Impact of *veA* on the development, aggressiveness, dissemination and secondary metabolism of *Penicillium expansum*

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SUMMARY

Penicillium expansum, the causal agent of blue mould disease, produces the mycotoxins patulin and citrinin amongst other secondary metabolites. Secondary metabolism is associated with fungal development, which responds to numerous biotic and abiotic external triggers. The global transcription factor VeA plays a key role in the coordination of secondary metabolism and differentiation processes in many fungal species. The specific role of VeA in *P. expansum* remains unknown. A null mutant PeΔ*veA* strain and a complemented PeΔ*veA*:*veA* strain were generated in *P. expansum* and their pathogenicity on apples was studied. Like the wild-type and the complemented strains, the null mutant PeΔ*veA* strain was still able to sporulate and to colonize apples, but at a lower rate. However, it could not form coremia either *in vitro* or *in vivo*, thus limiting its dissemination from natural substrates. The impact of *veA* on the expression of genes encoding proteins involved in the production of patulin, citrinin and other secondary metabolites was evaluated. The disruption of *veA* drastically reduced the production of patulin and citrinin on synthetic media, associated with a marked down-regulation of all genes involved in the biosynthesis of the two mycotoxins. Moreover, the null mutant PeΔ*veA* strain was unable to produce patulin on apples. The analysis of gene expression revealed a global impact on secondary metabolism, as 15 of 35 backbone genes showed differential regulation on two different media. These findings support the hypothesis that VeA contributes to the pathogenicity of *P. expansum* and modulates its secondary metabolism.

Keywords: apples, citrinin, pathogenicity, patulin, *Penicillium expansum*, secondary metabolism, veA.

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INTRODUCTION

Penicillium expansum is a ubiquitous fungus of soil origin that can be found on the peel of pome and stone fruits. During post-harvest processing and storage, this phytopathogen develops on wounds inside the fruit provoking maceration and spoilage. *Penicillium expansum* is the primary cause of blue mould and decay of apples, making them unfit for consumption and causing serious economic losses (Nunes, 2012). In addition, *P. expansum* produces a variety of toxic secondary metabolites, known as mycotoxins. Patulin, one of the main products of the secondary metabolism of *P. expansum*, is detected not only in fruits, but also in apple-based products. Patulin has several deleterious effects on human health (Assunção *et al.*, 2016; Puel *et al.*, 2010; Zouaoui *et al.*, 2016). As a result of its toxicity, the maximum permitted level of patulin is regulated in most European countries (EC Commission Regulation, 2006).

In addition to patulin, *P. expansum* produces numerous secondary metabolites and mycotoxins *in vitro*, such as citrinin, which has also been found in fruits (Martins *et al.*, 2002). Citrinin has nephrotoxic and teratogenic effects on mammals and chickens, respectively (Ciegler *et al.*, 1977). In addition to other secondary metabolites, *P. expansum* is able to synthesize roquefortine C (Andersen *et al.*, 2004), chaetoglobosins (Andersen *et al.*, 2004), expansolides (Massias *et al.*, 1990), fumaryl-DL-alanine (Birkinshaw *et al.*, 1942), andrastin (Kim *et al.*, 2012) and communesins (Andersen *et al.*, 2004). Although the ecological roles of secondary metabolites often remain unclear, some are involved in virulence during infection, defence against other microorganisms and communication (Macheleidt *et al.*, 2016). For instance, patulin plays a role in pathogenicity during apple infection as a cultivar-dependent aggressiveness factor that promotes the colonization of apples (Snini *et al.*, 2016). Genes encoding enzymes and proteins involved in the biosynthesis of secondary metabolites are often grouped into clusters. The patulin cluster comprises 15 genes (*patA*–*patO*) (Li *et al.*, 2015; Tannous *et al.*, 2014) and the putative cluster of citrinin includes nine genes (Pexp_005510–Pexp_005590) (Ballester *et al.*, 2015; He and Cox, 2016). A gene encoding a specific transcription factor, often located inside the cluster, activates all the genes in each cluster: *patL* (*P. expansum*) (Ballester *et al.*, 2015; Snini *et al.*, 2016) and *mrl3*/*ctnA*

(*P. citrinum/P. expansum*) (He and Cox, 2016; Li *et al.*, 2017) for patulin and citrinin clusters, respectively. Bioinformatics analysis has shown that the genome of *P. expansum* contains other identified or predicted clusters of secondary metabolites (Ballester *et al.*, 2015; Nielsen *et al.*, 2017).

Growth, morphological development and the production of secondary metabolites are interconnected and controlled by global transcription factors encoded by genes located outside the biosynthesis gene clusters (Calvo *et al.*, 2002). These genes respond to numerous environmental stimuli, including pH, oxidative stress, temperature, nitrate, carbon source, iron and light. Among them, VeA is a phosphoprotein that belongs to the Velvet family, which is composed of three other proteins: VelB, VosA and VelC. VeA is involved in the regulation of several cellular processes: morphogenesis, response to oxidative stress, light-dependent control of sexual or cleistothecial/sclerotial formation, asexual development or conidiation and secondary metabolism, but sometimes also virulence (Kim *et al.*, 2002; Rauscher *et al.*, 2016). In the dark, VeA is transported, together with VelB, to the nucleus, where they form a heterotrimer in association with LaeA, which acts as a positive regulator of sexual development and secondary metabolism (Kumar *et al.*, 2017a; Stinett *et al.*, 2007). The disruption of the *laeA* gene in *P. expansum* leads to a drastic reduction in patulin (Kumar *et al.*, 2017a).

In most cases, VeA acts as a positive regulator of the biosynthesis of secondary metabolites, such as aflatoxin in *Aspergillus parasiticus* and *Aspergillus flavus* (Duran *et al.*, 2007), as well as cyclopiazonic acid and aflatrem in *A. flavus* (Calvo and Cary, 2015), and ochratoxin in *Aspergillus carbonarius* (Crespo-Sempere *et al.*, 2013).

How *veA* affects the virulence of *P. expansum* in apples and its secondary metabolism remains unknown. In the present study, a null mutant PeΔ*veA* strain and a complemented PeΔ*veA*:*veA* strain were generated in *P. expansum*. Pathogenicity was studied on apples. Special emphasis was placed on patulin and citrinin production *in vitro*. The expression of genes belonging to the two corresponding clusters was analysed. The study was then extended to the expression of all genes encoding backbone enzymes of secondary metabolites found in the genome of *P. expansum*.

RESULTS

A PeΔ*veA* mutant was generated by replacing the *veA* gene with a hygromycin resistance marker (Parts S1 and S2, see Supporting Information). This mutant was used to characterize the role of VeA, to understand how this protein affects the virulence of *P. expansum* and how it influences the production of several secondary metabolites.

Deletion of *veA* affects *in vitro* growth, macroscopic and microscopic morphology

After 7 days of incubation, no difference was observed between the radial growth of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains, with colonies of 42 $\pm$ 2, 44 $\pm$ 3 and 43 $\pm$ 2 mm in diameter on potato dextrose agar (PDA) and of 39 $\pm$ 2, 37 $\pm$ 1 and 38 $\pm$ 3 mm on Czapek Dox agar (CzA), respectively. The diameters of the colonies of the WT, null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains grown on malt extract agar (MEA) were 37 $\pm$ 1, 41 $\pm$ 2 and 38 $\pm$ 1 mm, respectively. The diameter of the null mutant colony was thus slightly larger (10%) on this medium. The diameters of the colonies of the WT, null mutant and complemented strains were 50 $\pm$ 4, 44 $\pm$ 3 and 52 $\pm$ 5 mm, respectively, on Czapek glucose agar (CGA). The growth of the mutant PeΔ*veA* strain decreased by 12%.

The morphological aspects of the WT and complemented strains differed from those of the null mutant strain. Macroscopically, all the colonies displayed the usual *P. expansum* blue–green colour in conidial areas. A marked white margin was observed in both WT and complemented strains, but was lacking in the null mutant strain. A bright yellow ring was observed on the edge of the colony on MEA and CzA (Fig. 1A and Part S3, see Supporting Information). Radial furrows were also more pronounced and complete in the null mutant strain than in the WT strain (Fig. 1A and Part S3). With regard to texture, the colonies of the null mutant strain presented reduced fasciculation compared with the other strains (Fig. 1A and Part S3). The surface of the null mutant colony thus appeared planar. The texture was velutinous with conidiophores arising from submerged hyphae, but no coremia, whereas the surface of the other strains displayed aggregation of conidiophores that gave the surface a granular appearance. In colonies of WT and complemented strains, coremia were present on all media (Fig. 1A and Part S3). One other particularity of the null mutant was its inability to produce exudates whatever the medium used (Fig. 1A and Part S3).

Microscopically, the structures of the strains also presented some differences. The WT strain had terverticillate conidiophores with branches tied to the main axis (Fig. 1B). The null mutant strain frequently presented biverticillated conidiophores with inflated metulae (Fig. 1B). The null mutant also showed abnormally larger conidia that resembled ramoconidia (data not shown).

The macroscopic and microscopic characteristics of the complemented strain resembled those of the WT strain (Fig. 1B).

Deletion of *veA* affects colonization, development and dissemination

To assess the impact of *veA* deletion, freshly picked Golden Delicious apples were inoculated with the WT, PeΔ*veA* and

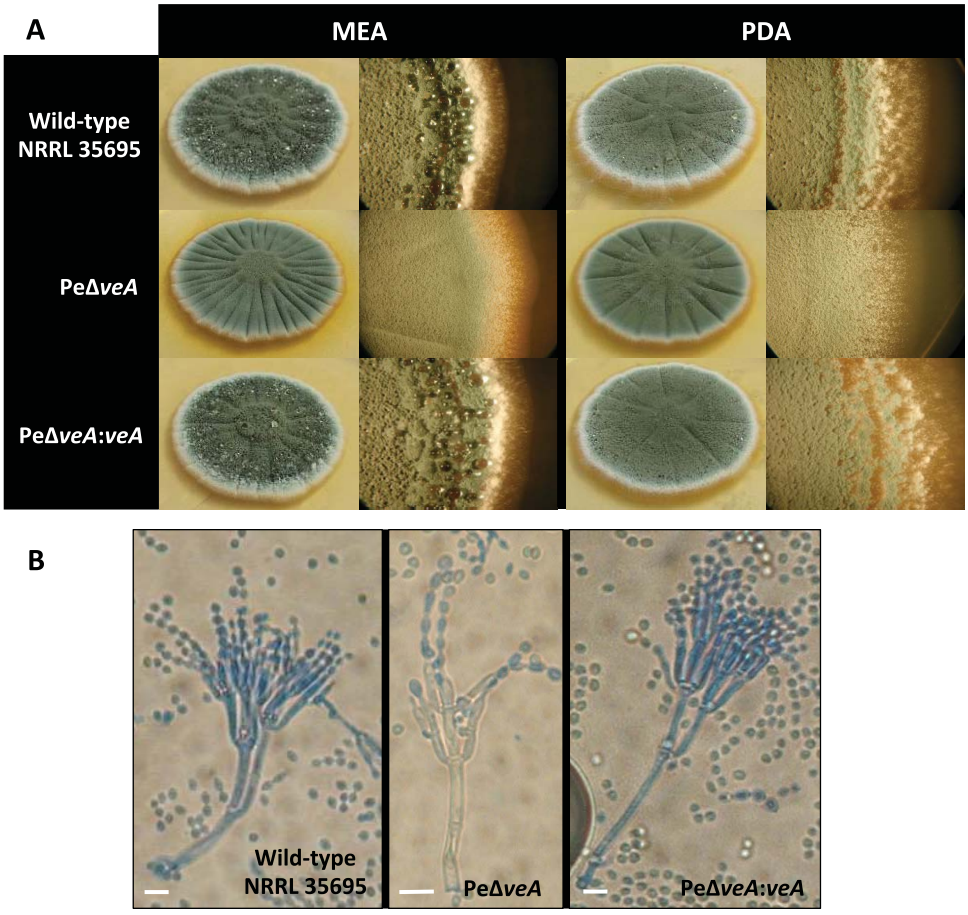

Fig. 1 Morphological appearance of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains. (A) Macroscopic appearance and observations under a stereomicroscope (12×) of colonies after 10 days of culture at 25 °C in the dark on potato dextrose agar (PDA) and malt extract agar (MEA) inoculated with 10^4 spores. (B) Microscopic appearance of conidiophores and conidia (400×) after 7 days of culture at 25 °C in the dark on MEA inoculated with 10^4 spores. The white bars represent 10 μm.

PeΔ*veA*:*veA* strains. During the first 4 days post-inoculation (dpi), the WT and null mutant strains showed the same development profile. A decrease in the speed of development of the null mutant strain was observed from 5 dpi. The development profile of the complemented PeΔ*veA*:*veA* strain resembled that of the WT strain (Fig. 2A). Growth rates calculated from growth curves (Fig. 2B) showed that the null mutant PeΔ*veA* strain grew at a rate of 0.15 cm/day, whereas the WT strain grew at a rate of 0.28 cm/day. No significant differences in growth rate were observed between the WT and complemented strains. At the end of the incubation period, the rot volume was calculated as described previously by Baert *et al.* (2007). The rot volume of apples infected with the null mutant PeΔ*veA* strain was 50% smaller than that of apples infected with either the WT or the complemented strain (Fig. 2C).

The implication of *veA* in the formation of coremia and synnemata was evaluated in the presence or absence of light *in vitro* and *in vivo*. After 7 days on Czapek yeast extract agar (CYA) and MEA, no impact of light or darkness on growth was observed (Part S4, see Supporting Information). On MEA, although PeΔ*veA* did not produce coremia, this strain was able to sporulate (Part S4). Apples were inoculated with spores of the WT and null mutant strains and then incubated for 1 month in the light or in the dark. In the presence of light, *P. expansum* appeared to be unable to pierce the wall of apples or to emerge from the fruit to complete its cycle (Fig. 3A,B). In the dark, the formation of coremia and synnemata was induced in the WT strain (Fig. 3C–E) in contrast with the null mutant strain (Fig. 3F–H). The *veA* gene positively regulates the release of the fungus through the formation of coremia on apple substrate.

Deletion of *veA* depresses mycotoxin production *in vivo* and *in vitro*

The production of patulin was measured in Golden Delicious apples at 14 dpi. Patulin concentrations were 99 ± 20 and 70 ± 21 μg/g fresh weight in apples infected with the WT and complemented strains, respectively (Fig. 4). By contrast, no patulin was detected in apples infected with the null mutant strain.

The capacity of the different strains to produce patulin and citrinin was assessed *in vitro* on two different culture media (Fig. 5). After 5 days, large amounts of patulin were detected in both the WT (24.3 ± 2 mg/g on MEA and 31.1 ± 2.5 mg/g on PDA) and the complemented (29.2 ± 2.4 mg/g on MEA and 22.2 ± 1.6 mg/g on PDA) strains (Fig. 5A). Citrinin was also

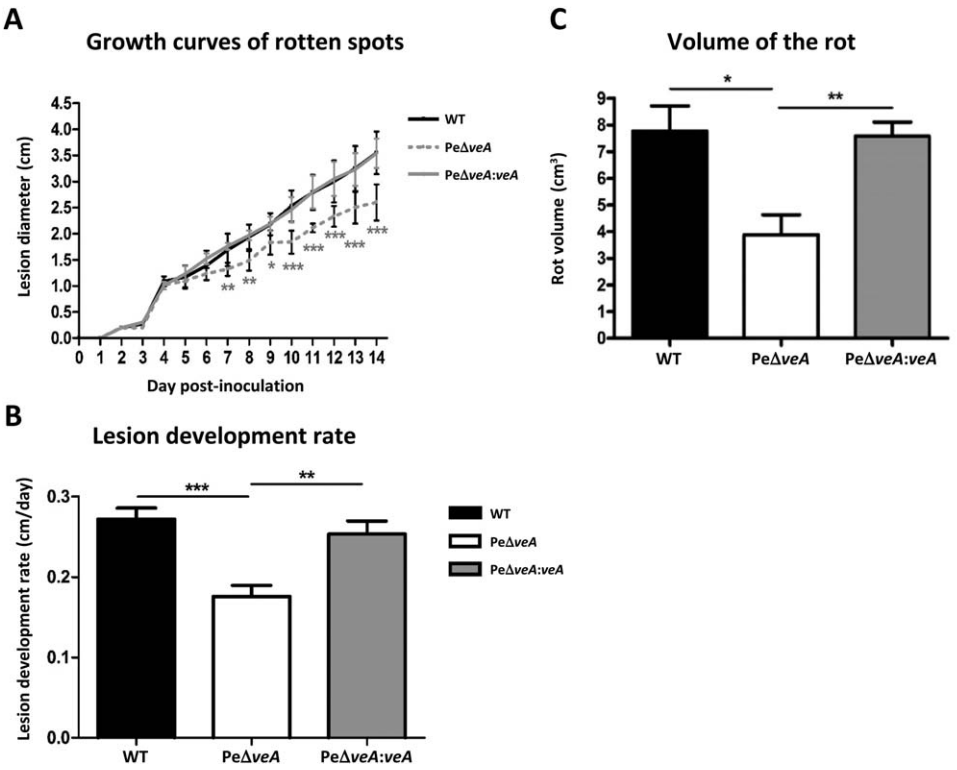

Fig. 2 Growth curves of the rotten spots and rot volume in Golden Delicious apples. (A) Diameters of the rotten spots measured every day of the incubation period. (B) Lesion development rates based on measurements of the rotten spot diameters on Golden Delicious apples. Measurements were taken every day for 14 days. (C) At 14 days post-incubation, rot volumes were calculated using the method described by Baert *et al.* (2007). Apples were inoculated with 10 μL of a 10^4 conidia/mL suspension of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* or complemented PeΔ*veA*:*veA* strain. Apples were kept at 25 °C in the dark. Graphs show the mean ± standard error of the mean (SEM) from six replicates. Asterisks denote significant differences between the WT, null mutant and complemented strains.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

detected, but to a lesser extent in both the WT (3.7 ± 0.25 mg/g on MEA and 0.6 ± 0.2 mg/g on PDA) and the complemented (4.4 ± 0.3 mg/g on MEA and 0.2 ± 0.1 mg/g on PDA) strains (Fig. 5B). Neither patulin nor citrinin was detected in PeΔ*veA* cultures.

The expression of the genes involved in the biosynthesis of patulin (15 genes) and citrinin (nine genes) was assessed on two media (MEA and PDA) and compared in the null mutant, WT and complemented strains. The null mutant strain showed marked down-regulation of all genes compared with the WT strain (Fig. 6). The expression of all genes was restored in the complemented strain (Part S5, see Supporting Information). Amongst the genes that were down-regulated in the null mutant strain, the lowest expression was observed on MEA for *patK*, *patJ*, *patI*, *patO*, *patN*, *patM*, *patA*, *patB* and *patC* in the patulin biosynthesis cluster (Fig. 6A). In the citrinin biosynthesis cluster, *citS* (*Pexp_005520*), *citA* (*Pexp_005530*), *citB* (*Pexp_005540*), *orf6* (*Pexp_005570*) and *citE* (*Pexp_005580*) were the genes most down-regulated on MEA (Fig. 6B). However, *patL* and *ctnA* genes, encoding the transcription factors of the patulin and citrinin clusters, respectively, were only repressed by 40%. The results of the analysis of gene expression on PDA were similar (Part S5).

veA modulates secondary metabolite gene expression

According to the results of bioinformatics analysis and other specific studies, the genome of *P. expansum* exhibits other gene clusters of predicted secondary metabolites. In addition to patulin and citrinin, *P. expansum* produces several secondary metabolites. We analysed the effect of *veA* on the expression of the genes involved in the biosynthesis of these metabolites. We assessed the expression of 35 gene clusters of the 63 that were identified by the antiSMASH 3.0 program from *P. expansum* T01 (GenBank: AYHP00000000.1) and d1 (GenBank: JQFY01000069.1) strain genomes. Backbone genes included polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) and dimethylallyl tryptophan synthases (DMATSs), as categorized by antiSMASH.

The expression of backbone genes was measured in the WT, null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains on two media, MEA and PDA.

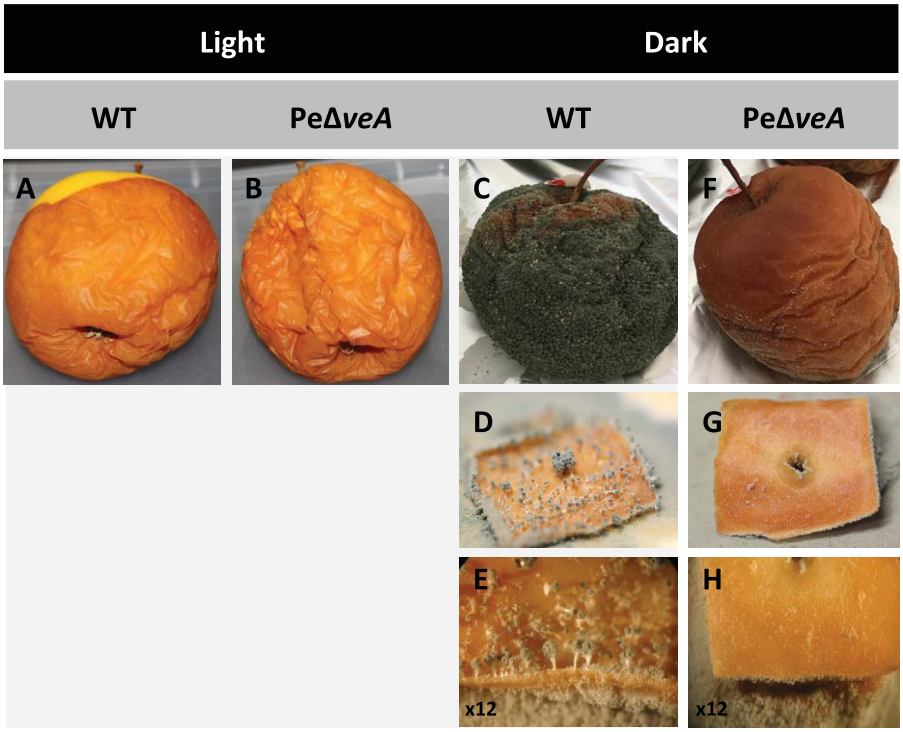

Fig. 3 Implication of *veA* in the development and dissemination of *Penicillium expansum* on Golden Delicious apples. Apples inoculated with the wild-type NRRL 35695 (WT) strain (A) and the null mutant PeΔ*veA* strain (B) stored at 25 °C in the light for 1 month. (C) An apple inoculated with the WT strain and stored at 25 °C in the dark for 1 month. (D) A piece of inoculated apple with the WT strain placed on potato dextrose agar (PDA) and stored for 5 days at 25 °C in the dark. (E) Same sample as in (D) taken under a stereomicroscope (×12). (F) An apple inoculated with the null mutant PeΔ*veA* strain and stored at 25 °C in the dark for 1 month. (G) A piece of apple inoculated with the null mutant PeΔ*veA* strain placed on PDA and stored for 5 days at 25 °C in the dark. (H) Same sample as in (G) taken under a stereomicroscope (×12). Golden Delicious apples were inoculated with 10 μL of a 10^4 conidia/mL suspension of each strain.

After quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis, 35 backbone genes were expressed in either one medium or in both media, and therefore expressed by at least one of the strains used. The expression of seven backbone genes, Pexp_000130, *citS* (Pexp_005520), Pexp_008740, Pexp_015170, Pexp_028920, Pexp_093210 and *patK* (Pexp_094460), was altered by the deletion of *veA* on both media (Fig. 7 and Part S6, see Supporting Information). Conversely, seven backbone genes, Pexp_000410, Pexp_013580, *cnsI* (Pexp_030540), Pexp_037250, Pexp_078820, Pexp_086670 and Pexp_094810, were up-regulated in the null mutant PeΔ*veA* strain on both media. Pexp_094810 and Pexp_000410 were shown to be the two most down-regulated genes by VeA (Fig. 7 and Part S6).

Four of these backbone genes, *citS* (Pexp_005520), *cnsI* (Pexp_030540), Pexp_093210 and *patK* (Pexp_094460), are involved in the biosynthesis of citrinin, communesin, an uncharacterized monodictyphenone-like compound and patulin, respectively (Kumar *et al.*, 2017a; Tannous *et al.*, 2018). The identity of the other clusters remains unknown. The expression of *patK* (Pexp_094460) was somewhat higher on PDA than on MEA, in agreement with the patulin level measured in the two media.

The backbone genes *roqA* (Pexp_030090), Pexp_047050, Pexp_076580, Pexp_095540 and Pexp_096630 showed no difference in their expression between the WT and null mutant strains regardless of the medium used. Pexp_030090 belongs to the cluster involved in the biosynthesis of roquefortine, which was thus not regulated by *veA* on either of the media tested.

Interestingly, regulation by *veA* appeared to be medium dependent in some of the gene clusters. The backbone gene Pexp_074060 was not expressed on PDA, whereas it was down-regulated in the null mutant strain on MEA. Moreover, the

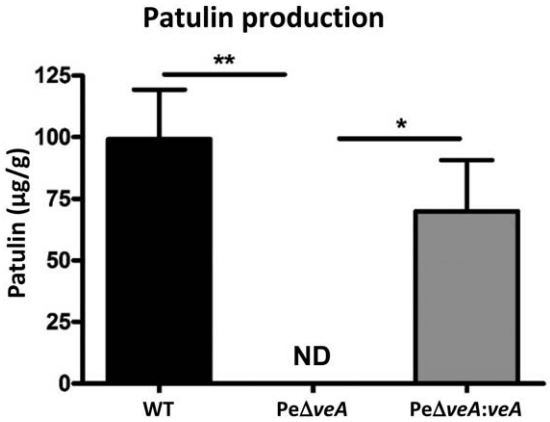

Fig. 4 Patulin production on Golden Delicious apples. Patulin production was measured by high-performance liquid chromatography with a diode array detector (HPLC-DAD) at 277 nm, 14 days post-inoculation on Golden Delicious apples as described previously (Snini *et al.*, 2016). Apples were inoculated with 10 μL of a 10^4 conidia/mL suspension of the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* or complemented PeΔ*veA*:*veA* strain. Apples were kept at 25 °C in the dark. Graphs show the mean ± standard error of the mean (SEM) of eight replicates. Asterisks denote significant differences between the NRRL35695 (WT), null mutant and complemented strains. $*P < 0.05$; $**P < 0.01$; ND, not detectable.

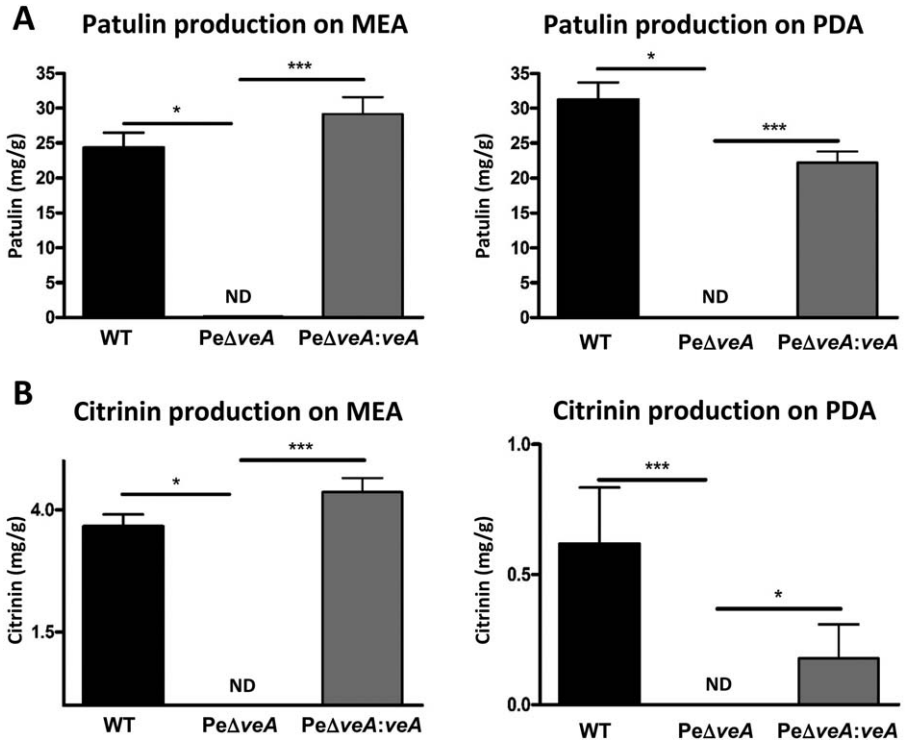

Fig. 5 Patulin and citrinin production on synthetic media by the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains. (A) Production of patulin on malt extract agar (MEA) and potato dextrose agar (PDA). (B) Production of citrinin on malt extract agar (MEA) and potato dextrose agar (PDA). Patulin and citrinin production was measured by high-performance liquid chromatography by a diode array detector (HPLC-DAD) at 277 and 327 nm, respectively, at 5 days post-inoculation at 25 °C in the dark (10^4 spores). Graphs show the mean ± standard error of the mean (SEM) of eight replicates. $^*P < 0.05$; $^{***}P < 0.001$; ND, not detectable.

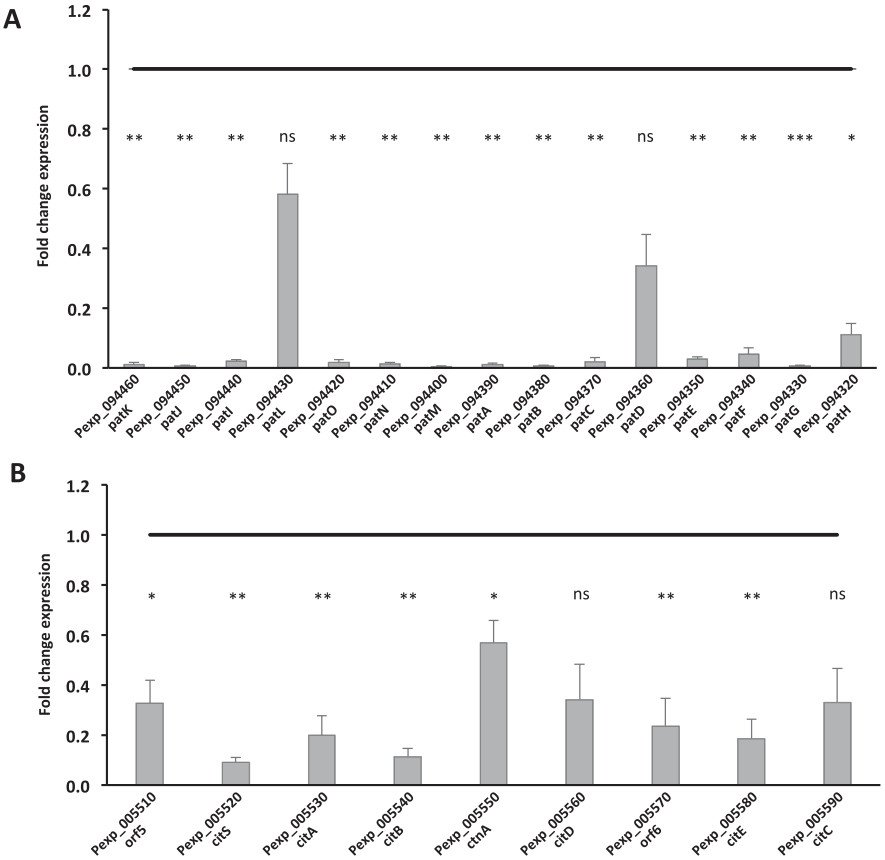

Fig. 6 Fold change expression of genes belonging to the clusters involved in citrinin and patulin biosynthesis in the wild-type NRRL 35695 (WT) and null mutant PeΔ*veA* strains. (A) Patulin cluster (adapted from Tannous *et al.*, 2014). (B) Citrinin cluster (adapted from He and Cox, 2016). The black line represents the expression level of genes in the WT strain. Strains were grown for 5 days at 25 °C in the dark on malt extract agar (MEA). Values were normalized to β-tubulin as housekeeping gene. Graphs show the mean ± standard error of the mean (SEM) from eight replicates. Asterisks denote significant differences between the WT and null mutant PeΔ*veA* strains. ns, no significant changes; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

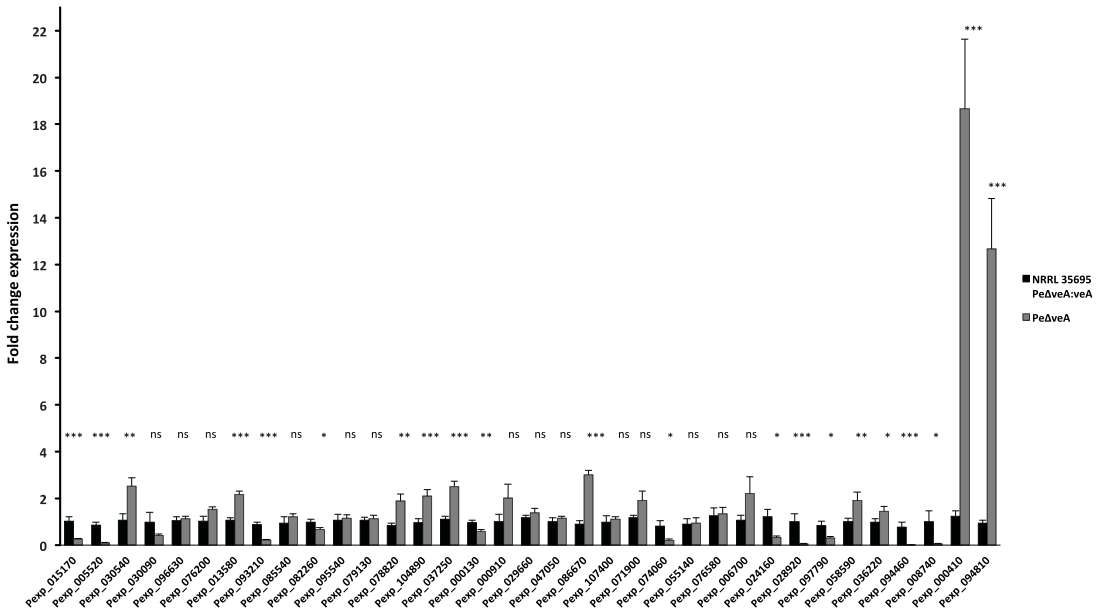

Fig. 7 Relative expression of 35 genes encoding backbone enzymes involved in secondary metabolite biosynthesis. Relative expression of polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) and dimethylallyl tryptophan synthases (DMATs) identified on the sequenced genomes of *Penicillium expansum* T01 and d1 strains grown on malt extract agar (MEA). Gene expression was evaluated in the wild-type NRRL 35695 (WT), null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains. Primers were designed using Primer Express 2.0 software and tests were carried out using a ViiA7 Real-Time PCR System. Black bars represent the combined expression level of genes in the WT and complemented PeΔ*veA:veA* strains after culture in the dark for 5 days at 25 °C. Grey bars represent the level of gene expression in the null mutant PeΔ*veA* strain grown under the same conditions. Graphs show the mean ± standard error of the mean (SEM) of eight replicates. Asterisks denote significant differences between the WT and null mutant PeΔ*veA* strains. ns, no significant changes; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

backbone genes Pexp_071900, Pexp_076200 and Pexp_079130 were up-regulated by VeA on PDA, but not regulated on MEA in the WT and null mutant strains. Moreover, four backbone genes were up-regulated on MEA, but down-regulated (Pexp_082260), not expressed (Pexp_074060) or not regulated (Pexp_024160, Pexp_097790) on PDA.

Pexp_029660, encoding the NRPS of the loline-like biosynthetic cluster (Ballester *et al*., 2015), Pexp_055140, Pexp_082260, Pexp_085540 and Pexp_107400 were up-regulated in the PeΔ*veA* strain on PDA, but not regulated on MEA, except Pexp_082260, which was up-regulated on MEA. Likewise, Pexp_036220, Pexp_058590 and Pexp_104890, which encodes the backbone gene belonging to a siderophore-like cluster (Ballester *et al*., 2015), were down-regulated by VeA on MEA, but not regulated by VeA on PDA. The genes in the complemented strain were regulated in the same way as those in the WT strain.

LaeA and *velB* were expressed in the WT, null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains on the two media tested. Expression of *velB* was not affected by the deletion of *veA* on both media. Expression of *laeA* was not significantly different in the null mutant PeΔveA relative to the WT and complemented strains on both media, but increased slightly on MEA (Part S7, see Supporting Information).

DISCUSSION

The global regulation factor VeA positively or negatively controls a large number of secondary metabolite pathways in fungi, including *A. nidulans* (Bayram and Braus, 2012; Calvo 2008; Rauscher *et al*., 2016) and *A. flavus* (Cary *et al*., 2015). VeA positively affects the production of fumigaclavine C, fumagillin and gliotoxin in the opportunistic human pathogen *Aspergillus fumigatus* (Dhingra *et al*., 2013). In *Fusarium* species, VeA orthologues are required for the synthesis of fumonisins and fusarins in *F. verticillioides* (Myung *et al*., 2012), trichothecenes in *F. graminearum* (Merhej *et al*., 2012) and gibberellins, fumonisins and fusarin C in *F. fujikuroi* (Wiemann *et al*., 2010). Conversely, VeA represses the production of the dark-coloured pigment bikaverin in *F. fujikuroi* (Wiemann *et al*., 2010). In *Penicillium* species, VeA activates the production of penicillin in *Penicillium chrysogenum* (Kopke *et al*., 2013) and represses the production of ML-236B (compactin) in *Penicillium citrinum* (Baba *et al*., 2012). Its role in *P. expansum* remains unknown. In this study, the positive regulation of patulin and citrinin biosynthesis by VeA was demonstrated. Both toxins have been reported to be positively regulated by LaeA, another member of the trimeric Velvet complex in *P. expansum* and *Monascus ruber*, respectively (Kumar *et al*.,

2017a; Liu *et al*., 2016). The lack of *veA* activity is not caused by *laeA* or *velB* expression changes. VeA acts at several levels including the Velvet complex. Although *veA* did not affect *laeA* and *velB* expression, we cannot rule out *veA* activity via the Velvet complex on patulin biosynthesis. Given the impact of VeA and LaeA on this biosynthesis pathway, the evaluation of the Δ*velB* mutant with regard to the expression of *pat* genes will strengthen the hypothesis that a functional Velvet complex is necessary for the production of this toxin.

In addition to patulin and citrinin, *P. expansum* produces several secondary metabolites with different toxic effects (Tannous *et al*., 2018). With regard to other known *P. expansum* secondary metabolites, backbone genes that belong to the clusters of roquefortine and a gliotoxin-like compound showed no difference in expression between the WT and null mutant strains, suggesting that *veA* does not regulate the synthesis of these metabolites, at least under the conditions of the present study.

Kumar *et al*. (2017a) compared the expression of *P. expansum* backbone genes when Δ*laeA* and WT strains were grown on Czapek yeast extract liquid medium (CY) and the apple-based apple puree agar medium (APAM). Among these genes, 32 and 29 genes that were expressed on MEA and PDA, respectively, were identified in the null mutant *veA* and WT strains. Among these common genes, 11 were up-regulated by LaeA, five on both media, but only five were also up-regulated by VeA: Pexp_028920 and *patK* (Pexp_094460) were expressed on both MEA and PDA, Pexp_074060 was expressed only on MEA, and Pexp_071900 and Pexp_079130 were only expressed on PDA. Pexp_071900 is the backbone gene of a biosynthetic cluster of a putative epipolythiodioxopiperazine-like (ETP-like) compound (Ballester *et al*., 2015). Pexp_107400, which encodes a PKS, was down-regulated by VeA on PDA and by LaeA on all media tested (Kumar *et al*., 2017a). Our results showed that VeA regulates Pexp_015170 positively on both media, whereas LaeA does not regulate it. Pexp_015170 encodes an NRPS displaying 91% identity with HcpA, an NRPS responsible for fungisporin synthesis (Ali *et al*., 2014). Although an HcpA orthologous gene exists in the genomes of all *Penicillium* and some *Aspergillus* species (Klitgaard *et al*., 2015; Nielsen *et al*., 2017), no physiological role of this peptide is known. Pexp_000410 was highly down-regulated by the *veA* gene on both media used, whereas it was up-regulated by LaeA on CY. In addition, Pexp_094810, which was also strongly down-regulated by VeA on both media, was not expressed in either the WT or Δ*laeA* strains whatever the medium (Kumar *et al*., 2017a). Among the genes down-regulated by LaeA, Pexp_076200, whose expression showed no significant difference between the null mutant PeΔ*veA* and the WT strains on MEA, was up-regulated by VeA on PDA. Contrary to the observations with LaeA, Pexp_008740 was up-regulated by VeA on both media. Pexp_029660, Pexp_055140 and Pexp_085540 were down-regulated by VeA, but up-regulated by LaeA (Kumar *et al*., 2017a).

The backbone gene of the citrinin (*citS*/Pexp_005520) cluster was down-regulated in the PeΔ*veA* strain, and genes of the communesin (*cnsI*/Pexp_030540) cluster and another ETP-like (Pexp_058590) cluster were up-regulated in the PeΔ*veA* strain on at least one medium tested, whereas none of the three were regulated by LaeA (Kumar *et al*., 2017a). Pexp_093210, corresponding to the cluster of the monodictyphenone-like compound, was up-regulated by VeA on both media, but was not expressed in the Δ*laea* strain or the WT strain on either medium. Pexp_104890, the backbone gene involved in siderophore-like biosynthesis (Gründlinger *et al*., 2013), was not regulated by LaeA on either medium or by VeA on MEA, but was down-regulated by VeA on PDA. The expression of most genes differed between the PeΔ*veA* and Δ*laeA* strains, but we cannot exclude the possibility that the discrepancies originated in the media used. VeA and LaeA, which sometimes interact (complex with VelB), also appeared to act independently, regulating some secondary metabolite clusters differently.

The null mutant PeΔ*veA* strain developed like the WT strain in Golden Delicious apples up to 4 dpi, but the rot progression rate decreased over the following 10 days. The same behaviour has been reported in a PeΔ*patL* strain, which was unable to produce patulin (Snini *et al*., 2016). In the previous study, patulin was only detected from 4 dpi with the WT strain. In the present study, the absence of *veA* completely halted the production of patulin *in vivo* and drastically affected *patL* expression *in vitro*. These results reinforce the hypothesis that patulin is not indispensable for the initiation of the disease (Ballester *et al*., 2015; Li *et al*., 2015), but plays a role as a cultivar-dependent aggressiveness factor (Kumar *et al*., 2017b).

Based on the behaviour of the null mutant strain in apple, *veA* is not required for the initiation of the disease. Knowledge of the pathogenicity determinants that allow the penetration and colonization of the fungus in apple pericarp is very limited. The toxins constitute only some weapons of the *P. expansum* arsenal. Some secreted elements, such as D-gluconic acid (Hadas *et al*., 2007; Prusky *et al*., 2004), fumaric acid (Vilanova *et al*., 2014) and polygalacturonase (Jurick *et al*., 2010), have been reported to contribute to the pathogenicity of *P. expansum*. Recently, it has been found that PeΔSte12 mutants show a significant decrease in *P. expansum* virulence (Sánchez-Torres *et al*., 2018). A possible modulation of one of these virulence factors by the VeA factor has yet to be demonstrated. The importance of *veA* in the virulence of some fungal pathogens has already been demonstrated. For example, BcVEL1 or BcVeA, orthologous to *veA* in *Botrytis cinerea*, is required for the full virulence of this species on bean leaves and grape berries (Schumacher *et al*., 2015; Yang *et al*., 2013). Maize plants inoculated at the seed stage with a null mutant Δ*veA* strain

of *F. verticilloides* showed no disease symptoms, whereas, under the same experimental conditions, plants infected by a WT strain exhibited symptoms (Myung *et al.*, 2012).

VeA is involved in sexual development and is also known to be a negative regulator of asexual reproduction. Deletion of the corresponding gene was thus expected to stimulate the production of conidia. However, in most species, reduced conidiation was observed in the Δ*veA* strain compared with the WT strain on synthetic media under both light and dark conditions (Calvo *et al.*, 2004; Crespo-Sempere *et al.*, 2013; Duran *et al.*, 2007; Merhej *et al.*, 2012; Wiemann *et al.*, 2010). Increased sporulation of the Δ*veA* strain compared with the WT strain has been reported less frequently (Jiang *et al.*, 2011; Park *et al.*, 2012; Rauscher *et al.*, 2016) and sometimes no difference has been observed between the WT strain and the Δ*veA* strain (Estiarte *et al.*, 2016). The different findings, even in the same species, could be partly linked to the different media used. For instance, Kato *et al.* (2003) reported a medium-dependent role of *veA* in conidiation.

Here, conidiophore structure was affected by *veA* in *P. expansum* grown on various synthetic media. The biggest difference was the absence of coremia in the null mutant strain, both *in vitro* and *in vivo*. The null mutant PeΔ*veA* strain, grown on apples in the dark, was still able to sporulate *in vitro* but, because of a lack of coremia formation, was unable to pierce the peel of apples and to emerge from the fruit again to complete its life cycle, in contrast with the WT and complemented strains. The storage of apples, which is usually in the dark and in ventilated cold rooms, favours the development and propagation of fungal species. Coremia are relatively rigid structures made up of conidiophores grouped into small columns that are fertile apically. *Penicillium expansum* has the ability to produce coremia when the fungus is grown on standard laboratory media, but also on natural substrates such as apples. The fresh isolates sometimes also produce synnemata, another fasciculate structure, which consist of appressed fertile hyphae along their entire length. *Penicillium expansum* usually produces conspicuous synnemata in its natural habitats, but tends to lose this ability when grown on synthetic media (Raper *et al.*, 1968). Little is known about the mechanisms that lead to the formation of coremia. Tinnel *et al.* (1977) showed that the presence of manganese is very important for coremia formation in *Penicillium vulpinum* (syn. *Penicillium claviforme*) and *Penicillium clavigerum*, both patulin-producing species. A specific manganese requirement for patulin biosynthesis has already been demonstrated. Of eight metal ions, only manganese strongly influenced patulin production in *Penicillium griseofulvum* (syn. *Penicillium urticae*) (Scott *et al.*, 1986a). This observation was confirmed in a more recent work (Dombrink-Kurtzman and Blackburn, 2005). Through the use of actinomycin D and cycloheximide, respectively, a transcription and translation inhibitor, manganese, has been suggested to have an effect on patulin biosynthesis at the transcription level (Scott *et al.*, 1986b). The similarity between coremia formation and patulin biosynthesis suggests a common regulation. The fact that neither PeΔ*patL* nor PeΔ*veA* produces patulin or coremia renders the hypothesis of a manganese-dependent signalling pathway shared by patulin biosynthesis and coremia formation plausible. To our knowledge, no relationship between manganese and the Velvet complex has been reported to date. This hypothesis deserves further attention in future work. Generally, secondary metabolism and morphological development are intimately associated in fungi, particularly in *Aspergillus* and *Penicillium* species (Calvo *et al.*, 2002). VeA is a major component of the global regulatory mechanisms involved in the formation of cleistothecia in homothallic *Aspergillus* species, such as *A. nidulans* (Bayram *et al.*, 2008), but also in sclerotial morphogenesis in some heterothallic *Aspergillus* species, such as *A. flavus* (Calvo and Cary, 2015). Sclerotia are considered to be vestiges of cleistothecia that have lost their ability to generate ascospores. As cleistothecia/sclerotia and coremia/synnemata are linked to sexual reproduction and asexual reproduction, respectively, except for VeA, there is no evidence to support a common regulatory mechanism.

In conclusion, our results shed light on the role of VeA in *P. expansum* and the *veA* gene. VeA upregulates the production of patulin and citrinin. Because of the different media used, our results also highlighted the induction or repression of the expression of several backbone genes involved in the biosynthesis of other secondary metabolites with known or still unknown functions. From a physiological and ecological point of view, our results show that VeA is important in the final step of fungal cycle development on natural substrates, enabling the spread of spores and hence the colonization of new substrates.

EXPERIMENTAL PROCEDURES

Strains, media and culture conditions

The *P. expansum* wild-type strain NRRL 35695 (WT) used in this study was originally isolated from grape berries in the Languedoc Roussillon region in France. The compositions of the media are detailed in Part S8. Unless otherwise specified, the strains used in this study were cultured on PDA (Sigma-Aldrich, Saint Quentin Fallavier, France), MEA (Biokar Diagnostics, Allonne, France) and CYA (Sigma-Aldrich) for 5 days at 25 °C in the dark.

For the morphological study, strains were plated on MEA, PDA, CzA (Oxoïd, Dardilly, France) and CGA (Sigma-Aldrich), and grown for 7 days at 25 °C in the dark.

For the preparation of protoplasts, we used 250 mL of yeast extract peptone dextrose (YPD) medium (Sigma-Aldrich) inoculated with a 3×10^7 conidial culture. The culture was grown on an orbital shaker (150 rpm) set at 25 °C for 12 h. The PeΔ*veA* gene disruption cassette was obtained using *Saccharomyces cerevisiae* FY1679: MATa/MATα ura3–52/ura3–52; trp1Δ 63/TRP1; leu2Δ 1/LEU2; his3Δ 200/HIS3; GAL2/GAL2 by

homologous recombination. This strain was grown on solid YPD medium at 30 °C for 5 days.

Construction and molecular characterization of the null mutant PeΔ*veA* and complemented PeΔ*veA:veA* strains

In order to characterize the role of the *veA* gene, to understand how it affects the virulence of *P. expansum* and how it influences the production of a wide diversity of important secondary metabolites, a gene deletion strategy was applied by replacing the coding region with the hygromycin selection marker, as described previously (Snini *et al.*, 2016). A more detailed description is available in Part S1. The veA deletion was validated by PCR screening and Southern blot as described in Part S2.

In vitro growth, macroscopic and microscopic morphology

WT, null mutant and complemented strains were plated on PDA. After 7 days of incubation, a spore suspension of each strain was prepared according to Adjovi *et al.* (2014). The concentrations of spore suspensions were quantified using a Malassez cell. An inoculum of 10^4 spores was plated centrally on MEA, PDA, CzA and CGA, and the plates were incubated at 25 °C in the dark for 10 days. The experiment was performed in triplicate for each strain. After 7 days, colony diameters were measured and microscopic characteristics (conidiophore branching pattern, phialide and conidia shape, etc.) were observed under an optical microscope [Olympus CX41 (×400 and ×1000, Rungis, France]. After 10 days, macroscopic features (colour of conidial areas, colony margin, colony texture, pigmentation, exudate, reverse colour, etc.) were studied using a stereomicroscope (Olympus SZX9, ×12–120).

Analysis of patulin production *in vivo*

To assess the production of blue mould and patulin on apples in the WT, null mutant and complemented strains, an *in vivo* study was performed using Golden Delicious apples. The apples were purchased from a supermarket in Toulouse (Carrefour, France), and were free of defects or injuries. The apples were surface sterilized in a 2% sodium hypochlorite solution and rinsed with water, as described previously by Sanzani *et al.* (2012). The apples were then wounded with a sterile toothpick. For each strain, 10 μL of a suspension at a concentration of 10^4 conidia/mL were deposited into the wounds of apples. Contaminated apples were then incubated at 25 °C in the dark for 14 days. The diameters of the rotten spots were measured every day during the incubation period to determine the growth rate, and the rot volume was calculated using the method described previously by Baert *et al.* (2007). After the incubation period, apples were ground using a blender until a homogeneous apple puree was obtained, as described previously by MacDonald *et al.* (2000) and detailed by Snini *et al.* (2016). Patulin production was determined by high-performance liquid chromatography with a diode array detector (HPLC-DAD) based on a standard calibration curve. Eight biological replicates were performed for each strain.

Analysis of secondary metabolism

The WT, null mutant and complemented strains were grown on two different media: MEA and PDA. A spore suspension of each isolate was prepared from a previous culture on PDA and incubated at 25 °C in the dark for 7 days. Petri dishes containing the medium were overlaid with a sterile cellophane layer and inoculated with 10 μL of a 10^6 spores/mL solution of each isolate.

Extraction of secondary metabolites

After a 5-day incubation period, mycelium was separated from the cellophane layer and split into two equal parts. The first half of the mycelium was used for RNA extraction. The second half of the mycelium and the agar medium were macerated separately in 50 mL of ethyl acetate on a horizontal shaking table at 160 rpm at room temperature for 6 days.

The organic phase was then filtered through a Whatman filter paper and evaporated to dryness using a Zymark TurboVap (McKinley Scientific, Sparta, NJ, USA). The dried residue was dissolved in 400 μL of acetonitrile–water (50 : 50, v/v) and filtered through a 0.45-μm syringe filter into a clean 2-mL vial. Eight biological replicates were performed for each strain.

HPLC-DAD analysis of patulin and citrinin

The chromatography device used for the quantification of patulin and citrinin was an Ultimate 3000 HPLC system (Dionex/ThermoScientific, Courtaboeuf, France). Patulin was analysed as described by Snini *et al.* (2016). Citrinin was quantified using a 150 mm × 2 mm, Luna® 5 μm, C18 column (Phenomenex, Le Pecq, France) at 30 °C at a flow rate of 0.2 mL/min. Eluent A was water acidified with 0.05% formic acid and eluent B was acetonitrile acidified with 0.05% formic acid. The column was equilibrated with a mixture of 80% solvent A and 20% solvent B. Elution conditions were as follows: a 30-min linear increase in solvent B from 20% to 50%, a 5-min linear increase of solvent B from 50% to 90%, 90% solvent B for 10 min, a 5-min linear decrease of solvent B from 90% to 20%, 20% solvent B for 10 min. The presence of citrinin was monitored at a wavelength of 327 nm and confirmed by its retention time (min) according to the standard. Patulin and citrinin standards were purchased from Sigma-Aldrich.

Isolation of fungal RNA and RT-PCR

At the end of the incubation period, mycelia were separated from the cellophane, RNAs were extracted and cDNAs were synthesized as described by Tannous *et al.* (2014). The quality of the extracted RNA was verified by gel electrophoresis (1.2% agarose) and the concentrations were measured using a Dropsense apparatus (Trinean, Proteigene, Saint Marcel, France).

Design and validation of qPCR primers

Primer pairs corresponding to the different genes of the patulin cluster described previously by Tannous *et al.* (2014) were used. Primer pairs corresponding to the different genes of the citrinin cluster were designed (Part S5). Primer pairs corresponding to *laeA* and *velB* were designed (Part S7).

The Antibiotics-Secondary Metabolite Analysis SHell (antiSMASH) program (Weber *et al.*, 2015) was used to identify the different clusters of secondary metabolites on the *P. expansum* genome using the T01 and d1 sequenced strains (GenBank: AYHP00000000.1 and GenBank: JQFY01000069.1, respectively). Sixty-three clusters were identified. The primer pairs of the different PKSs, NRPSs and DMATSs of these clusters were designed. Thirty-five backbone genes were expressed under our conditions (Part S6). The primers were designed and validated as described by Caceres *et al.* (2016). At least two biological replicates of *P. expansum* NRRL 35695 were used to validate the amplification specificity. Negative controls (without reverse transcriptase enzyme) were added to control for the absence of contamination.

Analysis of the expression of the genes linked to the secondary metabolites in *P. expansum*

Real-time PCR was used to quantitatively evaluate the expression of the gene clusters of patulin and citrinin and to measure the expression of the different secondary metabolite genes in each strain.

Experiments were carried out as detailed previously (Caceres *et al.*, 2016). β-Tubulin was used as housekeeping gene. Changes in gene expression of qPCR experiments were then analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Two separate experiments were performed, each including at least eight biological replicates of each condition.

Data analysis

Non-parametric Kruskal–Wallis and Mann–Whitney tests were used to analyse the differences in the WT, null mutant and complemented strains for each of the analyses of patulin production *in vivo*, and the gene expression of secondary metabolites using the RT-PCR technique. The differences were considered to be statistically significant when the *P* value was below 0.05. The statistical analysis of the data was carried out using GraphPad 4 software (GraphPad Software, La Jolla, CA, USA).

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SUPPORTING INFORMATION

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

Part S1 Construction of *veA* and *veA:veA* deletion cassette.

Part S2 Molecular characterization of the null mutant PeΔ*veA* and complemented PeΔ*veA*:*veA* strains

Part S3 Macroscopic morphology of the null mutant PeΔ*veA*.

Part S4 Dissemination of *Penicillium expansum*.

Part S5 Expression analysis of gene clusters involved in patulin and citrinin biosynthesis.

Part S6 Expression analysis of genes encoding backbone enzymes involved in secondary metabolite biosynthesis.

Part S7 Expression analysis of *laeA* and *velB*.

Part S8 Composition of the culture media.