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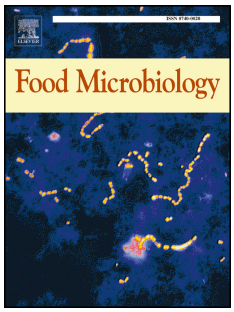

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Unravelling the contribution of the *Penicillium expansum PeSte12* transcription factor to virulence during apple fruit infection

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ABSTRACT

Blue mould disease caused by *Penicillium expansum* infection is one of the most important diseases of pome fruit accounting for important economic losses. In the present study, the *PeSte12* transcription factor gene was identified, and deletant mutants were produced by gene replacement. Knockout mutants showed a significant decrease of virulence during apple fruit infection. Virulence was affected by the maturity stage of the fruit (immature, mature and over-mature), and disease severity was notably reduced when the apples were stored at 0 ºC. The Δ*PeSte12* mutants resulted defective in asexual reproduction, producing less conidia, but this characteristic did not correlate with differences in microscopic morphology. In addition, the Δ*PeSte12* mutants produced higher quantity of hydrogen peroxide than the wild type strain.

Gene expression analysis revealed that *PeSte12* was induced over time during apple infection compared to axenic growth, particularly from 2 dpi, reinforcing its role in virulence. Analysis of transcriptional abundance of several genes in Δ*PeSte12* mutants showed that in most of the evaluated genes, *PeSte12* seemed to act as a negative regulator during axenic growth, as most of them exhibited an increasing expression pattern along the time period evaluated. The highest expression values corresponded to detoxification, ATPase activity, protein folding and basic metabolism. Gene expression analysis during apple infection showed that 3 out of 9 analysed genes were up regulated; thus, *PeSte12* seemed to exert a positive control to particular type of aldolase. These results demonstrate the *PeSte12* transcription factor could play an important role in *P. expansum's* virulence and asexual reproduction.

49 **Keywords:** apple, conidiation, fruit-fungal interaction, gene expression, transcription
50 factor, virulence.

1. Introduction

Blue mould, caused by *Penicillium expansum*, is the most important apple post-harvest disease. 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). This necrotrophic fungi is a pathogen that infects tissue through wounds, causing maceration and decay. Currently, the common method used to control this disease is the application of chemical fungicides, since they act quickly and effectively. However, the ability of fungal pathogens to become progressively resistant to fungicides and the negative impact of pesticides on human health has led to an increase in efforts to develop more effective control strategies. These strivings have been focus on unravelling fungal infection processes and pathogen strategies to infect the fruit. The current information about pathogenicity factors responsible for *P. expansum* decay are very scarce. In that sense, providing a good knowledge on the virulence factors could help finding targets for disease control.

Fungal-plant pathogens have developed several mechanisms to enhance their virulence (Perez-Nadales et al., 2014). In the case of *Penicillium* spp. the first attempt for infection during host-pathogen interaction are wounds present on the surface of the fruit, and nutrients and volatiles could stimulate spore germination (Droby et al., 2008). Then, the process is followed by penetration and colonization of the fruit tissue. *P. expansum* is able to cause decay at the low temperatures of fruit storage, and over-mature or long-stored fruits are more susceptible of being infected. Although the availability of the genomic sequences of several of *P. expansum* strains might help to identify virulence genes in this pathogen (Ballester et al., 2015), there is widespread ignorance about virulence elements that determine pathogen aggressiveness in this postharvest pathogenic fungus, including those than can subvert fruit defences. This

effect has been described in apples at different maturity stages infected with *Penicillium* spp. as host and non-host pathogens in which a suppression of hydrogen peroxide (H_2O_2) was observed (Buron-Moles et al., 2015). Moreover, Tian et al. (2013) found that proteins involved in the antioxidant metabolism, such as catalase and superoxide dismutase, are related with the pathogenicity of blue mould. It seems necessary to elucidate the different factors that mediate pathogen virulence in *Penicillium* spp. associated to fruit decay.

Previous studies have related the secretion of polygalacturonases, glutamate decarboxylase, calmodulin or C-4 methyl esterol oxidase with the pathogenicity of *P. expansum* in apples by studying genes differentially expressed during the infection process (Sánchez-Torres and González-Candelas, 2003). Qin et al (2007) also related polygalacturonases with the pathogenicity of *P. expansum* by analysing the cellular and extracellular proteomes. The hypothesis that *P. expansum* shows the ability to acidify the environment in colonized tissue by the accumulation of D-gluconic acid has also been proposed as mechanisms of pathogenicity in apples (Vilanova et al., 2014a).

P. expansum has great potential for the production of secondary metabolites. Secondary metabolism is commonly associated with sporulation processes and metabolite production. Patulin is included within secondary metabolites but its role in the development of blue mold decay on apples remains controversial. While some authors reported its implication in fungal pathogenesis (Barad et al., 2014; Sanzani et al., 2012), recently other authors described the absence of association between virulence and patulin production (Ballester et al., 2015; Li et al., 2015). Nevertheless, although patulin could not be required to infect apples, may act as an apple cultivar-dependent aggressiveness factor (Snini et al. 2016).

Virulence in plant pathogenic fungi is regulated by a network of cellular pathways that respond to signals present during host-pathogen interaction (Rispail and Di Pietro, 2010). Within the group of fungal transcription factors, the Ste12 factor has arisen as a relevant factor in these processes. The Ste12 protein is predominantly encountered as a target of the Fus3 mitogen-activated protein kinase (MAPK) cascade that controls mating (Hoi and Dumas, 2010).

The factor Ste12 is one of the most studied transcription factors and has an important role in sexual development and pathogenicity (Hoi and Dumas, 2010). Several reports describe the contribution of Ste12 homologs from many pathogenic fungi in pathogenicity/virulence. Example of that is the unsuccessful penetration of *Magnaporthe oryzae* defective MST12 mutants (Park et al., 2004). The effect in virulence /pathogenesis have also been described for *Colletotrichum lagenarium* (Tsuji et al., 2003), *Botrytis cinerea* (Schamber et al., 2010), *Fusarium graminearum* (Gu et al., 2015) and *Fusarium oxysporum* (García-Sánchez et al., 2010; Rispail and Di Pietro, 2009). Recently, the *P. digitatum* homologous Ste12 transcription factor has been reported, supporting its significance in fungal virulence and conidiation (de Ramón-Carbonell and Sánchez-Torres, 2017; Vilanova et al., 2016). Therefore, identification and evaluation of the Ste12 transcription factor in *P. expansum* opens the chance to get a better acquaintance of the mechanisms that control processes such as infection capacity or virulence.

In this work, we aimed to isolate and functionally characterize the *P. expansum Ste12* gene by loss-of-function to show that exerts an important role in conidiation and fungal virulence during apple infection.

2. Material and Methods

2.1. Microorganisms and culture conditions

Penicillium expansum strain CMP-1 (Ballester et al., 2015) was used as the wild type and parental strain of deletion mutants for the *PeSte12* gene. All strains were grown in potato dextrose broth (PDB; Liofilchem Laboratories, Roseto degli Abruzzi, Italy) or potato dextrose agar (PDA; Liofilchem Laboratories, Roseto degli Abruzzi, Italy). Cultures were incubated at 25 ºC for 1, 2 or 3 days (liquid cultures) depending of the further use or up to 1 week (solid media). Conidia were collected as previously described by Vilanova et al. (2016).

Escherichia coli DH5α was used for propagation material and *Agrobacterium tumefaciens* $C_{58}C_1$ was used for *P. expansum* transformation (de Ramón-Carbonell and Sánchez-Torres, 2017).

2.2. *Fruits*

Immature, mature and over-mature 'Golden Smoothee' apples were used to study the role of *PeSte12* in *P. expansum* virulence, conidiation and morphology. Apples (*Malus* × *domestica* Borkh.) were obtained from a commercial orchard in Mollerussa (Catalonia, Spain) and used immediately after harvest. Selected fruits with similar size, without injuries or infections were surface disinfected with 10 % sodium hypochlorite for 1 min, rinsed with tap water and air dried before experiments. Colour, firmness, starch index, soluble solids, and acidity were determined as quality parameters at each harvest date following the protocol described by Vilanova et al. (2012).

2.3. Nucleic acids manipulations

Genomic DNA of *P. expansum* strains was extracted as previously described by Marcet-Houben et al. (2012). All PCR DNA fragments obtained in this work were purified using the Ultra Clean TM PCR Clean-up (MoBio, Carlsband, CA, USA) and then sequenced with the appropriate primers using the fluorescent chain-terminating dideoxynucleotides method (Prober et al., 1987) and an ABI 377 sequencer (Applied

Biosystems, Madrid, Spain). DNA sequences were compared with those from the EMBL database with the Washington University-Basic Local Alignment Search Tool (WU-BLAST) algorithm (Altschul et al., 1997). When necessary, sequences were aligned using the ClustalX2 (version 1.64b) program (Larkin et al., 2007). Plasmid DNA preparations were purified using the UltraClean Mini Plasmid Prep kit (MoBio, Carlsband, CA, USA).

2.4. Construction of a P. expansum genomic DNA library

DNA was extracted from 1 g of frozen mycelium essentially as described by Moller et al (1992). Size-selected and end-repaired DNA was ligated into linearized fosmid pCC2FOS using the CopyControl HTP Fosmid Library Production kit (Epicentre Biotechnologies). Ligated DNA was packaged with the MaxPlax Lambda Packaging Extracts provided in the kit and transfected into *E. coli* EPI300 cells following the supplier's recommendations.

2.5. *Isolation of PeSte12*

Two PCR primers, PE1-PE2 (Table S1), were designed based on the nucleotide sequence of *Penicillium digitatum PdSte12* gene (PDIP_53990). The PCR reaction consisted of a first denaturation at 94ºC for 4 min, followed by 30 cycles at 94 ºC for 30 s, 56 ºC for 45 s and 72 ºC for 60 s, a final elongation step was carried out at 72 ºC for 10 min. The PCR amplicon was sequenced and primers were designed for the screening of the *P. expansum* CMP-1 genomic DNA library. The scrutiny took place as described by de Ramón-Carbonell and Sánchez-Torres (2017).

2.6. Construction of the vector pΔPeS12 and fungal transformation

Gene disruption by homologous recombination in *P. expansum* was done as earlier described by de Ramón-Carbonell and Sánchez-Torres (2017). A 1.5-kb fragment containing upstream flanking sequence of *PeSte12* was amplified from genomic DNA

of CMP-1 strain using primers PE7-PE8. Similarly, a 1.6-kb fragment containing downstream flanking sequence of *PeSte2* gene was amplified with primers PE9-PE10 and both fragments were cloned into the hph cassette of plasmid pRFHU2 (Frandsen et al., 2008) to generate the targeted gene deletion plasmid.

The resultant plasmid pΔPeS12, confirmed by sequencing, was introduced into a *A. tumefaciens* $C_{58}C_1$ strain. A pΔPeS12 transformed *A. tumefaciens* strain was then used to transform *P. expansum* CMP-1 following the protocol described by Marcet-Houben et al. (2012). Transformants were selected on PDA plates containing 150 µg/mL of hygromycin B. Verification of the disruption was done by PCR using genomic DNA from each monosporic isolate. Selected null mutants were analysed for absence of ectopic copies as described below.

2.7. *T-DNA copy number*

The number of T-DNA copies integrated in each selected transformant was evaluated by qPCR analysis with SYBR Green as a fluorogenic dye (Crespo-Sempere et al., 2013) using primers PE12-PE13 located in the promoter region of the *PeSte12 g*ene (Table S1). The *P. expansum* β-tubulin gene was used as a reference for normalization employing primers qPeTubF-qPeTubR (Table S2). DNA from the wild-type CMP-1 strain was used as a control. PCRs and data analysis were performed using a LightCycler 480 Real-Time PCR machine (Roche, Mannhein, Germany) and LightCycler software, version 4.0.

2.8. In vitro growth studies

Growth diameters of *P. expansum* wild type and *PeSte12* deletion mutants (ΔT6 and ΔT8) were determined after inoculating PDA Petri plates centrally with a 10 µL conidial suspension adjusted at 10^4 conidia/mL. After inoculation the plates were incubated at

25 ºC. Two radial measurements were made for each colony to a maximum of 7 days post inoculation with four replicates per strain.

Liquid cultures we carried out in 50 mL of PDB media at 24, 48 and 72 h inoculated with 10^5 conidia/ml of either CPM-1 wild type or ΔT6 and ΔT8 deletion mutants and incubated at 25 ºC and 200 rpm.

2.9. Fruit inoculation

For pathogenicity assays, immature, mature and over-mature apples were wounded twice with a nail (1 mm wide and 2 mm deep) on one side of each fruit and inoculated with 15 µL of aqueous conidia suspension of the wild type strain *P. expansum* or *PeSte12* deletion mutants (ΔT6 and ΔT8) at 10^4 conidia mL^{-1}. Inoculated fruits were stored at two different storage temperatures (20 and 0 ºC) and 85 % relative humidity (RH). Decay incidence and severity were evaluated at different times post inoculation. In both cases (20 °C and 0 °C), five apples constituted a biological replicate and four replicates were used for each treatment.

The severity of lesions (cm) at each time of measurement (day) were plotted and growth rates (cm/day) were obtained from the slopes by linear regression using Microsoft Excel (Microsoft Corporation, USA) following similar methodology described in other studies (Vilanova et al., 2012).

For RNA extraction, mature apples were used and twenty wounds were made on one side of each fruit using a nail and inoculated with 10 µL of aqueous conidia suspensions of either *P. expansum* wild type strain or ΔT6 mutant. Inoculated fruits were stored at 20 °C and 85 % RH for 24, 48 and 72 h. After each storage time, cylinders of peel and pulp encompassing the wounds (8 mm inside diameter and 3 mm deep) were removed using a cork borer. Each biological replicate consisted of 200 disks pooled from 10 fruits and three replicates were collected at each sampling. All samples were

immediately frozen in liquid nitrogen, lyophilized for 5 days and then ground to a fine powder for subsequent RNA extraction.

2.10. Conidiation assessment

Conidiation degree of the wild type and *PeSte12* mutants on the surface of decayed apples was evaluated following a 0-5 scaled described for oranges and adapted to apples and a conidiation index was used to obtain an absolute frequency chart (Vilanova et al., 2016). The scale numbers used in the conidiation index indicated: 0, soft lesion but no conidia or mycelium present; 0.5, mycelium but no conidia present; 1, <5%; 2, 5–30%; 3, 31–60%; 4, 61–90% and 5, >91% of the fruit surface covered with conidia. Five apples constituted a biological replicate and four replicates were used for each strain.

2.11.Microscopy morphology

Immature apples, inoculated as described in the virulence studies, were stored at 20 ºC and 85 % RH for different times post inoculation. After each stored period, fruit disks (16 mm diameter and 5 mm thickness) encompassing the wounds were removed from apples using a cork borer and placed into sterile Petri plates. Samples were visualized with both a Leica MZ16F stereoscope and a Leica DM5000 microscope. Images were acquired using a Leica colour digital camera (Leica DFC 420).

2.12. Hydrogen peroxide quantification

Twenty wounds were made in mature apples as described above. Wounds were inoculated with 10 µL of sterile water or aqueous conidia suspension of the ΔT6 mutant or wild type strain at 10^4 conidia mL^{-1}. Inoculated fruits were stored at 20ºC and 85 % RH. Cylinders of peel and pulp (8 mm diameter and 3 mm deep) encompassing the wounds were removed using a cork borer from four fruit at different times post inoculation. Pooled samples were frozen and powdered in liquid nitrogen, and immediately used for hydrogen peroxide (H_2O_2) determination. H_2O_2 production was

measured using a PeroxiDetect™ Kit (Sigma-Aldrich, Saint Louis, USA) following the methodology described by Buron-Moles et al. (2015). The H_2O_2 content was expressed as µmol Kg^{-1} of fresh weight (FW) and each value was the mean of four replicates constituted by four apples each one.

2.13. Quantification of relative gene expression

Total RNA from mycelium of *P. expansum* was obtained from frozen mycelium by using Trizol (Invitrogen, Carlsband, CA, USA). Total RNA during apple infection was extracted from fruit peel discs (section 2.8) as described previously Vilanova et al. (2014b). Synthesis of the first strand of cDNA and relative gene expression was carried out as previously described by Ballester et al. (2015). Thermal profile was: activation step (95 ºC for 5 min), amplification step (45 cycles of 95 ºC for 10 s, annealing temperature (T_{an}) 58ºC for 5 s, and 72 ºC 10 s), melting curve program (95 ºC for 5 s, 65ºC for 1 min, and heat to 97 ºC at 0.1 ºC/s rate), and cooling step (40 ºC for 10 s). Two technical repeats were conducted for each experiment, and three independent biological replicas were carried out. Oligos qPeSte12F and qPeSte12R were used for *PeSte12* gene and genes coding for fungal β-tubulin (AY674401) (qPeTubF-qPeTubR), ribosomal protein 37S PEX1_068590 (qPe37F-qPe37R) and histone H3 PEX1_049570 (qPeHis3F-qPeHis3R) were simultaneously used as independent reference genes (Table S2). The Relative Gene Expression ('RGE') was calculated using the modified equation EGOI^(-CqGOI)/EREF^(-CqREF) from Pfaffl (Pfaffl 2001) as described before by Ballester et al. (2015).

2.14. Statistical analysis

Data regarding quality parameters, incidence, initial day of visible rot, lesion growth rate (cm/day), cumulative frequency of conidiation index and H_2O_2 content were

analysed for statistical significance by analysis of variance (ANOVA) with the JMP 8 (SAS Institute Inc., Cary, USA) statistical package. Statistical significance was judged as $P<0.05$; when the analysis was statistically significant, a Tukey test for separation of means was used.

3. Results

3.1. Isolation and structural analysis of the P. expansum PeSte12 gene

An amplicon of 763 bp was obtained by PCR using PE1-PE2 primers (Table S1). The deduced amino acid sequence of the fragment showed homology to Ste12 genes from different fungi. The screening of the CMP-1 *P. expansum* genomic library using primers PE1-PE2 led to the obtaining of a full genomic gene and more than 10Kb of flanking regions. The sequence contains an open reading frame of 2354 bp encoding a putative protein of 693 amino acids, and was designated *PeSte12* (*P. expansum* Ste12 homolog= PEX1_013250). The gene is interrupted by four introns of 53, 54, 49, and 53 bp, placed at positions 19, 174, 1832, and 2018 of the coding region, respectively. The positions of the introns were verified by cDNAs amplification using pairs of primers that flank the predicted position of each intron (data not shown). The promoter region contains the consensus sequence TGAAACA, designated as the pheromone responsive element (PRE), located 1160 bp upstream from the ATG.

The protein PdSte12 contains two putative DNA-binding motifs, an homeodomain (amino acids 54–163) and two Cys2His2 zinc fingers are located at the C-terminal region (amino acids 567–589 and 597–617). The homeodomain exhibited a match with the consensus STE domain present in all the proteins of the Ste12-family when scanned against the Pfam protein families.

3.2. Deletion of the P. expansum PeSte12 gene

The plasmid pΔPeS12 (Supplementary Fig. 1A-B) was used to transform *P. expansum* CMP-1. Transformants were confirmed using PCR amplification with HygRt-PE10 primers (Table S1) from monosporic isolates. In the CMP-1 wild type strain there was no amplification, while the transformants amplify a fragment of 2.6 Kb (Supplementary Fig. 1B). Deletion of the targeted gene was analysed with primers HygRt-PE11 (Table S1). In the wild type strain and non replaced transformants there was no amplification, while true deletant mutants amplify a fragment of 2.8kb (Supplementary Fig. 1C). Two Δ*Ste12* mutants (ΔT6 and ΔT8) that contained only a single T-DNA integration (quantified by T-DNA copy number) were selected for further analysis.

Both deletant mutants exhibited the same phenotypic traits in axenic growth (PDA plates) compared to the parental strain CMP-1, with the same growth rate and similar conidiation (Supplementary Fig.1D).

3.3. *Fruit Quality parameters*

Significant differences in quality parameters were found among harvests (Table 1). Total soluble solids, starch index and colour increased as the harvest date progressed, while a decrease was observed in titratable acidity and flesh firmness.

3.4. Virulence of P. expansum strains on apples

The effect of *PeSte12* deletion during apple infection was analysed by inoculating both ΔT6 and ΔT8 mutants and *P. expansum* wild type CMP-1. Both Δ*Ste12* mutants were pathogenic to apples, but depending on storage conditions (20 ºC and 0 ºC) the symptoms caused by these mutants showed different behaviour compared to the wild type (Fig. 1). Lesion development on 'Golden Smoothee' apples inoculated with the *P. expansum* wild type or ΔT6 and ΔT8 mutants showed always a linear pattern, independently of fruit maturity or storage temperature.

At 20 ºC, decay incidence and severity of lesions did not show significant differences between the wild type and both *PeSte12* mutants (Fig. 1A-C). No differences were also found in growth rate and in visible initial rotting day among the wild type and both deletion mutants (Table 2).

After 42 days at 0 ºC, decay incidence in apples inoculated with the wild type was higher compared to ΔT6 and ΔT8 mutants in immature (57, 33 and 31 %, respectively), mature (70, 35 and 26 %, respectively) and over-mature (74, 38 and 36 %, respectively) harvests (data not shown). Moreover, significant differences were also detected at immature, mature and over-mature harvests between the growth rate of the wild type (0.056, 0.078 and 0.078 cm d^{-1}, respectively) compared to both ΔT6 (0.026, 0.045 and 0.047 cm d^{-1}, respectively) and ΔT8 (0.030, 0.048 and 0.041 cm d^{-1}, respectively) mutants (Fig. 1 and Table 2). Similarly, the visible initial rotting day appeared before in apples inoculated with the wild type (24, 25 and 24 days, respectively) than in apples inoculated with ΔT6 (34, 32 and 30 days, respectively) and ΔT8 (36, 32 and 32 days, respectively) at all harvest stages assayed (Table 2).

3.5. Conidia formation in P. expansum wild type and ΔT6 *and* ΔT8 *mutants on apples*

Phenotype differences between the wild type and both *PeSte12* mutants were mainly detected in conidiation during an advanced stage of apple decay (Fig. 2). Fruit stored for 18 days at 20 ºC showed that 100 % of apples at all maturity stages inoculated with both mutants showed < 5 % of the fruit surface covered with conidia. However, in the case of immature, mature and over-mature apples inoculated with the wild type, only 40, 0 and 0 %, respectively of the fruit showed < 5 % of surface covered with conidia. Moreover, 67 % of mature and 92 % of over-mature apples inoculated with the wild type showed >61 % of fruit surface covered with conidia, while in the case of apples inoculated with the mutants, a conidiation index of 4 was never reached.

Fruit inoculated with wild type and stored for 102 d at 0 ºC showed < 5 % of fruit surface covered with conidia in 95 % of immature, 27 % of mature and 27 % of over-mature apples. On the contrary, apples inoculated with both deletant mutants exhibited < 5 % of fruit surface covered with conidia in 100 % of immature and mature and more than 80 % of over-mature apples. Moreover, in 17 % of over-mature apples inoculated with the wild type, conidia covered more than 61 % of the apple surface while none of the mutants reached this level of conidiation.

3.6. *Microscopy morphology of the P. expansum wild type and ΔT6 and ΔT8 mutants on apples*

Differences in morphology between the *P. expansum* wild type and ΔT6 *and* ΔT8 mutants during apple colonization at 20 ºC were evaluated using a stereoscope and a light microscope (Fig. 3).

Independently if immature apples were inoculated with the wild type or with both deletion mutants, the first macerated symptoms were observed after 1 day post inoculation using a pathogen concentration of 10^4 conidia/mL (data not shown). Moreover, the superficial mycelium appeared at 3 days post inoculation without visual differences between the wild type and the mutants (data not shown). First conidia were visually detected at 13 days post inoculation on apples inoculated with the wild type (Fig. 3A and 3D), however only mycelium was observed in apples inoculated with ΔT6 (Fig 3B and 3E) and ΔT8 mutants (Fig. 3C and 3F). At longer storage periods, while the wild type covered completely the disk surface with mycelium and conidia, both *PeSte12* deletant mutants had only spores at the initial infection site (data not shown). Despite the clear visual differences between the wild type and both mutants, no differences in morphology were observed when the structures were visualised under 40x magnification with the light microscope throughout the different times evaluated (Fig.

372 3G-I). Both ΔT6 and ΔT8 mutants seemed to produce typical phialides and similar
373 length of metulae.

374 Since both mutants followed the same behaviour in virulence and conidiation only ΔT6
375 was used for further studies.

376 *3.7. Hydrogen peroxide quantification*

377 The production of H_2O_2 was measured in mature apples inoculated with either water
378 (control), ΔT6 mutant or the wild type *P. expansum* (Fig. 4). Apples inoculated with
379 water suffered a reduction in H_2O_2 after 4 hours of inoculation (30.3 µmol $Kg^{-1}FW$).
380 From this moment, H_2O_2 increased achieving 62.6 µmol $Kg^{-1}FW$ and then remained
381 without significant differences until 30 hpi. In the case of apples inoculated with both
382 wild type *P. expansum* and ΔT6, a similar pattern and tendency was observed, although
383 ΔT6 produced more H_2O_2. Both fungi seemed to stimulate H_2O_2 production during the
384 first 4 h (41.1 and 44.6 µmol $Kg^{-1}FW$, respectively), while during the time-curse
385 experiment they showed a H_2O_2 inhibition in comparison to water inoculation.
386 However, apples inoculated with ΔT6 mutant after 16, 24 and 30 h showed higher H_2O_2
387 production (45.7, 52.0 and 45.6 µmol $Kg^{-1}FW$, respectively) than apples inoculated with
388 the wild type (34.0, 36.3 and 35.4 µmol $Kg^{-1}FW$, respectively).

389 *3.8. Analysis of P. expansum PeSte12 gene expression*

390 Evaluation of *P. expansum PeSte12* gene expression was conducted using quantitative
391 RT-PCR (Fig. 5). We performed assays using *P. expansum* wild-type strain CMP-1 and
392 both deletant mutants at three different time points (1, 2 and 3 days). In axenic liquid
393 culture *P. expansum* wild type kept its expression more or less with the same rate over
394 time. During apple infection the transcription abundance increased over time,
395 particularly at 2 dpi in which the increment was 5 fold compared to 1 dpi (Fig. 5). The
396 gene expression during apple infection was induced after 2 dpi around two fold

compared to *in vitro* growth. As expected, no expression was detected in deletants mutants neither in axenic culture nor during apple infection (data not shown).

3.9. Analysis of the gene expression of several genes in ΔT6 *deletant mutant during in vitro growth*

The exploration of gene expression of several genes in ΔT6 deletant mutant was conducted by using qRT-PCR. The transcription abundance of 22 *P. expansum* genes (Table S2) was evaluated during axenic growth in liquid culture, comparing the wild type (WT) and ΔT6 deletant mutant (Fig. 6). Genes used in this study were selected based on their putative role during pathogen infection (Table S2).

Within these 22 genes, only 5: PEX1_034030, PEX1_094750, PEX1_018170, PEX1_034010 and PEX1_001890 showed repression in the ΔT6 mutant compared to the WT in at least one time. These 5 genes putatively encode a peptidase, an endoglucanase, a hypothetical protein, a fribillarin and a glutamine synthase, respectively. The grade of transcriptional abundance was very high with the exception of PEX1_001890 (glutamine synthase), which exhibited very low level of gene expression. In some cases, the t differences between ΔT6 and WT were restricted to a specific time, as is the case for PEX1_001890, PEX1_018170 and PEX1_034030, in which changes were identified at one time (1, 2 or 3 dpi, respectively) and PEX1_094750 (1 and 3 dpi). Only in PEX1_034010, the modifications were patent through all time points of the analysis (Fig. 6A).

The rest of the analysed genes (17 of the 22), showed induction in ΔT6 compared to WT. Within these genes, most of them (15 of 17) exhibited an increased expression throughout the three days, reaching the highest level of expression at 2-3 dpi. Nevertheless, transcriptional abundance varied from one gene to other. The highest expression values (>2500 RGE) were reached by PEX1_014410, PEX1_019060,

PEX1_032120 and PEX1_051270, which are putatively involved in detoxification, ATPase activity, protein folding and basic metabolism, respectively. PEX1_010730, PEX1_016620, PEX1_016860, PEX1_035540, PEX1_055640 and PEX1_069350, which code for a C2H2 type transcription factor, an alcohol dehydrogenase, a choline kinase, an aldolase, an endopolygalacturonase and a MFS transporter, respectively, reached medium values (90-1000 RGE) (Fig. 6B).

The lowest gene expression values corresponded to PEX1_018240, PEX1_047170, PEX1_078460, PEX1_083600 and PEX1_089030, which encode a hypothetically MFS transporter, a SLT2 like MAP kinase, a GAL_4 type transcription factor, a 3-hydroxyacyl-CoA dehydrogenase and ABC transporter, respectively (Supplementary Fig. 2).

Of 17 genes induced in ΔT6 compared to WT, only PEX1_056420 (G3PD) and PEX1_094410 (aldolase-type TIM barrel) showed a decreasing pattern of gene expression with time. Transcriptional abundance in PEX1_094410 decrease slightly with time, whereas PEX1_056420 gene expression dropped drastically 10-fold between 1 and 3 dpi (Supplementary Fig. 2).

3.10. Analysis of the gene expression of several genes in ΔT6 *deletant mutant during apple infection*

Of the 22 genes evaluated *in vitro,* 9 of them were selected to assess gene expression during apple infection (Table S2 in bold) based on their putative function and their expression profile in axenic growth. Surprisingly, transcriptional abundance during infection showed less differences between ΔT6 and WT than those observed *in vitro* (Fig. 7). Most of them (6 of 9) PEX1_19060, PEX1_032120, PEX1_034030, PEX1_051270, PEX1_069350 and PEX1_094750 showed slight differences between the ΔT6 mutant and WT, and exhibited lower grade of gene expression during infection

compared to axenic growth reducing one or two orders of magnitude in some cases (Fig. 7A). On the contrary, PEX1_018170 (Hypothetical protein), PEX1_034010 (fibrillarin) and PEX1_035540 (aldolase-type TIM barrel), exhibited intensification higher relative expression level during apple infection than during in vitro growth. PEX1_035540 (aldolase-type TIM barrel) showed the largest differences on expression between WT and ΔT6, but displayed a pattern opposite to the one observed during *in vitro* growth (Fig. 7B). A Higher level of expression in the WT was also observed in PEX1_018170 (Hypothetical protein), but only at 1 dpi. No differences were observed in PEX1_034010 (fribrillarin) between ΔT6 and WT.

Moreover, in order to confirm if *PeSte12* could regulate patulin production, the relative expression levels of three relevant genes in patulin pathway, *patK* (PEX1-002430) coding for 6-methylsalicylic acid synthase, the first enzyme of the patulin pathway, *patL* (PEX1-002400) encoding a transcription factor, and *patN* (PEX1_5160) coding for a isoepoxydon dehydrogenase, catalyzing one of the last steps of the pathway (White et al., 2006), were evaluated comparing the wild-type and ΔT6 deletant mutant during apple infection. No differences were observed in any of the three genes assayed between the wild-type and the mutant ΔT6 (Fig. 7C).

4. Discussion

This work accounts for the functional characterization of *PeSte12*, a particular type of C2H2 fungal transcription factor, in the pome fruit postharvest pathogen *P. expansum*. Examination of the *PeSte12* sequence showed high similarity to other fungal Ste12 genes and demonstrated the existence of the consensus sequence TGAAACA in the promoter. This sequence was identified as the pheromone responsive element (PRE), which suggests that in the presence of certain stimuli it could trigger a response as part

of the activation of cellular cascades. In yeast, the presence of this PRE is correlated with the interaction with the Ste12 protein which is sufficient to confer pheromone responsiveness (Yuan and Fields, 1991). Additionally, the *PeSte12* gene contains two typical C-terminally placed strongly linked C2H2 zinc fingers that are also found in other Ste12 genes (de Ramón-Carbonell and Sánchez-Torres, 2017).

The possibility of new breakthroughs in the control of this pathogen involves a better understanding of the virulence mechanisms deployed by *P. expansum*. Pathogen aggressiveness is controlled by the interactions of several genes that react to signals that appear during host-pathogen interactions (Schamber et al., 2010). The phosphorylation of Fus3/Kss1 MAP kinase can stimulate the transcriptional regulator Ste12, as part of cellular responses, which in turn could act as a specific regulator of pathway-specific genes (Rispail and Di Pietro, 2010). The involvement of *P. expansum PeSte12* in virulence was proved through loss of function where the deletants showed a decrease in aggressiveness, as shown by lower incidence and growth rate and longer visible initial rotting day on apple at 0 ºC of storage temperature. Consequently, our results are in concordance with earlier studies of Ste12 mutants that demonstrated their importance in conidiospore production and disease establishment in the closely related pathogen *P. digitatum* (de Ramón-Carbonell and Sánchez-Torres, 2017; Vilanova et al., 2016) and in another plant pathogenic fungi (García-Sánchez et al., 2010; Rispail and Di Pietro, 2009).

In this study, the deletant mutants were able to infect apple tissue, but decay progression, determined as lesion diameter, was 3-fold lower compared to the wild-type strain, indicating that *PeSte12* affects disease severity more than disease incidence in apples stored at 0 ºC. Similar results were found in citrus fruit infected with *P. digitatum* (de Ramón-Carbonell and Sánchez-Torres, 2017; Vilanova et al., 2016) and

497 in tomato plants, apple fruits (Rispail and Di Pietro, 2009) and common bean seedlings
498 (García-Sánchez et al., 2010) infected with two different *F. oxysporum* strains.
499 During fruit-pathogen interactions, *P. expansum* produces asexual spores (conidia) as
500 starting point to ensure successful infection. Like many plant pathogenic fungi, the
501 infectious process of *P. expansum* is initiated with germination of conidia in the
502 wounds' surface of pome fruits. Although, in this work *PeSte12* disruption mutants did
503 not show visible alterations in either colony morphology or conidia production during
504 axenic growth, they had impaired sporulation ability during infection of fruit as it was
505 previously reported in *P. digitatum* by Vilanova et al. (2016). Spores appeared only at
506 longer times of infection (more than 18 days post-inoculation) while the wild-type
507 CMP-1 fully covered the apple fruit with spores much earlier. Therefore, the prevention
508 of sporulation is clearly restricted to *PeSte12* gene elimination.
509 Our results showed that conidiation was impaired in both mutants (ΔT6 and ΔT8) and
510 the effect was more perceptible at 0 ºC, in which *P. expansum* infection progress was
511 slower. Stereoscope magnification revealed differences between wild type and both
512 disruption mutants and whereas the wild type strain was able to completely cover the
513 apple surface and produce clear sporulation, deletant mutants produced much less
514 spores. Microscopy evaluation did no clarify such differences in conidiation since both
515 mutants presented phialides and metula similar to the wild type. The mechanisms of
516 conidiogenesis in *P. expansum* has not been reported so far, but they are of particular
517 importance for the development of new strategies for blue mould control. To date,
518 several genes have been associated with conidiation in the closely related pathogen, *P.*
519 *digitatum* (Harries et al., 2015; Ma et al., 2016; Wang et al., 2015). Distinct stages of
520 conidiogenesis in *P. digitatum* can be related to the transcription factors PdbrlA,
521 PdabaA and PdwetA that control the formation of conidiophores, phialides and conidia

(Wang et al., 2015). In addition, Ma et al. (2016) reported that deletion of *PdSteA* (actually *PdSte12*) prevent the expression of PdwetA and conidial formation. Although the expression of *PdSte12* apparently was not regulated by Pd*MpkB* (Fus 3 MAPK of *P. digitatum*), probably, *PdSte12* could interact with Pd*MpkB* that act as a global regulator of cell proliferation and conidial formation as described in *Fusarium graminearum* (Gu et al., 2015). This might be a plausible explanation for conidiation impairment on *Pe*Δ*Ste12.*

One of the first responses to a pathogen attack is the production of reactive oxygen species (ROS), which are differentially produced depending on compatible or non-host pathogens. Moreover, ROS production have a special interest because involve different functions in the plant defence strategy. Concretely, ROS are involved in membrane peroxidation, cross-linking of cell wall proteins, induction of hypersensitive response, lignification process and expression of a wide array of defence-related proteins. Few studies have reported the production of ROS when fungal pathogens invade fruit tissues (see review of Tian et al. (2016)), and fewer studies have been focused on elucidating the mechanisms that regulate the fruit response after the oxidative burst occurs. Moreover, very little is known about the implication of the transcription factor Ste12 in the production of hydrogen peroxide (H_2O_2). To our knowledge only one study has demonstrated the association between the reduced virulence of the *PdSte12* mutant with higher H_2O_2 production in oranges in comparison to the wild type strain (Vilanova et al., 2016). In the present work we have found similar results, in which the production of H_2O_2 in response to *PeSte12* inoculation was higher than that obtained with the wild type strain. Moreover, our results are in agreement with Buron-Moles et al. (2015), who correlated the reduced virulence of the non-host pathogen *P. digitatum* with higher levels of H_2O_2 in apples compared to the compatible pathogen *P. expansum*. These

results could suggest that a reduction in pathogen virulence mediated by silencing Ste12 gene could affect the ability of *P. expansum* to suppress H_2O_2.

Expression of *PeSte12* during apple infection increased as infection progressed, showing the highest level at 3 dpi. This in contrast to what was observed in *PdSte12* from *P. digitatum*, whose expression was maximum at the first stages of infection (de Ramón-Carbonell and Sánchez-Torres, 2017). Expression analysis showed clearly the relevance of *PeSte12* in fungal virulence, since transcriptional abundance was doubled during apple infection compared to axenic growth from 2 dpi.

Nevertheless, the role of PeSte12 in virulence could be originated most likely through the control of other genes that affect infectivity rather than for being a virulence factor (Odds et al., 2001). In *P. digitatum*, *PdSte12* regulates both positively and negatively the expression of several genes, triggering multiple responses such as detoxification, oxidative burst or virulence (Vilanova et al., 2016). In *P. expansum*, *PeSte12* expression analysis in axenic growth suggested that it acts mostly as negative regulator (17 of 22 genes evaluated) and in the majority of analysed genes with an increase pattern of gene expression. The genes that showed higher rate of gene expression were correlated to ATPase activity, carbon metabolism, chaperones, aldolases and to detoxification. ATPases function as integral membrane proteins and move solutes across the membrane, typically against their concentration gradient (Lee et al., 2004). PEX1_032120 codes for a heat-shock protein and is described as an essential protein of the stress response, where it mainly functions as a molecular chaperone. This type of protein are described as being protein disaggregating machines, acting side by side with other chaperones and proteases to ensure protein quality control in the cell (Doyle and Wickner, 2009). Detoxification was represented by glutathione transferases (GSTs), which are an extended protein family involved in detoxification and tolerance to

572 oxidative stress. Some of them have been reported as essential for aggressiveness of
573 plant pathogenic fungi (Calmes et al., 2015). Although all these genes seem to be
574 negatively regulated by *PeSte12*, they showed lower expression in the ΔT6, and their
575 expression profiles, rate of repression and behaviour throughout the time evaluated was
576 very different from the wild type.

577 Genes with medium rate of gene expression included diverse functions that ranged from
578 metabolism (carbon, amino acid metabolism), pectinase, fungal transporter (MFS) or
579 transcription factor. The low rate of gene expression was observed for genes encoding
580 transporters (MFS or ABC), signal transduction (MAPK or transcription factor) and
581 metabolism.

582 Positive regulation by *PeSte12* was only observed in five genes, from which only 4 had
583 a predicted function: peptidase, endoglucanase, fibrillarin and glutamine synthase. No
584 correlation in expression pattern was found among them. The highly expressed did not
585 match with any known function. Endoglucanase and glutamine synthase (GS) were
586 expressed at the first stages. The endoglucanase had very high rate of expression and
587 generally is reported as many cell wall degrading enzymes (CWDE) for being relevant
588 for fungal infection. Glutamine synthase had an essential regulatory role in the nitrogen
589 regulation network and secondary metabolism (Wagner et al., 2013), although in *P.*
590 *expansum* its expression was very low.

591 Surprisingly, when some of these genes were evaluated during apple infection, most of
592 them revealed not to be significant for fungal infection, since their expression level was
593 lower than during *in vitro* growth. Only PEX1_034010 (fibrillarin), PEX1_018170
594 (Hypothetical protein) and PEX1_035540 (aldolase-type TIM barrel) showed higher
595 expression (3, 10 and 4 times respectively). Moreover, only PEX1_035540 appeared to

596 be regulated by *PeSte12*, since its expression in the ΔT6 deletant was much lower than
597 in the WT.
598 In the case of the three genes involved in patulin pathway, *PeSte12* has no effect in their
599 regulation since both wild type and deletant mutant exhibited the same rate of gene
600 expression and no induction during apple infection was observed comparing to previous
601 results shown in *P. expansum* CMP-1 in axenic growth (Ballester et al., 2015)
602 confirming the hypothesis of the absence of association between virulence and patulin
603 production.
604 We observed differences in the gene expression patterns between *in vitro* and *in vivo*
605 assays. In the particular case of PEX1_035540 (aldolase-type TIM barrel), several genes
606 that belong to the same group family showed a gene expression pattern that clearly
607 varied depending on pH and environmental conditions (Barad et al., 2016). pH
608 modulation seems to be one of the main ways to control pathogen success during host-
609 pathogen interaction and in *P. expansum* many genes are controlled by the
610 environmental pH. This effect might be occurred because genes are triggered depending
611 on their optimal environmental situations (pH, nutrients) to allow the best use of
612 enzymes (Barad et al., 2016).
613 In summary, this study illustrates the contribution of *PeSte12* in conidiation and
614 virulence in *P. expansum*. The mode of action at medium stage of the fruit-fungus
615 interaction might indicate that it administrates the activation or repression of specific
616 genes that might be important for the infection progress. The approach of targeting the
617 production of conidia, which are crucial for fungal life and for the development of
618 fungal disease, may result an alternative for controlling postharvest fungal pathogens,
619 and opens up new expectations within this field.

620 **5. Acknowledgements**

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6. References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Ballester, A.R., Marcet-Houben, M., Levin, E., Sela, N., Selma-Lázaro, C., Carmona, L., Wisniewski, M., Droby, S., González-Candelas, L., Gabaldón, T., 2015. Genome, transcriptome, and functional analyses of *Penicillium expansum* provide new insights into secondary metabolism and pathogenicity. Mol. Plant Microbe Interact. 28, 232-248.

Barad, S., Horowitz, S.B., Kobiler, I., Sherman, A., Prusky, D., 2014. Accumulation of the mycotoxin patulin in the presence of gluconic acid contributes to pathogenicity of *Penicillium expansum*. Mol. Plant Microbe Interact. 27, 66-77.

Barad, S., Sela, N., Kumar, D., Kumar-Dubey, A., Glam-Matana, N., Sherman, A., Prusky, D., 2016. Fungal and host transcriptome analysis of pH-regulated genes during colonization of apple fruits by *Penicillium expansum*. BMC Genomics 17, 330.

Buron-Moles, G., Torres, R., Teixidó, N., Usall, J., Vilanova, L., Viñas, I., 2015. Characterisation of H_2O_2 production to study compatible and non-host pathogen interactions in orange and apple fruit at different maturity stages. Postharvest Biol. Technol. 99, 27-36.

644 Calmes, B., Morel-Rouhier, M., Bataillé-Simoneau, N., Gelhaye, E., Guillemette, T.,
645 Simoneau, P., 2015. Characterization of glutathione transferases involved in the
646 pathogenicity of *Alternaria brassicicola*. BMC Microbiology 15, 123.
647 Crespo-Sempere, A., Selma-Lázaro, C., Martínez-Culebras, P.V., González-Candelas,
648 L., 2013. Characterization and disruption of the *cipC* gene in the ochratoxigenic fungus
649 *Aspergillus carbonarius*. Food Res. Int. 54, 697-705.
650 de Ramón-Carbonell, M., Sánchez-Torres, P., 2017. The transcription factor *PdSte12*
651 contributes to *Penicillium digitatum* virulence during citrus fruit infection. Postharvest
652 Biol. Technol. 125, 129-139.
653 Doyle, S.M., Wickner, S., 2009. Hsp104 and ClpB: protein disaggregating machines.
654 Trends in Biochemical Sciences 34, 40-48.
655 Droby, S., Eick, A., Macarisin, D., Cohen, L., Rafael, G., Stange, R., McColum, G.,
656 Dudai, N., Nasser, A., Wisniewski, M., Shapira, R., 2008. Role of citrus volatiles in
657 host recognition, germination and growth of *Penicillium digitatum* and *Penicillium*
658 *italicum*. Postharvest Biol. Technol. 49, 386-396.
659 Frandsen, R.J.N., Andersson, J.A., Kristensen, M.B., Giese, H., 2008. Efficient four
660 fragment cloning for the construction of vectors for targeted gene replacement in
661 filamentous fungi. BMC Mol. Biol. 9, 70.
662 García-Sánchez, M.A., Martín-Rodrigues, N., Ramos, B., de Vega-Bartol, J.J., Perlin,
663 M.H., Díaz-Mínguez, J.M., 2010. fost12, the *Fusarium oxysporum* hornolog of the
664 transcription factor Ste12, is upregulated during plant infection and required for
665 virulence. Fungal Genet. Biol. 47, 216-225.
666 Gu, Q., Zhang, C., Liu, X., Ma, Z., 2015. A transcription factor FgSte12 is required for
667 pathogenicity in *Fusarium graminearum*. Mol. Plant Pathol. 16, 1-13.

668 Harries, E., Gandía, M., Carmona, L., Marcos, J.F., 2015. The *Penicillium digitatum*
669 protein *O-mannosyltransferase* Pmt2 is required for cell wall integrity, conidiogenesis,
670 virulence and sensitivity to the antifungal peptide PAF26. Mol. Plant Pathol. 16, 748-
671 761.
672 Hoi, J.W.S., Dumas, B., 2010. Ste12 and Ste12-like proteins, fungal transcription
673 factors regulating development and pathogenicity. Eukaryot. Cell 9, 480-485.
674 Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A.,
675 McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D.,
676 Gibson, T.J., Higgins, D.G., 2007. Clustal W and clustal X version 2.0. Bioinformatics
677 23, 2947-2948.
678 Lee, S.H., Singh, A.P., Chung, G.C., Ahn, S.J., Noh, E.K., Steudle, E., 2004. Exposure
679 of roots of cucumber (*Cucumis sativus*) to low temperature severely reduces root
680 pressure, hydraulic conductivity and active transport of nutrients. Physiol. Plant 120,
681 413-420.
682 Li, B.Q., Zong, Y.Y., Du, Z.L., Chen, Y., Zhang, Z.Q., Qin, G.Z., Zhao, W.M., Tian,
683 S.P., 2015. Genomic characterization reveals insights into patulin biosynthesis and
684 pathogenicity in *Penicillium* species. Mol. Plant Microbe Interact. 28, 635-647.
685 Ma, H., Sun, X., Wang, M., Gai, Y., Chung, K.-R., Li, H., 2016. The citrus postharvest
686 pathogen *Penicillium digitatum* depends on the PdMpkB kinase for developmental and
687 virulence functions. Int. J. Food Microbiol. 236, 167-176.
688 Marcet-Houben, M., Ballester, A.-R., de la Fuente, B., Harries, E., Marcos, J.F.,
689 González-Candelas, L., Gabaldon, T., 2012. Genome sequence of the necrotrophic
690 fungus *Penicillium digitatum*, the main postharvest pathogen of citrus. BMC Genomics
691 13.

692 Moake, M.M., Padilla-Zakour, O.I., Worobo, R.W., 2005. Comprehensive review of
693 patulin control methods in foods. Comprehensive Reviews in Food Science and Food
694 Safety 4, 8-21.
695 Moller, E.M., Bahnweg, G., Sandermann, H. and Geiger, H.H. (1992) A simple and
696 efficient protocol for isolation of high-molecular-weight DNA from filamentous fungi,
697 fruit bodies, and infected-plant tissues. Nucleic Acids Res. 20, 6115–6116.
698 Morales, H., Sanchis, V., Rovira, A., Ramos, A.J., Marín, S., 2007. Patulin
699 accumulation in apples during postharvest: Effect of controlled atmosphere storage and
700 fungicide treatments. Food Control 18, 1443-1448.
701 Odds, F.C., Gow, N.A.R., Brown, A.J.P., 2001. Fungal virulence studies come of age.
702 Genome Biol. 2, reviews: 1009.1001–1009.1004.
703 Park, G., Bruno, K.S., Staiger, C.J., Talbot, N.J., Xu, J.R., 2004. Independent genetic
704 mechanisms mediate turgor generation and penetration peg formation during plant
705 infection in the rice blast fungus. Mol. Microbiol. 53, 1695-1707.
706 Park, G., Xue, G.Y., Zheng, L., Lam, S., Xu, J.R., 2002. MST12 regulates infectious
707 growth but not appressorium formation in the rice blast fungus *Magnaporthe grisea*.
708 Mol. Plant Microbe Interact. 15, 183-192.
709 Perez-Nadales, E., Nogueira, M.F., Baldin, C., Castanheira, S., El Ghalid, M., Grund,
710 E., Lengeler, K., Marchegiani, E., Mehrotra, P.V., Moretti, M., Naik, V., Oses-Ruiz, M.,
711 Oskarsson, T., Schäfer, K., Wasserstrom, L., Brakhage, A.A., Gow, N.A., Kahmann, R.,
712 Lebrun, M.H., Perez-Martin, J., Di Pietro, A., Talbot, N.J., Toquin, V., Walther, A.,
713 Wendland, J., 2014. Fungal model systems and the elucidation of pathogenicity
714 determinants. Fungal Genet. Biol. 70, 42-67.
715 Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time
716 RT–PCR. Nucleic Acids Res, 29:e45.

717 Prober, J., Trainor, G., Dam, R., Hobbs, F., Robertson, C., Zagursky, R., Cocuzza, A.,
718 Jensen, M., Baumeister, K., 1987. A system for rapid DNA sequencing with fluorescent
719 chain-terminating dideoxynucleotides. Science 238, 336-341.
720 Qin, G.Z., Tian, S.P., Chan, Z.L., Li, B.Q., 2007. Crucial role of antioxidant proteins
721 and hydrolytic enzymes in pathogenicity of *Penicillium expansum* - Analysis based on
722 proteomics approach. Molecular & Cellular Proteomics 6, 425-438.
723 Rispail, N., Di Pietro, A., 2009. *Fusarium oxysporum* Ste12 controls invasive growth
724 and virulence downstream of the Fmk1 MAPK cascade. Mol. Plant Microbe Interact.
725 22, 830-839.
726 Rispail, N., Di Pietro, A., 2010. The homeodomain transcription factor Ste12:
727 connecting fungal MAPK signalling to plant pathogenicity. Commun. Integr. Biol. 3,
728 327-332.
729 Sánchez-Torres, P., González-Candelas, L., 2003. Isolation and characterization of
730 genes differentially expressed during the interaction between apple fruit and *Penicillium*
731 *expansum*. Mol. Plant Pathol. 4, 447-457.
732 Sanzani, S.M., Reverberi, M., Punelli, M., Ippolito, A., Fanelli, C., 2012. Study on the
733 role of patulin on pathogenicity and virulence of *Penicillium expansum*. Int. J. Food
734 Microbiol. 153, 323-331.
735 Schamber, A., Leroch, M., Diwo, J., Mendgen, K., Hahn, M., 2010. The role of
736 mitogen-activated protein (MAP) kinase signalling components and the Ste12
737 transcription factor in germination and pathogenicity of *Botrytis cinerea*. Mol. Plant
738 Pathol. 11, 105-119.
739 Snini 2016. Patulin is a cultivar-dependent aggressiveness factor favouring the
740 colonization of apples by *Penicillium expansum*. Mol. Plant Pathol 17, 920–930.

741 Tian, S., Torres, R., Ballester, A.R., Li, B., Vilanova, L., González-Candelas, L., 2016.
742 Molecular aspects in pathogen-fruit interactions: virulence and resistance. Postharvest
743 Biol. Technol. 122, 11-21.
744 Tian, S.P., Qin, G.Z., Li, B.Q., 2013. Reactive oxygen species involved in regulating
745 fruit senescence and fungal pathogenicity. Plant Molecular Biology 82, 593-602.
746 Tsuji, G., Fujii, S., Tsuge, S., Shiraishi, T., Kubo, Y., 2003. The *Colletotrichum*
747 *lagenarium* Ste12-like gene CST1 is essential for appressorium penetration. Mol. Plant
748 Microbe Interact. 16, 315-325.
749 Vilanova, L., Teixidó, N., Torres, R., Usall, J., Viñas, I., 2012. The infection capacity of
750 *P. expansum* and *P. digitatum* on apples and histochemical analysis of host response.
751 Int. J. Food Microbiol. 157, 360-367.
752 Vilanova, L., Teixidó, N., Torres, R., Usall, J., Viñas, I., Sánchez-Torres, P., 2016.
753 Relevance of the transcription factor *PdSte12* in *Penicillium digitatum* conidiation and
754 virulence during citrus fruit infection. Int. J. Food Microbiol. 235, 93-102.
755 Vilanova, L., Viñas, I., Torres, R., Usall, J., Buron-Moles, G., Teixidó, N., 2014a.
756 Acidification of apple and orange hosts by *Penicillium digitatum* and *Penicillium*
757 *expansum*. Int. J. Food Microbiol. 178, 39-49.
758 Vilanova, L., Wisniewski, M., Norelli, J., Viñas, I., Torres, R., Usall, J., Phillips, J.,
759 Droby, S., Teixidó, N., 2014b. Transcriptomic profiling of apple in response to
760 inoculation with a pathogen (*Penicillium expansum*) and a non-pathogen (*Penicillium*
761 *digitatum*). Plant Mol. Biol. Rep. 32, 566-583.
762 Wagner, D., Wiemann, P., Huß, K., Brandt, U., Fleißner, A., Tudzynski, B., 2013. A
763 sensing role of the glutamine synthetase in the nitrogen regulation network in *Fusarium*
764 *fujikuroi*. PLoS One 8, e80740.

765 Wang, M., Sun, X., Zhu, C., Xu, Q., Ruan, R., Yu, D., Li, H., 2015. PdbrlA, PdabaA
766 and *PdwetA* control distinct stages of conidiogenesis in *Penicillium digitatum*. Res.
767 Microbiol. 166, 56-65.
768 White, S., O'Callaghan, J., Dobson, A.D.W., 2006. Cloning and molecular
769 characterization of *Penicillium expansum* genes upregulated under conditions
770 permissive for patulin biosynthesis. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.
771 255, 17-26.
772 Yuan, Y.L., Fields, S., 1991. Properties of the DNA-binding domain of the
773 *Saccharomyces cerevisiae* STE12 protein. Mol. Cell. Biol. 11, 5910-5918.
774 rulence and DMI resistance in *Penicillium digitatum*. Microbiol. Res. 168, 211-222.

775

Fig. 1. Lesion diameter (cm) caused by the inoculation of *Penicillium expansum* wild type (grey circle), and ΔT6 (white square) and ΔT8 (black triangle) deletion mutants at different days post inoculation (dpi). 'Golden Smoothee' apples were harvested at three different maturity stages: immature (A and D), mature (B and E) and over-mature (C and F) and after the inoculation with conidial suspensions (10^4 conidia mL^{-1}) were stored at 20 ºC (A, B and C) or 0 ºC (D, E and F) and 85 % RH. Each treatment consisted of four replicates of five apples each one.

Fig. 2. Absolute frequency of conidiation index of the *P. expansum* wild type (WT) and the ΔT6 and ΔT8 mutants on immature (A) mature (B) and over-mature (C) 'Golden Smoothee' apples. The conidition index used was categorized such as: (■) 0, soft lesion but no conidia or mycelium present; (▧) 0.5, mycelium but no conidia present; (▩) 1, <5%; (■) 2, 5–30%; (▨) 3, 31–60%; (□) 4, 61–90% and (▪) 5, >91% of the fruit surface covered with conidia. Fruits were inoculated with 10^4 conidia mL^{-1} and stored at 20 ºC and 85 % RH for 18 days. Each treatment consisted of four replicates of five apples each one.

Fig. 3. Immature 'Golden Smoothee' apples wounded and inoculated with the wild type *P. expansum* (WT) and ΔT6 and ΔT8 mutants at 10^4 conidia/mL and observed after 13 days post inoculation at different stereoscope magnification: A-C corresponds to 7.1x and D-F corresponds to 16x; or microscope magnification: G-I corresponds to 40x.

Fig. 4. Production of hydrogen peroxide (H_2O_2) in mature 'Golden Smoothee' apples in response to water, wild type *P. expansum* or ΔT6 inoculation. Apples were inoculated with 10 µL of water or conidial suspensions (10^4 conidia/ mL) and incubated at 20 ºC and 85 % RH for 4, 16, 24 and 30 hours post inoculation. Points with the same letter are not significantly different ($P<0.05$) according to Tukey test at each dpi. Each value is the mean of four replicates constituted by four apples each one ± SE.

Fig. 5. Analysis of *PeSte12* relative gene expression (RGE). Time course evaluation of gene expression of CMP-1 in PDB liquid culture at 24ºC or during apple infection with 10 µL of conidial suspensions (10^4 conidia/ mL) and incubated at 20 ºC. In all cases, d1, d2 and d3 correspond to 1 dpi, 2 dpi and 3 dpi. The expression levels are relative to three reference genes β–tubulin, histone 3 and 37S ribosomal protein. Error bars indicate standard deviations of three biological replicates.

Fig.6. Analysis of *in vitro* relative gene expression (RGE) in wild type (WT) and ΔT6 mutant. Time course evaluation of gene expression of WT (black columns) and deletant mutant ΔT6 (white columns) grown in PDB liquid culture at 24ºC. In all cases, d1, d2 and d3 correspond to 1 dpi, 2 dpi and 3 dpi. The expression levels are relative to three reference genes β–tubulin, histone 3 and 37S ribosomal protein. Error bars indicate standard deviations of three biological replicates.

Fig.7. Analysis of in vivo relative gene expression (RGE) in wild type (WT) and ΔT6 mutant. Time course evaluation of gene expression of WT (black columns) and deletant mutant ΔT6 (white columns) during apple infection with 10 µL of *P. expansum* conidial suspensions (10^4 conidia/ mL) and incubated at 20 ºC. **A**: Gene expression patterns of those genes with lower grade of gene expression during infection compared to axenic growth. **B**: Gene expression patterns of those genes that were induced during apple infection. **C**: Gene expression analysis of three genes involved in patulin synthesis. In all cases, d1, d2 and d3 correspond to 1 dpi, 2 dpi and 3 dpi. The expression levels are relative to three reference genes β–tubulin, histone 3 and 37S ribosomal protein. Error bars indicate standard deviations of three biological replicates.

Supplementary Fig. 1. Construction and analysis of *Penicillium expansum* knock-out *ste12* transformants. (A) Map of plasmid pΔPeS12. (B) Diagram of wild-type locus and

the *ste12* replacement with the HygR selectable marker from pΔPeS12 by homologous recombination to generate the Δ*ste12* mutants. (C) Polymerase chain reaction (PCR) analysis to confirm deletant transformants using HygRt-PE11 primers. Lanes correspond to nine transformants and wild-type CMP-1 strain. (D) Axenic growth on PDA plates during 6 days at 25ºC of *P. expansum* wild type CMP-1 and ΔT6 and ΔT8 mutant strains.

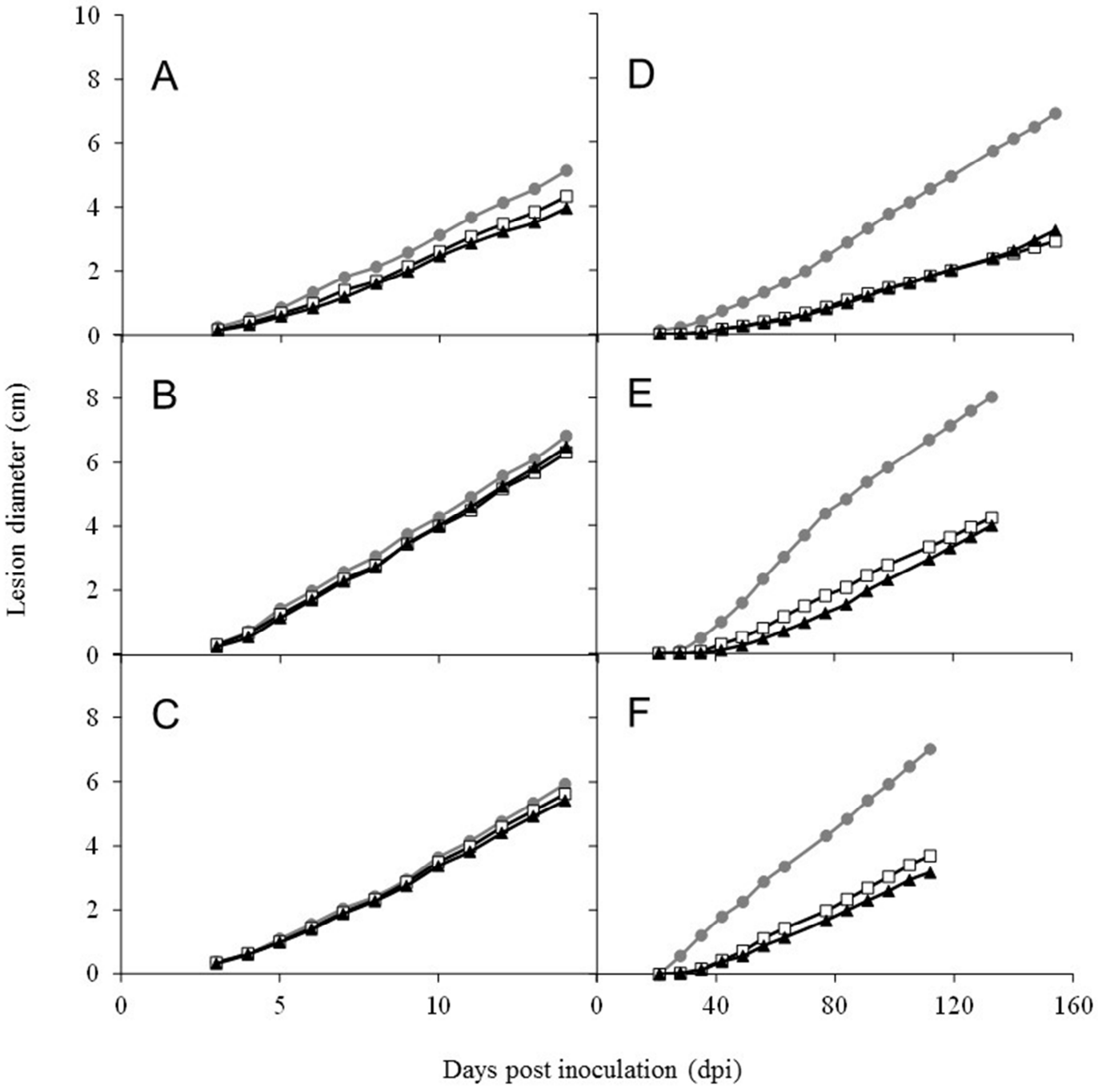
Supplementary Fig.2. Evaluation of *in vitro* relative gene expression (RGE) in WT and ΔT6 mutant. Time course evaluation of gene expression of WT (black columns) and deletant mutant ΔT6 (white columns) grown in PDB liquid culture at 24ºC. In all cases, d1, d2 and d3 correspond to 1 dpi, 2 dpi and 3 dpi. The expression levels are relative to three reference genes β–tubulin, histone 3 and 37S ribosomal protein. Error bars indicate standard deviations of three biological replicates.

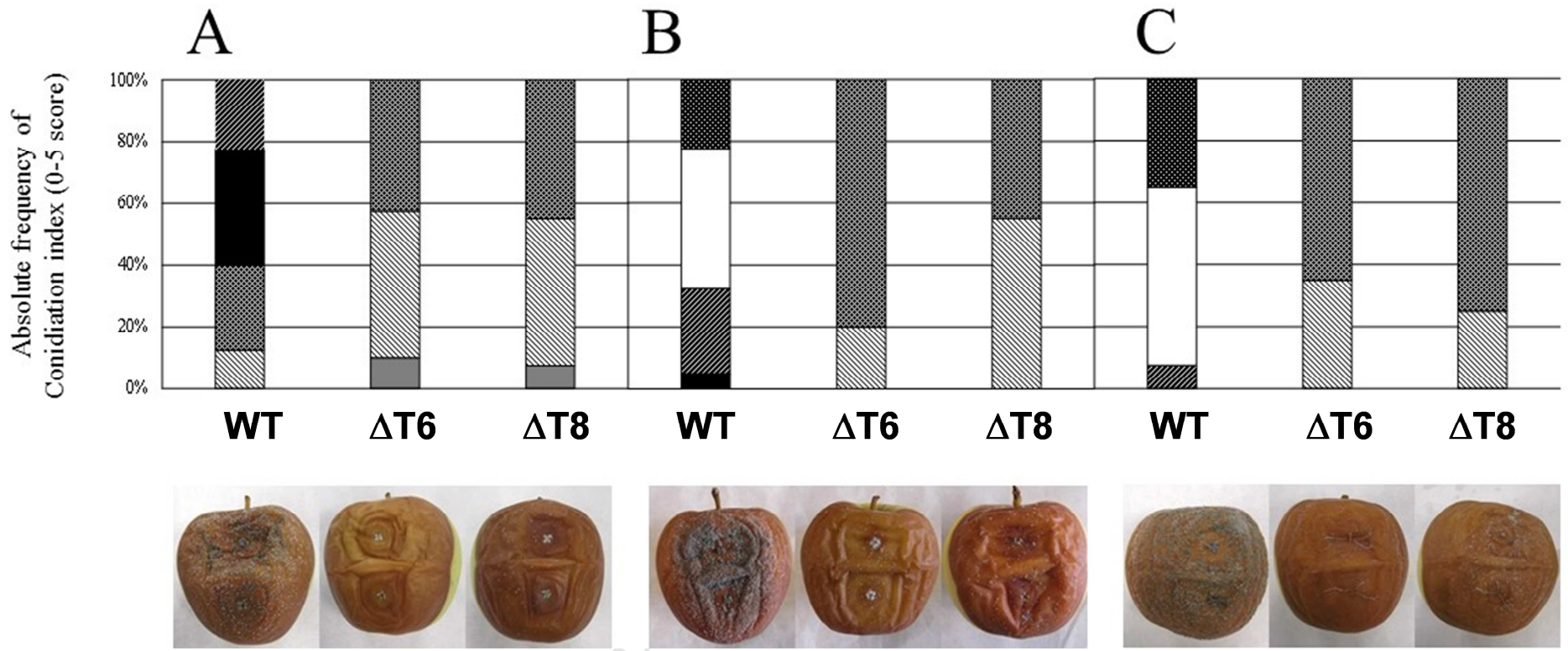
Table 1. Effect of harvest date on fruit quality parameters of 'Golden Smoothee' apples. Values for harvest dates with the same letter are not significantly different ($P < 0.05$) according to the Tukey test.

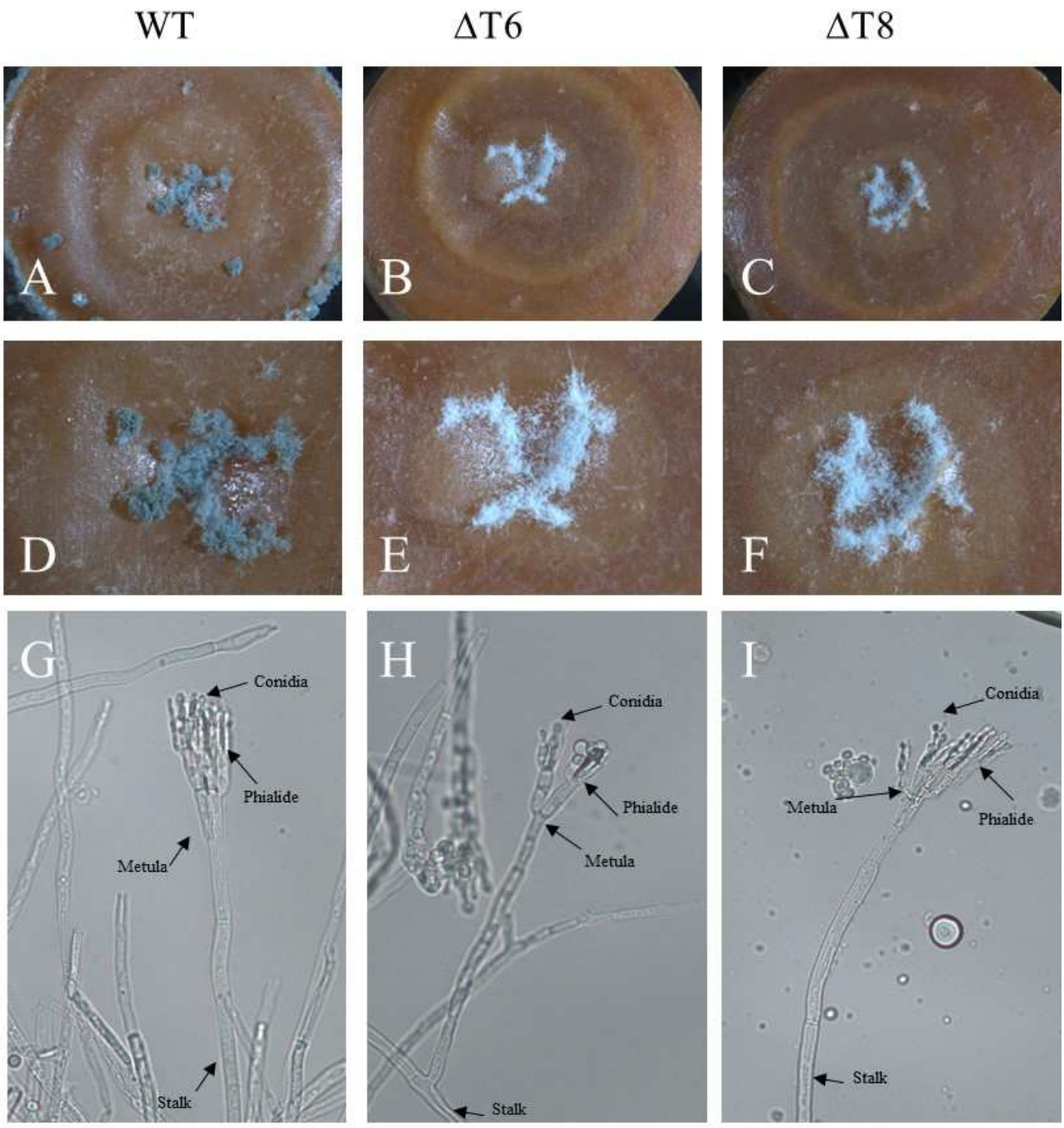
Harvest	**Total soluble solids (TSS in %)**	**Titratable Acidity (gL^{-1} malic acid)**	**Flesh firmness (N)**	**Starch index**	**(a*+b*)**
Immature	9.6 c	5.0 a	71.7 a	3.0 c	25.8 b
Commercial	12.3 b	5.2 a	64.6 b	5.3 b	27.6 b
Over-mature	14.2 a	4.4 b	57.5 c	10.0 a	30.4 a

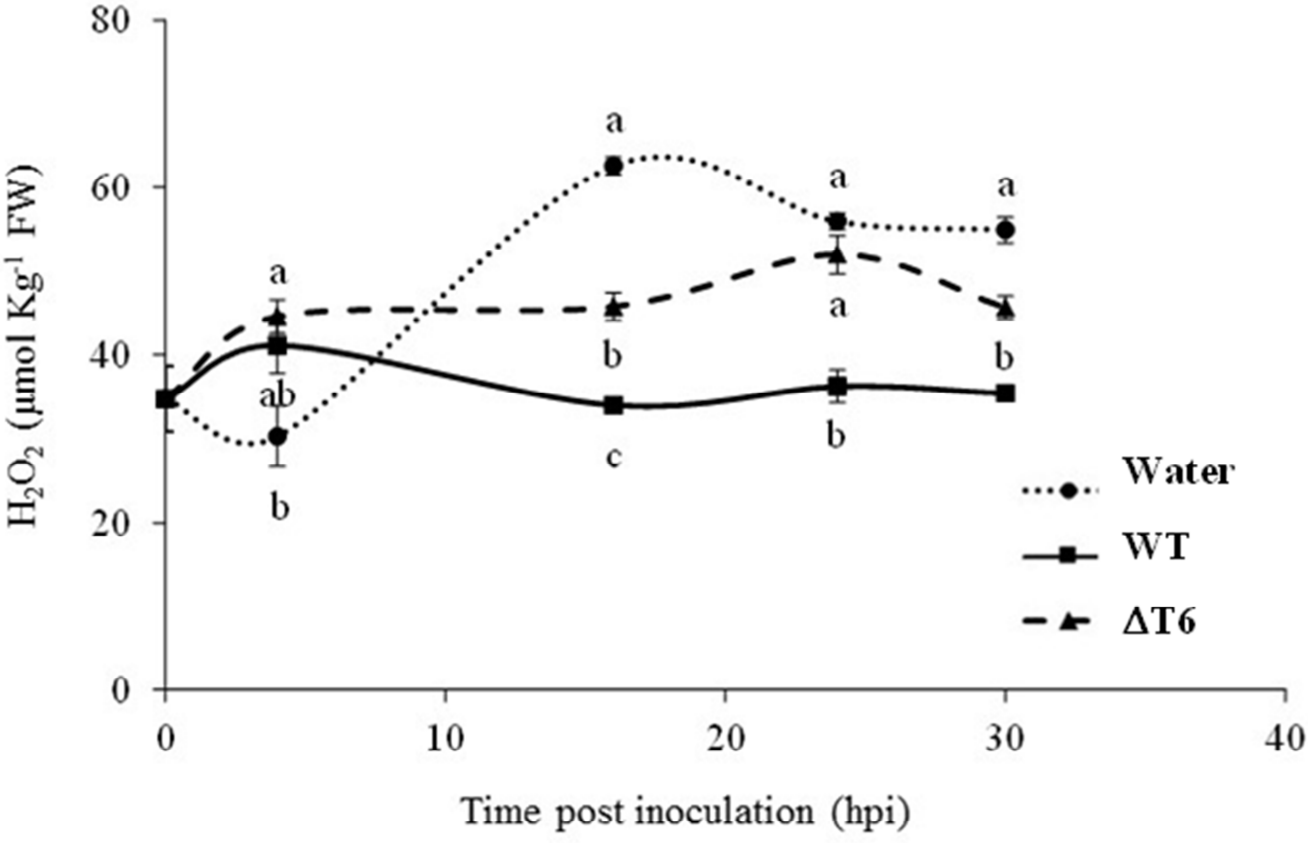
Table 2. Growth rates and visible initial rotting day of *Penicillium expansum* in Golden 'Smoothee apples' at four different inoculum concentration, different harvests and two different storage temperatures. For each inoculum concentration, harvests with different letters are statistically different according to the Tukey test (P<0.05).

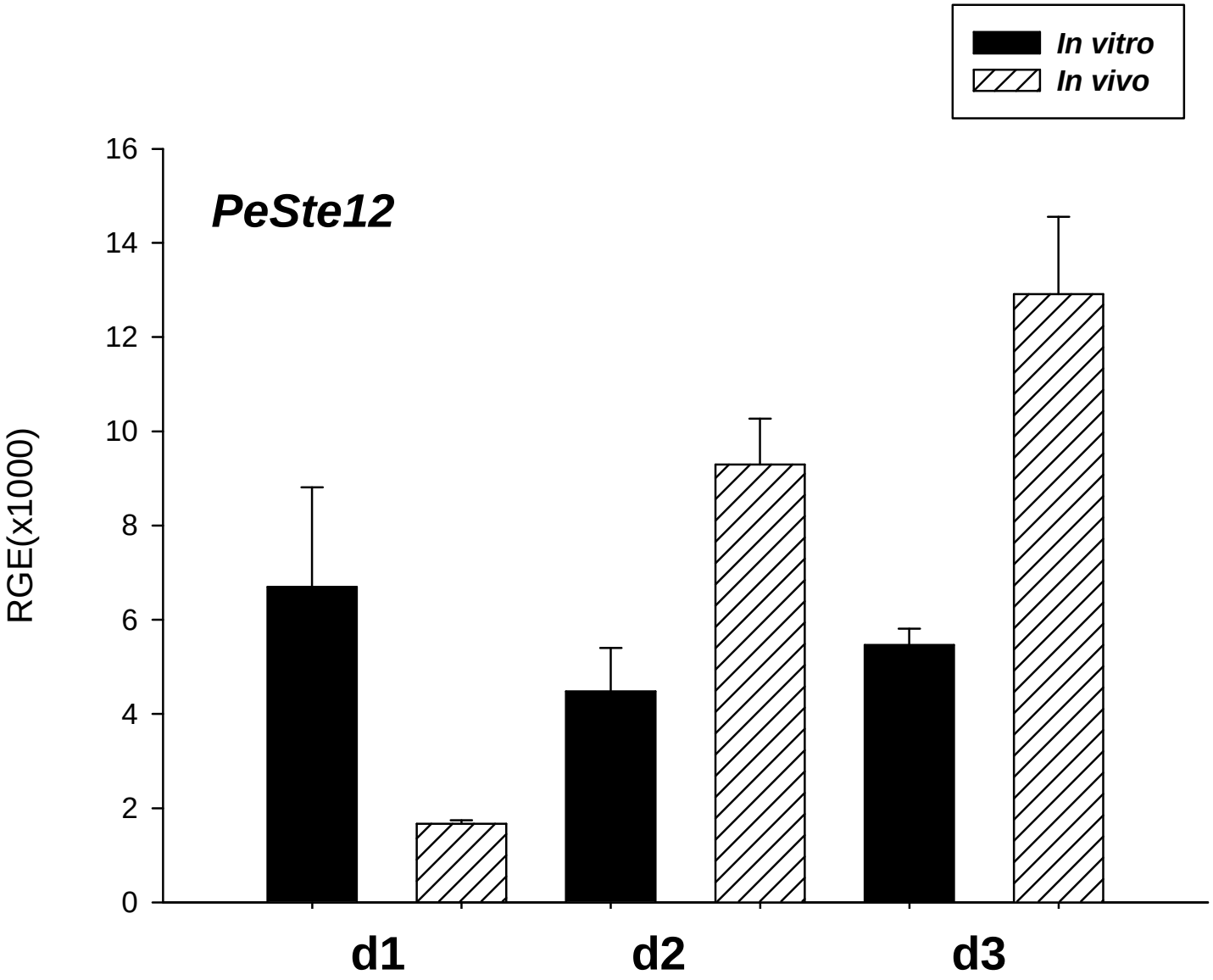
Harvest	Pathogen	20 ºC		0 ºC	
		Growth rate (cm d^{-1})	Visible initial rotting day (d)	Growth rate (cm d^{-1})	Visible initial rotting day (d)
Immature	Wild type	0.491 a	3.0 a	0.056 a	24.0 b
	Δ*Ste12*T6	0.436 a	3.0 a	0.026 b	34.0 a
	Δ*Ste12*T8	0.394 a	3.0 a	0.030 b	36.5 a
Commercial	Wild type	0.599 a	3.0 a	0.078 a	25.0 b
	Δ*Ste12*T6	0.558 a	3.0 a	0.045 b	32.2 a
	Δ*Ste12*T8	0.591 a	3.0 a	0.048 b	32.2 a
Over-mature	Wild type	0.533 a	3.0 a	0.075 a	24.0 b
	Δ*Ste12*T6	0.510 a	3.0 a	0.047 ab	30.2 a
	Δ*Ste12*T8	0.491 a	3.0 a	0.041 b	32.2 a

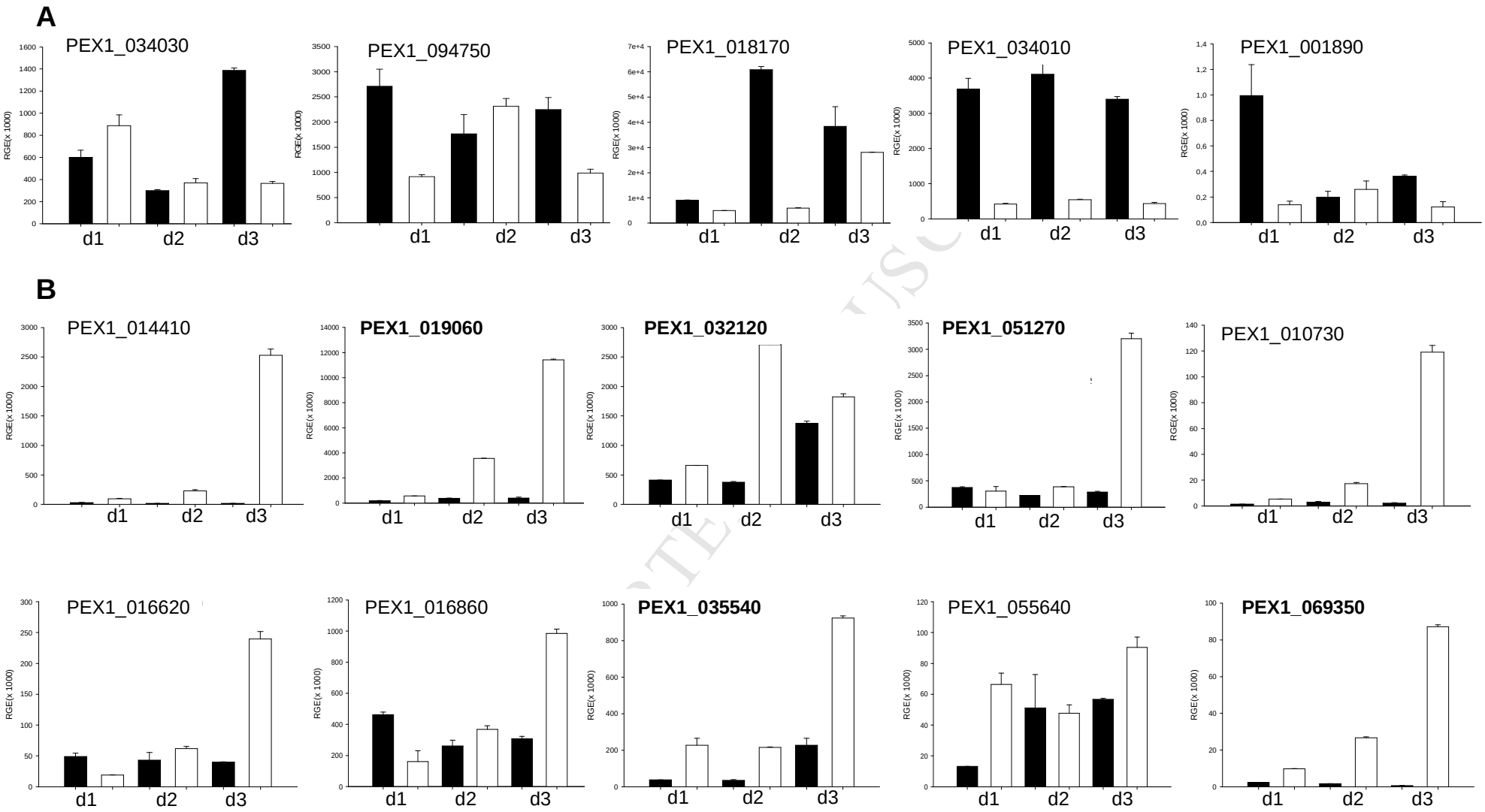

A
B
C
D
E
F
Lesion diameter (cm)
Days post inoculation (dpi)
0
2
4
6
8
10
0
5
10
0
40
80
120
160

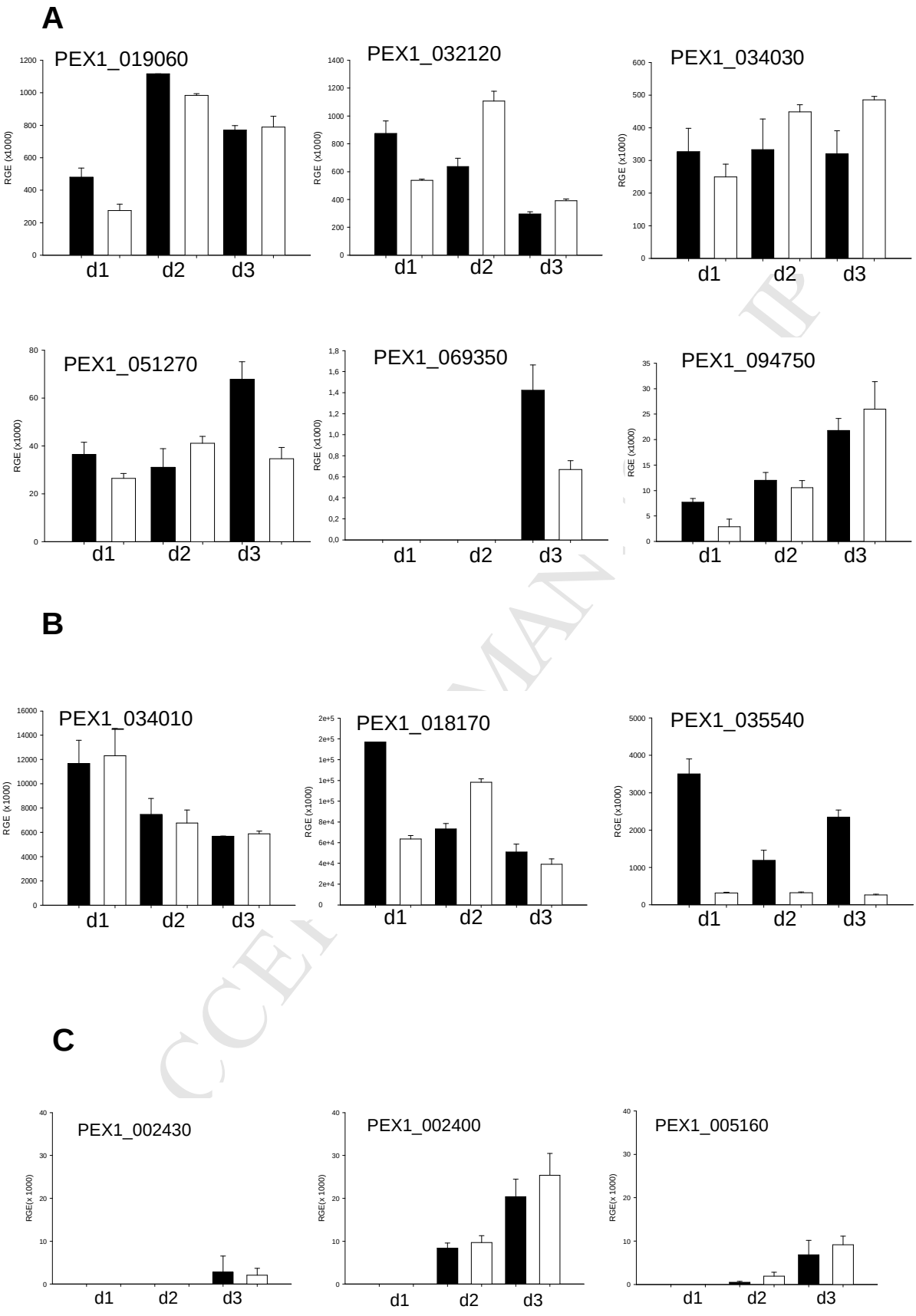

A
B
C
Absolute frequency of Conidiation index (0-5 score)
100%
80%
60%
40%
20%
0%
WT
ΔT6
ΔT8
WT
ΔT6
ΔT8
WT
ΔT6
ΔT8


WT
ΔT6
ΔT8
A
B
C
D
E
F
G
Conidia
Phialide
Metula
Stalk
H
Conidia
Phialide
Metula
Stalk
I
Conidia
Metula
Phialide
Stalk


80
60
40
20
0
H_2O_2 (µmol Kg^{-1} FW)
0
10
20
30
40
Time post inoculation (hpi)
a
b
ab
a
b
c
a
a
b
a
a
b
Water
WT
ΔT6






A
PEX1_019060
PEX1_032120
PEX1_034030
PEX1_051270
PEX1_069350
PEX1_094750
RGE (x1000)
d1
d2
d3
B
PEX1_034010
PEX1_018170
PEX1_035540
C
PEX1_002430
PEX1_002400
PEX1_005160
RGE(x 1000)

Highlights

Identification of a *P. expansum* transcription factor Stel2

PeSte12 is relevant for virulence and asexual reproduction.

Reduction in virulence by silencing Ste12 gene affect *P. expansum* H_2O_2 suppression.

PeSte12 is induced at medium stages of apple infection.

PeSte12 acts mostly as negative regulator during *in vitro* growth.