Transformants were compared with v23.1.3 by a Dunnett multiple comparison test ($\alpha = 0.05$). All statistical analyses were performed using XLStat 7.5 software.

deletions, as in *F. oxysporum*, *A. nidulans*, *F. graminearum* and *F. culmorum*, with less aerial mycelium and stunted colonies (Lysøe *et al*., 2011; Ohara and Tsuge, 2004; Pasquali *et al*., 2013). Since the observation of Clutterbuck (1969), showing that StuA mutants of *A. nidulans* are altered in conidiophore development, conidia production has also systematically been shown to be reduced in all fungi in which this gene has been mutated. StuA has also been reported to be required for the formation of perithecia in *F. graminearum* and *G. cingulata* (Lysøe *et al*., 2011; Tong *et al*., 2007), and for mating in *U. maydis* (García-Pedrajas *et al*., 2010).

We examined the pathogenicity of the transformants by inoculating a susceptible cultivar of *B. napus* (Westar). Pathogenicity assays were performed on cotyledons of 15-day-old plantlets, as described by Balesdent *et al*. (2006). Symptoms were scored on 10–12 plants at 14 dpi using the IMASCORE rating scale comprising six infection classes (ICs) (Balesdent *et al*., 2006), with two biological replicates. Although conidia production was low for the transformants, we were able to obtain the concentration used for pathogenicity assays (10^7 conidia/mL). Typical sporulating lesions were observed for the WT. In contrast, all silenced *LmStuA* transformants failed to cause disease and induced a typical black resistance response at the point of inoculation. The symptoms did not evolve with time to cause disease on the leaves. This phenotype is consistent with that which has been described in other phytopathogens, such as *G. cingulata*, *U. maydis*, *M. oryzae*, *S. nodorum*, *F. graminearum* and *F. culmorum* (García-Pedrajas *et al*., 2010; IpCho *et al*., 2010; Lysøe *et al*., 2011; Nishimura *et al*., 2009; Pasquali *et al*., 2013; Tong *et al*., 2007), whereas StuA is considered to be dispensable for pathogenicity in *F. oxysporum* (Ohara and Tsuge, 2004). Thus, except for StuA of *F. oxysporum*, the importance of StuA in pathogenicity seems to be conserved among phytopathogenic fungi, whatever their pathogenic behaviour, including dimorphic transition (*U. maydis*) or the requirement for appressoria to penetrate host leaves (i.e. *M. oryzae* and *G. cingulata*). However, the role of LmStuA in *L. maculans* pathogenicity could be indirect and mainly caused by morphological defects which block fungal development after spore germination.

Table 2 Effect of *LmStuA* silencing on the expression of selected effector genes *AvrLm1*, *AvrLm4-7* and *AvrLm6 in planta*.

Isolate/transformant	Mean normalized expression during plant infection*		
	AvrLm4-7	*AvrLm1*	*AvrLm6*
v23.1.3	3.0	1.7	4.6
s.*LmStuA*-21	2.9×10^{-3}	†	†
s.*LmStuA*-49	7.6×10^{-2}	2.3×10^{-1}	9.8×10^{-2}
s.*LmStuA*-64	1.2×10^{-3}	†	†

*Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of expression was performed in the wild-type strain v23.1.3 and in three transformants silenced for *LmStuA* at 7 days post-infection of oilseed rape leaves. Gene expression levels are relative to *elongation factor 1α* (*EF1α*) and calculated as described by Muller *et al*. (2002). Each value is the average of two biological replicates (two extractions from different biological material) and two technical replicates (two RT-PCRs).

†No expression detected.

We investigated the expression of three effector genes by qRT-PCR in the WT isolate and in the three selected silenced transformants at 7 dpi (corresponding to the peak of expression of effector genes in the WT strain). Silencing of *LmStuA* completely abolished the expression of *AvrLm1*, *AvrLm6* and *AvrLm4-7*, or drastically reduced their expression at 7 dpi compared with the WT strain (Table 2). Effector genes are major pathogenicity determinants of *L. maculans*. Notably, the loss of *AvrLm4* function is associated with a fitness cost: *avrLm4* virulent isolates produce fewer lesions of smaller diameter than do *AvrLm4* avirulent iso-

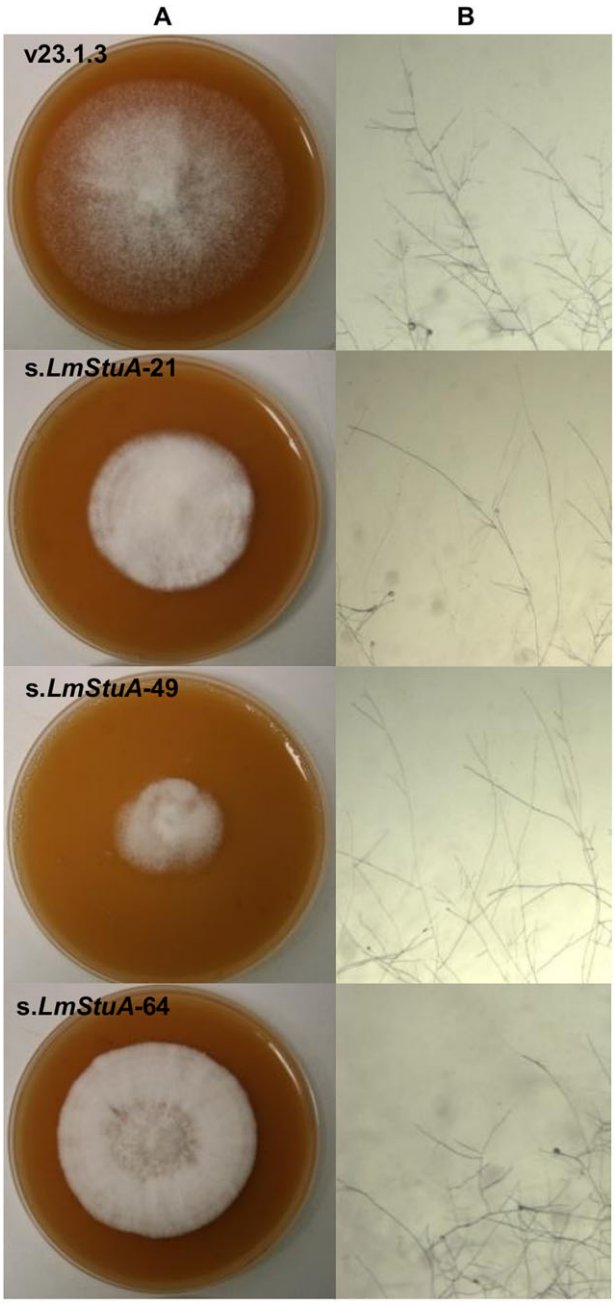

Fig. 2 Effect of *LmStuA* silencing on hyphal morphology of *Leptosphaeria maculans*. Transformants silenced for *LmStuA* and the v23.1.3 isolate were grown on V8 medium (A) and minimal medium MMII (B) agar plates. Observations of hyphae on MMII were made at ×4.

lates (Huang *et al*., 2006). Silenced *LmStuA* transformants showed pathogenicity defects which may be explained by this decrease in effector gene expression. In *F. graminearum* and *F. culmorum*, StuA is required for the expression of secondary metabolite gene clusters (Lysøe *et al*., 2011; Pasquali *et al*., 2013). In contrast, the production of secondary metabolites in *F. oxysporum* was not affected in the *FoStuA* mutants (Ohara and Tsuge, 2004) and, as these mutants showed no pathogenicity defect, we can hypothesize that there is an association between the effects triggered by StuA on the production of effector/secondary metabolites and pathogenicity. Interestingly, and closer to the *L. maculans* model, effector gene expression was also reduced in the *SnStuA* mutant of *S. nodorum* (IpCho *et al*., 2010), a closely related Dothideomycete relying on proteinaceous effector production to induce necrotrophic symptoms. Thus, it seems that the role of StuA in regulating effector gene expression is conserved. However, whether or not StuA regulates effector gene expression directly remains unclear. Indeed, both *SnStuA* and *LmStuA* mutants showed impaired growth and sporulation (IpCho *et al*., 2010; this study); thus, it is also likely that *in planta* development of the fungi is blocked at a step at which the expression of effector genes is not yet initiated. In *LmStuA* mutants, the amount of effector gene expression at 7 dpi is lower than the expression of effector genes at 3 dpi in the WT isolate (Fudal *et al*., 2007; Gout *et al*., 2006; Parlange *et al*., 2009), supporting the hypothesis that parasitic growth is blocked early in the leaves.

We have shown that *LmStuA* is required for normal growth, perithecia formation, pathogenicity on oilseed rape leaves and expression of effector encoding genes in *L. maculans*. *LmStuA* silencing triggers drastic effects on the morphogenesis and pathogenicity of *L. maculans*, indicating that it may affect a large number of genes and pathways. Further work is required to identify the set of genes regulated directly by *LmStuA*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Nucleotide sequence of the genomic region encoding *LmStuA* and amino acid sequence of the corresponding predicted protein. 5' and 3' untranslated regions of the gene are indicated as red and dark green, respectively. Introns are indicated as green.
Table S1 List of primers used in this study.