Molecular Microbiology (2007) **64**(1), 68–82 doi:10.1111/j.1365-2958.2007.05639.x

Regulation and role of a STE12-like transcription factor from the plant pathogen *Colletotrichum lindemuthianum*

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Summary

In phytopathogenic fungi, *STE12*-like genes encode transcription factors essential for appressorium-mediated host penetration. However, their regulation and downstream targets are still unknown. In the present study, a *STE12*-like gene (*CLSTE12*) from *Colletotrichum lindemuthianum* was isolated. We identified a spliced variant whose expression was negatively regulated during early stages of pathogenesis, whereas the correctly spliced mRNA remained expressed up to the penetration step, suggesting distinct roles for these two transcripts. Indeed, the full-length sequence was able to complement a yeast *STE12* mutant, whereas overexpression of the transcript variant had a dominant-negative effect on yeast invasive growth and *C. lindemuthianum* pathogenicity. To further investigate the downstream genes that could be regulated by *CLSTE12*, disruption mutants were generated. Phenotypic analyses of the mutants revealed reduced pectinase activity and conidial adhesion to polystyrene. Analysis of cell surface proteins allowed the identification of a major protein, Clsp1p, which was absent from the mutants. Clsp1p belongs to a new family of wall-associated proteins only found in euascomycetous fungi. Overall, these results suggest that the activity of *CLSTE12* can be modulated by a regulated alternative splicing mechanism and that this factor is involved in the production of cell surface proteins and host cell wall degrading enzymes.

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Introduction

Colletotrichum is a large genus comprising devastating plant pathogens causing anthracnose or blight on a wide range of important crops and ornamental plants (Prusky *et al*., 2000). To infect their hosts, most of them develop a series of infection structures (Perfect *et al*., 1999). Upon germination of the conidia at the surface of plant tissues, the germ tube differentiates into a specialized penetration structure called the appressorium in which high turgor pressure is generated to breach the plant cuticle. An infection peg then emerges from the appressorium, and the fungus subsequently develops a biotrophic interaction with the host through the production of an infection vesicle and primary hyphae. After colonizing one or a few cells, the fungus switches to a necrotrophic phase through the differentiation of secondary hyphae (Perfect *et al*., 1999).

Mutagenesis of *Colletotrichum* has led to the identification of several genes essential for pathogenicity. Among them, genes encoding an MAPK pathway, such as CgMEK1 from *Colletotrichum gloeosporioides* (Kim *et al*., 2000) and CMK1 from *Colletotrichum lagenarium* (Takano *et al*., 2000) were found. CMK1 is a homologue of the yeast MAPK KSS1 that regulates invasive (filamentous) growth and activates the transcription factor Ste12p. Based on this knowledge, a *STE12*-like gene, *CST1*, was recently isolated from *C. lagenarium* and disrupted (Tsuji *et al*., 2003). *cst1Δ* strains were not pathogenic, indicating an essential role of this transcription factor in the infection process. Similar results were obtained for the rice pathogen *Magnaporthe grisea* (Park *et al*., 2002) and the human parasite *Cryptococcus neoformans* (Chang *et al*., 2001). However, the regulation of Ste12p-like proteins by an upstream MAPK pathway is not well understood in filamentous fungi. Mutation of a putative MAP kinase phosphorylation site did not modify the activity of Mst12p in *M. grisea* (Park *et al*., 2004) suggesting that the molecular mechanisms regulating Ste12p activity in yeast

could be different from those regulating Ste12p homologues in filamentous fungi. A striking feature of Ste12p homologues isolated so far from filamentous fungi and basidiomycete yeasts is the presence of a C_2/H_2-Zn^{2+} finger domain whose role is still unclear. Recent studies suggested that this domain was dispensable for DNA binding but essential for some biological functions of the protein (Chang *et al*., 2004; Park *et al*., 2004). Although it is now clear that Ste12p homologues play a role in the pathogenicity of filamentous fungi, both their regulation and target genes remain to be elucidated.

In the course of studying the regulation of extracellular protein production in the bean pathogen *Colletotrichum lindemuthianum*, we recently identified regulatory elements similar to those recognized by Ste12p in the promoter of an endopolygalacturonase gene (Herbert *et al*., 2002). These elements were shown to be essential for induction of gene expression during growth in the presence of pectin as the sole carbon source, and during pathogenesis. This finding led us to isolate a *STE12*-like gene from *C. lindemuthianum*. An alternatively spliced variant of this gene encoding a protein with a truncated zinc finger domain was then isolated. Here we show that the full-length protein and the truncated protein bind to the same DNA motif. However, they display opposite effects on invasive growth when expressed in yeast and in *C. lindemuthianum*. A *CLSTE12* mutant was obtained and phenotype analysis revealed that the mutant was impaired in the production of a major cell surface protein which defined a new family of small secreted cysteine rich proteins found only in euascomycetous fungi. These results demonstrate that two proteins differing in their zinc finger domain and having opposite functions can be produced in fungal cells through an alternative splicing mechanism and suggest that they modulate the production of cell surface proteins possibly involved in pathogenesis.

Results

Isolation of CLSTE12 *and expression analyses*

Two degenerate oligonucleotides were designed from conserved regions of Ste12p-like proteins from filamentous fungi and used for polymerase chain reaction (PCR) on *C. lindemuthianum* genomic DNA, allowing the amplification of a 780 bp fragment. This fragment was used to probe a *C. lindemuthianum* genomic library (Dufresne *et al*., 1998) and a positive clone was further characterized. Sequencing of 3450 bp in the insert revealed an open reading frame of 2452 bp containing four exons and interrupted by three putative introns (Fig. 1A). The sequence encodes a 705 amino acid protein showing a strong level of identity with other Ste12p-like proteins isolated from filamentous fungi (data not shown). A highly conserved homeodomain was found in the N-terminal region and a pair of C_2/H_2-Zn^{2+} fingers in the C-terminal region. The gene was named *CLSTE12* (*Colletotrichum lindemuthianum STE12*). Southern blot analyses showed that *CLSTE12* was present in the genome of *C. lindemuthianum* as a single copy (data not shown). The cDNA was amplified by reverse transcription (RT)-PCR and cloned in the pGEM-T vector. Restriction analysis of 10 independent clones showed the presence of two types of fragments. Sequencing revealed that one type of cDNA (*CLSTE12*) corresponded to the four predicted exons from the gene sequence, whereas the other type of cDNA (*CLSTE12ΔE3*) represented a truncated version, where the third exon was skipped (Fig. 1A). The protein encoded by the shorter mRNA, Clste12ΔE3p, lacks 28 amino acid residues located in the C_2/H_2-Zn^{2+} finger domain (Fig. 1B). Reading frame conservation between exon 2 and exon 4 allowed the reconstitution of one single C_2/H_2-Zn^{2+} finger in Clste12ΔE3p.

To characterize the expression of *CLSTE12*, oligonucleotides were designed to specifically amplify either *CLSTE12* or *CLSTE12ΔE3* cDNAs, and their relative abundance was evaluated by real-time quantitative RT-PCR experiments on RNAs extracted from the mycelium grown in the presence of glucose for 48 h and then transferred to glucose medium or on bean leaves, at the onset of pathogenesis (Fig. 1C). The assay showed that during saprophytic growth in glucose medium *CLSTE12* transcript was about 20 times more abundant than *CLSTE12ΔE3* whatever the considered time point. Both transcripts were upregulated 6 h after the transfer onto bean leaves. However, a strong downregulation in the level of *CLSTE12ΔE3* occurred after 12 h whereas the amount of *CLSTE12* remained high, up to at least 24 h. At that particular time corresponding to appressorium formation in *C. lindemuthianum*, the ratio between the two transcripts was modified up to threefold as compared with growth on glucose medium.

DNA binding activity of Clste12p, Clste12ΔE3p and Clste12ΔZnp and functional analyses in yeast

In yeast, it has been reported that Ste12p binds to pheromone response elements (PREs) and to filamentation and invasion response elements (FREs). Two PREs (TGAAACA) are necessary for efficient binding of Ste12p. FREs are a combination of a single PRE and of a sequence (CATTCC) that binds a transcription factor named Tec1p (Madhani and Fink, 1997). Ste12p can bind an FRE only when dimerized with Tec1p (Baur *et al*., 1997; Madhani and Fink, 1997). A typical sequence of FRE is represented by a fragment of the retrotransposon *Ty1* whose expression requires the *TEC1* gene (Laloux

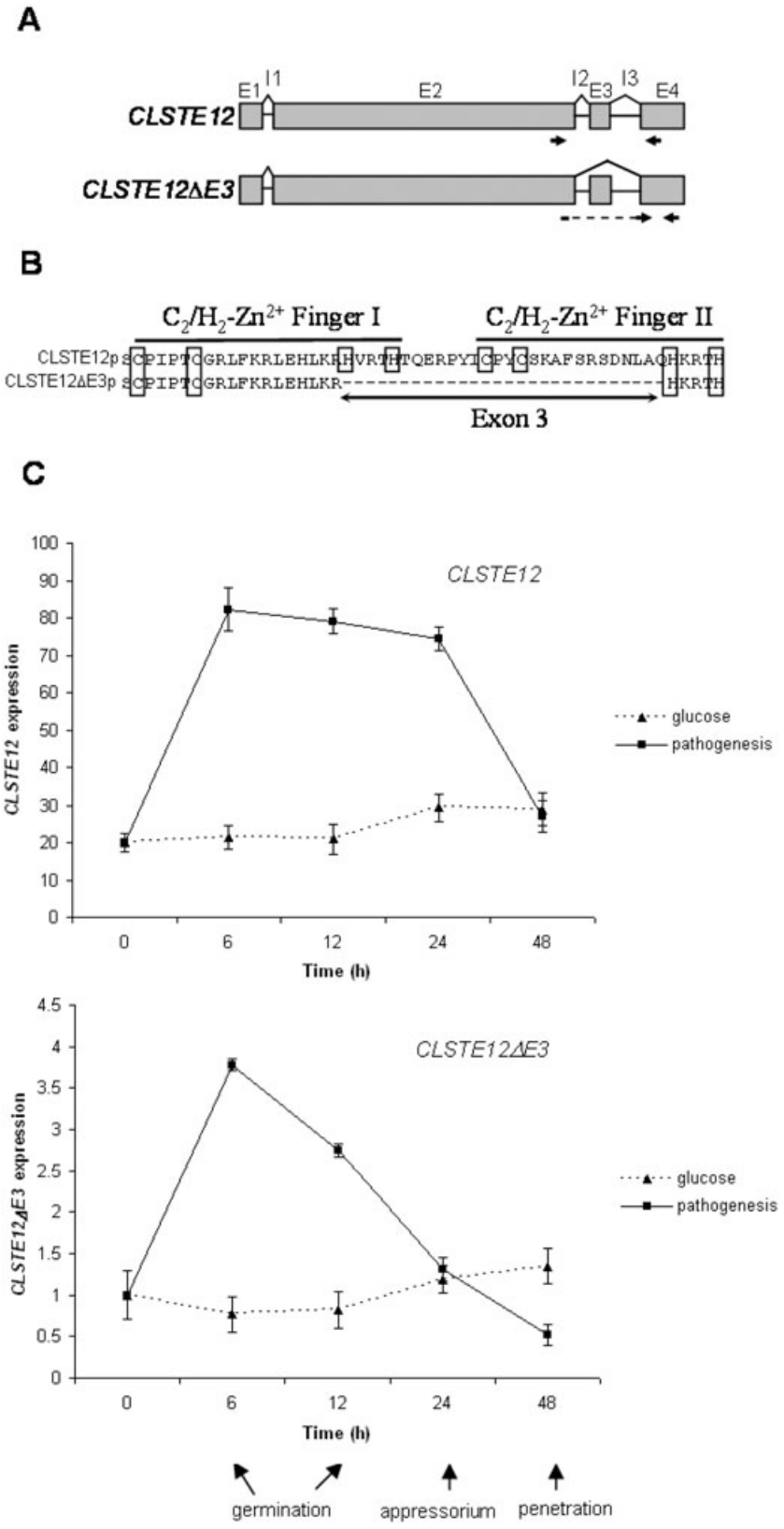

Fig. 1. *CLSTE12* gene structure and expression.
A. Structure of *CLSTE12* gene and location of the exons (E1, E2, E3 and E4) and introns (I1, I2 and I3). Illustrated is the splicing pattern leading to *CLSTE12* mRNA (excision of introns I1, I2 and I3) and *CLSTE12ΔE3* mRNA (excision of introns I1, I2, I3 and exon E3). Arrows indicate the position of the primer sets used for quantitative RT-PCR shown in C to specifically amplify *CLSTE12* or *CLSTE12ΔE3* mRNA. The dashes mean that the primer sits astride exons 2 and 4.
B. Primary structure of Clste12p zinc finger domain. The cysteine and histidine residues involved in the zinc fingers are squared. Position of exon 3 is indicated by a double-head arrow.
C. Quantitative RT-PCR analysis of time-course expression of *CLSTE12* and *CLSTE12ΔE3* during growth on glucose (▲) and upon transfer of the fungus onto bean leaves (pathogenesis, ■). The expression ratios were normalized by using EF1α gene as an internal control, according to the ΔΔCt method. An arbitrary value of 1.0 was affected to the transcript level of *CLSTE12ΔE3* at the time 0 point. Data are from three independent experiments ± standard error. Developmental stages of *C. lindemuthianum* corresponding to the time points are indicated by arrows: conidial germination (6–12 h), appressorium formation (24 h), host cell penetration (48 h).

et al., 1994). The proteins corresponding to Clste12p, Clste12ΔE3p and a version lacking the zinc finger domain (Clste12ΔZnp) were produced in *E. coli* as gene fusions with the *malE* gene coding for the maltose-binding protein (MBP) (Fig. 2A) and purified. The DNA binding capabilities of these proteins were studied using a DNA fragment containing an FRE. Because in yeast Ste12p binds an FRE only in cooperation with Tec1p, Tec1p produced in *E. coli* as an MBP fusion protein was added in the assays. All three proteins were able to form a protein-DNA complex with this probe showing that the zinc finger domain was not essential for FRE binding of Clste12p *in vitro* (Fig. 2B). Removal of Tec1p from the reaction mixtures did not modify Clste12p binding on FRE (data not shown), thereby showing that interaction of Clste12p with Tec1p was not essential for binding on FRE. A mutagenesis analysis showed that each base pair from the DNA sequence TGAAACA recognized by the yeast Ste12p was important for the binding except the last A (Fig. 2C).

Because Clste12p and Clste12ΔE3p were able to recognize the yeast FRE *in vitro*, we then looked for the ability of *CLSTE12* and *CLSTE12ΔE3* to complement a yeast *STE12* mutant. A yeast strain (JCY600 in the Σ1278b background) lacking *STE12* is unable to invade agar (Cook *et al*., 1996). A yeast expression vector containing either Clste12p or Clste12ΔE3p coding sequences under the control of a galactose-induced promoter was introduced into JCY600 (*ste12Δ*) or JCY100 (*STE12*) cells. The transformed strains were assessed for invasive growth using the standard agar penetration assay on glucose or galactose medium. Figure 2D shows that on the inductive medium containing galactose, *CLSTE12* was able to complement *ste12Δ* cells, whereas *CLSTE12ΔE3* blocked invasive growth in the wild-type strain. The expression study of two genes previously described as being induced during yeast invasive growth (Madhani *et al*., 1999), *PGU1* (Fig. 2E) and *FLO11* (Fig. 2F) was carried out in the yeast cells. Both *PGU1* and *FLO11* expressions were downregulated in JCY100 expressing *CLSTE12ΔE3*, and upregulated in JCY600 expressing *CLSTE12*, which indicated that stickiness to agar was indeed related to the invasive growth process.

Functional analysis of Clste12p and Clste12ΔE3p in C. lindemuthianum

To validate the dominant negative effect of Clste12ΔE3p in *C. lindemuthianum*, the wild-type strain was transformed with constructs comprising either a modified *CLSTE12* gene lacking introns 2 and 3 (named *ΔI2,I3*) or lacking introns 2 and 3 and exon 3 (named *ΔI2,I3,E3*) (Fig. 3A). These constructs were obtained by replacing an ApaI–ClaI fragment encompassing intron 2 and intron 3 from the genomic fragment by the ApaI–ClaI fragment originating either from a cloned cDNA fragment corresponding to *CLSTE12* or to *CLSTE12ΔE3* mRNA. The two modified *CLSTE12* genes were placed under the control of the 5′ and 3′ non-coding sequences of *CLSTE12* and used to transform the wild-type strain through *Agrobacterium*-mediated transformation. Southern blot analyses were carried out to select three transformants for each construct having integrated a single insert of the transgene (data not shown). Quantitative RT-PCR experiments showed that these genes were similarly and correctly expressed indicating that a silencing effect did not occur in the transgenic strains (Fig. S1). Pathogenicity tests were conducted by recording the appearance of anthracnose symptoms upon inoculation of bean leaves. All transformants expressing the modified *CLSTE12* gene without exon 3 (*ΔI1,I2,E3*) showed a reduction of pathogenicity, producing smaller and fewer anthracnose lesions than the wild-type strain or the strains expressing the complete *CLSTE12* cDNA (*ΔI1,I2,E3*; Fig. 3B).

Clste12 mutants are deficient in pathogenesis, pectinase production and adhesion to polystyrene

To look for Clste12p targets in *C. lindemuthianum*, disruption mutants were generated (Fig. 4A). The occurrence of gene replacement was confirmed by genomic DNA blot analysis (Fig. 4B). A single 5 kb band was present in the wild type, whereas a 7.5 kb band was present in transformants B1 and B77 as expected for a proper gene replacement event. The absence of *CLSTE12* and *CLSTE12ΔE3* mRNAs was confirmed in these mutants (Fig. 4B). As expected from previous results obtained with two other plant pathogens, *M. grisea* and *C. lagenarium* (Park *et al*., 2002; Tsuji *et al*., 2003), the mutants were non-pathogenic (Fig. 4C) and appeared to be blocked in the penetration stage even though mature melanized appressoria with basal penetration pores were differentiated on bean leaves similar to those of the wild type (Fig. 4D).

In yeast, Ste12p controls the expression of a polygalacturonase gene, *PGU1* (Madhani *et al*., 1999). Based on the occurrence of putative *cis*-elements for a STE12-like factor in the promoter of *CLPG2* (Herbert *et al*., 2002), an endopolygalacturonase gene from *C. lindemuthianum*, the pectinolytic activity of the wild type and mutant strains was assessed by a pectinase-plate assay. As shown on Fig. 5A, disrupted *CLSTE12* strains had a reduced activity, compared with the wild type and to the ectopic transformant (Fig. 5A). In addition, Ste12p also controls the expression of genes encoding secreted yeast proteins, notably the cell surface protein Flo11p, which mediates adhesion of the cells to plastic surfaces such as polysty-

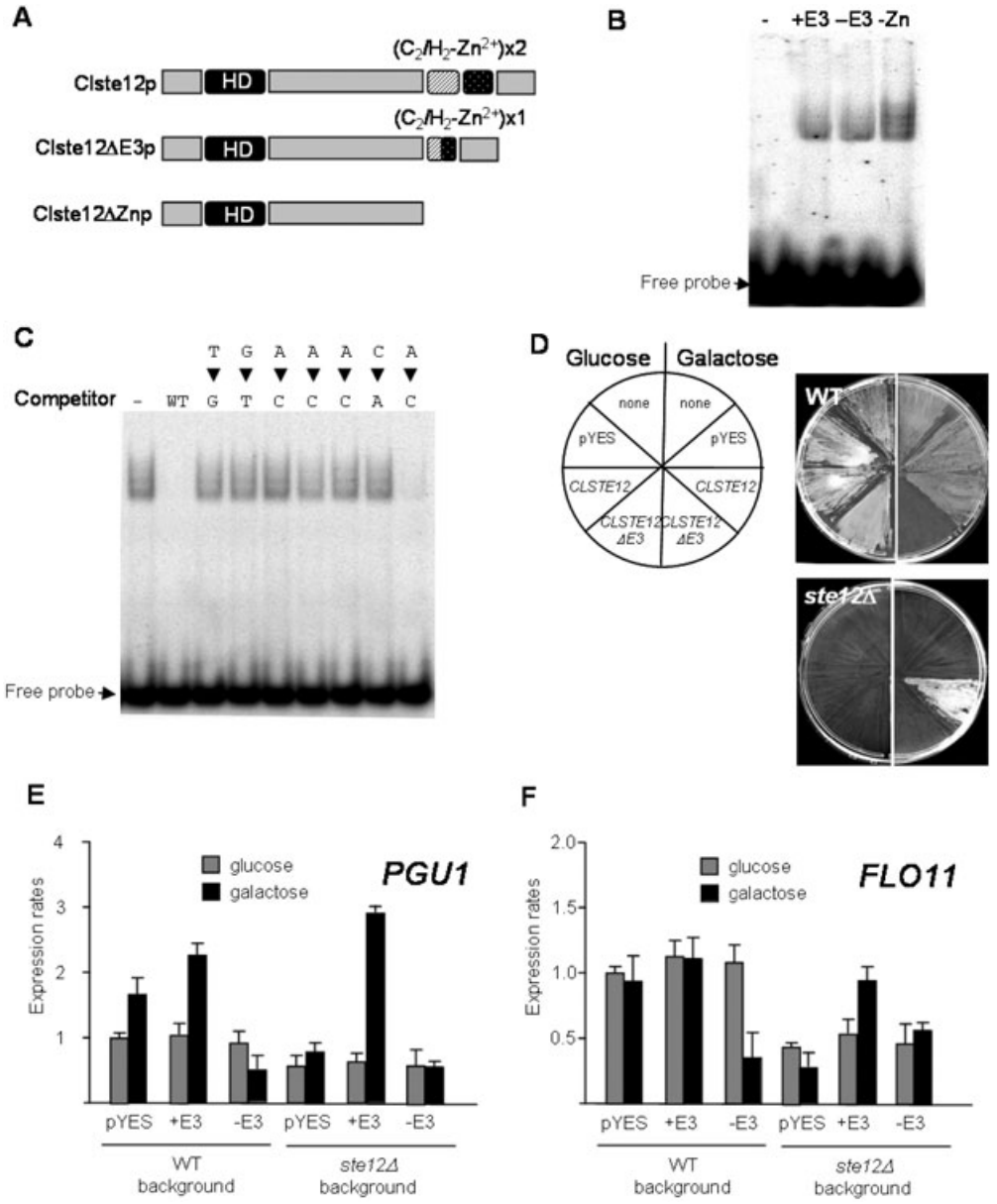

Fig. 2. DNA binding activity of Clste12p and Clste12ΔE3p and functional analysis in yeast.
A. Schematic representation of Clste12p variants produced in *E. coli*. Location of the homeodomain (HD) and the zinc finger domain (C_2/H_2-Zn^{2+}) are shown.
B. DNA binding assay using no protein (–), Clste12p (+E3), Clste12ΔE3p (–E3) or Clste12ΔZnp (–Zn). The recombinant purified proteins (40 fmoles) were incubated with the radiolabelled probe (40 fmoles) corresponding to an FRE. Tec1p produced in *E. coli* was added to the reaction mixtures.
C. Mutagenesis analysis of the DNA sequence required for Clste12p binding. Wild-type or DNA fragments mutated on single nucleotides (as indicated on top of the figure) were used as cold competitors, in a 10-fold molar excess.
D. Functional analysis of *CLSTE12* and *CLSTE12ΔE3* in yeast. *CLSTE12* and *CLSTE12ΔE3* cDNAs were cloned in the yeast expression vector pYES. Each construct and the empty vector were introduced into the yeast strains JCY100 (wild type) or JCY600 (*ste12Δ*) as indicated. The strains without the plasmid (none), transformed with the empty vector (pYES) or with plasmids containing *CLSTE12* cDNAs (*CLSTE12* or *CLSTE12ΔE3*) were plated on agar medium containing either glucose or galactose (repressive or inductive of *GAL1*-driven expression respectively) as shown on the left panel, and incubated for 48 h at 30°C. Invasion assays were performed by rinsing the plate under a stream of water.
E. *PGU1* expression analysis in transformed yeast strains. Quantitative real-time RT-PCR experiments were carried out on RNAs extracted from yeast grown for 2 days on agar medium containing either glucose or galactose. The expression ratios were normalized by using *ACT1* gene as an internal control, according to the ΔΔCt method. An arbitrary value of 1.0 was affected to the transcript level of the wild-type yeast strain transformed with the empty pYES vector, grown on glucose medium. Data are from three independent experiments ± standard error.
F. *FLO11* expression analysis in transformed yeast strains. Same as E, but considering *FLO11* expression. The expression ratios were calculated in the same way as described in E.

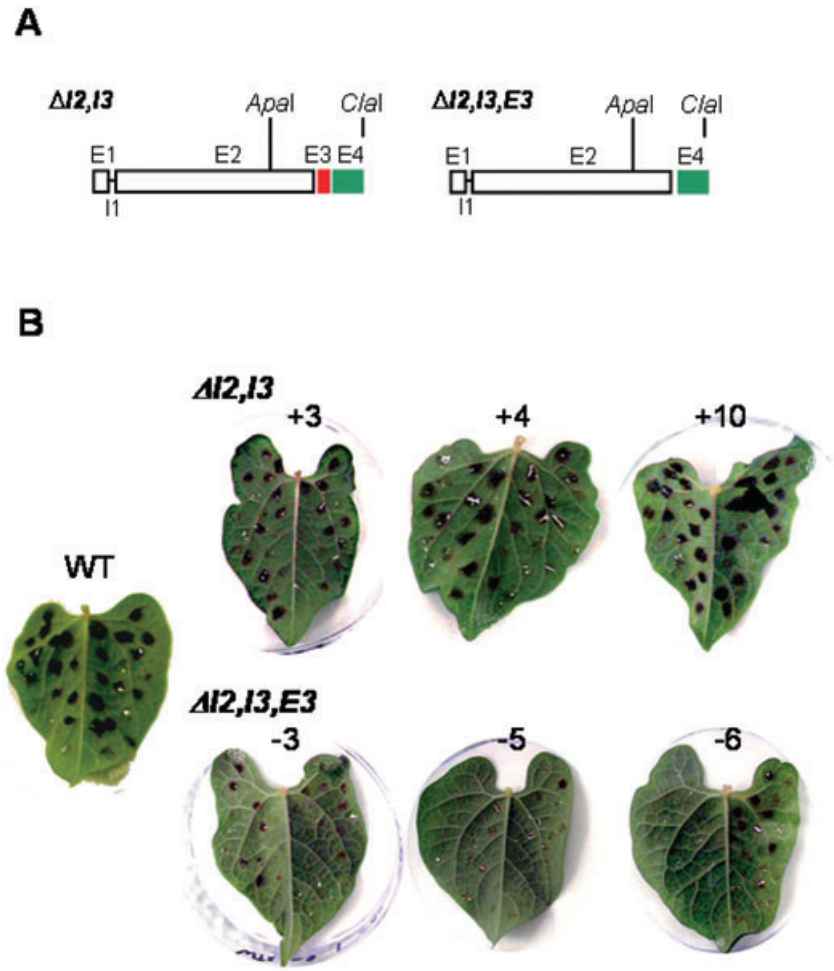

Fig. 3. Characterization of transformants overexpressing *CLSTE12* or *CLSTE12ΔE3* in *C. lindemuthianum*.
A. Modified versions of the *CLSTE12* gene were constructed by replacing a genomic ApaI–ClaI fragment by the corresponding ApaI–ClaI fragment obtained from *CLSTE12* or *CLSTE12ΔE3* cDNAs. The resulting constructs had the native *CLSTE12* promoter and terminator sequences but lacked introns I2 and I3 (*ΔI2,I3*) or introns I2, I3 and exon E3 (*ΔI2,I3,E3*).
B. Bean leaves were inoculated by spotting drops of conidial suspensions from transformants, and the presence of anthracnose symptoms was recorded after 7 days. Independent transformants expressing each construct were tested, namely +3, +4 and +10 for construct *ΔI2,I3* and transformants –3, –5 and –6 for construct *ΔI2,I3,E3*.

rene (Reynolds and Fink, 2001). To check the adhesion properties of *C. lindemuthianum*, conidia were resuspended in sterile water, transferred into wells of a polystyrene ELISA plate, left to settle and then the plate was washed. Polystyrene was chosen for this assay because it has been shown that its hydrophobicity was very similar to that of host tissue (Hughes *et al*., 1999). In the test conditions, around 70% of the conidia from the wild type, ectopic transformant and complemented strain remained attached to polystyrene after extensive washing (Fig. 5B). In contrast, disrupted *CLSTE12* strains showed a severe reduction in adhesion, because only 30% of their conidia remained attached to the surface, suggesting that these strains were defective in the production of a cell surface adhesion component.

A major cell surface protein, CLSP1 is absent in the clste12Δ *mutants*

As a first approach to solubilize cell wall associated proteins, we adapted a protocol which was formerly used to extract plant cell wall components (Fry, 1988). The mycelium was washed in KCl buffer to remove proteins not specifically associated to the extracellular matrix and ground in liquid nitrogen. Then, cytoplasmic and membrane proteins were removed from the insoluble material by a buffer containing a detergent (deoxycholate) and the cell wall residue was incubated in an aqueous phenol acetic acid solution (PAW). The PAW solution has proven to solubilize proteins which are not covalently bound to cell wall polysaccharides (Fry, 1988).

Comparison of the cell surface protein patterns obtained from the wild-type strain and the *clste12Δ* mutant revealed a major protein of 16 kDa that was absent from the mutant strain (Fig. 6A). The MALDI-TOF MS spectrum acquired from the trypsin digest of the protein showed four high intensity ions (1205.6744 m/z, 1476.7695 m/z, 1607.8136 m/z and 1623.8075 m/z), which were absent in the corresponding fingerprint from the mutant strain (Fig. 6B). An aliquot of the trypsin digested protein was analysed by LC-MS/MS to determine their amino acid sequence. Two sequence tags were obtained: xxVPEWT$^{L}/_{I}$EGFTR (Tag 1) from the fragmentation of doubly charged ion 738.856 corre-

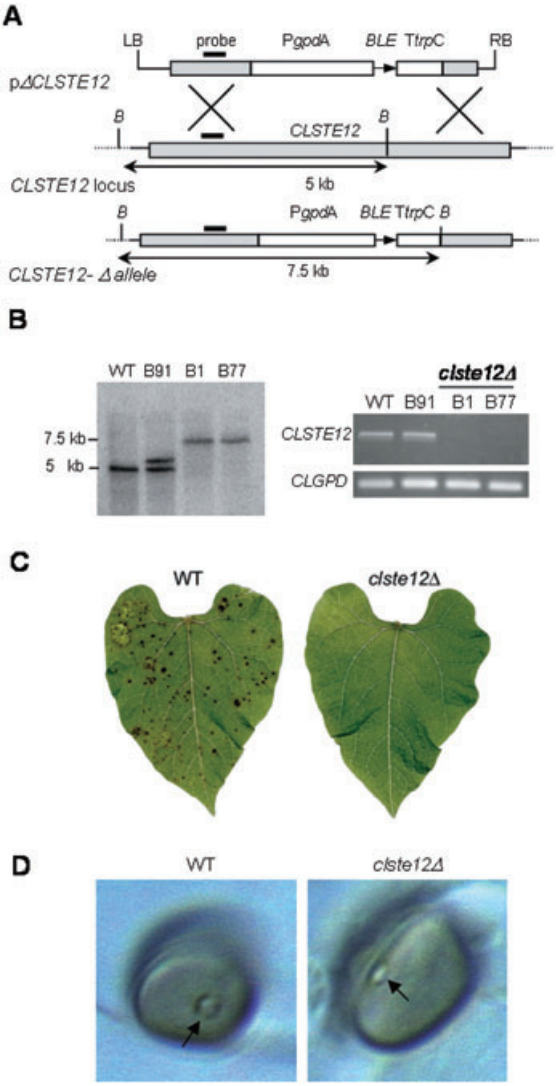

Fig. 4. Phenotype of *CLSTE12* knock-out mutants.
A. Predicted restriction map of *CLSTE12* disrupted by homologous recombination with the replacement vector p*ΔCLSTE12*. Through double crossing over, a 5.0 kb BamHI fragment of the wild-type *CLSTE12* is predicted to be replaced by a 7.5 kb BamHI fragment containing the *BLE* phleomycin resistance gene. *B*: BamHI restriction site. The position of the probe is indicated.
B. DNA blot analysis of transformants (left panel) and *CLSTE12* expression (right panel). Genomic DNA was digested by BamHI and probed with a *CLSTE12* genomic fragment. In the wild-type (WT), a 5.0 kb band was detected. In transformant B91 the 5.0 kb band was intact and an additional band was revealed showing an ectopic insertion of the replacement construct. In transformants B1 and B77, the 5.0 kb band was replaced by the expected 7.5 kb band. To analyse *CLSTE12* expression by RT-PCR, total RNAs were extracted from the wild-type strain and from transformants B91, B1 and B77 grown on glucose medium. A fragment of *CLSTE12* cDNA was amplified by RT-PCR. No signal was detected in the knock-out mutants B1 and B77. The constitutively expressed *CLGPD* gene was used as an internal control.
C. Bean infection assays with *C. lindemuthianum* strains, wild type (WT) and a *clste12Δ* mutant. Note the absence of symptoms produced by the *CLSTE12* mutant.
D. Microscopic observation of appressoria differentiated on bean leaves. Penetration pores are indicated by the arrows.

sponding to 1476.7695 ion (MH^+), and $^{L}/_{I}{}^{L}/_{I}$VWPAYTDK (Tag 2) from the fragmentation of doubly charged ions 603.311, 804.384 and 812.378 corresponding to 1205.6744, 1607.8136 and 1623.8075 ions (MH^+) respectively. Following BLAST analysis, both tags best matched with the same predicted protein of unknown function from the human pathogen *Chaetomium globosum*, CHGG_05278.1.

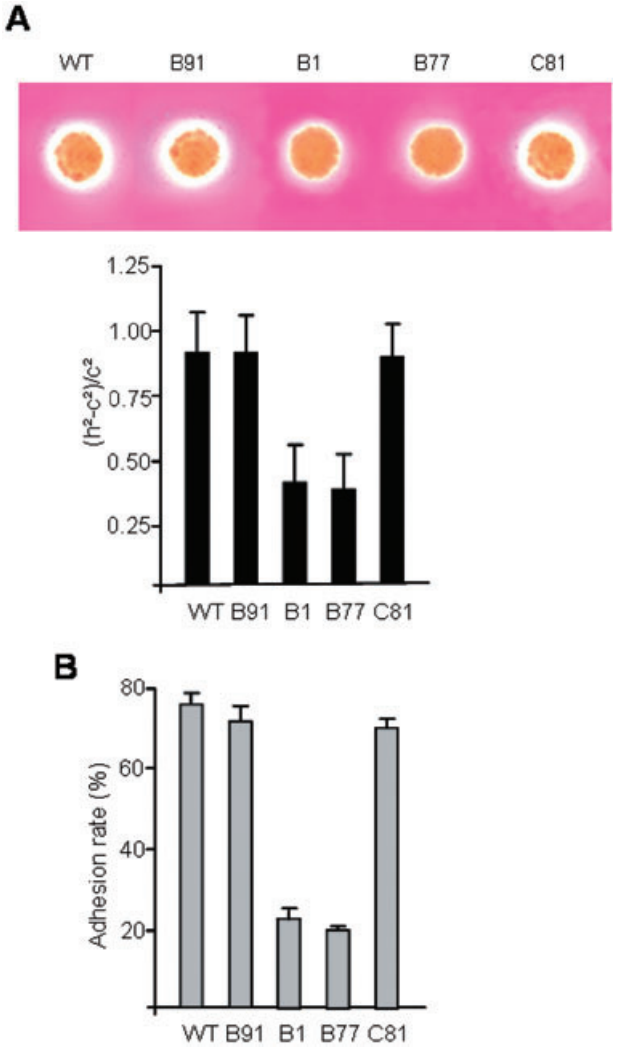

Fig. 5. Phenotype of *CLSTE12* knock-out mutants.
A. Pectinase production of the wild-type strain (WT), the ectopic *CLSTE12* transformant (B91), the *clste12Δ* mutants (B1 and B77) and the complemented strain (C81) was evaluated on a medium containing 1.5% pectin. Pectinase activity was quantified by calculating $(h^2 - c^2)/c^2$ (*h*, halo diameter; *c*, colony diameter).
B. Adhesion of *C. lindemuthianum* conidia to polystyrene. Quantification of attachment to polystyrene was performed with conidia obtained from the wild-type strain (WT), the B91 ectopic transformant, B1 and B77 mutants and the complemented strain (C81). Percentage of conidia adhesion was assessed after 16 h at 23°C. Data are from three independent experiments ± standard error.

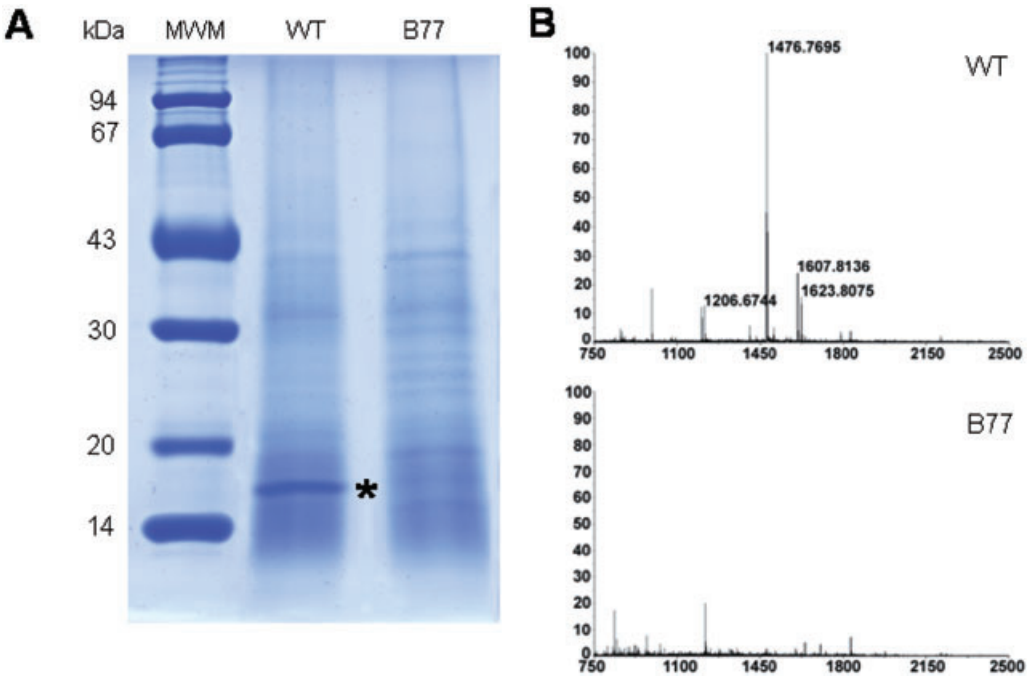

Fig. 6. Cell surface protein analysis.
A. Monodimensional analysis of cell surface proteins produced in the wild-type strain compared with *CLSTE12* mutant B77. The asterisk shows a major band in the wild type, which is absent from mutant B77.
B. MALDI-TOF mass spectrometry of the trypsin digested band after excision from the gel. Note that major ions present in the wild type are absent in the mutant profile.

Clsp1p belongs to a new fungal family of small secreted proteins

Using degenerate primers designed inside the sequenced tags, a 278 bp fragment was amplified, cloned and named *CLSP1* (*C*olletotrichum *l*indemuthianum *S*urface *P*rotein 1). Orthologues of this gene were found in other filamentous fungi, either saprophytic (*Aspergillus nidulans*) or pathogenic (*Fusarium graminearum*, *M. grisea*). Interestingly, similar proteins were not detected in yeasts such as *Saccharomyces cerevisiae* or *Candida albicans*, but only in euascomycetes. The deduced amino acid sequence was aligned with orthologues from filamentous fungi (Fig. 7A). Clsp1p belongs to a family of small-sized cysteine-containing proteins (approximately 15 kDa) which are predominantly electronegatively charged (*p*I ranging from 4.3 to 4.8), except for FG045831 which has a net charge of 5 and a *p*I of 9.1. In this respect, the Clsp1p fragment exhibits a *p*I of 6 and a net charge of −1. These structurally conserved proteins possess a signal peptide of about 20 residues and predominantly consist of β-sheet structures, as predicted in their hydrophobic cluster analysis (HCA) profiles (Fig. 7B). They all exhibit four extremely conserved cysteine residues that are most probably involved in disulphide bonds. Additionally, a few proteins, e.g. CHGG052781 and MGG07973, contain a putative *N*-glycosylation site occurring in a well-exposed region and are thus presumed to be glycosylated. As shown from the HCA plots, the occurrence of charged exposed loop regions along the polypeptide chain supports the possible interaction of these proteins with other charged cell proteins to fulfil some signalling function.

Expression of *CLSP1* was studied by quantitative RT-PCR in mycelium grown in glucose or pectin medium, and during pathogenesis. In the wild-type strain, the ectopic *CLSTE12* transformant (B91) and the complemented strain (C81), *CLSP1* expression is slightly induced during pathogenesis, as compared with the expression when the fungus grows as a saprophyte in glucose or pectin medium. However, *CLSP1* transcript accumulation was dramatically reduced in the *clste12Δ* mutants (Fig. 8).

Discussion

While several *STE12*-like genes have been characterized in various filamentous fungi, little is known about the activity of this family of transcription factors. In addition to a highly conserved homeodomain, two tandem zinc finger motifs near the C-terminus were found in the Ste12p-like proteins from filamentous fungi, which were absent from their orthologues in ascomycetous yeasts. Here we show that an alteration in this zinc finger domain can occur through an alternative splicing mechanism and that this

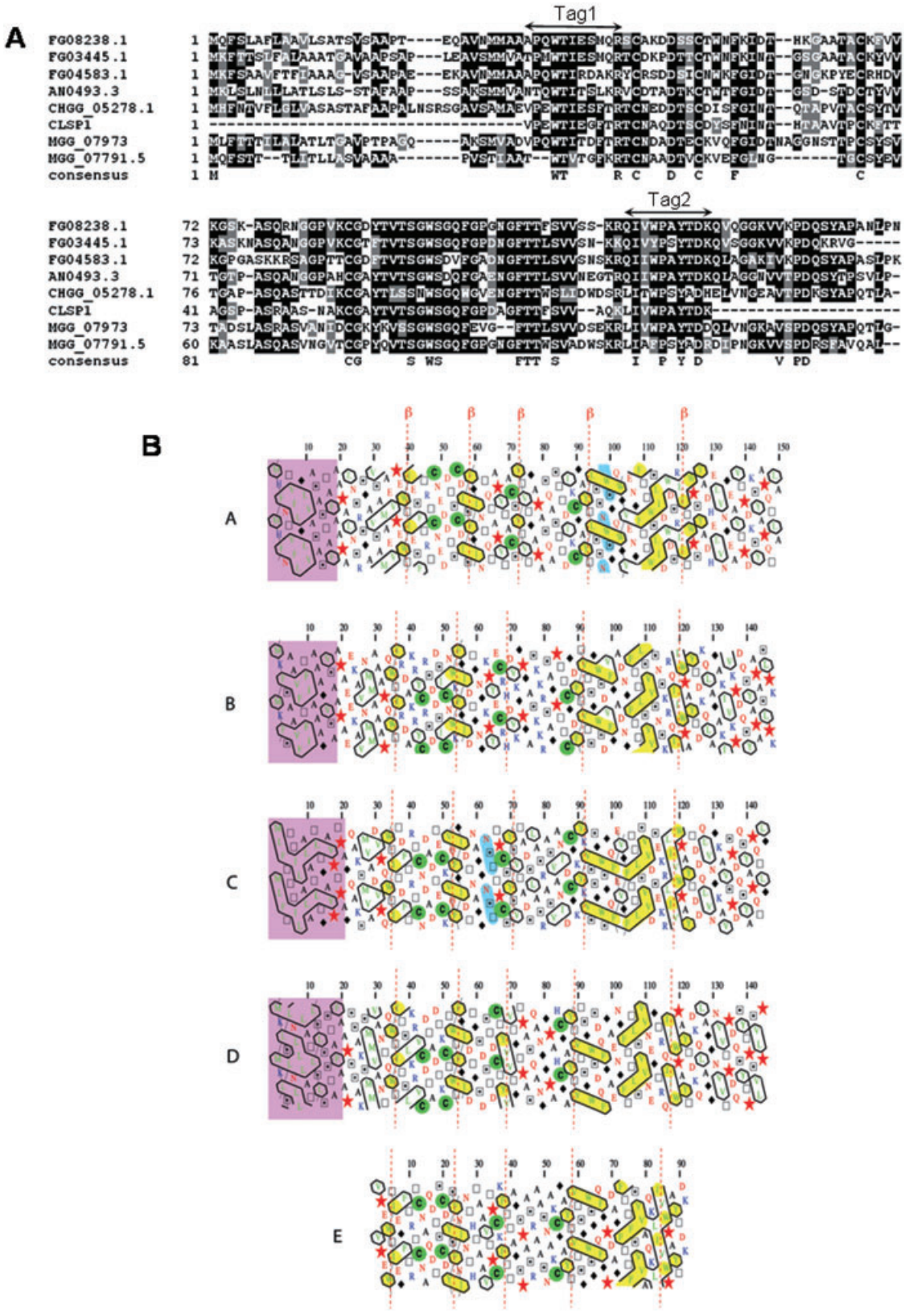

Fig. 7. Comparison of CLSP1 orthologues in filamentous fungi.
A. Alignment of the CLSP1 homologues in *Fusarium graminearum* (FG08238.1, FG03445.1, FG4583.1), *Aspergillus nidulans* (AN0493.3), *Chaetomium globosum* (CHGG_05278.1), *Colletotrichum lindemuthianum* (CLSP1) and *Magnaporthe grisea* (MGG_07973, MGG_07791.5). Identical residues are shown in black (conserved in >80% of aligned proteins) or in grey (conserved in >50% of aligned proteins) backgrounds. The peptides obtained by sequencing (Tag 1 and Tag 2) are indicated by double-head arrows.
B. Comparison of the HCA plots of CHGG052781 (A), FG082381 (B), MGG07973 (C), AN04933 (D) and CLSP1 (E). Clusters of hydrophobic residues (I, L, M, V, F, Y, W) are circled black and special symbols are used for Gly (◆), Ser (⊡), Thr (□), Pro (☆) and Cys (**C**). Conserved residues of hydrophobic clusters and cysteine residues are yellow and green circled respectively. The vertical oriented cluster regions predicted as corresponding to β-strands are indicated by red dashed lines (β). The signal peptide is labelled pink and the putative *N*-glycosylation sites are blue-circled.

modification leads to a dominant-negative control of invasive growth when expressed in fungal cells. Moreover, we provide evidence that supports a role for *CLSTE12* in regulating the production of extracellular proteins during pathogenesis.

Two different forms of Clste12p, having opposite functions in fungal invasive growth, are produced through an alternative splicing mechanism

Cloning of *CLSTE12* cDNA led to the identification of an alternative transcript arising from skipping of the third exon (*CLSTE12ΔE3*). Expression of both types of mRNAs was studied using specific oligonucleotides. The proportion of each mRNA remained unchanged during saprophytic growth of the fungus, *CLSTE12* mRNA being the major form. At the onset of pathogenesis, expression of both *CLSTE12* and *CLSTE12ΔE3* was induced. However, while the amount of *CLSTE12* mRNA remained high up to at least 24 h, *CLSTE12ΔE3* mRNA rapidly decreased. This suggests that *CLSTE12* alternative splicing could be developmentally regulated during the penetration step of infection, and that Clste12ΔE3p could have a negative role in this process.

As a first step to analyse the function of the two protein variants, their DNA binding properties were determined. It was found that the two proteins bind to the same sequence, corresponding to the consensus recognized by the yeast Ste12p. This is the first identification of the target sequence of a Ste12p-like protein from a filamentous fungus, opening the way to a search for the genes controlled by this transcription factor by promoter analysis. Recently, it was shown that Ste12αp, a Ste12-like protein from the human pathogen *C. neoformans* was able to bind to a DNA fragment *in vitro* corresponding to a fragment of a promoter of *CAP59*, a gene associated with virulence (Chang *et al*., 2004). However, the binding motif was not determined. In all *STE12*-like genes cloned so far, a high homology was found in the N-terminal homeodomain region. The finding that the same consensus binding site was found for Clste12p, Clste12ΔE3p and Ste12p is consistent with the high conservation of this region shown to be involved in the DNA binding activity of Ste12p (Yuan and Fields, 1991).

To further analyse the activity of Clste12p and Clste12ΔE3p, we studied the effect of these proteins on invasive growth in wild-type yeast and *STE12* mutants. Expression of *CLSTE12* was able to complement the *STE12* mutant whereas expression of *CLSTE12ΔE3* inhibited invasive growth in yeast. Confirmation that the observed phenotypes were related to invasive growth was obtained by assessing the expression of two yeast invasive growth marker genes, *PGU1* and *FLO11*, in the yeast transformants. Because Clste12ΔE3p binds the same DNA sequence as Clste12p, a likely explanation for the dominant-negative effect could be that the truncated protein competed with Ste12p for FRE binding, but unlike Ste12p, was unable to activate gene expression. To check if this dominant negative activity of Clste12ΔE3p occurred in *C. lindemuthianum, CLSTE12* or *CLSTE12ΔE3* were expressed in the wild-type strain under the control of the *CLSTE12* regulatory sequences. All transformants expressing *CLSTE12ΔE3* showed reduced invasive growth within the host and failed to produce anthracnose lesions on inoculated bean leaves. Thus, Clste12ΔE3p plays a negative regulatory role in parasitic development probably by repressing expression of genes involved in key steps of pathogenesis.

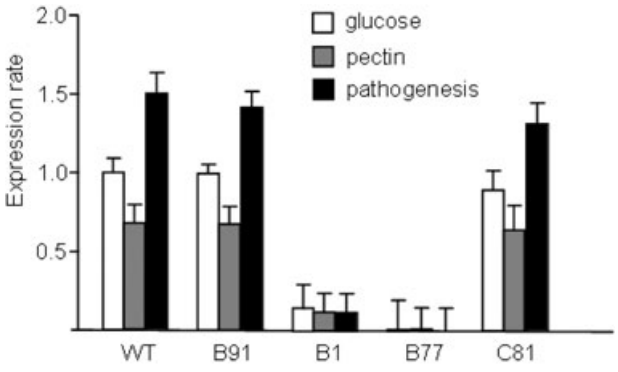

Fig. 8. Expression levels of *CLSP1*. Total RNAs were extracted from mycelium cultivated on glucose, pectin medium or on bean leaves for 2 days. The expression of *CLSP1* in the wild type (WT), the ectopic *CLSTE12* transformant (B91), the *clste12Δ* mutants (B1 and B77), and the complemented strain (C81) were assessed by quantitative RT-PCR. Expression rates were calculated by giving an arbitrary value of 1.0 to the wild type grown on glucose medium. Data are from three independent experiments ± standard error.

It is noteworthy that *CLSTE12* undergoes alternative splicing in one of its functionally important regions and that production of the alternatively spliced pre-mRNA is negatively regulated during interaction with the host plant. While the occurrence of this alternatively spliced mRNA was not reported in other filamentous fungi, the position of the last two introns which interrupt the sequence encoding the zinc finger motifs is highly conserved in all *STE12* homologues isolated so far from euascomycetous fungi (Fig. S2). Moreover, in the event of skipping the third exon by alternative splicing, the reading frame between the encompassing exons would be conserved in all these genes. Thus, the extreme conservation of the structure of *STE12*-like genes in this region suggests an essential role in the regulation of this important family of transcription factors.

CLSTE12 *disruption mutants are impaired in the production of extracellular proteins*

To determine which pathogenicity factors could be modulated by Clste12p, *CLSTE12* gene-disruption mutants were produced. These mutants proved to be non-pathogenic, consistent with previous reports on the plant pathogens *M. grisea* and *C. lagenarium* (Park *et al*., 2002; Tsuji *et al*., 2003) and the human pathogens *C. albicans* and *C. neoformans* (Lo *et al*., 1997; Chang *et al*., 2000; 2001). Because the cellular and molecular events underlying the loss of pathogenicity were not previously identified, several experimental approaches were undertaken to characterize the *clste12Δ* mutants. Based on the identification of *cis*-regulatory elements putatively recognized by a Ste12p-like factor in the promoter of the *C. lindemuthianum* endopolygalacturonase gene *CLPG2* (Herbert *et al*., 2002), we first looked for pectinase activity in the mutant. Enzymatic assays revealed that the *clste12Δ* mutant was impaired in pectinase production. A further approach showed that germinating conidia from *CLSTE12* mutants were deficient in adhesion to polystyrene substrata, suggesting that *CLSTE12* regulates the expression of cell surface components involved in this process. Examination of cell wall associated proteins of the wild-type strain compared with the *clste12Δ* mutant led to the identification of a major protein, Clsp1p. *CLSP1* is not expressed in *clste12Δ* mutants, suggesting that *CLSP1* expression could be controlled by *CLSTE12*. Because neither *CLSP1*-like genes nor related proteins had previously been described in fungi, a search for orthologous genes was undertaken. Screening sequence databases revealed the presence of *CLSP1*-like genes in filamentous fungi (euascomycota) only, notably the plant pathogens *M. grisea* and *F. graminearum*, but not in yeasts. The structural characteristics of Clsp1p and its location at the cell surface suggest a possible role for this new protein family during interaction with the host.

Altogether, these results highlight the role of STE12-like factors from filamentous fungi in the production of extracellular proteins, including cell wall degrading enzymes and cell surface proteins putatively involved in the interaction of fungal cells with abiotic or biotic surfaces. Future work will focus on the characterization of *CLSP1* to obtain better insight into its possible function in the pathogenicity of *C. lindemuthianum*.

Experimental procedures

Fungal strains and growth conditions

Yeast strains constructed in the Σ1278b background JCY100 (*MAT*a *leu2Δ::hisG his3Δ::hisG trp1Δ::hisG ura3-52*) and derivative JCY600 (*ste12Δ::leu2*) were a gift from J. Thorner. Standard growth media and procedures for yeast transformation were used (Ausubel *et al*., 2003). *Colletotrichum lindemuthianum* (Sacc. et Magn.) Briosi and Cav. race β was maintained on synthetic agar medium (Bannerot, 1965). Bean (*Phaseolus vulgaris* cv. Early Wax) infection was done by spraying or spotting 5 μl droplets of a conidial suspension (10^6 conidia ml^{-1}) onto excised cotyledonary leaves. The leaves were placed in Petri dishes under high relative humidity at 23°C in the dark. Anthracnose lesions were observed 5–7 days post inoculation.

Colletotrichum lindemuthianum Agrobacterium tumefaciens*-mediated transformation*

Conidia obtained from 7-day-old cultures were transformed (Mullins *et al*., 2001) using *A. tumefaciens* strain AGL1 bearing the gene replacement vector. *A. tumefaciens* was grown in minimal medium supplemented with rifampicin, ampicillin and kanamycin (50 μg ml^{-1} each). Cocultures were plated on cellophane membranes placed on solid medium in the presence of acetosyringone. After a 2 day incubation at 23°C in the dark, the cellophane membranes were transferred onto ANM (2% malt extract, 0.1% bactopeptone, 2% dextrose) solid selection medium containing phleomycin (25 μg ml^{-1}) or hygromycin (50 μg ml^{-1}) and cefotaxime (200 μM). A thin layer of ultra-low-gelling ANM medium (ANM, 1.5% ultra-low melting agarose) containing phleomycin (50 μg ml^{-1}) or hygromycin (100 μg ml^{-1}) was poured over the membrane and the dishes were left to set at 4°C for 1 h. Transformants appearing after 5 days at 23°C were isolated on solid ANM medium.

Isolation of CLSTE12 *gene and cDNAs*

Two degenerate PCR primers, clste12-sensB (5′-AAGAAYTCHAARAARTTYGAGGARGG-3′) and clste12-revC (5′-TCYTCRTTGGCRATRTAWGCRGGYT-3′), were designed according to the conserved amino acid sequences KNSKKFEE and PAYIANE among Ste12p proteins from *A. nidulans* (Vallim *et al*., 2000), *Penicillium marneffei* (Borneman *et al*., 2001) and *Neurospora crassa* (Li *et al*., 2005). These primers were used to amplify a partial 780 bp *CLSTE12* fragment from *C. lindemuthianum* race β genomic

DNA. The PCR product was cloned into pGEM-T vector (Promega), sequenced and used as a probe to screen a *C. lindemuthianum* genomic DNA library kindly provided by M. Dufresne and T. Langin (Dufresne *et al*., 1998). Recombinant phages were plated on *Escherichia coli* strain Q358 according to standard procedures (Sambrook *et al*., 1989). Plaques were blotted on a nylon membrane and screened by hybridization. Plaques identified as positive clones were screened by two additional rounds of hybridization for purification. Clones were analysed by PCR and Southern blot. A genomic fragment containing the full-length gene was subsequently sequenced. mRNAs extracted from mycelium grown in liquid ANM medium were used to obtain *CLSTE12* and *CLSTE12ΔE3* cDNAs by RT-PCR.

Plasmid constructions

To construct p*ΔCLSTE12*, the *CLSTE12* gene replacement vector, a 2.1 kb amplified fragment of the gene was obtained using Clste12-LBs (5′-GTCGAAGAGCTCATCTCTCGCTTG AGT-3′) and Clste12-RBr (5′-GGTGCTCTAGACGCTTG AAGAGACGAC-3′) primers, digested by SacI and XbaI and subcloned in the binary vector pDHt (Mullins *et al*., 2001). The BstEII–BamHI *CLSTE12* fragment was replaced by the phleomycin resistance cassette borne by plasmid pAN8-1 (Punt *et al*., 1987). To construct the complementation vector pDHt-*CLSTE12*, PCR primers were designed to amplify the gene including the promoter and the terminator sequences, Clste12promXba (5′-CC**TCTAGA**TTCCACACCCACGCCCT CGCCCCACG-3′) and Clste12termH3 (5′-CC**AAGCTT** CATGGTTACACACAAGAGTACGCCC-3′), generating a 4 kb PCR product, which was cloned in pGEM-T vector (Promega). The XbaI–HindIII *CLSTE12* fragment was then cloned in a pDHt vector carrying a hygromycin resistance cassette. For the expression of *CLSTE12* in yeast, *CLSTE12* cDNA was cloned into the shuttle vector pYES2 (Invitrogen) between HindIII and XbaI sites. Primers Clste12-pYESs (5′-CG**AAGCTT**ATGCTGAGCACCGAGGC-3′) and Clste12-pYESr (5′-GC**TCTAGA**TCATTGAACGTCGCCCGGGGCCA TGC-3′) were used for the amplification of the cDNA. For the expression of *CLSTE12* and *CLSTE12ΔE3* in *E. coli*, the corresponding cDNAs were cloned into pMAL-c2 (Biolabs) between EcoRI and HindIII sites. Clste12-pMALs (5′-**GAAT TC**CTGAGCACCGAGGCCCAGCAGGCGCTCCCC-3′) and Clste12-pMALr (5′-**AAGCTT**GGGGTTCACTCCACACTCCA CAGTCTACAG-3′) were used as primers for the amplification of *CLSTE12* or *CLSTE12ΔE3* cDNAs. To get the *CLSTE12ΔZn* construct, the *CLSTE12* construct was digested by EcoRI and BamHI and ligated into an empty pMAL vector between the same restriction sites. For the expression of *CLSTE12* or *CLSTE12ΔE3* mRNAs in *C. lindemuthianum*, *CLSTE12* and *CLSTE12ΔE3* cDNAs were digested with ApaI and ClaI and the fragments were ligated with the pDHt-*CLSTE12* plasmid restricted with ApaI and ClaI enzymes.

Partial cloning of CLSP1 gene

A set of degenerate PCR primers (5′-GTNCCNGA RTGGACNATHGARGGNTT-3′ and 5′-TSYTTRTCNGT YTTNGCNGGCC-3′) designed according to the sequenced peptides VPEWTIEGF and WPAYTDK, were used on wild-type *C. lindemuthianum* genomic DNA as a template to amplify a 278 bp fragment, which was subsequently cloned into pGEM-T vector.

Expression and purification of recombinant proteins

The pMAL-c2 plasmid (Biolabs) containing *CLSTE12, CLSTE12ΔE3 or CLSTE12ΔZn* cDNAs was introduced into *E. coli* BL21. The expression of CLSTE12 versions fused to *malE* and the purification of the resulting recombinant MBP-Clste12p, MBP-Clste12ΔE3p and MBP-Clste12ΔZnp proteins were carried out according to the manufacturer's instruction manual.

Gel mobility shift assays

Gel mobility shift assays were carried out as previously described (Herbert *et al*., 2002), except that DNA-protein complexes were separated on a 4% non-denaturating polyacrylamide gel in 0.5 × Tris Borate EDTA Buffer. The probe used for the binding assays corresponds to an FRE motif (CA**CATTCT**TCTGTTTTGGAAGC**TGAAAC**GTC) or a repetition of three yeast Ste12p recognition motifs: (C**TGAAACA**C**TGAAACA**TCC**TGAAACA**C).

Yeast invasive growth assays

YPD medium was used as a control medium for invasive growth tests according to (Roberts and Fink, 1994). The same medium containing 2% galactose and 1% raffinose instead of 2% dextrose was used as inductive medium for the expression of the gene cloned in pYES vector, under the control of *GAL1* promoter.

Conidial adhesion assay

Conidia from 7-day-old cultures were collected and washed twice in sterile distilled water. They were then left to settle for 16 h in 96-well polystyrene plates at a concentration of 10^5 conidia ml^{-1} at 23°C in the dark. An inverted microscope was used to count the number of spores in 15 random fields of view at 100 × magnification within each well (3 wells per fungal strain). The liquid was then thrown away, the conidia were washed three times in sterile distilled water. The number of conidia was scored in 15 random fields. Percentage adhesion was calculated by dividing the average number of conidia that remained attached by the average number which had originally settled onto the polystyrene well.

Pectinase activity assay

Solid synthetic medium containing 1.5% apple pectin was inoculated with a suspension of 10^4 conidia, in a final volume of 10 µl. Following an incubation of 3 days at 23°C, unhydrolysed pectin was precipitated overnight at room temperature with 1% CTAB, and then counterstained for 30 min with the pectin-binding dye ruthenium red (0.1%).

Extraction and separation of cell wall proteins

The mycelium was cultivated for 5 days at 23°C in synthetic medium containing 1% glucose and washed three times in deionized water to remove the medium contaminants. Following incubation for 2 h at 4°C in 50 mM KCl, the mycelium was ground in liquid nitrogen, vigorously resuspended in 20 mM Hepes pH 7.5, 1% deoxycholic acid, and washed two additional times in the same buffer and then in deionized water. The cell wall residue was then incubated overnight at 4°C in phenol-acetic acid-water (PAW, 100:50:50, w/v/v). The extracted proteins were precipitated in acetone and resuspended in 10 mM Tris-HCl pH 6.8. Monodimensional SDS-PAGE was performed according to the procedure described by Laemmli (1970).

Mass spectrometry analysis and HCA plots

For identification by peptide mass mapping, in-gel digestion of proteins by trypsin and MALDI mass spectra were carried out as described by Borderies *et al*. (2003). For *de novo* sequencing, the trypsin-digested proteins were analysed with an LC-MS/MS system including a nano-HPLC (LC-Packings) and a Q-TRAP mass spectrometer (Applied Biosytems/MDS Sciex), following chromatography gradient conditions and acquisition parameters as described by Boudart *et al*. (2005). Manual interpretation of fragmentation spectra in MS/MS analysis was facilitated by using the Analyst Software (Applied biosystems/MDS Sciex). The HCA (Gaboriaud *et al*., 1987) plots were generated using the HCA server (http://bioserv.rpbs.jussieu.fr/RPBS/cgi-bin/Ressource.cgi?chzn_lg=fr&chzn_rsrc=HCA).

Quantitative PCR assays

Real-time PCR was carried out in 384-well optical plates. The fluorescence was quantified with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Each run was assayed in triplicates in a total volume of 10 μl containing the template, 1 × SybrGreen Master Mix (Eurogentec) and 100 nM each gene-specific primer. The thermal profile recommended by Applied Biosystems was used for amplification (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min). To check amplification of one single specific target DNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer (95°C for 15 s, 60°C for 15 s, followed by a ramping to 95°C for 20 min). The generated data were analysed with SDS 2.0 software (Applied Biosystems).

Gene expression monitoring

Total RNAs from *Colletotrichum* mycelium was prepared with the RNeasy Plant Mini Kit (Qiagen). Total RNAs from yeast was extracted as described by Ausubel *et al*. (2003). RNAs were treated with RQ1 DNase (Promega) according to the manufacturer's protocol. The cDNAs were then generated by reverse transcription with SuperscriptII First Strand cDNA synthesis kit (Invitrogen) and the oligod$T_{(20–25)}$ primers. Gene expression was assessed by quantitative RT-PCR experiments, using 2 μl of 10 × diluted RT product as template. Primers E3sens (5′-CATACACAAGAAAGACCCTACATCT GCCC-3′) and 3252rev (5′-CCGGAGAAGTTGAGCGC CGCCTCGC-3′), were used for the expression of *CLSTE12* mRNA; E2-4sens (5′-CGAGCACCTCAAGCGACACAAGCG-3′) and 3322rev (5′-CTCTGAATTGGGAGACGCCTCCTCG-3′) for the expression of *CLSTE12ΔE3* mRNA. To assess the expression of CLSP1, primers 5′-TTCACCCGCACCT GCAACGC-3′ and 5′-TTGTCGGTTTTTGCGGGCC-3′ were used. The expression levels of *PGU1* and *FLO11* in yeast were evaluated by using PGU1sens (5′-AACCATGT TATCAACTCTGACAACG-3′) and PGU1rev (5′-ATTGCC AGTTAGTAGCGTTTTTCAC-3′) or FLO11sens (5′-AGTC ATCTGTTGGTACTAACTCCG-3′) and FLO11 rev (5′-TT CCAAGAACCTTGATATTAGCAGC-3′). The Ct values were normalized according to the ΔΔCt method, using ribosomal elongation factor 1α as a constitutively expressed gene for *C. lindemuthianum* (EF1αsens 5′-GAAGACTCACATCAA CGTCG-3′ and EF1αrev 5′-TTGAAGGAACCCTTGCCGAG-3′), and actin for the expression in *S. cerevisiae* (ACT1sens 5′-CGTCGGTAGACCAAGACACC-3′ and ACT1rev 5′-CAGC AGTGGTGGAGAAAGAG-3′).

Accession numbers

Nucleotide and amino acid sequence data from this article are available in the EMBL/GenBank databases under Accession no. AJ459778 (*CLSTE12*) and AM398202 (*CLSP1*).

Acknowledgements

We thank J. Thorner (University of California, Berkeley) and G. Fink (Whitehead Institute, Cambridge) for generous gift of yeast strains and plasmids. We are grateful to M. Dufresne and T. Langin (Orsay University, France) for providing us with the *C. lindemuthianum* genomic library and M.T. Esquerré-Tugayé for critically reading the manuscript. We thank 'CRGS Genopole Toulouse' for giving access to real-time PCR technology, 'Plate-forme protéomique Toulouse' for MALDI-TOF and LC-MS/MS analyses, and MilleGen (Labège, France) for sequencing facilities. Financial support from the French Foreign Office (Procope Programme) to J.W.S.H is gratefully acknowledged.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Analysis of transcript levels in transformants overexpressing CLSTE12 or CLSTE12DE3. A. Expression of CLSTE12 mRNAs in single copy transformants was assessed by quantitative RT-PCR using specific primers that specifically amplified CLSTE12 cDNA, obtained from the fungi grown for 48 h on glucose (grey) or pathogenesis (black) conditions. Expression rates were calculated by giving an arbitrary value of 1.0 to the wild type. B. Same as A, but considering expression of CLSTE12DE3.

Fig. S2. Schematic diagram showing the structure conservation of Ste12-like genes from euascomycetous fungi, including plant pathogens (*C. lindemuthianum*, *C. lagenarium*, *M. grisea*, *Fusarium graminearum*, *Stagonospora nodorum*, *Cryphonectria parasitica*), animal pathogens (*Penicillium marneffei*, *Histoplasma capsulatum*, *Coccidioides immitis*, *C. globosum*), and saprophytes (*N. crassa*, *A. nidulans*, *Uncinocarpus reesii*,), and basidiomycetous fungi (*C. neoformans* mating type a or a and *Coprinus cinereus*). The exons are symbolized by squares linked by lines representing the introns. The zones encoding the zinc finger motifs are indicated by * (C2) and ° (H2).

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