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Patulin is a cultivar-dependent aggressiveness factor favouring the colonization of apples by *Penicillium expansum*

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SUMMARY

The blue mould decay of apples is caused by *Penicillium expansum* and is associated with contamination by patulin, a worldwide regulated mycotoxin. Recently, a cluster of 15 genes (*patA–patO*) involved in patulin biosynthesis was identified in *P. expansum*. BLAST analysis revealed that *patL* encodes a Cys₆ zinc finger regulatory factor. The deletion of *patL* caused a drastic decrease in the expression of all *pat* genes, leading to an absence of patulin production. Pathogenicity studies performed on 13 apple varieties indicated that the Pe Δ *patL* strain could still infect apples, but the intensity of symptoms was weaker compared with the wild-type strain. A lower growth rate was observed in the Pe Δ *patL* strain when this strain was grown on nine of the 13 apple varieties tested. In the complemented Pe Δ *patL:patL* strain, the ability to grow normally in apple and the production of patulin were restored. Our results clearly demonstrate that patulin is not indispensable in the initiation of the disease, but acts as a cultivar-dependent aggressiveness factor for *P. expansum*. This conclusion was strengthened by the fact that the addition of patulin to apple infected by the Pe Δ *patL* mutant restored the normal fungal colonization in apple.

Keywords: aggressiveness factor, apples, mycotoxin, patulin, *Penicillium expansum*, post-harvest disease.

INTRODUCTION

Blue mould, caused by *Penicillium expansum*, is the most important apple post-harvest disease. *Penicillium expansum* is a pathogen that infects fruit tissues, causing maceration and decay. Blue mould represents a significant economic problem for the fresh fruit industry, causing up to 80% of decay in stored fruits (Morales *et al.*, 2007). Little is known about the pathogenicity determinants

of *P. expansum*. As for other post-harvest pathogens, a key factor in the pathogenicity of *P. expansum* is the secretion of pectolytic enzymes (Jurick *et al.*, 2010). The action of one class of these enzymes, the polygalacturonases, results in tissue maceration, accompanied by acidification, mainly caused by the production and secretion of gluconic acid and fumaric acid (Prusky *et al.*, 2004; Vilanova *et al.*, 2014). This acidification of apple tissue activates the transcription of polygalacturonases by the fungus.

The blue mould caused by *P. expansum* is generally associated with patulin production that affects the quality of apple products. Patulin is a toxic secondary metabolite that has been reported to cause intestinal disorders (McKinley and Carlton, 1980) with ulceration and bleeding (Speijers *et al.*, 1988). As a result of its cytotoxicity, genotoxicity and immunosuppressive properties (Moake *et al.*, 2005), the European Union (EU) has fixed the maximum tolerated level for patulin at 50 μ g/kg for fruit juice and derived products, and 25 μ g/kg for solid apple products. The maximum level in food intended for babies and young children was set at 10 μ g/kg (EC Commission Regulation, 2006).

The biological role of toxic secondary metabolites is not clear, but several studies have attributed to mycotoxins a role in virulence and aggressiveness in animal and plant fungal pathogens (Scharf *et al.*, 2014). 1,8-Dihydroxynaphthalene (DHN)-melanin is essential for the virulence of *Aspergillus fumigatus* in humans, but also for the virulence of *Magnaporthe oryzae* in rice (Langfelder *et al.*, 1998; Woloshuk *et al.*, 1980). Other fungal secondary metabolites, such as the well-known trichothecenes, deoxynivalenol (DON) and nivalenol (NIV), have been proven to have a well-established role in the onset of diseases in wheat by *Fusarium graminearum* (Maier *et al.*, 2006; Proctor *et al.*, 2002). In order to elucidate the biological role of mycotoxins, some studies have used the gene disruption technique to block the biosynthetic pathway at an essential step, thus completely turning it off (Proctor *et al.*, 1995; Tsai *et al.*, 1998). In filamentous fungi, the production of secondary metabolites is usually orchestrated by a series of genes generally organized in clusters (Keller and Hohn, 1997; Keller *et al.*, 2005). A large number of clusters, related to the

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production of secondary metabolites, have already been discovered, such as the clusters responsible for the biosynthesis of the mycotoxins aflatoxins (Yu *et al.*, 2004) and trichothecenes (Alexander *et al.*, 2009). Based on biochemical, genetic and molecular studies, different patterns of the biosynthesis of patulin have already been proposed (Puel *et al.*, 2010). Initially, the patulin biosynthesis cluster was characterized in *Aspergillus clavatus* (Artigot *et al.*, 2009). In this fungus, two genes encoding P450 cytochrome (Artigot *et al.*, 2009) and one encoding the 6-methylsalicylic decarboxylase (Snini *et al.*, 2014) were characterized. Very recently, the patulin biosynthesis cluster was identified in *Penicillium expansum*, the main producer of patulin in apple (Ballester *et al.*, 2015; Tannous *et al.*, 2014).

The biosynthetic pathways of fungal secondary metabolites are enzymatic cascades in which enzymes are activated at the same time and the newly synthesized products are consecutively metabolized by the next enzyme. This phenomenon is facilitated by the organization in clusters of the genes encoding the enzymes involved in the biosynthetic pathway. These genes are generally co-activated by a specific transcription factor, often located within the cluster, which is itself activated by other global transcription factors. Most of the transcription factors involved in fungal metabolite synthesis belong to the zinc finger protein family (Ehrlich *et al.*, 1999a,b). They are proteins that regulate gene expression by binding to specific gene promoter sites and play the role of an 'on/off' switch for the production of the mycotoxin. Among these positive-acting pathway and specific transcription factors, TRI5 and AFLR regulate positively trichothecene and aflatoxin/sterigmatocystin production, respectively (Payne *et al.*, 1993; Proctor *et al.*, 1995; Woloshuk *et al.*, 1994; Yu *et al.*, 1996). Located within the patulin biosynthesis cluster of *P. expansum*, the gene *patL* encodes a transcription factor containing a N-terminal Gal4-like Cys6 zinc binuclear cluster DNA-binding domain which activates patulin gene expression to produce patulin (Li *et al.*, 2015).

Based on the generation and use of the *PeΔpatL* deficient mutant and *PeΔpatL:patL* complemented strains, we report, in this study, the involvement of patulin as an aggressiveness factor favouring the colonization of apples by *P. expansum*.

RESULTS

patL gene deletion and complementation

In order to determine whether patulin plays an important role in the pathogenicity of *P. expansum*, *PeΔpatL* mutants were generated by the transformation of the gene replacement cassette *ΔpatL* shown in Fig. S1 (see Supporting Information) in the wild-type *P. expansum* NRRL 35695 strain. Fifty-one fungal transformants were isolated and purified by single-spore isolation. All the transformants were tested for their ability to produce patulin on Czapek-glucose broth, and only those which did not produce patu-

lin were selected for the following tests. Nineteen fungal transformants were analysed by polymerase chain reaction (PCR) to determine which mutant potentially carried a disrupted *patL* gene. First, the presence of the hygromycin cassette in the genome of the mutants was confirmed by amplification of the genomic DNA with *hygF* and *hygR* primers that are specific to the hygromycin sequence. Then, the presence of the disruption cassette at the *patL* locus was confirmed by amplification of the genomic DNA with *dPatLF3* and *dPatLR3* primers. The primers were designed to anneal on the *P. expansum* genome, but not on the *ΔpatL* disruption cassette. The wild-type strain presented a 5030-bp fragment, whereas mutants presented a 6681-bp amplicon. To confirm the presence of the disruption cassette at the *patL* locus, an enzymatic digestion of the amplicon obtained was performed using *NdeI*. Southern blot analysis confirmed that the *PeΔpatL1* mutant displays only one insertion of the disruption cassette at the *patL* locus (Fig. S2, see Supporting Information). For the following experiments, only this *PeΔpatL1* mutant was used. In order to verify that the phenotype exhibited by the mutant strain *PeΔpatL1* resulted from the deletion of the *patL* gene, the disrupted *PeΔpatL1* strain was transformed with a wild-type copy of *patL*, generating the complemented strain *PeΔpatL:patL*. In the latter, the wild-type gene could be detected, but not the hygromycin sequence, therefore suggesting that the homologous replacement of *patL* occurred at its initial locus. The capacity to produce patulin was assessed *in vitro* by apple puree agar medium (APAM), a permissive substrate for patulin production (Baert *et al.*, 2007). No patulin was detected for the mutant strain *PeΔpatL1* and a large amount of patulin was detected for both the wild-type (0.098 mg/mL) and the complemented strains (0.55 mg/mL) (Fig. S3, see Supporting Information). Finally, the expression of all the genes included in the cluster was analysed. As expected, expression was absent for all *pat* genes in *PeΔpatL1* at day 5, whereas the expression of all genes was restored in the complemented strain (Fig. S4, see Supporting Information). According to the report by Li *et al.* (2015), these results show that *patL* is necessary to trigger patulin production. Unlike the latter authors, we also generated a complemented strain, which provides the ultimate evidence.

Effect of *patL* deletion on *in vitro* growth, macroscopic and microscopic morphology

No significant difference was observed between the radial growth of *PeΔpatL1* and wild-type NRRL 35695 strains. Indeed, after 7 days of incubation on malt extract agar (MEA), colony diameters were 39.3 ± 0.6 mm and 40 ± 1 mm for wild-type and mutant strains, respectively. Similar results were observed on potato dextrose agar (PDA) with colony diameters of 41.3 ± 0.6 mm and 40.5 ± 0.7 mm for wild-type and mutant strains, respectively. These results show clearly that the absence of patulin production does not affect the *in vitro* growth of *P. expansum*. Overall, the

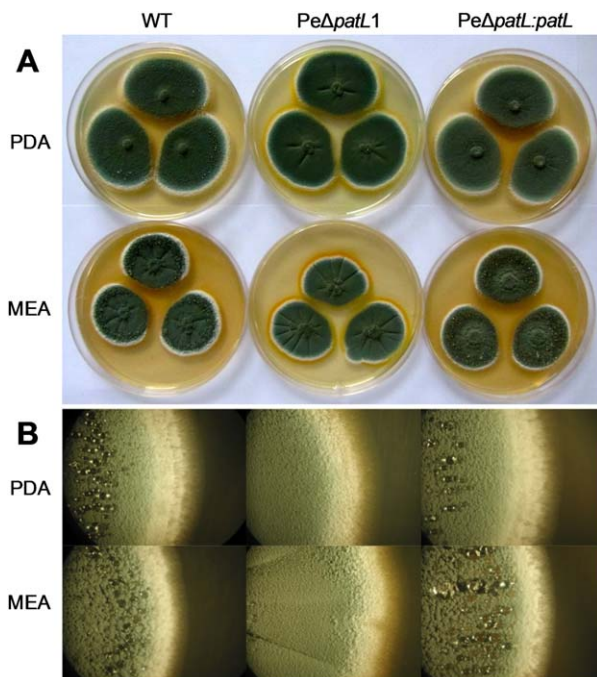


Fig. 1 Morphological aspect of the wild-type NRRL 35695 (WT), *PeΔpatL1* and complemented *PeΔpatL:patL* strains after 7 days of culture at 25°C on potato dextrose agar (PDA) and malt extract agar (MEA). (A) Macroscopic aspect of colonies. (B) Observations under a stereomicroscope (×12).

morphology of wild-type and mutant strains was very similar. Macroscopically, both colonies displayed a classical *P. expansum* blue–green colour with marked white margin (Fig. 1A). The texture of the colony was comparable with a clear fasciculation. The aggregation of conidiophores into erect bundles gave a granular appearance to the surface. Interestingly, the surface of the mutant colony appeared mildly planar with less definite coremia than that of the wild-type (Fig. 1B). The *PeΔpatL1* strain also presented other particularities: exudate was less abundant and a yellow pigment was clearly visible on MEA. Microscopically, both strain structures were similar and typical of *P. expansum*: conidiophores were hyaline, terverticillate, with the branches appressed against the main axis. The stipes were smooth-walled on PDA and finely roughened on MEA. Phialides were cylindrical with a short distinct neck. Conidia were ellipsoidal when young and then turned subglobose, greenish and smooth-walled (data not shown). The microscopic and macroscopic characteristics of the complemented strain *PeΔpatL:patL* were similar to those of the wild-type strain.

Effect of *patL* deletion on patulin production *in vivo*

In vivo, the mutant strain *PeΔpatL1* can cause blue mould on Golden Delicious apples, but not in the same way as the wild-type strain. The wild-type and mutant strains *PeΔpatL1* shared the same development profile for the first 4 days. A slowdown was

Growth curves of rotten spots

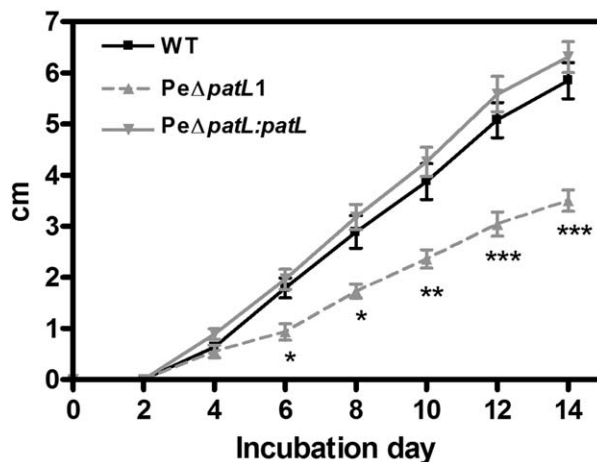


Fig. 2 Growth curves of the rotten spots on Golden Delicious. Golden Delicious apples were infected with each strain. The diameters of the rotten spots were measured every 2 days of the incubation period. Graphs show the mean \pm standard error of the mean (SEM) from six replicates. Asterisks denote significant differences between the wild-type NRRL 35695 (WT), the mutant *PeΔpatL1* and the complemented *PeΔpatL:patL* strains: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

then observed for the mutant strain *PeΔpatL1*. The complemented strain *PeΔpatL:patL* presented the same developmental profile as the wild-type strain. Growth rates were calculated from the growth curves. When compared with the wild-type strain, the growth rate was significantly lower after the fourth day for the mutant strain *PeΔpatL1* (0.27 ± 0.02 cm/day). Meanwhile, no significant differences were observed between the wild-type and the complemented strain (0.48 ± 0.03 cm/day and 0.50 ± 0.05 cm/day, respectively) (Fig. 2).

At the end of the incubation time, the rot volume was determined as described previously by Baert *et al.* (2007). The volume of rot in apples infected with the deleted strain was three times lower than that in apples infected with the wild-type strain or the complemented strain (Fig. 3). This decrease in growth in Golden Delicious apples has been observed with two other mutants (Fig. S5, see Supporting Information). Apples were ground and patulin production was assessed. In apples inoculated with the wild-type strain and the complemented strain, a large amount of patulin was detected. After 14 days of incubation, the patulin concentration detected averaged $4.9 \mu\text{g/g}$ apple for the wild-type strain and $11.3 \mu\text{g/g}$ apple for the complemented strain. As expected, no patulin was detected in apples infected with the deficient strain (Fig. 4).

Influence of apple varieties on the development of rot and on patulin production

In order to evaluate whether this higher capacity of patulin-producing *P. expansum* infecting apples was also conserved in

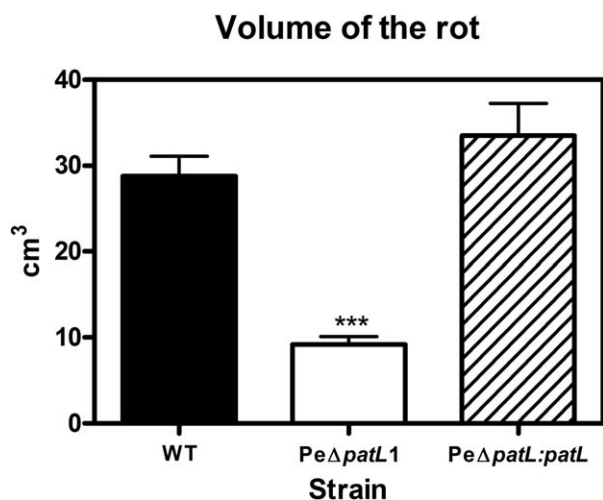


Fig. 3 Volume of rot on Golden Delicious apples. Following 14 days of incubation, rot volumes were calculated using the method described by Baert *et al.* (2007). Graphs show the mean \pm standard error of the mean (SEM) from six replicates. *** $P < 0.001$. WT, wild-type.

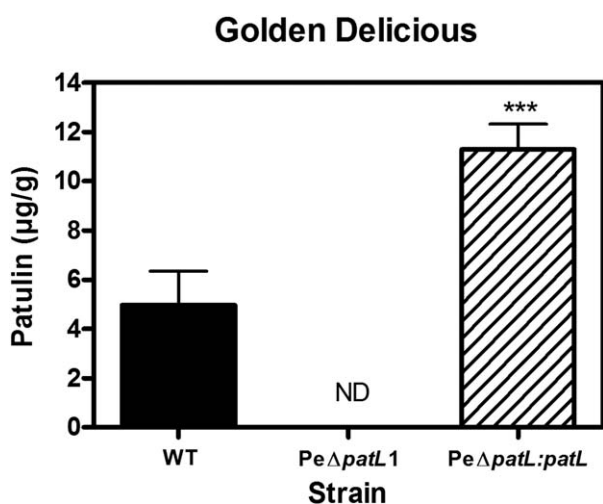


Fig. 4 Patulin production on Golden Delicious. Patulin production was evaluated by high-performance liquid chromatography with diode array detector (HPLC-DAD) at 270 nm after 14 days on Golden Delicious. Graphs show the mean \pm standard error of the mean (SEM) from six replicates. *** $P < 0.001$. ND, not detected; WT, wild-type.

different varieties of apple, in addition to Golden Delicious, 12 different varieties were infected by the mutant strain and the results were compared with apples of the same varieties infected by the wild-type strain.

Among the different apple varieties tested, most presented significant differences in the development of rotten spots, growth rates and rot volumes.

With regard to the development of lesion diameter, significant differences were observed on Pink Lady, Ariane, Marie Ménéard,

Saireline, Bedan, Fréquin Rouge, Avrolle and Petit Jaune between apples infected with the wild-type and mutant strains. The apple variety Reinette grise du Canada seems to present the same tendency (Fig. S6, see Supporting Information).

Concerning the growth rate of rotten spots, significant differences were observed in apples infected with the wild-type compared with the mutant for the following varieties: Pink Lady, Ariane, Marie Ménéard, Saireline, Fréquin Rouge, Avrolles and Petit Jaune (Table 1). At the end of the incubation time, the rot volume was also determined, as described previously by Baert *et al.* (2007). Significant differences were observed for Pink Lady, Ariane, Marie Ménéard, Fréquin Rouge, Avrolles and Petit Jaune apple varieties (Table 1). At the end of the experiment, apples were ground and patulin was extracted. In apples infected with the wild-type strain, large amounts of patulin were detected at day 14. Total amounts varied greatly (from 1.11 to 37.39 $\mu\text{g/g}$) depending on the variety. As expected, patulin was not detected in apples infected with the deleted strain (Table 2).

Effect of exogenous patulin application on virulence and aggressiveness

First, a kinetic study of patulin production was performed in order to determine the time at which patulin reached a detectable limit. Only the rotten part of the apples was extracted and patulin was quantified. No patulin was detected in apples infected by the wild-type strain for the first 3 days; then, 1.6, 4.3 and 6.3 $\mu\text{g/g}$ of patulin were detected at days 4, 5 and 6, respectively (Fig. 5). These results are in accordance with the developmental profiles of blue mould observed on apples, suggesting that the growth rate increases in the presence of patulin.

In order to assess whether patulin restores the aggressiveness of the mutant strain, patulin was added directly to the wound used to inoculate the apples at day 4, the first day on which patulin was detected in the kinetic study. The development of the rotten spot and the growth rate were evaluated. As demonstrated previously, the mutant strain Pe Δ patL1 causes blue mould on Golden Delicious apples, but not in the same way as the wild-type strain. However, when 50 μg of patulin were added to the infection point, the growth rate was restored, and the mutant strain grew as rapidly as the wild-type strain (Fig. 6). This result demonstrates that patulin is an aggressiveness factor, facilitating the growth of the fungus in the flesh of the fruit.

DISCUSSION

Although the mycotoxin patulin is produced by different genera and species, the main producer is *P. expansum*, which is responsible for blue mould decay on apples. *Penicillium expansum* infects apples before or during harvest via injuries. However, symptoms, such as rotten spot, decay and patulin production, appear only later during storage.

Table 1 Analysis of the growth rate and rot volume for apples infected with the wild-type NRRL 35695 (WT) and mutant *PeΔpatL1* strains.

Apple variety	Growth rate (cm/day)		Rot volume (cm ³)		
	WT	<i>PeΔpatL1</i>	WT	<i>PeΔpatL1</i>	
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	
Dessert apples	Pink Lady	0.51 ± 0.01	0.25 ± 0.01*	33.11 ± 0.40	6.69 ± 0.30*
	Ariane	0.52 ± 0.02	0.37 ± 0.01*	27.77 ± 2.46	15.74 ± 3.68*
	Granny Smith	0.48 ± 0.04	0.44 ± 0.02	30.82 ± 2.24	27.11 ± 3.12
	Reinette grise du Canada	0.45 ± 0.04	0.37 ± 0.04	24.86 ± 0.12	21.39 ± 1.3
	Braeburn	0.49 ± 0.01	0.49 ± 0.02	20.72 ± 2.32	26.25 ± 3.48
	Fuji	0.53 ± 0.01	0.49 ± 0.01	21.05 ± 1.92	17.64 ± 0.41
Cider apples	Marie Ménard	0.46 ± 0.01	0.37 ± 0.04*	31.27 ± 3.52	13.51 ± 4.26*
	Saireline	0.41 ± 0.04	0.21 ± 0.01*	21.83 ± 5.8	4.2 ± 0.95
	Bedan	0.39 ± 0.01	0.33 ± 0.02	12.91 ± 0.66	11.66 ± 0.76
	Fréquin Rouge	0.40 ± 0.02	0.24 ± 0.01**	17.26 ± 1.9	3.22 ± 0.44*
	Avrolles	0.40 ± 0.01	0.30 ± 0.01**	16.79 ± 1.1	6.15 ± 0.07**
	Petit Jaune	0.47 ± 0.05	0.18 ± 0.02**	26.64 ± 7.08	2.77 ± 0.64*

Growth rates were calculated from the growth curves of the rotten spots, and the rot volumes were calculated using the method described by Baert *et al.* (2007). Results show the mean ± standard error of the mean (SEM) from three replicates. Asterisks denote significant differences highlighted in bold between the wild-type NRRL 35695 and mutant *PeΔpatL1* strains: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2 Analysis of patulin production in apples infected with the wild-type NRRL 35695 (WT) and mutant *PeΔpatL1* strains.

Apple variety	Patulin production (µg/g)		
	WT	<i>PeΔpatL1</i>	
	Mean ± SEM	Mean ± SEM	
Dessert apples	Pink Lady	2.77 ± 0.44	ND
	Ariane	10.37 ± 2.30	ND
	Granny Smith	1.11 ± 0.1	ND
	Reinette grise du Canada	1.96 ± 0.22	ND
	Braeburn	8.47 ± 3.11	ND
	Fuji	22.8 ± 2.6	ND
Cider apples	Marie Ménard	37.39 ± 2.02	ND
	Saireline	9.24 ± 0.07	ND
	Bedan	2.01 ± 0.75	ND
	Fréquin Rouge	15.44 ± 1.99	ND
	Avrolles	2.64 ± 0.25	ND
	Petit Jaune	3.54 ± 1.81	ND

ND, not detected; SEM, standard error of the mean.

Patulin is the final product of a succession of enzymatic reactions constituting the biosynthetic pathway. Genes encoding these enzymes are gathered in a 40-kb DNA cluster (Tannous *et al.*, 2014). In this cluster, some genes code for enzymes that are directly involved in the biosynthetic pathway, whereas others encode one transcription factor and three transporters. BLAST analysis has revealed that the *patL* gene encodes a protein which presents several characteristic patterns of a Cys₆ zinc finger regulatory factor. Generally, in a mycotoxin's biosynthetic cluster, a specific transcription factor plays the role of an 'on/off' switch between production and no-production states. To date, the biolog-

Kinetics of patulin production in Golden Delicious

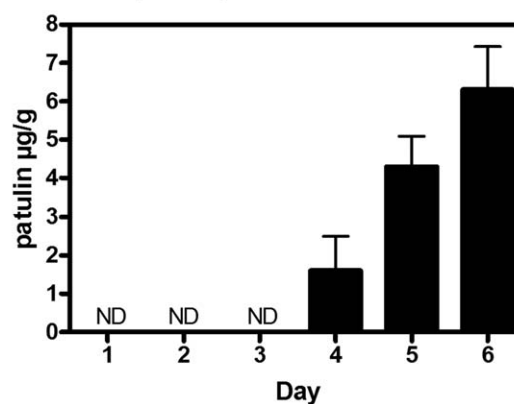


Fig. 5 Kinetic study of patulin production in apples. For each post-inoculation period, the mouldy part of the apples was extracted and patulin production was quantified by high-performance liquid chromatography with diode array detector (HPLC-DAD). Graphs show the mean ± standard error of the mean (SEM) from three replicates. ND, not detected.

ical role of fungal secondary metabolites has not been elucidated, but some studies have suggested a role in pathogenicity during host infection. For example, *Fusarium graminearum* strains which produce more DON are more virulent than others (Boddu *et al.*, 2007). Another notable example of a polyketide involved in plant pathology is the T-toxin produced by the fungal pathogen *Cochliobolus heterostrophus*, which imparts to this species a virulence towards maize (Yang *et al.*, 1996).

Supported by the fact that the importance of mycotoxins in plant pathogenesis has been described previously for other diseases, some studies have focused on the role of patulin in the

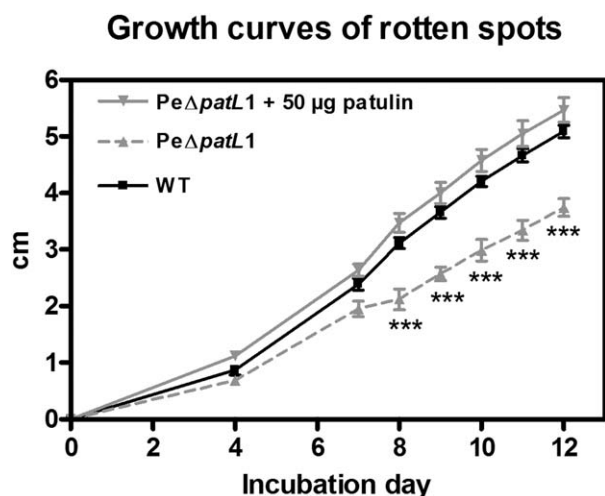


Fig. 6 Effect of exogenous patulin application on aggressiveness. Golden Delicious apples were infected with each strain and 50 μ g of patulin were added after the fourth day of incubation. Diameters of rotten spots were measured during the incubation period and growth rates were calculated. Graphs show the mean \pm standard error of the mean (SEM) from seven replicates. *** $P < 0.001$. WT, wild-type.

aggressiveness of *P. expansum*. A recent study has shown that patulin may be involved in the pathogenicity and virulence of *P. expansum* (Sanzani *et al.*, 2012). These authors disrupted the *patK* gene encoding the biosynthetic enzyme 6-methylsalicylic acid synthase (*Pe*pk5) in order to study the role of patulin in the virulence of *P. expansum*. The mutants obtained still produced patulin, but to a lesser extent than the wild-type strain. On Golden Delicious apples inoculated with the mutants, disease incidence and severity were lower than in apples inoculated with the wild-type strain. The authors restored the wild-type phenotype by the artificial addition of patulin to the inoculation point, suggesting that patulin acts as a virulence factor. By using the RNAi (ribonucleic acid interference) technique, Barad *et al.* (2014) generated *idh*-RNAi mutants exhibiting a down-regulation of *idh* and showing reduced patulin production. As in the previous study, the authors observed a reduction in disease incidence and severity. However, in both studies, mutants generated by either knock-out or RNAi still produced residual patulin amounts; thus, disease incidence was still observed, and it was difficult to draw definitive conclusions.

In the present study, in order to clearly determine the involvement of patulin in the ability of *P. expansum* to cause disease, the gene *patL*, encoding the specific regulatory factor of the patulin biosynthetic pathway, was deleted. The deficient Pe Δ patL mutant was then tested on 13 different apple varieties, seven dessert apples intended for direct consumption and six for cider production. The results of the present study clearly demonstrate that patulin acts as an aggressiveness factor for the vast majority of varieties. First, we observed that the absence of the *patL* gene in *P. expansum* caused a total abolition of expression of all the

genes within the cluster, and no patulin was produced, as shown previously by Li *et al.* (2015). In addition, the present study included the complemented Pe Δ patL:*patL* strain which strengthened the results.

When the mutant was used to inoculate Golden Delicious apples, we showed that it did not produce patulin in the apple, but, in the first 4 days, induced disease in a manner similar to the wild-type strain. Then, a decrease in the rate of rot progression was observed for the mutant compared with the wild-type strain. At this stage, the rate of progression of the rotten spot was correlated with patulin production. The reintroduction of the gene restored the wild-type phenotype and behaviour. According to the definition of Van der Plank (1965) (virulence is a qualitative term defining the ability to cause disease and aggressiveness describes the severity of disease), our results show that patulin can definitely be considered as an aggressiveness factor, but not as a virulence factor. This conclusion was strengthened by the fact that the addition of patulin to a Pe Δ patL mutant restored its rate of progression in apples. Towards the end of the present work, two very recently published studies using *patL* and *patK* gene knockout mutants demonstrated that patulin is not required by *P. expansum* to infect Golden Delicious and Fuji apples, respectively (Ballester *et al.*, 2015; Li *et al.*, 2015). The results obtained in the present study provide additional information to their conclusions. The subtleties of interpretation between the different studies can be explained as follows. Although they used Golden Delicious apples in their pathogenicity assays, Ballester *et al.* (2015) only measured disease incidence (i.e. percentage of infected wounds) at 5 days post-inoculation, therefore concluding that patulin is not a virulence factor indispensable for a successful infection of apples by *P. expansum*. A post-inoculation period that is too short (5 days), a different wild-type strain and differences in the conditions of their pathogenicity assays might explain why these authors did not notice a growth decrease for the deletion mutants. In the study by Li *et al.* (2015), the only apple variety studied was the Fuji cultivar. In the present study, we observed that this cultivar belongs, with the Granny Smith and Braeburn varieties, to the limited group of apples in which no difference is observed between the wild-type and Pe Δ patL strains.

The results of our study clearly demonstrate that patulin acts as an aggressiveness factor in most apple varieties. The importance of mycotoxins in the aggressiveness of some fungal pathogens has already been described. For example, DON produced by *F. graminearum* is necessary for disease severity, but not for the initial infection of wheat ears (Bai *et al.*, 2001), and is considered as an aggressiveness factor (Maier *et al.*, 2006; Proctor *et al.*, 2002). Another example is that of phytotoxins produced by *Botrytis cinerea*. In this case, botrydial and botcinic acid are required to kill host cells and for the colonization of plant tissues (Dalmais *et al.*, 2011). In the case of patulin, the toxin is produced after the third day, and clearly facilitates the colonization of Golden

Delicious and most of the apple varieties tested by *P. expansum*. Currently, only one report has described the effect of patulin on apple cells. Patulin's ability to directly induce cell death was tested and the results indicated a three- to four-fold increase in cell death in apple tissue (Barad *et al.*, 2014). This could explain the positive effect of patulin on the development of disease.

The morphological changes in *P. expansum* between the wild-type and the mutant *PeΔpatL1* strains are surprising. The gene *patL* is expected to encode only the specific regulatory factor for the patulin biosynthetic pathway, but the mutant strain *PeΔpatL1* presented some morphological changes. Exudate is only present in the wild-type strain culture and is mainly composed of patulin (data not shown). Two hypotheses can be proposed: the first is that the gene *patL* is a specific transcription factor, but, although it is located within the patulin biosynthetic gene cluster, it affects the expression of numerous genes outside of the cluster. Such a regulation has already been observed for the *Tri6* gene in the wheat pathogen *F. graminearum*. This gene is located in the trichothecene gene cluster, but regulates not only the genes within the cluster, but also numerous other genes involved in growth and development (Nasmith *et al.*, 2011; Seong *et al.*, 2009). The second hypothesis is that patulin influences the development of *P. expansum*. However, to date, no study has demonstrated the positive involvement of mycotoxins in fungal development, and so the first hypothesis is more probable.

In conclusion, among the studies on the involvement of patulin in the pathogenicity of *P. expansum*, all, except one, were conducted on Golden Delicious apples. In this study, several apple varieties were tested in addition to Golden Delicious: six apple varieties intended for direct consumption and six apple varieties used for the production of cider. Our study clearly demonstrates that patulin is not required to infect apples, but acts as an aggressiveness factor in most apple cultivars. We also observed that *P. expansum* behaved in a similar manner on apple cultivars sharing the same genetic background. Indeed, a decrease in growth rate was observed on Golden Delicious cultivar apples and its descendants, Pink Lady and Ariane (Brown and Maloney, 2005; Noiton and Alspach, 1996). On the contrary, no difference was observed on Granny Smith and its descendants, Braeburn and Fuji. These observations may suggest the existence of an inheritable trait explaining these results. One can also imagine that the composition of apples (sugars, phenolic compounds, such as flavonoids) and the oxidant ability of the host in general will greatly influence the development of the disease.

EXPERIMENTAL PROCEDURES

Chemicals

Patulin was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All solvents used in the extraction and high-performance liquid

chromatography (HPLC) were analytical grade. Water for HPLC with the diode array detector (DAD) was purchased from Merck (Darmstadt, Germany). Water for the molecular biology procedures was purified using a Millipore MilliQ purification system (Billerica, MA, USA).

Strains, media and culture conditions

Penicillium expansum NRRL 35695 (USDA-ARS National Center for Agricultural Utilization Research, Peoria, IL, USA), initially isolated from grape berries in Languedoc Roussillon (France), was used in this study. It was cultured on PDA medium (Sigma-Aldrich) at 25°C for 7 days in the dark. Cultures intended for the preparation of protoplasts were grown for 12 h in 250 mL of yeast extract peptone dextrose (YPD) medium (20 g dextrose, 10 g yeast extract, 20 g bacto-peptone per litre) on an orbital shaker set at 25°C and 150 rpm. Cultures were inoculated with 3×10^7 conidia.

Saccharomyces cerevisiae FY1679: MATa/MAT α ura3-52/ura3-52; trp1 Δ 63/TRP1; leu2 Δ 1/LEU2; his3 Δ 200/HIS3; GAL2/GAL2 was used in order to obtain the Δ *patL* gene disruption cassette by homologous recombination.

Escherichia coli Top 10F (Invitrogen, Carlsbad, CA, USA) was used for plasmid propagation.

Apple varieties

Two sorts of apples were used in this study: apples designated for direct consumption (seven varieties) and apples designated for cider production (six varieties). Apples designated for direct consumption were purchased from a supermarket in Toulouse (France), and cider apples were provided by the Institut Français des Productions Cidricoles (IFPC, Sées, France) and analysed 7 days after harvest.

Strategy to obtain *PeΔpatL* mutant and complemented strain

Construction of the Δ *patL* gene disruption cassette

The cluster of genes involved in the biosynthesis of patulin has been sequenced recently from *P. expansum* NRRL 35695 (Tannous *et al.*, 2014). Specific primers of the *patL* gene were designed from the *P. expansum* patulin cluster sequence deposited in GenBank (KF899892), and all other primers used in this study are listed in Table S1 (see Supporting Information).

A gene replacement cassette Δ *patL* carrying the hygromycin resistance cassette, flanked by DNA sequences corresponding to the sequences located upstream and downstream of the *patL* gene, was constructed using homologous recombination in *S. cerevisiae*. The strategy employed is detailed in Fig. S7 (see Supporting Information). The fragments required for the construction of the gene disruption cassette were amplified by PCR and assembled by homologous recombination on the pRS426 cloning vector after transformation of the fragment and the vector in *S. cerevisiae*. Briefly, a 1179-bp DNA fragment matching with the promoter and the 5' untranslated region of the *patL* gene and a 1049-bp fragment matching with the 3' untranslated region of the *patL* gene were amplified from the genomic DNA of *P. expansum* NRRL 35695 strain using two pairs of primers, 5fdebPatLPex/DebPatLPex1Rhygro and FinPatLPex1Fhygro/3rfinPatLPex, respectively. The pair of primers hphF1/hphR1 was used to amplify a 4107-bp fragment encoding the hygromycin resistance cassette,

containing the *hph* gene under the control of the *Aspergillus nidulans* *gpdA* promoter and *A. nidulans* *tpcC* terminator. This cassette was amplified from the pAN7.1 expression vector (accession number Z32698). The sequences underlined in primers DebPatLPex1Rhygro and FinPatLPex1Fhygro (Table S1) were added at their 5' ends. These sequences are identical to the extremities of the PCR product generated by the primers hphF1/hphR1 in order to promote recombination between PCR products. The sequences underlined in primers 5fdebPatLPex and 3rfinPatLPex were added at their 5' ends. These sequences are identical to the extremities of the pRS426 cloning vector used for the homologous recombination in *S. cerevisiae*. The three amplicons were purified using a GenElute™PCR Clean-Up Kit (Sigma-Aldrich), as described in the procedure specified by the manufacturer. The PCR conditions for the amplification of the 5' junction and 3' junction were as follows: denaturation at 98°C for 30 s, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. A final extension step at 72°C for 10 min was performed. The PCR conditions for the amplification of the hygromycin resistance cassette were the same as described previously, except for the cycle extension, which was performed at 72°C for 2 min. The Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Illkirch, France) was used for PCRs.

The two junctions were co-transformed into yeast, together with the hygromycin resistance cassette and the pRS426 cloning vector. Homologous recombination creates a circular construct. The procedure allowing yeast transformation was achieved according to Colot *et al.* (2006) with a few changes. Before yeast transformation, the pRS426 cloning vector was digested by *Bam*HI and *Hind*III and, for yeast DNA extraction, the Fast-prepsystem (MP Biomedical, Illkirch-Graffenstaden, France) with lysing matrix C (MP Biomedical) in yeast lysis buffer was used instead of 0.5-mm glass beads.

After yeast DNA extraction, the resulting linear deletion cassette (6229 bp) was amplified from the pooled yeast DNA with the nested primers dPatL-NestedF and dPatL-NestedR. The following PCR conditions were used: 40 cycles with denaturation at 98°C for 10 s, annealing at 61°C for 30 s and extension at 72°C for 3.25 min. An initial denaturation step at 98°C for 30 s and a final extension step at 72°C for 10 min were performed. The Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used.

This DNA fragment carrying the hygromycin resistance cassette fused to the *patL* flanking regions was used to transform protoplasts of the *P. expansum* NRRL 35695 strain in order to generate the mutated PeΔ*patL*1 strain.

For the complementation experiment, dPatLF3/dPatLR3 primers were used to generate the DNA fragment containing the wild-type *patL* gene from the wild-type strain to transform protoplasts of the PeΔ*patL*1 strain.

Protoplast preparation, transformation and transformant selection

In order to prepare protoplasts of *P. expansum*, a culture grown in shaken YPD liquid medium for 12 h at 25°C was filtered through polyethylene filter (Sigma-Aldrich), washed with NH₄Cl and treated with lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich) (20 mg/mL of MgSO₄, 1.2 M, pH 5.8) for 3 h at 26°C on an orbital shaker set at 60 rpm. After incubation, protoplasts and cellular debris were deposited at the bottom of a

tube containing MS buffer (0.6 M sorbitol and 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.3) as described by Merhej *et al.* (2011). Cellular debris and protoplasts were separated by centrifugation. The white interphase containing protoplasts was withdrawn and washed by centrifugation with TMS buffer (1 M sorbitol and 10 mM MOPS, pH 6.3). Protoplasts were harvested and resuspended in TMS buffer (1 M sorbitol, 10 mM MOPS, 40 mM CaCl₂, pH 6.3).

To 100 μL of protoplasts in TMS buffer (1 × 10⁷ protoplasts/mL), 5 μg of target DNA were added in 60 μL Tris-EDTA-CaCl₂ buffer [10 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid (EDTA), 40 mM CaCl₂, pH 7.5] and 160 μL of 60% polyethylene glycol (PEG) solution were added and mixed. This mixture was added to 50 mL of TopAgar YPDS (10 g/L of yeast extract, 20 g/L of bactopectone, 182 g/L of sorbitol and 7.5 g/L agar) and spread on 10 YPDS plates (10 g/L of yeast extract, 20 g/L of bactopectone, 182 g/L of sorbitol and 15 g/L agar). The plates were incubated in the dark at 25°C for 24 h. For complementation, the wild-type DNA fragment obtained by PCR was co-transformed with 2 μg of the pSM334 plasmid (Marek *et al.*, 1989) containing the geneticin resistance cassette. The following day, 5 mL of TopAgar YPDS containing hygromycin (150 μg/mL) or geneticin (100 μg/mL) were added to the plates, which were then placed at 25°C. Transformants were purified by single-spore isolation and screened for successful insertion as described below.

Nucleic acid manipulation

To confirm the insertion of the hygromycin resistance cassette at the *patL* locus in genomic DNA, PCR followed by enzymatic digestion was performed. First, genomic DNA was extracted as described previously (Puel *et al.*, 2005), and transformants containing the hygromycin cassette were screened using the hygromycin-specific primers hygF and hygR. Then, a PCR with the pair of primers dPatLF3/dPatLR3 was carried out on the selected transformants. These primers are located upstream and downstream of the 5' and 3' junctions of the disruption cassette, respectively. The following PCR conditions were used: 35 cycles of denaturation at 98°C for 10 s, annealing at 61°C for 30 s and extension at 72°C for 3 min and 45 s. An initial denaturation step of 30 s at 98°C and a final extension step at 72°C for 10 min were performed. The Phusion High-Fidelity DNA Polymerase was used.

For the wild-type strain, this PCR generated a 5030-bp fragment, whereas the PeΔ*patL*1 strain generated a 6681-bp fragment. Enzymatic digestions of these PCR products with *Nde*I produced three fragments (122, 1030 and 3878 bp) for the wild-type strain and four fragments (122, 1030, 1570 and 3959 bp) for the PeΔ*patL*1 strain.

Southern blot analysis was performed on 20 μg of genomic DNA digested with *Stu*I restriction enzyme, separated by electrophoresis in a 0.8% agarose gel and transferred onto a nylon membrane Hybond N⁺ (GE Healthcare, Velizy-Villacoublay, France). The membrane was hybridized with biotin-labelled probes synthesized according to the procedure specified by the manufacturer (Li-Cor, Lincoln, NE, USA). The Hyg DNA probe (homologous to the hygromycin resistance cassette) and the PatL DNA probe (homologous to the 3' junction of the *patL* gene) were generated using the primer pairs hygF/hygR and probePatLPeF1/probePatLPeR1, respectively (Table S1). The Hyg DNA probe was amplified from the pAN7.1 expression vector (accession number Z32698) and the PatL DNA probe from the genomic DNA of *P. expansum* NRRL 35695 strain.

Comparative analysis between wild-type, mutant and complemented strains

In vitro growth, and macroscopic and microscopic morphology

All strains of interest were stored on MEA at +4°C. Before the experiments, the strains were grown on MEA plates for 7 days at 25°C. Spore suspensions were prepared according to Adjovi *et al.* (2014). In order to compare radial growth, a calibrated inoculum of 10⁴ spores was centrally placed on both MEA and PDA, incubated at 25°C in the dark and colony diameters were measured after 7 days. For macroscopic and microscopic examinations, a three-point culture was performed on the same media. Macroscopic (colour of conidial areas, colony margin, colony texture, pigmentation, exudate, reverse colour, etc.) and microscopic (conidiophore branching pattern, phialide and conidia shape, ornamentation, etc.) characteristics were observed using a stereomicroscope (Olympus SZX9, ×12–120, Rungis, France) and an optical microscope (Olympus CX41, ×400 and ×1000), respectively.

Analysis of relative expression of the genes from the patulin cluster and patulin production *in vitro*

The culture conditions used for the analysis of the relative expression of the genes from the patulin cluster were the same as described previously by Tannous *et al.* (2014). APAM, a permissive substrate for patulin production, was used (Baert *et al.*, 2007), and plates were incubated for 5 days in the dark at 25°C. Cultures were also performed on Czapek agar medium. Following mycelial growth, total RNA extraction and reverse transcription were performed as described previously by Tannous *et al.* (2014). Real-time PCR was used to quantitatively evaluate the expression of the gene cluster in each strain. The primers used for the housekeeping gene (β -tubulin) and all 15 *pat* genes and the real-time PCR conditions were the same as described by Tannous *et al.* (2014). After incubation, the agar medium was extracted as described previously by Tannous *et al.* (2014), and patulin production was assessed by HPLC-DAD. Six biological replicates were performed for each strain.

Patulin production analysis *in vivo*

The ability to cause blue mould and patulin production by the wild-type and mutant strains was assessed *in vivo* on apples. As described previously by Sanzani *et al.* (2012), apples were surface sterilized in 2% sodium hypochlorite solution and rinsed with water. Apples were then wounded with a sterile toothpick. For each isolate, 10 μ L of a suspension at a concentration of 10⁴ conidia/mL were deposited into the wound. Apples were incubated at 25 °C in the dark for 14 days. The diameters of the rotten spots were measured every 2 days of the incubation period, and the growth rate and rot volume were determined. Patulin was extracted from apples and quantified by HPLC. To determine the rot volume, the method described previously by Baert *et al.* (2007) was used.

At the end of the incubation period, apples were ground in a blender, as described previously by MacDonald *et al.* (2000). An aliquot (10 g) of each sample was digested overnight at room temperature with 150 μ L of *Aspergillus aculeatus* pectinase enzyme (Sigma-Aldrich) and 10 mL of distilled water. Then, the samples were centrifuged at 4500 *g* for 5 min and the supernatants were collected and extracted with 50 mL of ethyl acetate. After overnight shaking, the organic phase was filtered through Whatman paper and evaporated with a ZymarkTurboVap (McKinley Scien-

tific, Sparta, NJ, USA) set at 60°C. The residue was taken up in 300 μ L of methanol–water (80/20, v/v), and this suspension was filtered through a 0.45- μ m pore size filter before injection (25 μ L) into the chromatography apparatus. Patulin production was determined by HPLC-DAD analysis. Six biological replicates were analysed for each strain on Golden Delicious. The same experiment was conducted on different apple varieties with three biological replicates for each of the wild-type and mutant strains.

Kinetics of patulin production in apples by the wild-type strain NRRL 35695

In order to determine the time at which *P. expansum* starts to produce patulin after infecting apples, a kinetic study was performed. Eighteen Golden Delicious apples were inoculated as described above and incubated at 25°C. Every 6 days, three apples were analysed. The diameters of the rotten spots were measured and the rotten part was cut with a margin of 1 cm in the healthy part in order to quantify the patulin produced. Samples were ground and used to quantify patulin as described above. Patulin production was determined by HPLC-DAD analysis.

HPLC-DAD analysis

The chromatography apparatus used for patulin quantification was an Ultimate 3000 HPLC system (Dionex/Thermo Scientific, Courtaboeuf, France). Analyses were performed using a 250 mm × 4.60 mm Gemini 5- μ m C6-Phenyl column (Phenomenex, Torrance, CA, USA) at a flow rate of 0.9 mL/min employing the following gradient elution programme: eluent A was water acidified with 0.2% acetic acid and eluent B was HPLC-grade methanol (Fisher Scientific, Illkirch, France). The elution conditions used were as follows: 0 min, 100% B; 10 min, 90% B; 30 min, 50% B; 45 min, 90% B; from 45 to 50 min, 90% B; from 60 to 65 min, 0% B. The presence of patulin was monitored at a wavelength of 277 nm. Patulin was confirmed by its retention time (min) according to a standard (Sigma-Aldrich), and quantified by measuring the peak area according to a standard curve.

Effect of exogenous patulin application on virulence and aggressiveness

The effect of the exogenous application of patulin on the virulence and aggressiveness of the mutants was assessed. As described previously, apples were surface sterilized, wounded and inoculated with each strain. Apples were incubated at 25°C in the dark. Four days after inoculation, 50 μ g of patulin were added to the wound of the apples inoculated with the mutated strain and incubated for another 8 days. Disease incidence and severity were assessed as described previously.

Statistics

Following Fischer test on equality of variance, one-way analysis of variance (ANOVA) was used to analyse the differences between the different fungal strains. The statistical analysis of the data was carried out with GraphPad Prism 4 software (GraphPad Software, La Jolla, USA). Differences were considered to be statistically significant when the *P* value was lower than 0.05.

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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 Construction of the *PeΔpatL* mutant. Map of the *patL* wild-type (WT) locus and the replacement construct *ΔpatL* containing a hygromycin resistance cassette. The primers used to elaborate a polymerase chain reaction (PCR) strategy to distinguish between wild-type and locus integrated transformants are indicated with arrows. Black bars and grey bar indicate the position of the PatL DNA and Hyg DNA probes, respectively, used in the Southern blot. The *NdeI* and *StuI* sites are also represented.

Fig. S2 Southern blot analysis of genomic DNA of the wild-type strain NRRL 35695 (WT), the *PeΔpatL1* transformant and the complemented transformant *PeΔpatL:patL*. DNA was digested with *StuI* and hybridized with the PatL probe (A) and with the Hyg probe (B), both indicated in the map in Fig. 1. T+ corresponds to the *ΔpatL* cassette of 6.7 kb. (A) The *PeΔpatL1* transformant shows a unique fragment of 4.7 kb consistent with the replacement of the *patL* gene by the *ΔpatL* cassette. The complemented strain shows one fragment of 3 kb corresponding to the replacement of the hygromycin cassette by the wild-type copy of *patL*. (B) Only the *PeΔpatL1*

transformant shows a unique fragment of 4.7 kb consistent with the replacement of the *patL* gene by the *ΔpatL* cassette. No hybridization is observed for the wild-type and the complemented strain, as expected if the *ΔpatL* cassette is absent.

Fig. S3 Patulin production on apple puree agar medium (APAM). Patulin production was evaluated by high-performance liquid chromatography with diode array detector (HPLC-DAD) at 277 nm after 5 days on APAM. Graphs show the mean \pm standard error of the mean (SEM) from six replicates. Asterisks denote significant differences between the wild-type strain NRRL 35695 (WT) and the complemented strain *PeΔpatL:patL*: *** $P < 0.001$. ND, not detected.

Fig. S4 Expression of the patulin biosynthesis genes in the wild-type strain NRRL 35695 (WT), the mutant strain *PeΔpatL1* and the complemented strain *PeΔpatL:patL*. Values were normalized to β -tubulin as reference gene. Data are represented as the mean \pm standard error of the mean (SEM) from six replicates.

Fig. S5 Growth curves of rotten spots, growth rate and volume of rot on Golden Delicious infected with the wild-type strain NRRL 35695 (WT), the mutated strain *PeΔpatL1* and two other mutants (*PeΔpatL2* and *PeΔpatL4*).

Fig. S6 Growth curves of rotten spots on various apple varieties. Apples were infected with the wild-type strain NRRL 35695 (WT) and the mutated strain *PeΔpatL1*. Diameters of rotten spots were measured every 2 days of the incubation period. Graphs show the mean \pm standard error of the mean (SEM) from three replicates. Asterisks denote significant differences between the wild-type strain NRRL 35695 and the mutated strain *PeΔpatL1*: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. S7 Strategy used to construct the deletion cassette *ΔpatL*. The 5' junction (5J) and 3' junction (3J) were amplified from the wild-type strain. Primers A and D 5'-extremities are identical to the extremities of the linearized pRS426 cloning vector. Primers B and C 5'-extremities are identical to the extremities of the PCR product generated by primers E and F. Primers E and F were used to amplify the hygromycin resistance cassette. Primers G and H were used to amplify the deletion cassette *ΔpatL* from the DNA extracted from transformed *S. cerevisiae*. A, 5fdebPatLPex; B, DebPatLPex1Rhygro; C, FinPatLPex1Fhygro; D, 3rfinPatLPex; E, hphF1; F, hphR1; G, dPatL-NestedF; H, dPatL-NestedR.

Table S1 Primers used in this study.