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Molecular, biochemical and pathological approaches to unravel the defence responses of apples and oranges against Penicillium spp.

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Dipòsit Legal: L.859-2014
http://hdl.handle.net/10803/145981

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Universitat de Lleida

Escola Tècnica Superior d'Enginyeria Agrària

Departament de Tecnologia d'Aliments

Molecular, biochemical and pathological approaches to unravel the defence responses of apples and oranges against *Penicillium* spp.

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Lleida, Mayo de 2014

Los estudios presentados en esta tesis doctoral se realizaron en el laboratorio de Patología del programa de Postcosecha del IRTA (Institut de Recerca i Tecnologia Agroalimentàries) de Lleida.

Para la realización de este trabajo se recibió apoyo económico del Ministerio de Ciencia e Innovación mediante la beca pre-doctoral BES-2009-027752, la estancia breve EEBB-2011-43395 y los proyectos nacionales AGL2008-04828-CO3 y AGL2011-30519-CO3. Y del Ministerio de Economía y Competitividad por la estancia breve EEBB-I-13-06397.

A mis padres

No hay que temer a nada en la vida, solo hay que comprender.

Marie Curie (1867-1934)

La vida es una unión simbiótica y cooperativa que permite triunfar a los que se asocian.

Lynn Margulis (1938-2011)

Observar sin pensar es tan peligroso como pensar sin observar. Vivimos en un país en que el talento científico se desconoce a sí mismo.

Ramón y Cajal (1852-1934)

AGRADECIMIENTOS

Y justo en este momento en el que empiezo a redactar la parte más leída de cualquier tesis doctoral es donde quiero dejar la ciencia a un lado para dar paso a las personas que han participado, de una manera u otra, en este trabajo. Sois muchas las personas que me habéis acompañado en esta experiencia y por eso quiero daros las gracias.

En primer lugar, quiero dar las gracias a mis directoras de tesis, Inma y Charo. A Inma porque confiaste ciegamente en mí y me diste la oportunidad de dar un paso más en el mundo de la investigación. Por solventar cualquier duda o problema siendo tremendamente eficiente. A Charo, por estar siempre a mi lado en cada paso, en cada error y en cada logro. Por tu apoyo, consejos, correcciones, pero sobretodo por preocuparte tanto por mí, tanto en el día a día como en la distancia. También tengo mucho que agradecer a Josep y a Neus, quienes han sacado el tiempo de debajo de las piedras para solventar cualquier duda o problema. Por vuestro ejemplo de unión y fuerza. A Maribel, por tu carácter, por preocuparte de todo y por todos. A Lucia, por los consejos que me diste nada más llegar y por tu positivismo. Gracias a todos por vuestras incansables palabras de ánimo, vuestra confianza y cercanía. Y sobretodo quiero agradecer la oportunidad que me brindasteis de formar parte, más que de un grupo de investigación, de una gran familia.

A Rosa Vilaplana, por estar siempre dispuesta a solventar mis dudas y por ser un sol de persona. Te agradezco todas las canciones que me has dedicado, el tiempo juntas en el laboratorio y sobretodo tus risas. Te deseo lo mejor del mundo mundial! A Carla, por tu risa contagiosa y positivismo. Recuerdo como si fuera ayer el primer día que nos presentaron. Desde entonces supe que iba a tenerte un cariño especial. Tus consejos, optimismo y alegría han hecho mucho más fácil la parte dura de la tesis. Y sí, sigo sintiendo muchísimo haber llamado a la grúa para que se llevara tu coche, pero a quién se le ocurre aparcar en mi vado! A ti Cris, por ser simplemente tú. Por estar a mi lado en lo bueno y en lo malo, incluso cuando no tenías porqué. En lo científico

me quito el sombrero y en lo personal…eres muy grande, y un ejemplo a seguir! A Cèlia, por tu corazón y energía. No puedo imaginarme a nadie mejor con quien compartir el día a día, las prisas y las risas. Ha sido y será un honor trabajar a tu lado. Y déjame decirte algo más, sé que en el fondo echarás de menos que te repita unas cincuenta veces al día lo del 'Cèlia, para compte'! A Rosa Altisent, por el tiempo que he pasado a tu lado, por la paz y tranquilidad que transmites, y sobre todo por haberme soportado este último periodo de redacción de tesis! Ahora, después de todo, puedo decir que he disfrutado muchísimo y trabajado como nunca, pero sobretodo que ha sido un placer haber pasado todo este tiempo a vuestro lado.

También tengo mucho que agradecer al Servicio de genómica y proteómica de la UdL, especialmente a Isabel y M. Alba con quien he tenido el placer de trabajar. Gracias por vuestro interés, dedicación y cercanía. Sinceramente creo que la colaboración establecida ha sido enormemente provechosa para mi formación.

To Mark W. Davey, for the opportunity to work in your lab in Leuven (Belgium). I appreciate sincerely your valuable suggestions on my second paper. To Francis Amoako-Andoh, for introducing me into the proteomics world. For everything I learnt working with you related to science and life. I cannot forget my colleagues at the KUL: Bruno, Mauricio, Tuyet, Dihn, Ifigenia, Veerly, Stijn, Carlo, Federica, for their help in my every-day life in Leuven and for those lovely conversations.

To Michael and all his family, for making me feel like at home. I cannot forget my colleagues at the USDA Jay, Erick, Jin, Roger, Elizabeth, Ralph, Tim, and especially Elena. I wanted to recognize that I fell in love with Shepherdstown (West Virginia, USA). The time that I spent there was awesome!

A mi queridísima Sara, y a toda su familia. Gracias Sara por abrirme las puertas de tu casa, por nuestras conversaciones, paseos y horas cocinando, pero sobre por cuidarme como lo haría una madre. A Hope y John, quienes junto a sus tres hijos, me hicieron sentir como

una más de su familia estando tan lejos de la mía. Nunca podré agradecer el trato recibido por todos ustedes. Que dios les bendiga!

Volviendo a mi grupo, quiero dar las gracias a todas mis compañeras doctorandas, todas y cada una de vosotras me habéis enseñado algo. Han sido años de risas, alegrías, celebraciones, pero también de agobios, deadlines, y campañas a las que hemos sobrevivido. Gracias a todas: Isa, Laura, Lourdes, Marcia, María Bernat, María Sisquella, Pilar y Vivi. Y no me puedo olvidar de Carlos! A tod@s gracias por la ciencia, ciencia y ciencia!

A todo el personal con quien he podido coincidir de una manera u otra. Todos me habéis echado una mano de forma directa e indirecta así que gracias a todo@s: Elena, Claudia, Neus, Robert, Marina, Sveta, Teresa, Isabel Alonso, Isabel Escuer, Just, Rubén, Billy…y perdón si me dejo a alguien! También a todos aquellos que han pasado en un momento u otro por el laboratorio Vanessa (Uruguay), Mariela (Argentina), Stefano (Italia), Rocío 'Boris' (Méjico). A todos gracias por el tiempo en que trabajamos juntos.

Ya fuera del laboratorio me gustaría agradecer a mi familia todo el apoyo que me han brindado durante todos estos años. En especial a mis padres, Inma y Alfredo, a quienes no solo les debo la vida sino todo lo que soy, ya que sin su esfuerzo, dedicación y cariño esto no hubiera sido posible. A mi hermano Sergio, y al resto de mi familia, quienes aunque siguen sin entender qué hago, y porqué llevo tanto tiempo estudiando, me siguen queriendo y apoyando.

A mis amigas, Anna, Cris, Esther, Noe y Patricia, por serlo y por haberme apoyado en los momentos más difíciles, pero sobretodo por celebrar mis éxitos como si fueran vuestros. A la pequeña Carla, que en su corta vida ha sido, sin saberlo, un remanso de paz en este último período de redacción de la tesis.

Y por supuesto a Pablo, quien desde hace mucho tiempo ha sido mi amigo, compañero, confidente, y hasta revisor! No miento cuando digo que sin ti esta tesis no hubiera sido posible. Gracias por estar a mi lado tanto en la vida como en lo científico. Nos queda mucho camino por recorrer y, por duro que pueda ser, será un placer recorrerlo a tu lado.

ABBREVIATIONS

2D	Two-dimensional
AM	Apple medium
APX	Ascorbate peroxidase
ATMT	*Agrobacterium tumefaciens*-mediated transformation
BCA	Biological control agents
CAT	Catalase
CBB	Coomassie brilliant blue
DAB	3,3´-diaminobenzidine tetrahydrochloride
DD	Differential Display
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
EF	Elongation Factor
EST	Expressed sequence tag
FAO	Food and Agriculture Organization of the United Nations
GFP	Green fluorescent protein
GO	Gene ontology
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
IATA	Instituto de Agroquímica y Tecnología de Alimentos
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
LSD	Least Significant Difference
MALDI	Matrix-assisted laser desorption/ionization
TOF	Time-of-flight
MM	Molecular mass
MS	Mass spectrometry
MT	Million tons
OSA	Orange serum agar
PAGE	Polyacrylamide gel electrophoresis
PD	*Penicillium digitatum*
PDA	Potato dextrose agar
PE	*Penicillium expansum*

p*I*	Isoelectric point
PMF	Peptide mass fingerprint
PR	Pathogenesis-related
PTM	Post-translational modification
mRNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real time-quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
UV	Ultraviolet

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Summary/Resumen/Resum

SUMMARY

Despite the current use of chemical fungicides, *Penicillium digitatum* and *P. expansum* still are the most devastating pathogens of citrus and pome fruits respectively, causing important economic losses during postharvest handling worldwide. To obtain new rational and environmentally friendly control alternatives, it is fundamental to unravel the molecular mechanisms underlying the fruit defence responses and the pathogen virulence factors.

The main objective of this thesis was to gain insights into the fruit-pathogen interactions, especially into the oranges and apples defence responses against compatible and non-host pathogens. To achieve this goal, we combined molecular, biochemical and pathological approaches. This is the first time that green fluorescent protein (GFP)-tagged strains of *P. digitatum* and *P. expansum* were used to visualise both interactions in apples and oranges (Chapter 1). Then, we characterised the H_2O_2 effect on the pathogens and in the fruits (Chapter 2). Finally, to uncover the global defence mechanisms of apple fruit in response to abiotic (wounding) and biotic (*P. expansum* and *P. digitatum*) stresses, we used a proteomic approach (Chapters 3 and 4).

The results obtained demonstrated that the GFP transformation does not affect the ecophysiology, the pathogenicity and the sporulation index of *P. digitatum* and *P. expansum*. Thereafter, we used these GFP-tagged strains to visualise the compatibility/non-compatibility of both pathogens in apples and oranges. It is noteworthy that *P. expansum* was able to infect mature 'Lanelate' oranges, whereas the *P. digitatum* growth in 'Golden Delicious' apples was restricted to the wound site. The importance of characterising these GFP-tagged strains relies on the possibility of studying other interactions, as well as of carrying out colonization studies in other environments.

The compatible and non-host fruit-pathogen interactions have been traditionally explored through already known defence response

mechanisms, such as the reactive oxygen species (ROS) production. Among ROS, we evaluated the H_2O_2 role in these compatible and non-host interactions. We studied: (i) its *in vitro* effect on the *P. digitatum* and *P. expansum* ecophysiology and (ii) its *in vivo* apple and orange production in response to abiotic (wounding) and biotic (compatible and non-host pathogen) stresses, and at different maturity stage.

The *in vitro* treatment with high concentrations of H_2O_2 revealed an almost lethal effect in both pathogens. Nevertheless, *P. digitatum* was more resistant to H_2O_2 than *P. expansum*, especially at 25 ºC. In immature 'Golden Smoothee' apples, there was a biphasic H_2O_2 production after wounding and, to a lesser extent after pathogen inoculation. This biphasic H_2O_2 production decreased with ripening, increasing the apple susceptibility to be infected by its non-host pathogen (*P. digitatum*). Therefore, these results confirmed the H_2O_2 importance in immature apple resistance reactions. In 'Valencia' oranges, the differences observed in H_2O_2 accumulation appear to be more related with ripening than with the kind of stress suffered. Although our *in vitro* studies revealed that the H_2O_2 founded levels in oranges must be almost lethal for *P. expansum*, the fungus was able to cause infection at the three tested maturity stages. Overall, our findings suggests that the H_2O_2 fruit production in response to both stresses could be involved in signalling functions -mediating the cross-linking of cell wall proteins or other induced resistance responses-, in addition to inhibit the pathogen spore germination.

The current availability of high-throughput molecular methods also provides an unprecedented opportunity to conduct global screenings of the major determinants of the fruit defence mechanisms. In this thesis, we optimized a protein extraction protocol for apple, improving this crucial step for further proteomic studies, as well as for analysing other apple-pathogen interactions. Thereafter, a proteomic approach was conducted to study the apple protein changes (in terms of abundance and oxidation) in response to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses. On the one hand, we identified generic responses, such as metabolism modifications and

increments of defence proteins. Specifically, the PR-protein Mal d 1.03A increased its abundance in response to both stresses. On the other hand, some protein changes were specifically induced after the inoculation with the non-host pathogen (*P. digitatum*), including abundance changes of the Mal d 1.03E and the elongation transcription factor EF-Tu, as well as oxidative changes in the ACC oxidase (ACO) and the glutamine synthetase (GS). Unexpectedly, in our experimental conditions, we did not identify those proteins responsible for ameliorating oxidative stress such as catalase (CAT). In general, the results obtained suggested that apple induces a metabolic deficiency through protein oxidative damage, a post-translational modification (PTM), mainly caused by ROS, that may starve pathogens, especially *P. digitatum*, until the activation of further defence responses. Additionally, to corroborate that these protein changes were not regulated at the transcriptional level, we evaluated the correlation between the transcript and protein abundances of six genes involved in wound and pathogen responses. Importantly, only one of the six selected genes showed a significant correlation at the transcript and protein level (Mal d 1.03E), supporting the idea that studies only based on transcriptional changes may provide a partial and biased view of the fruit response against external stresses.

The knowledge obtained in this thesis establishes the basis towards the improvement of disease control strategies, not only for the pathogens studied here, but also in other important postharvest diseases.

RESUMEN

A pesar del uso de fungicidas químicos, *Penicillium digitatum* y *P. expansum* continúan siendo los patógenos más devastadores en postcosecha de cítricos y frutas de pepita, respectivamente, causando importantes pérdidas económicas en todo el mundo. Para obtener nuevas alternativas de control, racionales y respetuosas con el medio ambiente, es fundamental descifrar los mecanismos moleculares subyacentes a las respuestas de defensa del fruto y los factores de virulencia de los patógenos.

El principal objetivo de esta tesis fue obtener información sobre las interacciones fruta-patógeno, especialmente de las respuestas defensivas de naranjas y manzanas a patógenos compatibles y no compatibles. Para lograr este objetivo, combinamos enfoques moleculares, bioquímicos y patológicos. Por primera vez, se han utilizado cepas marcadas con proteína verde fluorescente o GFP (del inglés Green Fluorescent Protein) de *P. digitatum* y *P. expansum* para visualizar ambas interacciones en manzanas y naranjas (Capítulo 1). Después, caracterizamos el efecto del H_2O_2 sobre los patógenos y en los frutos (Capítulo 2). Finalmente, para desvelar los mecanismos de defensa globales en manzana frente a diferentes tipos de estrés, como el abiótico (herida) y el biótico (*P. expansum* y *P. digitatum*), utilizamos un enfoque proteómico (Capítulos 3 y 4).

Los resultados obtenidos demostraron que la transformación con GFP no afecta a la ecofisiología, la patogenicidad y el índice de esporulación de *P. digitatum* y *P. expansum*. Posteriormente, se utilizaron estas cepas marcadas con GFP para visualizar la compatibilidad/no compatibilidad de ambos patógenos en manzanas y naranjas. Cabe destacar que *P. expansum* fue capaz de infectar naranjas 'Lanelate' maduras, mientras que el crecimiento de *P. digitatum* en manzanas 'Golden Delicious' se vio restringido a la zona de la herida. La caracterización de estas cepas marcadas con GFP posibilitará estudiar otras interacciones, así como realizar estudios de colonización en otros ambientes.

Las interacciones compatibles y no compatibles fruta-patógeno se han estudiado tradicionalmente a través de los mecanismos de defensa ya conocidos, como la producción de especies reactivas de oxígeno o ROS (del inglés Reactive Oxygen Species). Entre las ROS, evaluamos el papel del peróxido de hidrógeno (H_2O_2) en estas interacciones compatible y no compatible. Se estudió: (i) su efecto *in vitro* en la ecofisiología de *P. digitatum* y *P. expansum* y (ii) su producción *in vivo* en manzana y naranja en respuesta a un estrés abiótico (herida) y uno biótico (patógeno compatible y no compatible), y a diferentes estados de madurez.

El tratamiento *in vitro* a altas concentraciones de H_2O_2 mostraron un efecto casi letal en ambos patógenos. No obstante, *P. digitatum* fue más resistente al H_2O_2 que *P. expansum*, especialmente a 25 ºC. En manzanas 'Golden Smoothee' inmaduras, hubo una producción de H_2O_2 bifásica después de la herida y, en menor medida, después de la inoculación del patógeno. Esta producción bifásica de H_2O_2 disminuyó con la maduración, lo que aumentó la susceptibilidad de las manzanas a ser infectadas por su patógeno no compatible (*P. digitatum*). Por tanto, estos resultados confirmaron la importancia del H_2O_2 en la resistencia de las manzanas inmaduras. En naranjas 'Valencia', las diferencias observadas en la acumulación de H_2O_2 parecen estar más relacionadas con la maduración del fruto que con el tipo de estrés sufrido. Aunque nuestros estudio *in vitro* mostraron que los niveles de H_2O_2 encontrados en naranjas deberían ser casi letales para *P. expansum*, el hongo fue capaz de causar infección a los tres estados de madurez evaluados. En conjunto, nuestros resultados sugieren que la producción de H_2O_2 en fruta frente a ambos estreses podría estar implicada en funciones de señalización -mediando en el entrecruzamiento de proteínas en la pared celular o en otras respuestas de resistencia inducida-, además de inhibir la germinación de las esporas de los patógenos.

La disponibilidad actual de métodos moleculares de alto rendimiento también proporcionan una oportunidad sin precedentes para realizar análisis globales de los principales determinantes de los mecanismos de defensa de la fruta. En esta tesis, hemos optimizado un protocolo

de extracción proteica en manzana, el cual es un paso clave para posteriores estudios proteómicos, así como también para analizar otras interacciones manzana-patógeno. Utilizando este protocolo optimizado, se estudiaron los cambios en las proteínas de manzana (en términos de abundancia y oxidación) en respuesta a un estrés abiótico (herida) y uno biótico (patógeno compatible y no compatible). Por un lado, identificamos respuestas genéricas, tales como modificaciones del metabolismo e incremento de proteínas de defensa. Concretamente, la proteína PR Mal d 1.03A aumentó su abundancia en respuesta a ambos estreses. Por otro lado, algunos cambios proteicos se indujeron específicamente tras la inoculación con *P. digitatum*, incluyendo cambios en abundancia (ej. de Mal d 1.03E y del factor de transcripción de elongación EF-Tu), así como cambios en oxidación (ej. de la ACC oxidasa (ACO) y la glutamina sintetasa (GS)). Sorprendentemente, bajo nuestras condiciones experimentales, no se identificaron proteínas responsables de reducir el estrés oxidativo, por ejemplo, la catalasa (CAT). En general, los resultados de nuestro estudio sugieren que -hasta la activación de respuestas de defensa adicionales- la manzana induce la deficiencia metabólica a través de la oxidación proteica, una modificación postraduccional causada principalmente por las ROS, que podría limitar los nutrientes a los patógenos, especialmente a *P. digitatum*. Adicionalmente, para corroborar que estos cambios proteicos no fueron regulados a nivel transcripcional, se evaluó la correlación entre la abundancia del transcrito y la proteína de seis genes implicados en la respuesta a herida y patógeno. Es importante destacar que sólo uno de los seis genes estudiados mostró una correlación significativa entre el nivel de transcrito y proteína (Mal d 1.03E), corroborando la idea que estudios basados solamente en cambios transcripcionales pueden proporcionar una visión parcial y sesgada de las respuestas de la fruta frente a estreses externos.

El conocimiento obtenido en esta tesis podría ser un paso importante hacia la mejora de las estrategias de control de enfermedades, no sólo en los patógenos estudiados en este trabajo, sino también en otras enfermedades importantes de postcosecha.

RESUM

Malgrat l'ús de fungicides químics, *Penicillium digitatum* i *P. expansum* continuen sent els patògens més devastadors en postcollita de cítrics i fruites de llavor, respectivament, causant importants pèrdues econòmiques a tot el món. Per obtenir noves alternatives de control, racionals i respectuoses amb el medi ambient, és fonamental desxifrar els mecanismes moleculars subjacents a les respostes de defensa del fruit i els factors de virulència dels patògens.

El principal objectiu d'aquesta tesi va ser obtenir informació sobre les interaccions fruita-patogen, especialment de les respostes defensives de taronges i pomes enfront de patògens compatibles i no compatibles. Per aconseguir aquest objectiu, hem combinat enfocaments moleculars, bioquímics i patològics. En particular i per primera vegada, s'han utilitzat soques marcades amb proteïna verda fluorescent o GFP (de l'anglès, Green Fluorescent Protein) de *P. digitatum* i *P. expansum* per per visualitzar ambdues interaccions en pomes i taronges (Capítol 1). Després, vam caracteritzar l'efecte del H_2O_2 en els patògens i en els fruits (Capítol 2). Finalment, per revelar els mecanismes de defensa globals en poma enfront diferents tipus d'estrès, com l'abiòtic (ferida) i el biòtic (*P. expansum* i *P. digitatum*), vam utilitzar un enfoc proteòmic (Capítols 3 i 4).

Els resultats obtinguts van demostrar que la transformació amb GFP no afecta l'ecofisiologia, la patogenicitat i l'índex d'esporulació de *P. digitatum* i *P. expansum*. Posteriorment, es van utilitzar aquestes soques marcades amb GFP per visualitzar la compatibilitat/no compatibilitat d'ambdós patògens en pomes i taronges. Cal destacar que *P. expansum* va ser capaç d'infectar taronges 'Lanelate' madures, mentre que el creixement de *P. digitatum* en pomes 'Golden Delicious' es va veure restringit a la zona de la ferida. La caracterització d'aquestes soques marcades amb GFP possibilitarà estudiar altres interaccions, així com realitzar estudis de colonització en altres ambients.

Les interaccions compatibles i no compatibles fruita-patogen s'han estudiat tradicionalment mitjançant els mecanismes de defensa ja coneguts, com ara la producció d'espècies reactives d'oxigen o ROS (de l'anglès Reactive Oxygen Species). Entre les ROS, vam avaluar el paper del peròxid d'hidrògen (H_2O_2) en aquestes interaccions compatible i no compatible. Es va estudiar: (i) el seu efecte *in vitro* en l'ecofisiologia de *P. digitatum* i *P. expansum* i (ii) la seva producció *in vivo* en poma i taronja després d'un estrés abiòtic (ferida) i biòtic (patogen compatible i no compatible), i a diferents estats de maduresa.
El tractament *in vitro* amb altes concentracions de H_2O_2 va mostrar un efecte gairebé letal en ambdós patògens. No obstant, *P. digitatum* va ser més resistent al H_2O_2 que *P. expansum*, especialment a 25 ºC. En pomes 'Golden Smoothee' immadures, hi va haver una producció de H_2O_2 bifàsica després de la ferida i, en menor mesura, després de la inoculació del patogen. Aquesta producció bifàsica de H_2O_2 va disminuir amb la maduració, el que va augmentar la susceptibilitat de les pomes a ser infectades pel seu patogen no compatible (*P. digitatum*). Per tant, aquests resultats van confirmar la importància del H_2O_2 en la resistència de pomes immadures. En taronges 'Valencia', les diferències observades en l'acumulació de H_2O_2 semblen estar més relacionades amb la maduració del fruit que amb el tipus d'estrès patit. Encara que els nostres estudis *in vitro* van mostrar que els nivells de H_2O_2 trobats en taronges haurien de ser gairebé letals per *P. expansum*, el fong va ser capaç de causar infecció en els tres estats de maduresa avaluats. En conjunt, els nostres resultats suggereixen que la producció de H_2O_2 en fruita davant ambdós estressos podria estar implicada en funcions de senyalització –mitjançant l'entrecreuament de proteïnes a la paret cel·lular o en altres respostes de resistència induïda-, en comptes d'inhibir la germinació de les espores dels patògens.

La disponibilitat actual de mètodes moleculars d'alt rendiment també proporcionen una oportunitat sense precedents de realitzar anàlisis globals dels principals determinants dels mecanismes de defensa de la fruita. En aquesta tesi, hem optimitzat un protocol d'extracció de proteïnes per poma, el cual és un pas clau per a posteriors estudis proteòmics, així com també per analitzar altres interaccions poma-

patogen. Utilitzant aquest protocol optimitzat es van estudiar els canvis en les proteïnes de poma (en termes d'abundància i oxidació) en resposta a un estrès abiòtic (ferida) i biòtic (patogen compatible i no compatible). D'una banda, vam identificar respostes genèriques, com ara modificacions del metabolisme e increment de proteïnes de defensa. Concretament, la proteïna PR Mal d 1.03A va augmentar la seva abundància enfront ambdós estressos. D'altra banda, alguns canvis proteics es van induir específicament en resposta a la inoculació amb *P. digitatum*, incloent canvis en abundància (ex. de Mal d 1.03E i del factor de transcripció d'elongació EF-Tu), així com canvis en oxidació (ex. de l'ACC oxidasa (ACO) i la glutamina sintetasa (GS)). Sorprenentment, sota les nostres condicions experimentals, no es van identificar proteïnes responsables de reduïr l'estrès oxidatiu, com per exemple la catalasa (CAT). En general, els resultats del nostre estudi suggereixen que -fins l'activació de respostes de defensa addicionals- la poma indueix la deficiència metabòlica a través de l'oxidació proteica, una modificació postraduccional causada principalment per les ROS, que podria limitar els nutrients als patògens, especialment a *P. digitatum*. Addicionalment, per corroborar que aquests canvis proteics no van ser regulats a nivell transcripcional, es va avaluar la correlació entre l'abundància del transcrit i la proteïna de sis gens implicats en la resposta a ferida i patogen. És important destacar que només un dels sis gens estudiats va mostrar una correlació significativa entre el nivell de transcrit i proteïna (Mal d 1.03E), recolzant la idea que els estudis només basats en canvis transcripcionals poden proporcionar una visió parcial i esbiaixada de les respostes de la fruita enfront estressos externs.

El coneixement obtingut en aquesta tesi podria ser un pas important cap a la millora de les estratègies de control de malalties, no només en els patògens estudiats en aquest treball, sinó també en altres malalties importants de postcollita.

Introduction

1. Importance of orange and apple fruit

Oranges and apples are essential products grown worldwide. The European Union is the third largest worldwide orange producer, and Spain contributed with 2.8 million tons (MT) in 2011, according to Food and Agriculture Organization of the United Nations (FAO). As an important apple producer, Spain contributed to the apple worldwide production (over 76 MT) with 0.7 MT in 2011 (Figure 1) [1].

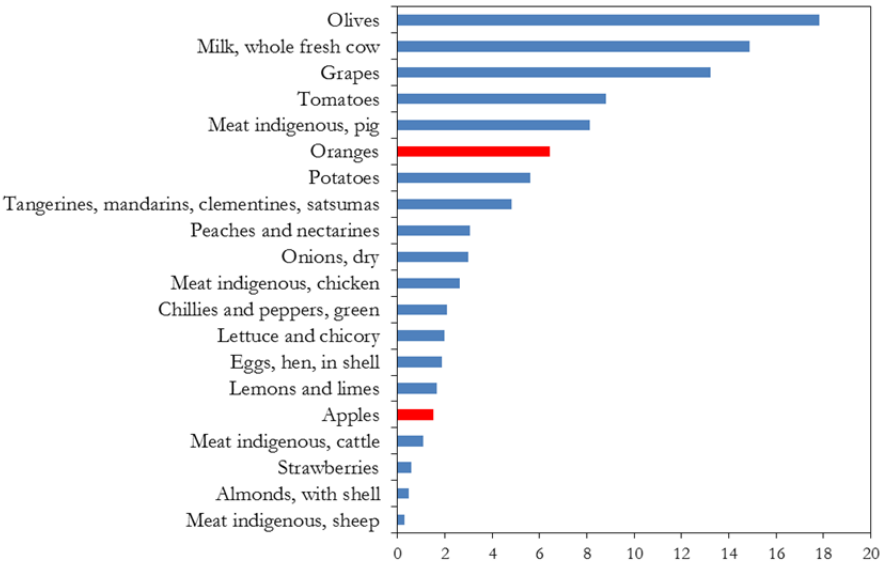

Figure 1. The production (percentage of the total production) of most important food and agricultural commodities in Spain during 2011 [1].

In addition to sales in the fresh market, fruit production and continuous supply of the commodities for human consumption is an important part of the market economy. To enable the steady year-round supply of high-quality fruit, the produce must be stored for weeks or months prior to marketing. Any waste during the postharvest phase, results in significant economic losses. During this postharvest period, apple fruits are stored in controlled-atmosphere chambers where ethylene-induced ripening is delayed. Orange fruits however, are usually stored only at the recommended optimal temperature (3-5 °C).

Based on the profile of ethylene production, two types of fruit ripening can be defined: non-climateric or climateric. Non-climateric fruits, such as orange (*Citrus x sinensis* (L.) Osbeck), are characterised by an ethylene-independent ripening. Ethylene is not involved in regulating internal ripening parameters of the fruit (e.g. acidity, taste, aroma, nutritional value), but it will stimulate ripening-related processes in the peel tissue such as destruction of the green chlorophyll and accumulation of orange/yellow carotenoids. Exposure of the fruit to exogenous ethylene will enhance respiration and ethylene production in the fruit. In contrast, climateric fruits, such as apples (*Malus x domestica* Borkh), are characterised by a dramatic increase in ethylene production, which is responsible for the typical respiratory burst during internal ripening [2] (Figure 2). On the one hand, apples are usually stored for periods of up to 12 months, at the recommended optimal temperature (0 ºC), to ensure a steady year-round supply of high-quality fruit. On the other hand, oranges can be stored shorter period of time (from 8 to 12 weeks).

A.Orange (Citrus) B. Apple (Malus)

Figure 2. A) Non-climateric and B) climateric fruits.

One of the main orange varieties is 'Valencia', which is a late-season fruit. Actually, 'Valencia' orange is a popular variety when another important variety such as 'Navel' oranges is out of season. The most important apple variety of the 21th century is 'Golden Delicious'.

2. Main postharvest diseases of orange and apple fruit

Although quality deterioration of fresh postharvest fruits and vegetables is the result of a number of different factors, microbial activity is by far the most important one [3]. In the developed countries, approximately 10-30% of harvested fruits and vegetables are lost due to postharvest spoilage, and in the developing countries the losses are estimated to be 30-50% annually due to the lack of sanitation and postharvest refrigeration facilities [4].

Among fungal diseases of citrus fruits are: *Geotrichum candidum aurantii*, the cause of sour rot, stem-end rots such as *Alternaria citri* , *Deplodia natalensis*, *Phomopsis citri*, and brown rot caused by *Phytophthora citrophthora* [5], while of pome fruits are: *Botrytis cinerea*, *Phytophthora* spp., *Venturia inequaelis* and *Alternaria alternata* [6]. *Rhizopus stolonifer* mainly affects stone fruit, but to a lesser extent it can also affect apples [7].

However, the most common causes of citrus and pome fruit decay worldwide are the *Penicillium* rots. *Penicillium* is the more diverse genus (in terms of numbers of species and range of habitats) out of the Deuteromycetes class, a phylogenetic class for which no sexual reproduction is known (apparently these fungi reproduce only asexually, haploid spore→haploid mycelium→haploid spore) [8]. *Penicillium* rots are one of the most common and destructive postharvest diseases, affecting wide variety of fruits and vegetables.

Whereas *Penicillium italicum* and *P. digitatum* the cause of blue and green moulds in oranges, respectively [9], *P. expansum* is the principal cause of blue mould in apples [10] (Figure 3).

A. Blue and green moulds in oranges B. Blue mold in apple

Figure 3. A) *Penicillium italicum* and *P. digitatum* in 'Valencia' oranges and B) *P. expansum* in 'Golden Smoothee' apple.

In particular, the necrotrophic fungi *P. digitatum* and *P. expansum*, enters tissues through wounds, which readily occurs during handling and harvesting, accounting for up to 80% of decay in transit, in storage, and in the market [7]. Whereas *P. digitatum* is a specialist species, affecting only citrus fruit, *P. expansum*, however, has the ability to infect a broader range of different fruit, including apples, pears, strawberries, grapes, peaches, nectarines, plums, apricots and tomatoes [5]. It is also worth noting that postharvest infection with *P. expansum*, *P. funiculosum*, and occasionally other species of *Penicillium* may lead to significant levels of patulin contamination in stored apples, constituting a serious health hazard for humans [11].

3. Postharvest disease control

P. digitatum and *P. expansum* are the most devastating postharvest pathogens of citrus and pome fruits, causing important economic losses during postharvest handling worldwide, extensive efforts have been made to develop effective and safe control methods.

Fungicides and storage technologies were the primary means used to control these important postharvest diseases. However, the high levels of fungicides residues in fruits and the development of fungicide resistance [12], have fostered a significant interest in the development of alternative methods to manage postharvest diseases.

Environmental-friendly systems that integrate pre-harvest, harvest and postharvest practices should be considered as important components that influence the complex interaction between host, pathogen, and environmental conditions.

Pre-harvest practices include the application of chemical treatments in orchards or minimizing fruit injuries, which are necessary for these important fungi infection. Other promising alternative strategies after harvest include physical, chemical, and biological control methods. Alternative control measures of *P. digitatum* include the use of UV light [13], which induces formation of the phytoalexin scoparone [14, 15], heat (both hot air and hot water dipping) treatments, which induces tissue healing [16-18] [16, 18] and ozone exposure [19]. A wide variety of alternative control measures have been suggested for *P. expansum*, including the use of deoxyglucose [20, 21], chlorine dioxide in wash waters [22], wash water filtration [23], calcium chloride infiltration under increased temperature and pressure [24], low toxicity compounds such as sodium bicarbonate [25], or a combination of some of the above strategies [26].

Biological control agents (BCA) against the major postharvest pathogens of citrus and pome fruit have become an active research area. An extensive list of the antagonists described for biological control of postharvest diseases of orange and apple was provided by Teixidó *et al.* [27] including *Candida oleophila* [28], *Metschnikowia fructicola* [29], *Pantoea agglomerans* [30], *Pichia guilliermondii* [31] for *P. digitatum* in orange, and *Candida sake* [32], *Metschnikowia pulcherrima* [33, 34], *Pantoea agglomerans* [35], *P. ananatis* [36], and more recently, *Pichia caribbica* [37] for *P. expansum* in apple. Furthermore, Morales *et al.* [38] demonstrated that the use of two BCAs (*Candida sake* and *Pantoea agglomerans*) have a positive effect on decay control in *P. expansum*-infected apples, and on patulin accumulation after cold storage. As discussed in several reviews, the combination of BCA with other alternative control methods can be a promising approach to overcome some drawbacks of BCA activity, and enhancing their efficacy [39, 40].

Many challenges still need to be addressed in order to develop a commercially successful postharvest biocontrol product. Nonetheless, numerous challenges and opportunities still exist as this field of research matures.

Despite the use of commercial fungicides and the implementation of new alternative strategies, green mould in citrus and blue mould in pome fruits remain critical diseases of these stored fruits worldwide, with strong trends focused on finding new rational and environmentally friendly control alternatives.

4. Understanding fruit-pathogen interactions

Together with the development of alternative control strategies at both pre and postharvest, novel approaches are needed to acquire knowledge on fruit-pathogen interactions. In particular, new studies must be based on: (i) understanding fruit's defence responses to both pathogens and non-host pathogens and (ii) elucidating pathogen's virulence and specificity mechanisms. This knowledge will provide opportunities to develop novel, safe and more effective control strategies.

Fruit defence responses against pathogens and non-host pathogens

Fruit defence mechanisms appear to be highly effective against most fungi, and only a relatively few genera and species are able to invade and cause serious losses [41]. In general, fruits defend themselves against pathogens by a combination of constitutive (passive) and inducible (active) mechanisms. For example, waxy cuticles avoid the pathogen ingress (constitutive mechanism), while biochemical reactions prevent pathogen development (inducible mechanism) [8]. The constitutive and inducible mechanisms are different for each host-pathogen interaction, and even vary with the fruit maturity stage.
In most cases, the onset of ripening and senescence render fruits more susceptible to infection by pathogens [42-46]: the pH of the tissue

increases, skin layers soften, soluble carbohydrates build up and defence barriers weaken.

In many instances, the differences in the outcome of a host-pathogen interaction depends on a rapid and efficient deployment of defence responses [47], which will be modulated depending on whether the host-pathogen interaction involves a compatible or incompatible pathogen. A compatible interaction takes place if a pathogen overcomes fruit defence barriers and establishes disease symptoms, whereas in an incompatible or non-host pathogen interaction, fruit deploy an array of defences that prevent or significantly limit pathogen growth [48, 49]. Following the above definition, the *P. digitatum*-orange and *P. expansum*-apple may be categorized as a compatible or host-pathogen interactions, while *P. digitatum*-apple and *P. expansum*-orange as non-host pathogen interactions. Unravelling the determinants of these specificities can provide valuable insights into the nature of the fruit defence responses against compatible and non-host pathogen. However, the defence mechanisms underlying the natural resistance of fruits at different maturity stages to fungal pathogens remain unclear yet.

The induction of an active response is triggered by the mutual fruit-pathogen recognition, and can be localized or systemic. These responses include the hypersensitive response (HR; based on rapid localized cell death at the site of infection), which increased expression of defence-related genes such as pathogenesis-related (PR) genes, and the oxidative burst by reactive oxygen species (ROS) production [50].

Current knowledge in host-pathogen interaction, especially on plants, suggests that ROS plays a crucial role in host defence [50-52]. The oxidative burst is one of the earliest and most rapid defence responses of plant against pathogen attacks, and involves two phases: phase I occur in both compatible and non-host pathogen interactions and causes a rapid, but weak, accumulation of ROS with a response time of a few minutes. In contrast, phase II has only been observed in non-host pathogen interactions and is characterised by prolonged

accumulation of ROS after a period of several hours following infection with the pathogen [51, 53]. The kinetics of ROS accumulation and cell death in host and non-host interactions is illustrated in Figure 4.

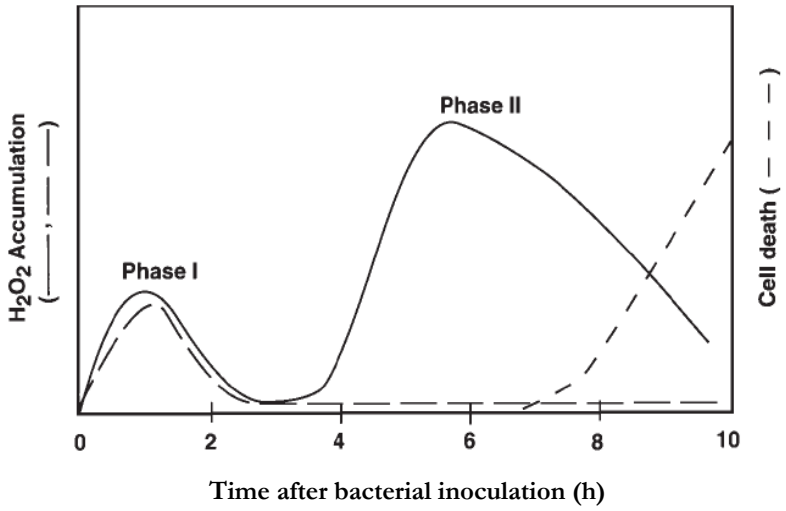

Figure 4. Kinetics for accumulation of H_2O_2 and induction of cell death in plant cells following bacterial inoculation. Cell death and H_2O_2 accumulation following inoculation with non-host pathogen (avirulent) and pathogen (virulent) [51].

Although oxidative burst implicate the production of superoxide anions and hydroxyl radicals, hydrogen peroxide (H_2O_2) is likely to be the most important ROS, because of its stability and slow reactivity with biological molecules. Within the different possible functions described in a plant's defence strategy, H_2O_2 may be involved in: membrane peroxidation, the cross-linking of cell wall proteins [51, 54], signalling after wounding [55, 56], mediating the induction of hypersensitive cell death, the expression of a wide array of defence-related genes in surrounding cells [53, 57-59], and in the spore germination inhibition of many fungal pathogens [60-62].

Production of H_2O_2 in response to wounding and/or pathogen attacks was shown to be integral to the induction of fruit defence mechanisms

[43, 44, 63-66]. Indeed, the H_2O_2 production has been reported to be associated with fruit development, ripening and senescence [67-71], as well as with the biosynthesis, polymerisation and deposition of lignin [72, 73]. Both features are known to influence fruit resistance in many different ways [74, 75]. For example, lignified cell walls can constitute a barrier preventing free nutrient movement and therefore help to starve the pathogen. All these possible alterations in the structure of the plant cell walls may contribute to the fruit resistance, either by directly stopping pathogen ingress or by slowing down the penetration process before inducing further defence mechanisms, such as the expression of PR-related genes [74].

Accumulation of H_2O_2 in response to wounding and/or pathogen attack has been demonstrated in tomato plants [58, 76], lemon [65] and, more importantly, in orange [66] and apple [43, 44, 63] fruits. Overall it is suggest that H_2O_2 plays a crucial role in fruit defence mechanisms during ripening in response to wounding and pathogen infection.

Pathogen's virulence and specificity mechanisms

Pathogens have developed several mechanisms to modulate their virulence [77, 78]. Among them, modification or adaptation to the host environment by the pathogen is being considered as a major factor determining successful colonization of host tissues by fungal pathogens [79-81].

One of the major environmental factors that have a profound effect on the host-pathogen interaction is the pH of the plant tissue. In fruits, pH is important for the control of postharvest disease because it directly affects the germination of conidia [82] and influences the virulence of pathogens through their colonization of host tissue [81, 83]. Some postharvest pathogenic fungi may enhance their virulence by locally modulating the host's ambient pH. *Penicillium* spp. colonization results in acidified citrus and apple tissues and is enhanced by low pH [83]. Prusky *et al.* [83] also suggested that host

acidification in apple and citrus fruit is a regulatory cue for processes linked to pathogenicity of postharvest pathogens, such as *Penicillium* spp., and that pectolytic genes are expressed as a result of this modified host pH created by the pathogens. In the apple-*P. expansum* interaction, Hadas *et al.* [84] have shown data that support the hypothesis that gluconic acid is a key factor contributing to fungal disease of apple. The tissue acidification by this acid enhanced the expression of pectolytic enzymes and the establishment of conditions of necrotrophic development of this fungus.

The role of antioxidant system has also been a matter of study in postharvest fruit-pathogen interactions. Ballester *et al.* [85] have analysed the spatial distribution of antioxidant enzymes in *P. digitatum*-infected fruits. All enzyme activities decreased as the pathogen progressed through the tissue, but following different kinetics, except for catalase and soluble peroxidase which showed a slight increase in the most colonized tissue. Qin *et al.* [86] have compared the cellular and extracellular proteomes of *P. expansum* in absence and presence of borate, which has been used in agriculture as a safe method for control fungi. They have showed that two antioxidant enzymes, catalase and glutathione S-transferase and the hydrolytic enzyme polygalacturonase may be a critical for the virulence of *P. expansum*. Macarisin *et al.* [65] have demonstrated that during infection of oranges, *P. digitatum* suppresses the H_2O_2-oxidative burst in host cells, while the non-pathogen *P. expansum* triggers a massive accumulation of H_2O_2 in citrus exocarp. However, as mentioned before, in *P. expansum*-infected apples there is an accumulation of gluconic acid probably derived from glucose by a fungal glucose oxidase. In this reaction, H_2O_2 is generated and its presence has been detected in the forefront of the macerated fruit tissue [84]. Although, it seems that different mechanisms operate in these two *Penicillium*-fruit interactions, knowledge is lacking about pathogenesis/virulence factors that mediate pathogen virulence in these postharvest pathogenic fungi, including how they could suppress fruit defences and if they effectively do so.

Therefore, the identification of the putative pathogenesis/virulence factors and fruit defence responses in both compatible and non-host pathogen interactions will provide opportunities to develop novel, safe and more effective control strategies.

Novel approaches for understanding fruit-pathogen interactions

From a molecular point of view, and taking advantage of well-established technologies, targeting pathogen virulence constitutes one of these novel approaches [87]. The strategy is to target essential virulence mechanisms that allow pathogen progression within the host and/or cause disease symptoms, rather than being directed to the essential cell survival machinery, as it happens with actual antimicrobials.

The use of fungal transformants expressing green fluorescent protein (GFP) has been used as a molecular tool to enhance the ability to study plant-pathogen interactions by both, detecting and visualizing the infection process *in situ* [88, 89]. Regarding fruit-pathogen interactions, the GFP has been successfully used as a marker of the infection process of *Botrytis cinerea*-strawberry [90], *Aspergillus carbonarius*-grapes [91], *Fusarium oxysporum*-tomato [92] and *Rosellinia Necatrix*-avocado [93].

For a variety of postharvest pathogens, the GFP-tagged fungal transformants are already available [90, 94, 95] (Figure 5). Although the genetic transformation of *P. digitatum* by *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been described [96], the GFP-tagged *P. digitatum* transformant has not been reported. Likewise, the genetic transformation of *P. expansum* remains a methodological challenge.

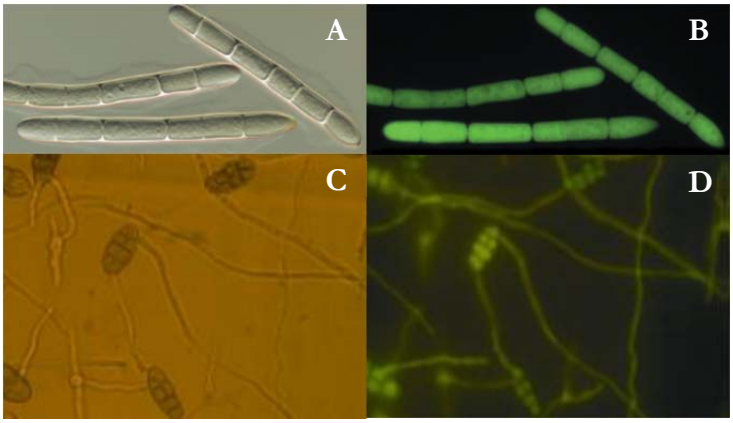

Figure 5. Micrographs of fungi transformed with green fluorescent protein. (A-B) A mint root infected with *Verticillium dahliae* [89] and (C-D) spores on cellulose membrane of *Alternaria citri* [95].

With the dramatic reduction in sequencing costs, high-throughput genomic and transcriptomic approaches have also gained increased popularity as tools to study a plethora of biological processes. For example, on the one hand, the overall response of citrus fruit to *P. digitatum* infection was analysed from a genomic perspective [97]. The results demonstrated how the orange fruit respond to *P. digitatum* modifying the metabolism towards secondary metabolism with ethylene being a major player in the process [98-102]. On the other hand, apple-*P. expansum* interaction by means of Differential Display (DD) revealed that several induced fungal genes are related to adaptation to acidic pH and the genes with the highest induction level code for polygalacturonases [103].

The availability of the complete genome sequence of *Malus x domestica* [104] has greatly aided the development of '-omic' approaches such as genomics, transcriptomics and proteomics in *Malus x domestica*. For example, a transcriptional approach of apple gene expression in response to compatible (*P. expansum*) and non-host (*P.digitatum*) pathogens has been recently conducted to characterise disease resistance mechanisms in apples [105]. Moreover, there is also an increasing trend in application of proteomic methods to identify fruit

protein changes in response to developmental and environmental signals, offering to the research community the opportunity to unravel complex sets of proteins [4].

5. Why proteomic approaches?

Although transcriptomic analyses have provided helpful insights into the apple resistance responses [105], it entails some limitations. Indeed, protein expression is controlled at different levels, including pre-transcriptional, post-transcriptional and post-translational regulatory mechanisms. Since transcript and protein levels may be uncorrelated [106-108], studies only focusing on regulatory changes at the transcriptional level may provide a partial bias view of the overall fruit defence mechanisms. For example, a number of defence-associated proteins, such as kinases, may not be responsive to defence-related signals at the transcriptional level, and thus the potential role of these proteins\genes could not be fully characterised in transcriptomic studies. A complementary approach is proteomics [109], which give a view of the ultimate responsible to carry out the molecular function: the proteins.

One of the main goals of proteome analysis is comparative proteomics, where the main aim is not to characterise the entire set of proteins in a specific sample, but rather to identify quantitative and qualitative changes between protein samples to gain deeper insights into biological processes and diseases.

The key steps of any proteomic study include: (i) protein extraction and separation, (ii) gel staining, image acquisition and analysis, (iii) and finally in-gel digestion and protein identification (Figure 6).

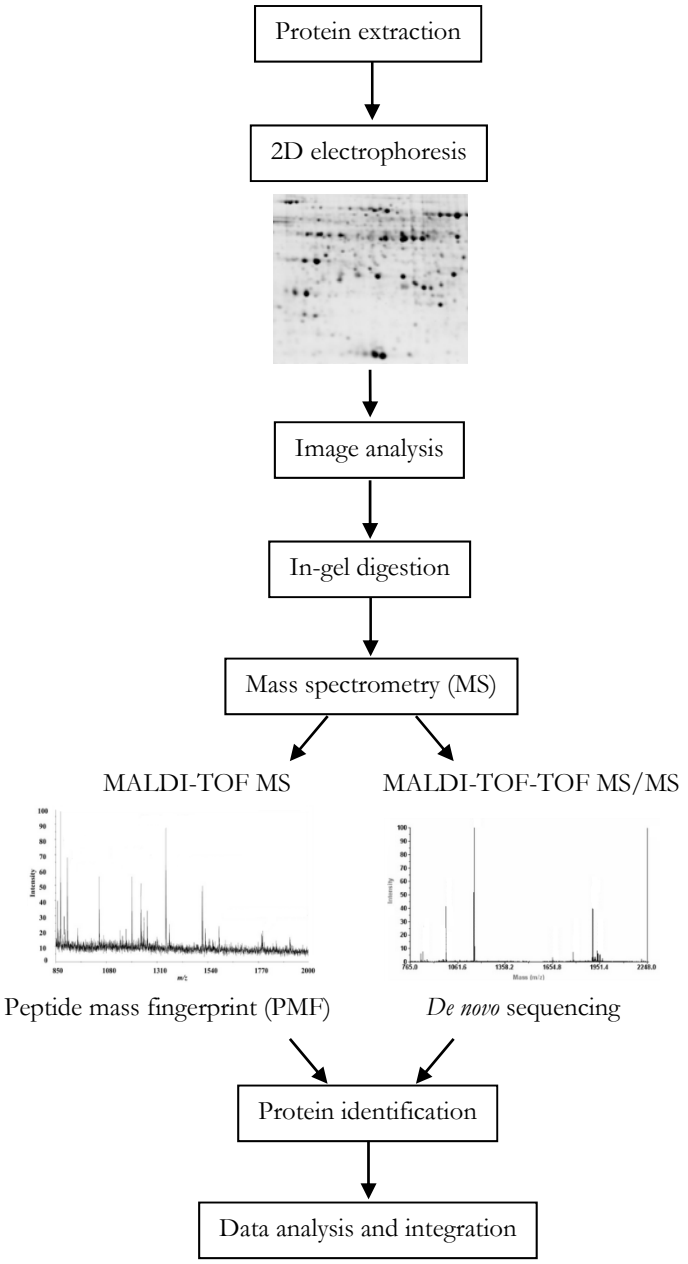

Figure 6. Overview of common steps involved in proteomics analysis.

Protein extraction and separation

Sample or protein extractions are the most critical steps in any proteomic study and each extraction strategy needs to be chosen according to the nature of the sample tissue [110, 111].

Fruit tissues are particularly problematic for proteomic procedures, especially because of the presence of compounds that severely interfere with protein extraction, including pigments, carbohydrates, polyphenols, polysaccharides and starch [112]. In fact, the prevalence of these interfering compounds possibly represents the most significant problem associated with fruit proteome analysis. To further complicate matters, it has been described that fruit have lower protein content than plants, i.e. leaves [113]. For that reason, many efforts have been performed to obtain suitable methodology for extracting fruit tissue proteins. Nowadays, the TCA/acetone and phenol-based protein extraction methods have been described as one of the most effective protocols for some fruits [114-116].

After protein extraction, the separation of the complex protein mixtures by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used for several decades [117, 118]. 2D is the most powerful separation method for proteins and involves two dimensions. Proteins are first separated based on their charge or isoelectric points (p*I*) by isoelectric focusing (IEF), and second, proteins are separated according to their molecular mass (MM) (Figure 7). The 2D provides hundreds proteins quantitatively different in expression, however, the technical drawback is the limited ability to detect low abundance proteins [119].

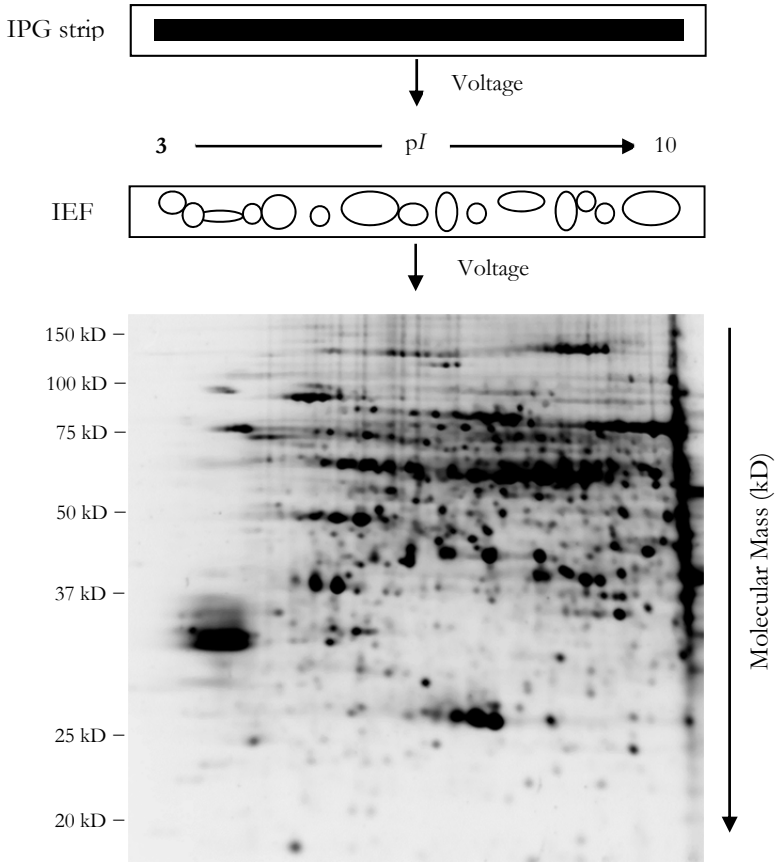

Figure 7. Schematic representation of 2D-SDS-PAGE.

Gel staining, image acquisition and analysis

After electrophoretic separation, proteins must be visualised in some manner. Among staining techniques, coomassie brilliant blue (CBB) and silver staining have been typically used because of their cost-effectiveness and the more or less suitability with subsequent protein identification by mass spectrometry (MS). Protein staining and image analysis allow us to compare each quantitative and qualitative protein changes of samples. To improve the MS compatibility, fluorescent staining, mostly SYPRO dyes, such as SYPRO Red and SYPRO Ruby,

and more recently DIGE technique, were developed; however, the main drawback is the high cost of these dyes.

Following staining, the next step is gel imaging that basically depends on the dye used. After coomassie or silver stains, flatbed scanners are cheaper and allow most rapid gel imaging. Comparison of digital images of the gels, spot detection and image analysis is the next step. Although several software packages are available to aid with the image analysis this part requires time investment and considerable manual intervention. Among others, the most used software packages are ImageMaster 2D Platinum (http://www.gelifesciences.com) and PDQuest 2D Analysis (http://www.bio-rad.com).

In-gel digestion and protein identification

Protein identification is performed by excision of 2D gel plugs containing the protein spot of interest, following by in-gel digestion with a site-specific protease (commonly trypsin), and finally MS analysis of the resultant eluted peptides (Figure 8).

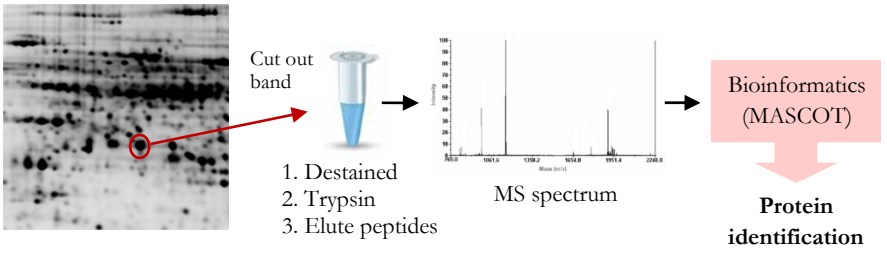

Figure 8. Schematic representation of in-gel digestion.

The most widely used method for protein identification is the matrix-assisted laser desorption/ionization attached to a time-of-flight mass spectrometer (MALDI-TOF MS).

The MALDI-TOF MS is commonly used to measure the masses of the peptides derived from the trypsinized protein spot, generating a 'peptide mass fingerprint' (PMF) of the protein. The protein then is identified by matching the measured peptide masses to corresponding peptide masses from protein or nucleotide sequence databases [120]. The series of peptide fragments is specific for only one protein and is referred to as a peptide fingerprint. After that, MASCOT mass search software is the most used, which compare experimental peptide masses with theoretical digestion of all protein sequences from a sequence database. In addition, the name of the enzyme used for proteolytic digestion must be specified, as well as the number of missed cleavage sites.

MS/MS or *de novo* sequencing is the alternative to the PMF approach, by coupling a MALDI source to tandem mass spectrometers, which provide the amino acid sequences of the selected tryptic peptides. The pros and cons of each approach have been described extensively in the following proteomics reviews [110, 111]. Summarizing, the MALDI-TOF/PMF approach has some advantages as the quickness, needs relatively little user expertise and is tolerant to contaminations. The main drawback is that the data are ambiguous if there are not genomic sequences, or at least a substantial EST collection, for the species under study. In contrast, MS/MS analysis is technically more challenging and allows the study of post-translational modifications; however, the main drawback is the high cost of this approach.

Nowadays, bioinformatics plays a fundamental role in plant science. Because the amount of data is growing exponentially, the development of bioinformatics tools and specific algorithms that allow data integration, modelling and prediction are very important. The methods, software packages, and databases used in plant science bioinformatics has been described extensively in the following review [121].

6. Fruit proteomics

Proteomic approaches have been widely used to study the fruit ripening of some fruits including tomato [122], grape [123], citrus [116, 124], prunus [125], papaya [115, 126], mango [127] and banana [128]. An extensive list of the proteomic studies of fruits ripening was provided by Palma *et al.* [2]. Ripening in fruit is the final step of development and involves genetically, biochemically and physiologically programmed processes. Many changes such as increased ethylene biosynthesis and respiratory activity, cell wall softening, degradation of chlorophyll and biosynthesis of pigments, degradation of starch, formation of volatiles, become evident [129]. Many proteins identified in fruit ripening appear to be common between different species studied. Specifically, there appears to be a general regulation of proteins involved in energy and carbon metabolism, with particular emphasis on glycolytic and TCA-cycle enzymes, stress and defence response.

Regarding apple proteomic studies, Guarino *et al.* [112] described the repertoire of proteins present in the pseudocarp tissues of three accessions of *Malus* × *domestica* Borkh. cv. 'Annurca', and more recently, changes in the protein profiles of 'Golden Delicious' during ripening and in response to exogenous ethylene treatment have also been reported [130].

7. Proteomics in response to abiotic stresses

Proteome changes in the vegetative portions of plants in response to a broad range of abiotic stress factors, such as cold, heat, drought, salinity, flooding, ozone treatment and mechanical wounding, have been extensively described [131, 132]. And even though the response of plant tissues to wounding has been studied for some time [133-135], few reports dealing with the response to wounding have been published [136-141].

For instance, an important role has been reported for thaumatin (PR-5), which was wound-inducible in poplar phloem exudates of plants

whose leaves had been wounded 24 h prior to collection [137]. Other authors have reported that thaumatin might also be involved in protecting against chilling injury in peach [142]. In this sense, data on the specific responses of fruit to abiotic stresses as wounding is lacking.

8. Proteomics in response to biotic stresses

Proteomic approaches have also been successfully used to study plant-pathogen interactions [143-145]. In particular, several proteomic studies focused on the pathogen side have been performed to understand fungal pathogenicity, i.e. *Ustilago maydis* [146] and *Phytophthora infestans* [147].

Despite the economic importance of some postharvest diseases, proteomics has only been applied in a few cases to study host-pathogen interactions in fruits. This include studies in peach [148], sweet cherry [149], jujube [150] and tomato [151] fruits, designed to characterise the biochemical interactions between fruits and pathogens subjected to different environmental conditions. Therefore, few studies used proteomic approaches focused on the *in vivo* fruit-pathogen interaction [148, 149, 151]. Although, from the pathogen side, in regard to *P. expansum*, extensive proteomic data are available [86, 152, 153], from the fruit (host) side, proteomic data are limited to the works mentioned above in sweet cherry, peach and tomato. In fact, many components of host resistance are not well known and there is a long way to go before we fully understand host and non-host resistance in fruit.

9. Post-translational modifications

As mentioned above, abiotic and biotic (i.e. wounding and pathogen attacks) stresses induced the ROS production, which is perceived by cells probably through post-translational modifications (PTMs) inasmuch as ROS are responsible of protein oxidation. This deleterious PTM or protein oxidative modification may affect

enzymatic activity, intracellular localization, protein–protein interactions and protein stability [154, 155]. Therefore, PTMs are often viewed as cellular switches and potential sensors of the complex redox regulation system [156]. However, few examples of PTM-based redox regulation were reported in plants.

Proteomics and advanced mass spectrometry have greatly aided to provide insight into mechanisms that involve the PTMs in plants [155]. In particular, one of the major challenges for the identification of PTMs is because are considered to play an important role in the adaptation of plants to different abiotic and biotic stimuli and in the operation and functioning of many host-pathogen interactions [157]. The level of protein oxidation has been used to assess protein oxidative damage in peach [158] and in sweet cherry fruit [159, 160] using two dimensional gel electrophoresis coupled with immunoblotting. However, different methods have been recently described for the detection and quantification of oxidised (carbonylated) proteins, including the use of fluorescent probes, such as Bodipy-Hz [161]. The use of Bodipy-Hz to detect carbonylated proteins presents many advantages. It saves time, because avoids Western-blot methods to detect protein oxidative damage. The method also provides the timely detection and excision of the protein spot after two-dimensional gel electrophoresis for further identification. Additionally, the use of fluorescent probes has the advantage of being able to detect a lower level of carbonylated proteins [154].

10. References

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Hypothesis and objectives/ Hipótesis y objetivos

P. digitatum and *P. expansum* are the two major pathogens of orange and apple fruit, respectively, not only in Spain but also worldwide. Although they are closely related species they show a different range of hosts. *P. digitatum* only infects citrus fruit, whereas *P. expansum* is found mainly associated to pome fruits and is non-pathogenic on citrus fruit. Control of these pathogenic fungi actually relays on the use of chemically synthesized fungicides. However, the actual trend, both from a legislative and consumer perspectives, is to reduce as much as possible the use of such chemicals. Our working hypothesis is that to design new alternative control strategies, we should first gather a deeper knowledge of these pathosystems, taking into account both, fungal pathogenicity mechanisms and host defence responses. On the pathogen side, we consider that targeting virulence determinants are essential for the development of new antimicrobials. On the host side, exploiting natural fruit defence responses, such as the oxidative burst, represents another alternative to improve disease control. Furthermore, host defence response mechanisms should be determined at the molecular level. In this regard, proteomics constitutes an excellent approach to unravel the key role played by the ultimate responsible of the molecular function: the proteins. Overall, we foresee that the route map we are trying to open with this work will lead the way to similar studies in other important postharvest diseases.

The main objective of this thesis is to increase the knowledge of fruit-pathogen interactions in both compatible and non-host pathogen interactions. In particular, defence responses of oranges and apples against compatible (orange-*P. digitatum* and apple-*P. expansum*) and non-host (orange-*P. expansum* and apple-*P. digitatum*) interactions have been studied by combining: molecular, biochemical and pathological approaches.

The specific objectives of this thesis are:

1. To determine the suitability of GFP-tagged strains to monitor *P. digitatum* and *P. expansum* infection in oranges and apples, both in compatible and non-host interactions, by checking their pathogenicity, germination and growth capacity compared to wild type strains.

2. To analyse the effect of H_2O_2 treatment as oxidative stress on ecophysiological behaviour of *P. digitatum* and *P. expansum* under *in vitro* conditions.

3. To detect and quantify the apple and orange H_2O_2 production in response to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses at different maturity stage of fruits.

4. To optimize a suitable methodology for extracting apple tissue proteins appropriate for (i) obtaining enough protein concentration and (ii) removing the interfering compounds, for further proteomic studies.

5. To describe the 'Golden Delicious' apple fruit proteins, as well as the changes in their abundance in response to abiotic stress (wounding).

6. To examine temporal changes in the proteome of 'Golden Smoothee' apple against compatible (*P. expansum*) and non-host (*P. digitatum*) pathogens as biotic stress.

7. To examine temporal changes in the oxi-proteome of 'Golden Smoothee' apple against compatible (*P. expansum*) and non-host (*P. digitatum*) pathogens as biotic stress.

8. To evaluate the transcriptional levels of several apple genes involved in wound and pathogen response chosen from proteomic studies.

9. To determine the correlation between the transcript and protein level of the selected genes.

P. digitatum y *P. expansum* son los dos principales patógenos de naranja y manzana, respectivamente, no sólo en España sino también en todo el mundo. Aunque son especies estrechamente relacionadas muestran un rango de huéspedes distinto. *P. digitatum* sólo infecta cítricos, mientras que *P. expansum* se encuentra asociado principalmente a frutas de pepita y no es patógeno de cítricos. El control de estos hongos patógenos actualmente recae en el uso de fungicidas sintetizados químicamente. Sin embargo, la tendencia actual, tanto desde las perspectivas legislativas como de consumo, es reducir al máximo el uso de dichos productos químicos. Nuestra hipótesis de trabajo es que para diseñar nuevas estrategias de control alternativas, primero debemos tener un mayor conocimiento de estos patosistemas, teniendo en cuenta tanto los mecanismos de patogenicidad de los hongos, como las respuestas de defensa del huésped. Por parte del patógeno, consideramos que determinar los factores de virulencia es esencial para desarrollar nuevos antimicrobianos. Por parte del huésped, entender y explotar las respuestas de defensa de la fruta, como la explosión oxidativa, representa otra alternativa para mejorar el control de la enfermedad. Además, los mecanismos de defensa del huésped deben determinarse a nivel molecular. En este sentido, la proteómica constituye un enfoque excelente para descifrar el papel clave que desempeña el último responsable de la función molecular: la proteína. En general, los estudios mostrados en esta tesis pueden ser la hoja de ruta a seguir en futuros estudios que se planteen en otras importantes enfermedades de postcosecha.

El objetivo principal de esta tesis es aumentar el conocimiento en las interacciones fruta-patógeno, tanto en las interacciones compatible como no compatible. En particular, se han estudiado las respuestas de defensa de naranjas y manzanas en sus interacciones compatibles (naranja-*P. digitatum* y manzana-*P. expansum*) y no compatibles (naranja-*P. expansum* y manzna-*P. digitatum*) combinando aproximaciones: moleculares, bioquímicas y patológicas.

Los objetivos específicos de esta tesis son:

1. Determinar la idoneidad del uso de cepas marcadas con GFP para visualizar la infección de *P. digitatum* y *P. expansum* en naranjas y manzanas, tanto en las interacciones compatibles como no compatibles, comparando su patogenicidad, germinación y capacidad de crecimiento con las cepas parentales.

2. Analizar el efecto del tratamiento con H_2O_2, como estrés oxidativo, en el comportamiento ecofisiológico de *P. digitatum* y *P. expansum* en condiciones *in vitro*.

3. Detectar y cuantificar en manzana y naranja la producción de H_2O_2 como respuesta a un estrés abiótico (herida) y uno biótico (patógeno compatible y no compatible), a diferentes estados de madurez.

4. Optimizar una metodología adecuada para la extracción de proteínas de tejido de manzana apropiada para (i) la obtención de suficiente concentración proteica y (ii) la eliminación de compuestos de interferencia, para realizar futuros estudios proteómicos.

5. Describir el proteoma de manzana 'Golden Delicious', así como también los cambios de abundancia proteica en respuesta al estrés abiótico (herida).

6. Examinar los cambios temporales en el proteoma de manzana 'Golden Smoothee' en respuesta a un patógeno compatible (*P. expansum*) y uno no compatible (*P. digitatum*), como estrés biótico.

7. Examinar los cambios temporales en el oxi-proteoma de manzana 'Golden Smoothee' en respuesta a un patógeno compatible (*P. expansum*) y uno no compatible (*P. digitatum*), como estrés biótico.

8. Evaluar en manzana los niveles de transcripción de varios genes implicados en la respuesta a herida y a patógeno, seleccionados a partir de los estudios proteómicos.

9. Determinar la correlación entre el nivel de transcrito y proteína en los genes seleccionados.

Chapter 1

Use of GFP-tagged strains of *Penicillium digitatum* and *Penicillium expansum* to study host-pathogen interactions in oranges and apples

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International Journal of Food Microbiology 160 (2012) 162-170

International Journal of Food Microbiology 160 (2012) 162–170

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

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ARTICLE INFO

Article history:
Received 30 July 2012
Received in revised form 8 October 2012
Accepted 9 October 2012
Available online 13 October 2012

Keywords:
ATMT
Population monitoring
Ecophysiology
Pathogenicity
Apple
Orange

ABSTRACT

Penicillium digitatum and *Penicillium expansum* are responsible for green and blue molds in citrus and pome fruits, respectively, which result in major monetary losses worldwide. In order to study their infection process in fruits, we successfully introduced a green fluorescent protein (GFP) encoding gene into wild type *P. digitatum* and *P. expansum* isolates, using *Agrobacterium tumefaciens*-mediated transformation (ATMT), with hygromycin B resistance as the selectable marker. To our knowledge, this is the first report describing the transformation of these two important postharvest pathogens with GFP and the use of transformed strains to study compatible and non-host pathogen interactions. Transformation did not affect the pathogenicity or the ecophysiology of either species compared to their respective wild type strains. The GFP-tagged strains were used for *in situ* analysis of compatible and non-host pathogen interactions on oranges and apples. Knowledge of the infection process of apples and oranges by these pathogens will facilitate the design of novel strategies to control these postharvest diseases and the use of the GFP-tagged strains will help to determine the response of *P. digitatum* and *P. expansum* on/in plant surface and tissues to different postharvest treatments.

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1. Introduction

Oranges and apples are both essential food crops cultivated in Spain and largely exported to Europe and other countries. *Penicillium digitatum* and *Penicillium expansum* are causal agents of green and blue molds in citrus and pome fruits, respectively, which result in major monetary losses worldwide.

Due to the development of resistance to fungicides, as well as concerns about the environment and consumer's health, the use of synthetic fungicides is becoming increasingly restricted (Viñas et al., 1993). In spite of fungicide use and the increased implementation of new alternative strategies, both green mold in citrus and blue mold in pome fruit continue to represent a major problem for stored fruits worldwide.

The development of a postharvest fungal disease partially depends on storage conditions and the physiological status of the fruit, as well as any inherent host defense mechanisms. These factors are intimately related, as fruit tend to become more susceptible to infection with physiological age (Su et al., 2011; Torres et al., 2003; Vilanova et al., 2012b). While the etiology of *Penicillium* rots are well understood, the physiological and biochemical bases of their host specificity is much less clear. Both *P. digitatum* and *P. expansum* are wound pathogens. The primary infection court for these pathogens is surface wounds where nutrients and volatiles stimulate conidial germination, which is followed by colonization of the fruit tissue (Droby et al., 2008; Eckert and Brown, 1986). This scenario suggests that adaptation to a particular host plays an important role in pathogenicity. For example, it has been shown that the presence of some oils facilitate infection in citrus (Rodov et al., 1995; Stange et al., 2002).

P. digitatum-orange and *P. expansum*-apple are considered compatible host-pathogen interactions. In contrast, *P. digitatum* has not been shown to cause postharvest disease on pome fruits nor has *P. expansum* been shown to cause postharvest rot of citrus. Thus these are considered to be non-host interactions. However, Vilanova et al. (2012b) working with *P. expansum* on oranges did demonstrate that a non-host pathogen interaction can become compatible when orange fruit were from commercial maturity stage.

P. digitatum is a pathogen very specific to citrus (Adams and Moss, 2000), whereas *P. expansum* has been isolated from a wide range of fruits other than pome fruits, including tomatoes, strawberries, avocados, grapes and a variety of others, indicating that it is a broad spectrum pathogen (Snowdon, 1990). The basis of host specificity for these postharvest pathogens remains unknown, so increased knowledge on compatible and non-host pathogen interactions for these pathogens could facilitate the development of new and safer control strategies.

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0168-1605/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2012.10.005

The use of fungal transformants expressing green fluorescent protein (GFP) is a very useful molecular tool that has enhanced our ability to study host-pathogen interactions and to both detect and visualize the infection process *in situ* (Horowitz et al., 2002). GFP-tagged fungal transformants have been obtained for a variety of postharvest pathogens (de Silva et al., 2009; Isshiki et al., 2003; Li et al., 2007). However, there are few reports on the genetic transformation of these two important postharvest pathogens, and to the best of our knowledge GFP-tagged strains of *P. digitatum* and *P. expansum* have not been reported so far.

The aim of this study was to introduce the *gfp* gene into wild type *P. digitatum* and *P. expansum* isolates by means of *Agrobacterium tumefaciens*-mediated transformation (ATMT) using hygromycin B resistance as the selectable marker. A protocol for ATMT of *P. digitatum* has been recently described (Wang and Li, 2008) but has not been reported for *P. expansum*. In order to check whether transformants maintained their pathogenicity, germination and growth capacity compared to wild type strains ecophysiological studies were conducted before using them to visualize the infection process in compatible and non-host pathogen interactions on oranges and apples.

2. Materials and methods

2.1. Plasmids

Two different plasmids containing two variants of the GFP were used for *Penicillium* transformation. The binary plasmid pRFHUE-eGFP (Crespo-Sempere et al., 2011) contains the eGFP, which differs from the native GFP from *Aequorea victoria* in a double amino acid substitution of Phe-64 to Leu, Ser-65 to Thr. The *egfp* gene included in this plasmid was obtained from plasmid pEGFPC3 and was cloned in plasmid pRF-HUE (Frandsen et al., 2008) under the control of the *Aspergillus nidulans gpdA* promotor. The second plasmid used was pCAMBgfp (Sesma and Osbourn, 2004). This plasmid contains the sGFP variant, in which there is a single amino acid substitution of Ser-65 to Thr with respect to the original GFP. In plasmid pCAMBgfp the expression of the *sgfp* gene is under control of *ToxA* gene promoter from *Pyrenophora tritici-repentis*. These two vectors were introduced into electrocompetent *A. tumefaciens* AGL-1 cells.

2.2. Fungal strains

Isolate Pd1 of *P. digitatum* (Pers.: Fr.) Sacc was obtained from a rotten "Navelina" orange at IVIA orchards that were not treated with fungicides and *P. expansum* Link CMP-1 was isolated from a decayed "Golden" apple after several months in storage. Wild type strains, *P. digitatum* and *P. expansum*, were grown on Petri dishes containing Potato Dextrose Agar medium (PDA: 200 mL/L boiled potato extract; 20 g/L dextrose, 20 g/L agar, pH 5.5) in the dark at 25 °C for 7–10 days to achieve conidia production. Transformed strains, *P. digitatum* and *P. expansum* were maintained on PDA containing 100 and 200 μg/mL of hygromycin B (Hyg B; InvivoGen, San Diego, USA), respectively. Transformed strains were also incubated at 25 °C in the dark during 7–10 days to obtain heavily sporulating cultures. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. The final conidia concentration was adjusted using a haemacytometer and diluted to different concentrations depending on each assay.

2.3. A. tumefaciens-mediated transformation (ATMT)

A. tumefaciens AGL-1 carrying the plasmid of interest was inoculated at 28 °C for 24 h in LC liquid medium (Hooykaas et al., 1977) with kanamycin (50 μg/mL), rifampicin (20 μg/mL) and carbenicillin (75 μg/mL). Bacterial cells were centrifuged, washed with induction medium (IM) (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.6 mM $CaCl_2$, 9 μM $FeSO_4$, 4 mM $(NH4)_2SO_4$, 10 mM glucose, 40 mM 2-[N-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol) and diluted to an OD_{600} of 0.15 in the same medium amended with 200 μM acetosyringone (AS). The culture was inoculated at 28 °C and 200 rpm until it reached an OD_{600} of 0.7–1.0.

Conidial suspensions of *P. digitatum* and *P. expansum* were washed twice with induction medium (IM) and adjusted to a concentration of 10^5 conidia/mL. Then, equal volumes of conidia and *A. tumefaciens* cells were mixed and spread onto nitrocellulose membrane filters (0.45 μm pore and 47 mm diameter, Albet, Dassel, Germany) that were placed on agar plates containing IM (containing 5 mM instead of 10 mM of glucose). After co-cultivation at 24 °C for 3 days, the filters were transferred to PDA plates containing hygromycin B (100 μg/mL or 200 μg/mL for *P. digitatum* and *P. expansum*, respectively) as the selection agent for fungal transformants, and 200 μg/mL of cefotaxime (Serva, Heidelberg, Germany) to inhibit growth of *A. tumefaciens* cells. Hygromycin resistant colonies obtained after 4 to 5 days of incubation were transferred to PDA plates containing hygromycin B and incubated at 24 °C for sporulation.

2.4. Genomic DNA extraction and PCR analysis

Conidia from transformants were transferred with a toothpick to a 1.5 mL Eppendorf tube containing 0.5 mL of Glucose Peptone Yeast (GPY: glucose 10 g/L, peptone 5 g/L, yeast extract 2 g/L, pH 7.5) medium supplemented with hygromicyn B and incubated with shaking at 24 °C for 48 h. The culture was centrifuged for 5 min at 12,000 rpm, the supernatant was removed and the pellet was resuspended with 300 μL of TNES (50 mM Tris HCl pH 8.0, 20 mM EDTA, 100 mM NaCl, 1% SDS). The sample was shaken during 2 min in a cell disruptor (BeadBeater, Biospec, Bartlesville, USA) with five stainless steel balls of 2.7 mm and centrifuged for 10 min at 12,000 rpm. Then, DNA was purified following the protocol described by Cenis (1992) and dissolved in 100 μL of TE.

To confirm the integration of the T-DNA in the genome, several transformants were randomly selected to analyze the presence of the hygromicin resistance gene. PCR was conducted using oligonucleotides HMBR1 (5′-CTGATAGAGTTGGTCAAGACC-3′) and HMBF1 (5′-CTGTCGAGAAGTTTCTGATCG-3′). DNA amplification was done under the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 62 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were separated in an agarose gel and were visualized under UV light.

2.5. Detection of GFP gene expression in transformants

To visualize the fluorescence of *P. digitatum* and *P. expansum* transformants obtained with each vector, they were grown on PDA plates with hygromicyn B at 24 °C for several days. A piece of mycelium from these plates was washed and resuspended in 20% glycerol. The fluorescence was visualized using a fluorescence microscope 90i (Nikon) equipped with a FITC B-2E/C filter (excitation from 450 to 490 mm and emission from 515 to 565 nm). Images were acquired with the Nikon's NIS-elements software.

2.6. Pathogenicity studies on fruit

Oranges (*Citrus sinensis* L. cv Lanelate) were obtained from a commercial orchard in Tortosa (Catalonia, Spain) and apples (*Malus domestica* L. cv Golden Delicious) from Lleida (Catalonia, Spain). Fruit were selected by hand prior to the application of any commercial postharvest treatment. Fruit were stored at an optimal storage temperature for each fruit, 4 °C for oranges and 0 °C for apples, until used. Prior to inoculation, the fruit were randomized, washed with tap water, and allowed to air-dry at room temperature. Each fruit was artificially wounded once with a nail (3 mm wide and

3 mm deep) on the equator and inoculated with 15 μL of an aqueous conidial suspensions of pathogen at $1 \cdot 10^6$ conidia/mL. The trial was performed with the wild type strains of *P. digitatum* and *P. expansum*, and the transformed strains eGFP-*P. digitatum* and sGFP-*P. expansum*, with their host, oranges and apples, respectively. This methodology was carried out individually for each pathogen and host. The treated fruit were incubated 7 days at 20 °C and 85–90% relative humidity (RH). After the incubation period, the percentage of infected wounds (incidence) and the lesion diameters (severity) caused by wild type and transformed strains were measured. Five fruits constituted a single replicate and each treatment was repeated four times. The average of five fruit was used as a single replicate and the experiment consisted of four biological replicates.

2.7. Sporulation assessment

The degree of *Penicillium* sporulation on the surface of decayed fruits was evaluated on a 0–5 scale described by Palou et al. (2003). A quantitative sporulation index was used in which the numbers 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. The index value for each fruit was treated as a biological replicate.

2.8. Ecophysiological characterization of strains

Conidia obtained from actively growing 7–10-day-old colonies of each strain grown on PDA media (wild types) and PDA media containing hygromycin B (transformants) were used for all ecophysiological studies.

Synthetic and semi-synthetic media were used for all ecophysiological studies. Synthetic media were PDA and Orange Serum Agar (OSA) with a pH of 5.5. Semi-synthetic Apple-based medium (AM) contained 10% apple juice sterilized through a nitrocellulose membrane filter (0.22 μm pore and 25 mm diameter, Millipore, Billerica, U.S.A.), glucose (4 g/L) and 2% agar with a final pH of 4.3.

2.8.1. Germination studies in vitro

Ten-microliter droplets of the conidia suspensions adjusted to $5 \cdot 10^5$ conidia/mL were inoculated on PDA and OSA for *P. digitatum* strains and PDA and AM for *P. expansum* strains. Petri dishes were incubated at 25 °C (*P. digitatum* and *P. expansum* strains), 4 °C (*P. digitatum* strains) and 0 °C (*P. expansum* strains). Periodically, depending on the temperature, three agar disks (5 mm diameter) coinciding with each of the placed drops were aseptically removed from each replicate using a cork borer. At each sampling time, disks from the same temperature and medium were placed into a sterile empty Petri dish, and conidia germination was immediately stopped by adding 3 mL of ammonia (NH_3 25%) onto a filter paper placed on the cover of each plate. Then, Petri dishes were stored at 4 °C until microscopic examination. Fifty single conidia per disk (150/replicate; 450/treatment) were microscopically examined (Leica DM5000B). Conidia were considered germinated when the germ tube was equal to or longer than the diameter of the conidia (Casals et al., 2010; Plaza et al., 2003). The variable measured was the percentage of germination at different temperatures and culture media against time. Experiments were carried out with three replicates per treatment.

2.8.2. Growth studies in vitro

Ten-microliter droplets of the conidia suspensions adjusted to $5 \cdot 10^5$ conidia/mL were single-point inoculated in the middle of Petri plates with different media. Wild type and transformed strains of *P. digitatum* were inoculated on PDA and OSA and *P. expansum* strains on PDA and AM. *P. digitatum* strains were incubated at 25 °C and 4 °C and *P. expansum* strains at 25 °C and 0 °C. Strains incubated at 25 °C were examined daily whereas strains incubated at cold conditions were examined every 7 days. Measurements were carried out for a maximum of 12 days at 25 °C and 77 days at 4 °C or 0 °C depending on the pathogen. Colony diameters were measured in two directions at right angles to each other (Marín et al., 2006) until the plate was fully covered. The variable measured was the colony diameter at different temperatures and culture media against time. Experiments were carried out with three replicates per treatment.

2.9. Visualization of fruit infected with Penicillium strains

Six fruit disks (16 mm diameter and 5 mm thickness) were removed from oranges and apples using a cork borer and placed into sterile Petri plates. One set of disks remained intact and another set was wounded once with a nail (3 mm wide and 3 mm deep) at the center. Both, intact and wounded fruit disks were inoculated with 15 μL of a conidia suspension of each strain. For compatible interactions, orange-*P. digitatum* and apple-*P. expansum*, 10^5 conidia/mL of *P. digitatum* or eGFP-*P. digitatum* and 10^4 conidia/mL of *P. expansum* or sGFP-*P. expansum* were inoculated. For non-host pathogen interactions, orange-*P. expansum* and apple-*P. digitatum*, the concentration in both cases was 10^7 conidia/mL. Disks inoculated with wild type and transformed strains of *P. digitatum* were stored at 20 °C and 4 °C. Disks inoculated with *P. expansum* strains were stored at 20 °C and 0 °C. The experiment was conducted for a maximum of 96 h at 20 °C and 32 days at 0 °C and 4 °C. After the incubation period, samples were examined using a stereoscope (Leica MZ16F) equipped with external light source and appropriate filter sets (excitation from 460 to 500 nm and 510 nm emission). Images were captured using the Leica's DFCTwain software.

2.10. Statistical analyses

Differences on the percentage of infected wounds (incidence), lesion diameters (severity), germination percentage, growth rate and sporulation index between the wild type and the transformed strains were evaluated by the *t*-test using the statistical package SAS (Microsoft). Differences between mean values were considered significant when $P \leq 0.05$.

Scores in the sporulation index were considered as a quantitative variable. In order to homogenize variances, each value in the sporulation data set was transformed to the square root of the value plus 0.5.

For the growth studies, growth rates (mm/day) were obtained from the growth data using linear regression of the linear parts of the temporal growth curves.

3. Results and discussion

3.1. Transformation

The major aim of this work was to obtain GFP-tagged *P. digitatum* and *P. expansum* transformants in order to study compatible (orange-*P. digitatum* and apple-*P. expansum*) and non-host pathogen (orange-*P. expansum* and apple-*P. digitatum*) interactions. In many fungal species transformation with the native *gfp* gene have resulted in non-fluorescent transformants (Fernandez-Abalos et al., 1998), probably due to inadequate codon usage. As *P. digitatum* transformants expressing this native version of the *gfp* gene did not show any fluorescence (data not shown), we used the modified versions encoded by *egfp* and *sgfp* genes for GFP tagging of *P digitatum* and *P. expansum*. These two GFP variants are present in plasmids pRFHUE-eGFP and pCAMBgfp, respectively. ATMT was used to transform *P. digitatum* and *P. expansum* with these two plasmids. Randomly selected hygromycin resistant colonies were analyzed by PCR to detect the presence of the hygromycin resistance gene and all of them were positive (data not shown). Microscopic analysis of GFP-tagged strains revealed homogeneity of the

fluorescent signal, which was clearly visible in the conidia and hyphae and stable for several hours during observations. No green autofluorescent background was observed in the wild type strains (Fig. 1).

Despite the economic importance of these two postharvest pathogens little attention has been paid to their physiological and genetic characterization. Genetic transformation of *P. expansum* has only been described twice (Dias et al., 1999; Sanzani et al., 2012), but to

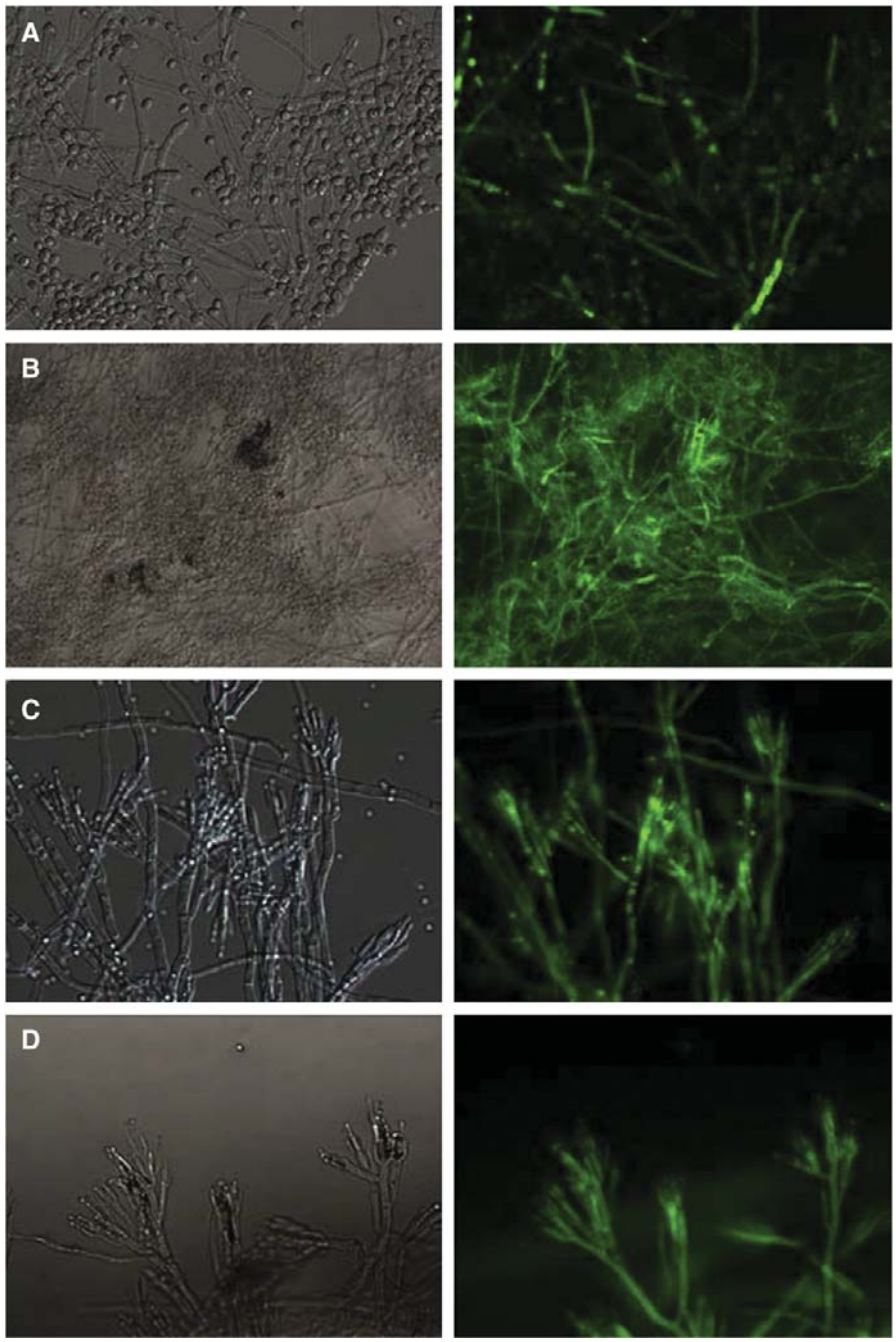

Fig. 1. Microscopy analysis of transformant strains eGFP-*Penicillium digitatum* (A) sGFP-*P. digitatum* (B), eGFP-*P. expansum* (C) and sGFP-*P. expansum* (D). Differential interference contrast (left) and fluorescence (right) images of conidiophores and conidia (A–D). A, C, and D, microscope magnification, ×400; B, microscope magnification, ×200.

our knowledge this is the first time that ATMT has been described in *P. expansum*. On the other hand, genetic transformation of *P. digitatum* has been used to study the mechanisms of fungicide resistance (Hamamoto et al., 2001; Nakaune et al., 1998; Nakaune et al., 2002) and ATMT has been described recently for this fungus (Wang and Li, 2008).

GFP-tagged fungal pathogens have been used to study the different stages of the progress of the fungus within a host (Horowitz et al., 2002; Isshiki et al., 2003; Lagopodi et al., 2002; Pliego et al., 2009; Talhinhas et al., 2008). Thus, the availability of GFP-tagged *P. digitatum* and *P. expansum* transformants constitutes a powerful tool for studying the interactions between *P. digitatum* and *P. expansum* and their fruit hosts. The GFP-transformed strains are also very useful in determining the response of *P. digitatum* and *P. expansum* on/in plant surface and tissues to different postharvest treatments.

3.2. Pathogenicity studies

To determine whether or not the transformants were altered in their ability to cause disease in their respective hosts as a result of integration of the transforming DNA within the genome, an assessment of pathogenicity of the wild type and transformed strains was conducted. For this purpose one transformant strain from each species was chosen and compared to the non-transformed wild type strain.

Seven days after inoculation in oranges, there was no significant difference in percentage of infected wounds (%) or lesion diameter (cm) at 20 °C ($P>0.05$) (data not shown). For the wild type and the eGFP-*P. digitatum* strains, the *Penicillium* rot was 95% and 100%, and the lesion diameter was 12.5 and 11.5 cm, respectively. As in oranges, no difference was found in the percentage of infected apple wounds (%) or lesion diameter (cm), after 7-day incubation at 20 °C ($P>0.05$) (data not shown). For the wild type and the sGFP-*P. expansum* strains, blue mold was observed in 100% inoculated apples, and lesion diameter was 3.4 cm.

GFP has been successfully used as a marker in a broad range of plant pathogens to study both leaf and root infections (Horowitz et al., 2002; Lorang et al., 2001; Maor et al., 1998; Morocko-Bicevska and Fatehi, 2011; van West et al., 1999; Visser et al., 2004). As described previously for other fungal species such as *Botrytis cinerea* in strawberry (Li et al., 2007), *Aspergillus carbonarius* in grapes (Crespo-Sempere et al., 2011), *Fusarium oxysporum* in tomato (Lagopodi et al., 2002) and *Rosellinia necatrix* in avocado (Pliego et al., 2009), the expression of a fluorescent protein does not affect the pathogenicity of the transformed fungi. In agreement with these previous reports, pathogenicity tests on oranges and apples revealed that the GFP-tagged strains of *P. digitatum* and *P. expansum* maintain the characteristics of the wild type strains and express the GFP *in vitro* and *in vivo* in both hyphae and conidia. Therefore, the GFP-tagged strains for *P. digitatum* and *P. expansum* represent a powerful tool for *in situ* analysis of the infection process in fruit-pathogen interactions.

3.3. Sporulation index

Results obtained for the sporulation index in oranges and apples were similar between wild type and transformed strains. Inoculated oranges reached a sporulation index of 2 indicating that 5–30% of the fruit surface was covered with conidia (data not shown). On inoculated apples, less than 5% of the fruit surface was covered with conidia which correspond to a sporulation index of 1 (data not shown). There were no significant differences in the sporulation index of wild type and GFP-tagged strains on either oranges or apples ($P>0.05$) (data not shown). Therefore, our results confirm that GFP transformation did not significantly affect the sporulation of either pathogen on their compatible hosts.

3.4. Ecophysiology studies

3.4.1. Germination studies in vitro

Results obtained in synthetic (PDA and OSA) media inoculated with wild type and transformed strains of *P. digitatum* are shown in Fig. 2. Overall patterns of germination were similar for *P. digitatum* and eGFP-*P. digitatum* strains. For instance, after 12 h of incubation on PDA at 25 °C, the percentage of germination for both strains was 85% and 88%, respectively. In addition, after 96 h of incubation at 4 °C, the percentage of germination also showed similar values (77% and 79%, respectively). In both cases, differences were not significant ($P>0.05$) (Fig. 2A–B). After 12 h of incubation on OSA at 25 °C, the percentage of germination for *P. digitatum* (76%) and eGFP-*P. digitatum* (90%) was significantly different ($P<0.05$) (Fig. 2C). The wild type strain of *P. digitatum* germinated slightly later than eGFP-*P. digitatum* at 4 °C on OSA. However, after 96 h no significant differences were observed (70% and 75% for *P. digitatum* and eGFP-*P. digitatum*, respectively) ($P>0.05$) (Fig. 2D). It is interesting to note that the percentage of germinated spores at both temperatures reached similar values, although there was a delay in germination at 4 °C.

Germination percentage was also similar for the wild type and transformed *P. expansum* strains in synthetic (PDA) and semi-synthetic (AM) media (Fig. 3). For example, after 12 h of incubation on PDA at 25 °C, germination percentage reached 100%, and no significant differences were observed between both strains ($P>0.05$) (Fig. 3A). Only in a few cases the differences were significant ($P<0.05$): first, after 144 h of incubation on PDA at 0 °C, the germination percentage was 90% for *P. expansum*, and 78% for sGFP-*P. expansum* (Fig. 3B). Second, after 12 h of incubation on AM at 25 °C, it was 84% and 70%, for the wild type and sGFP-*P. expansum* strains, respectively (Fig. 3C). Lastly, sGFP-*P. expansum* also germinated to a lower extent than *P. expansum* at 0 °C (Fig. 3D). However, the high germination capability exhibited by *P. expansum* and sGFP-*P. expansum* at 0 °C is noteworthy.

These results suggest that transformation with the *gfp* gene did not modify the overall germination pattern in *P. digitatum* and *P. expansum* transformants as compared to their respective wild type on different incubation media and temperatures.

In this study OSA and AM media were used because they are reasonably similar in composition to orange and apple fruit. Wyatt and Parish (1995) demonstrated that *P. digitatum* conidia did not germinate at 0 or 3 °C on Orange juice serum agar. Low temperatures used in this study are commonly used in storage rooms used to keep oranges (4 °C) and apples (0 °C) for several months. Our results showed 70% of *P. digitatum* germination after 96 h of incubation at 4 °C on OSA (Fig. 2D). These results differ from those obtained by Plaza et al. (2003), in which the germination percentage for *P. digitatum* was around 20% after 96 h of incubation at 4 °C on OSA. These differences could be due to the different isolates used in both studies.

The comparison of germination percentages at two different temperatures suggests that *P. digitatum* and *P. expansum* may be more difficult to control in susceptible foods stored at 4 and 0 °C, respectively, because both fungi show a high germination rate at low temperatures, thus cold storage does not prevent spoilage but only retards it.

3.4.2. Growth studies in vitro

No differences in colony morphology on synthetic and semi-synthetic media were observed at 25 °C, 4 °C and 0 °C. The effects of temperature and culture medium on the growth rate for wild type and GFP-tagged strains are presented in Tables 1 and 2. There were no significant differences between the wild type and the eGFP-*P. digitatum* strains, in any studied condition ($P>0.05$) (Table 1). For instance, both wild type and eGFP-*P. digitatum* strains had similar growth rates when cultured on PDA (8.30 and 8.48 mm/day, respectively) and OSA (8.77 and 8.41 mm/day, respectively) at 25 °C. These values are higher than reported by Plaza et al. (2003) for another isolate of *P. digitatum* incubated at the same temperature

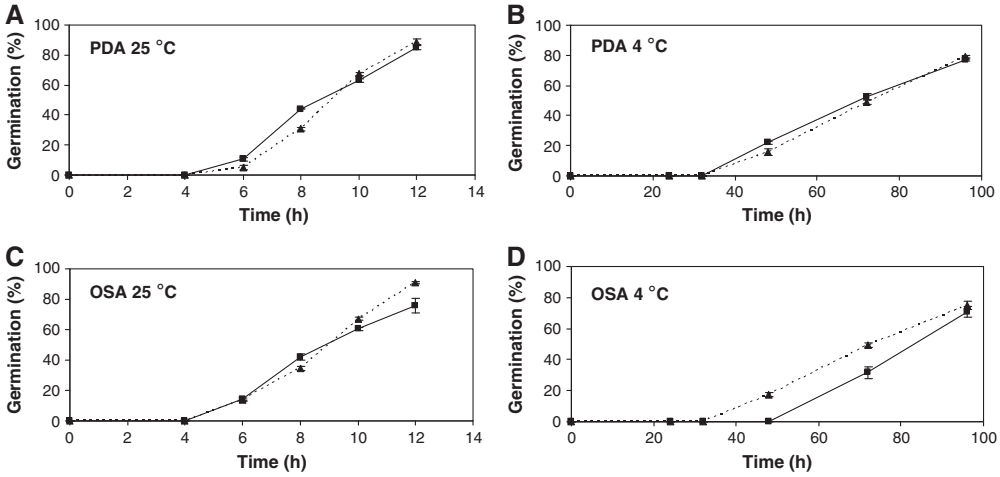

Fig. 2. Effect of temperature on the germination percentage of *Penicillium digitatum* and eGFP-*P. digitatum* strains on PDA and Orange Serum Agar (OSA) media. Strains are (■) *P. digitatum* and (▲) eGFP-*P. digitatum*. Values are the means of three replicates and 150 conidia per replicate. Vertical bars are the standard deviation.

(3.5 mm/day). As in the germination study, these differences could be due to the different isolates used in both studies. Growth rates of both strains were reduced when temperature varied from 25 to 4 °C. In such case, the growth rates were 0.38 and 0.32 mm/day on PDA, and 0.64 and 0.45 mm/day on OSA (Table 1).

Statistical analysis revealed that the growth rate was not different between the wild type and the transformed *P. expansum* strains, in any studied condition ($P>0.05$) (Table 2). At 25 °C, growth rates of *P. expansum* and sGFP-*P. expansum* strains were 8 mm/day on PDA, and 7.88 and 7.84 mm/day on AM. Growth rates of both strains were reduced when temperature varied from 25 to 0 °C. At 0 °C, the growth rates were 0.52 mm/day on PDA, and 0.63 mm/day on AM (Table 2). These results are in agreement with those obtained by Baert et al. (2007), who demonstrated that shortened growth rates were found when the temperature increased from 2 °C to 25 °C.

These results suggest that transformation with the *gfp* gene did not modify the growth rate in *P. digitatum* and *P. expansum* transformants as compared to their respective wild type strains on different incubation media and temperatures. It is difficult to extrapolate the results obtained *in vitro* to natural environment because other factors, such as pH, antifungal compounds of the peel, essential oils, may influence the development of the fungus within the host. Moreover, further studies are needed in order to provide detail knowledge on the ecological requirements of these species for colonizing and infecting the surface of oranges and apples.

3.5. Fluorescence visualization in vivo: compatible and non-host pathogen interaction

Once determined *in vitro* that from the ecophysiological point of view transformant and wild type are equivalent, other confirmation on fruit-pathogen interaction were done *in vivo*.

Fruits were inoculated with wild type and transformant strains to test whether GFP can help to visualize the pathogen on the fruit surface and the behavior of both transformants on host and non-host fruits. Colonization of the hosts by the pathogens in relation to the development of

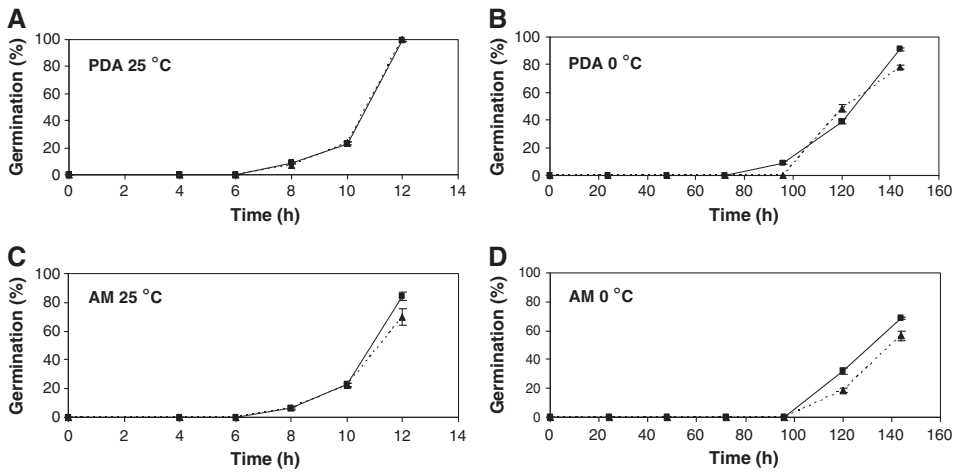

Fig. 3. Effect of temperature on the germination percentage of *Penicillium expansum* and sGFP-*P. expansum* strains on PDA and Apple-based medium (AM) media. Strains are (■) *P. expansum* and (▲) sGFP-*P. expansum*. Values are the means of three replicates and 150 conidia per replicate. Vertical bars are the standard deviation.

Table 1
Comparison of growth rate (mm/day) between the wild type and the eGFP-*P. digitatum* at different media (PDA and OSA) and temperature (25 °C and 4 °C).

		Growth rate (mm/day)[a]		
		P. digitatum	eGFP-*P. digitatum*	*P*-value
25 °C	PDA	8.30	8.48	0.70
	OSA	8.77	8.41	0.38
4 °C	PDA	0.38	0.32	0.34
	OSA	0.64	0.45	0.11

[a] Rate was determined as the slope of the linear portion of a curve produced by plotting distance of growth diameter (mm) *vs.* time (days). Each result is the mean of three replicates. Significant differences ($P<0.05$) were evaluated using *t*-test.

Table 2
Comparison of growth rate (mm/day) between the wild type and the sGFP-*P. expansum* at different media (PDA and AM) and temperature (25 °C and 0 °C).

		Growth rate (mm/day)[a]		
		P. expansum	sGFP-*P. expansum.*	*P*-value
25 °C	PDA	8.02	8.00	0.96
	AM	7.88	7.84	0.54
0 °C	PDA	0.52	0.52	0.63
	AM	0.63	0.63	0.59

[a] Rate was determined as the slope of the linear portion of a curve produced by plotting distance of growth diameter (mm) *vs.* time (days). Each result is the mean of three replicates. Significant differences ($P<0.05$) were evaluated using *t*-test.

green and blue mold in compatible and non-host pathogen interactions was determined using GFP-tagged strains (Figs. 4 and 5). Overall, wounded orange and apple disks with the transformed strains showed green fluorescence under the fluorescence stereomicroscope, whereas no fluorescence was observed in intact fruit disks confirming that both are wound pathogens. Additionally, re-isolation of GFP-tagged from inoculated wounded disks fruits on PDA with hygromycin B confirmed microscopic observations.

To visualize pathogens *in planta* it is important for whole fungi to express the GFP. This can be achieved by constitutive expression of GFP, as described above, which typically results in a cytoplasmically located protein occurring in all fungal morphotypes (hyphae, conidia, *etc.*) with no obvious effects on fungal growth or pathogenicity (Lorang et al., 2001; Maor et al., 1998; Spellig et al., 1996; van West et al., 1999). Compatible interactions are shown in Figs. 4A–C and 5A–C, whereas non-host pathogen interactions are shown in Figs. 4B–D and 5B–D. In this work, GFP transformants of phytopathogenic fungi were easily detected with fluorescence microscopy (Figs. 4C–D and 5C–D). In addition, images of *P. expansum* in orange revealed that primary infection takes place in a similar way as in the *P. digitatum*-orange interaction. Vilanova et al. (2012b) has previously shown that depending on a combination of factors (maturity stage and inoculum concentration), the *P. expansum*-orange interaction can change from non-host pathogen to compatible in Valencia and Navelina varieties. We have observed the same phenomenon in Lanelate oranges, supporting this previous finding. The maturity stage of the oranges we have used was mature to over-mature (data not shown). Therefore, maturity stage could affect the infection capacity in a non-host interaction in oranges. However, rot infection did not develop in the apple-*P. digitatum* interaction, although, as shown in Fig. 5B and D, *P. digitatum* is able to germinate inside the wounded apple tissue and produce a limited infection only in surrounding cells. It seems that apple is able to avoid the progress of *P. digitatum* by expressing an efficient defense response.

The phenomenon of selective stimulation of fruit pathogens by volatile compounds is known in other fruit pathogen systems. Droby et al. (2008) demonstrated that *P. digitatum* and *Penicillium italicum* are uniquely adapted to and stimulated by the volatile environment associated with citrus wounds, whereas this same environment is inhibitory to other non-citrus pathogens such as *P. expansum* and *B. cinerea*. However, the relative importance of each volatile component separately is not clear.

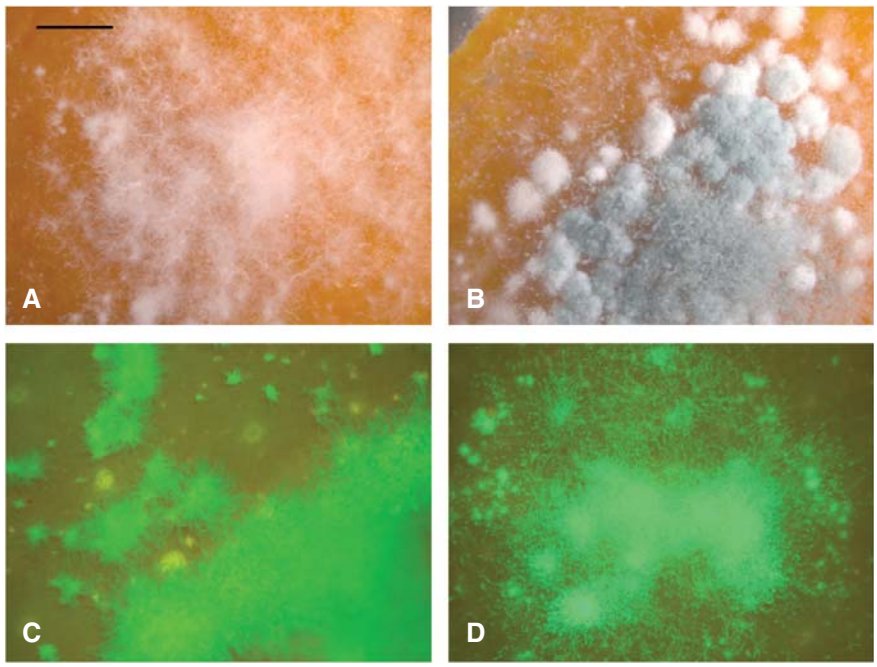

Fig. 4. *In situ* visualization of wild type and GFP-tagged strains on wounded oranges. (A) *Penicillium digitatum*, (B) *P. expansum*, (C) eGFP-*P. digitatum*, (D) sGFP-*P. expansum*. Light (A–B) and fluorescence (C–D). Scale bar = 2 mm.

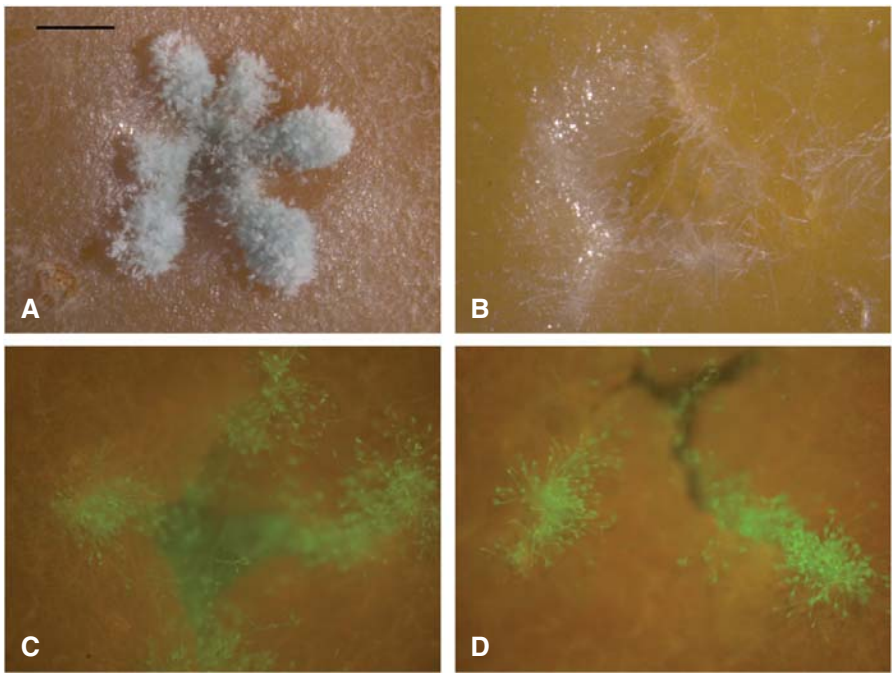

Fig. 5. *In situ* visualization of wild type and GFP-tagged strains on wounded apples. (A) *Penicillium expansum*, (B) *P. digitatum* (C) sGFP-*P. expansum*, (D) eGFP-*P. digitatum*. Light (A–B) and fluorescence (C–D). Scale bar = 1 mm.

We have confirmed the capacity of *P. expansum* to infect oranges (non-host pathogen) under specific conditions. Similarly, *P. digitatum* is able to germinate inside apple wounded tissue (non-host pathogen), but only causing a limited infection around the wound (Vilanova et al., 2012a).

To our knowledge, this is the first study that reports the transformation of *P. digitatum* and *P. expansum* with GFP and the use of transformed strains to study compatible and non-host pathogen interactions of these two important postharvest pathogens. Knowledge of these interactions is essential to offer novel insights into *P. digitatum* and *P. expansum* pathogenicity from the early stages of tissue colonization to advanced stages of the disease. In addition, knowledge of the infection process of apples and oranges by their pathogen and non-host pathogen is essential for the design of novel strategies to control these postharvest diseases and determine the response of *P. digitatum* and *P. expansum* on/in plant surface and tissues to different postharvest treatments.

Acknowledgments

We thank Ane Sesma (John Innes Center, UK) for providing us plasmid pCAMBgfp. The authors are grateful to the Spanish Government for its financial support with projects AGL2008-04828-C03-01 and AGL2008-04828-C03-02, for Ramón y Cajal Contract (R. Torres) and for the scholarships BES-2006-12983 (M. López) and BES-2009-027752 (G. Burón). We also want to thank the excellent technical assistance of Cèlia Sánchez.

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Chapter 2

Characterisation of H_2O_2 production to study compatible and non-host pathogen interactions in orange and apple fruit at different maturity stages

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Submitted to: Postharvest Biology and Technology

Characterisation of H_2O_2 production to study compatible and non-host pathogen interactions in orange and apple fruit at different maturity stages

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Research highlights

1. *P. expansum* is more susceptible to higher levels of H_2O_2 than *P. digitatum*
2. Level of H_2O_2 in response to abiotic and biotic stresses depended on the harvest date
3. Apple resistance to *P. digitatum* (non-host pathogen) declined clearly with ripening
4. Orange resistance to *P. expansum* (non-host pathogen) is slightly maturity independent

Abstract

Penicillium digitatum and *P. expansum* are the main postharvest pathogens of orange and apple fruit, respectively. These wound pathogens can overcome the fruit defences through injuries caused during harvest and postharvest handling, which annually lead to large economic losses.

Several studies have been published on the increase susceptibility of fruit to mechanical damage or infection during ripening, but few focussed on the fruit wound-induced defence responses, such as H_2O_2 production. In the present study, the characterisation of H_2O_2 production in the fruit defence response to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses at different maturity stages was investigated. Three maturity stages, from immature to over-mature, of orange (*C. sinensis* cv. 'Valencia') and apple (*M. domestica* L. cv. 'Golden Smoothee') were used. Previously, an *in vitro* study to characterise the ecophysiology of both fungi strains (*P. digitatum* and *P. expansum*) under an oxidative stress environment was also performed. Results indicated that under *in vitro* conditions, the potential antifungal effect of H_2O_2 in both pathogens depends on the temperature. In fact, *P. expansum* was more susceptible to higher levels of H_2O_2 (200 mM) than *P. digitatum* at both temperatures.

The lesion diameter in compatible interactions increased significantly with fruit ripening in apples and oranges. Particularly, the susceptibility of over-mature apples increase in non-host pathogen interaction. H_2O_2 production showed different patterns depending on the fruit. In apples, the higher resistance of immature harvested fruit to pathogen infection correlated with an increase in H_2O_2 production (biphasic oxidative burst), whereas in oranges, immature and commercial harvests exhibited a similar pattern of H_2O_2 production among treatments. H_2O_2 production in oranges and apples because of abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses depended on the harvest date.

Keywords:

Ecophysiology; *Penicillium digitatum*; *Penicillium expansum*; Hydrogen peroxide; Fruit ripening; Wounding.

1. Introduction

The most devastating postharvest disease in fruits is decay caused by fungi, which annually leads to large economic losses. *Penicillium digitatum* and *P. expansum* are causal agents of green and blue moulds in citrus and pome fruits, respectively (Pitt and Hocking, 1997), that are essential food crops cultivated in Spain and widely exported to Europe and other countries. Mechanical injury, which can occur during harvesting, packing-house operations, handling and transport, causes the fruit to be more susceptible to opportunistic infection by these postharvest pathogens (Van Zeebroeck *et al.* 2007).

The development of a postharvest fungal disease partially depends on storage conditions and on the physiological status of the fruit, as well as on any inherent host defence mechanisms. These factors are intimately related because fruits tend to become more susceptible to infection with their physiological age (Torres *et al.* 2003; Su *et al.* 2011; Vilanova *et al.* 2012a; Vilanova *et al.* 2012b). During fruit-pathogen interactions, the pathogens need to overcome the innate defences of the fruit, and the fruits should initiate an efficient defence response to overcome wound stress, and consequently avoid or prevent a possible pathogen infection. These wound-induced defence responses may be modulated by fruit ripening, which could be one of the main factors that determine the susceptibility of fruit to mechanical damage or to infection during postharvest storage (Mehdy, 1994; Torres *et al.* 2003; Su *et al.* 2011).

The association of reactive oxygen species (ROS) formation with the induction of defence responses has been demonstrated in many plant-pathogen interactions (Mehdy, 1994). In almost all host-pathogen interactions, one of the first events that is detected in attacked host cells is the oxidative burst, which is the rapid and transient generation of activated oxygen radicals, including superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2), at the site of pathogen infection (Lamb and Dixon, 1997; Wojtaszek, 1997). The crucial role of ROS in the host defence against pathogen attack has been generally described by two phases: phase I occurs in both compatible and non-host pathogen interactions and causes a rapid, but weak, accumulation

of ROS with a response time of a few minutes. In contrast, phase II has only been observed in non-host pathogen interactions and is characterised by an important and prolonged accumulation of ROS after a longer period, which is usually several hours (Levine *et al.* 1994; Baker and Orlandi, 1995). H_2O_2 is the most important ROS because of its stability and slow reactivity with biological molecules. Within the different possible functions in a plant's defence strategy, H_2O_2 may be involved in membrane peroxidation and in the cross-linking of cell wall proteins (Bradley *et al.* 1992; Lamb and Dixon, 1997). In addition to the role of H_2O_2 as a signalling molecule after wounding (Orozco-Cardenas *et al.* 2001; Nurnberger *et al.* 2004), H_2O_2 mediates the induction of hypersensitive cell death and the expression of a wide array of defence-related genes in surrounding cells (Levine *et al.* 1994; Grant and Loake, 2000; Bolwell *et al.* 2002; Borden and Higgins, 2002), and H_2O_2 has also been shown to be involved in the spore germination inhibition of many fungal pathogens (Peng and Kuc, 1992; Cerioni *et al.* 2010; Cerioni *et al.* 2013).

In many fruits, ROS, including H_2O_2, have been reported to be associated with fruit development, ripening and senescence (Brennan and Frenkel, 1977; Jimenez *et al.* 2002; Larrigaudiere *et al.* 2004; Vilaplana *et al.* 2006; Chiriboga *et al.* 2013). In addition, H_2O_2 is also primarily involved in the biosynthesis, polymerisation and deposition of lignin (Olson and Varner, 1993; Razem and Bernards, 2002). The lignification process may contribute to resistance in many different ways, although little is known regarding its role in fruit-induced resistance (Sticher *et al.* 1997; Valentines *et al.* 2005). The presence or accumulation of H_2O_2 in response to wounding and to pathogen attack may be a requirement in the establishment of the host defence (Mehdy, 1994; Wu *et al.* 1995; Orozco-Cardenas *et al.* 2001; Rea *et al.* 2002). In previous works, we have shown that H_2O_2 (Torres *et al.* 2003) and lignification (Vilanova *et al.* 2013; Vilanova *et al.* 2014) might play an important role in the disease resistance of 'Golden delicious' apple and 'Valencia' orange fruit. The potential role of H_2O_2 accumulation in response to wounding was described by Castro-Mercado et al. (2009) in unripe avocado fruit, whereas H_2O_2 accumulation in response to pathogen attack was studied in tomato

plants (Borden and Higgins, 2002; Mandal *et al.* 2008), apple (Castoria *et al.* 2003; Torres *et al.* 2003; Su *et al.* 2011), orange (Torres *et al.* 2011) and lemon (Macarisin *et al.* 2007) fruits.

H_2O_2 appears to play a crucial role in fruit defence mechanisms during ripening in response to wounding and to pathogen infection. Therefore, it would be important characterise H_2O_2 production in the fruit response against wounding, pathogens and non-host pathogens at different maturity stages. In this study, three maturity stages, from immature to over-mature, of orange (*C. sinensis* cv. 'Valencia') and apple (*M. domestica* L. cv. 'Golden Smoothee') were used to characterise H_2O_2 production in the resistance response to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses and to characterise H_2O_2 production during the infection process. Previously, an *in vitro* study to characterise the behaviour of both fungi strains (*P. digitatum* and *P. expansum*) under an oxidative stress environment was also performed.

2. Materials and Methods

2.1. Fungal cultures

The *P. digitatum* strain PDM-1 and *P. expansum* Link CMP-1 were isolated from decayed citrus and pome fruits, respectively. These isolates were the most aggressive in our collection (Pathology Laboratory, IRTA, Lleida). *P. digitatum* and *P. expansum* were grown on Petri dishes containing potato dextrose agar medium (PDA: 200 mL/L boiled potato extract; 20 g/L dextrose, 20 g/L agar, pH 5.5) in the dark at 25 ºC for 7-10 days to achieve conidia production. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of the cultures grown on PDA and by rubbing the surface of the agar with a sterile glass rod. The final conidia concentration was adjusted using a haemocytometer and diluted to different concentrations depending on each assay.

2.2. Ecophysiological pathogen response to environmental oxidative stress

In vitro assays of the resistance of the pathogens *P. digitatum* and *P. expansum* to H_2O_2 treatment were performed as follows: conidial suspensions ($5 \cdot 10^5$ conidia/mL) were added to flasks containing 25 mL of freshly prepared malt extract medium (ME: 30 g/L malt extract, 5 g/L peptone pepsique de viande USP, pH 5.5) alone or, alternatively, containing 2, 20, 50 or 200 mM H_2O_2 (PANREAC, Barcelona, Spain) and incubated for 1 h shaking at 150 rpm at 25 ºC. All treatments were performed in triplicates. After incubation, H_2O_2-treated conidia were filtered (0.22 μm pore and 25 mm diameter, Millipore, Billerica, U.S.A) and washed with sterile distilled water. To recuperate all spores, filters were resuspended in 4.5 mL of sterile distilled water with 0.01% (w/v) Tween-80 and vortexed for 2 min. Ten-microlitre droplets of the conidial suspensions were inoculated on PDA plates. Petri dishes were incubated at 25 ºC (*P. digitatum* and *P. expansum*), 4 ºC (*P. digitatum*) and 0 ºC (*P. expansum*). Periodically, depending on the temperature and coinciding with each of the placed drops, three agar discs (5 mm diameter) were aseptically removed from each replicate using a cork borer. At each sampling time, discs from the same temperature and medium were placed into a sterile empty Petri dish, and conidia germination was immediately stopped by adding 3 mL of ammonia (NH_3 25%, PANREAC, Barcelona, Spain) onto a filter paper placed on the cover of each plate. Then, Petri dishes were stored at 4 ºC until microscopic examination. The germination percentages of *P. digitatum* and *P. expansum* were evaluated as previously described by Buron-Moles *et al.* (2012). The variable measured was the percentage of germination at different temperatures against time. Experiments were performed with three replicates per treatment.

2.3. Fruit source

Apples (*Malus domestica* L. cv. 'Golden Smoothee') were harvested at different maturity stages from August to October 2010 (immature, 12th

August; commercial, 16th September; over-mature, 21th October) from a commercial orchard in Mollerussa (Catalonia, Spain). Apples were used immediately after harvest.
Oranges (*Citrus sinensis* cv. 'Valencia') were obtained at different maturity stages from March to June 2011 (immature, 17th March; commercial, 29th April; over-mature, 23th June), from a commercial orchard in Tortosa (Catalonia, Spain). Oranges were used immediately after harvest.

2.4. Determination of quality parameters

In 'Valencia' oranges, the loss of firmness, colour development, soluble solids and acidity were determined to evaluate the effects of different harvest dates on fruit quality. Orange firmness measurements were performed using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd., Surrey, UK), based on the millimetres of fruit deformation resulting from fruit response to 2 kg of pressure on the longitudinal axis at a constant speed of 2 mm s^{-1}. Colour was measured on two opposite sides of each fruit using a tri-stimulus colourimeter (Chromameter CR-200, Minolta, Japan). The mean values for the lightness (L*), red-greenness (a*) and yellow-blueness (b*) parameters were calculated for each fruit and expressed as a Colour index (CI) = (1000*a)/(L*b). Total soluble solids content (TSS) and titratable acidity (TA) were assessed in extracted juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator, respectively. The maturity index was calculated as a ratio of TSS/TA.

In 'Golden Smoothee' apples, the colour development, flesh firmness, starch index, soluble solids and acidity were determined to evaluate the effects of different harvest dates on fruit quality. Flesh firmness was measured on two opposite peeled sides using a penetrometer (Effegi, Milan, Italy), which was fitted with an 11-mm-diameter probe. Colour was measured using hue values, which were calculated from a* (red-greenness) and b* (yellow-blueness) parameters that were measured using a CR-200 chromameter (Minolta, Japan) on both the exposed

and the shaded sides of each fruit, with a standard CIE illuminant with an 8 mm viewing aperture diameter. The total soluble solids content (TSS) was determined by measuring the refractive index of the juice (Atago, Tokyo, Japan), and the data were expressed as percentages (g per 100 g fresh weight, FW). Titratable acidity (TA) was measured in 10 ml of juice, which was diluted in 10 ml H_2O and titrated with 0.1 N NaOH and 1% phenolphthalein as an indicator. Acidity was expressed in grams of malic acid per litre of juice. Starch hydrolysis was scored visually using a 1–10 scale (1, full starch; 10, no starch) (Planton, 1995) after staining an equatorial section with 0.6% (w/v) I2–1.5% (w/v) KI solution.
The data regarding the maturity indexes represent the means of 20 individual fruits.

2.5. Pathogenicity studies

Before inoculation, the fruits were randomised, washed with tap water, and allowed to air-dry at room temperature. At each harvest period, each fruit was artificially wounded once with a nail (3 mm wide and 3 mm deep) on the equator and inoculated with 15 μL of a conidia suspension of each pathogen. For compatible interactions, orange-*P. digitatum* and apple-*P. expansum*, 10^5 conidia/mL of *P. digitatum* and 10^4 conidia/mL of *P. expansum* were inoculated. For non-host pathogen interactions, orange-*P. expansum* and apple-*P. digitatum*, the concentration in both cases was 10^7 conidia/mL. Fruits were then incubated at 20 ºC and 85-90% relative humidity (RH) for seven days. After the incubation period, the percentage of infected wounds (incidence) and the lesion diameters (severity) were measured. Five fruits constituted a single replicate, and each treatment was repeated four times.

2.6. Preparation of the samples for biochemical analysis

At each harvest period, fruits were washed and air-dried as described above. Wounds were made at the equator of each fruit using a sterilised nail. Wounds were inoculated with 10 μL of sterile water

(control) or conidia suspension (pathogens). For compatible interactions, orange-*P. digitatum* and apple-*P. expansum*, 10^5 conidia/mL of *P. digitatum* and *P. expansum* were inoculated. For non-host pathogen interactions, orange-*P. expansum* and apple-*P. digitatum*, the concentration in both cases was 10^7 conidia/mL. All fruits were incubated at 20 ºC and 85% RH. For apples, twenty plugs of fruit tissue (8 mm in diameter and 3 mm deep) were removed using a cork borer from four fruits at 0, 4, 8, 16, 24 and 32 h after wounding. For oranges, twenty-eight plugs of fruit tissue (5 mm in diameter and 3 mm deep) were removed using a cork borer from four fruits at 0, 4, 8, 16, 24, 32 and 48 h after wounding. Samples were mixed, frozen and powdered in liquid nitrogen, and immediately used for H_2O_2 determination. Four fruits constituted a single replicate, and each treatment was repeated four times for each harvest date.

2.7. H_2O_2 determination in compatible and non-host pathogen interactions

Apple powdered fresh weight (2.5 g) was homogenised in 10 mL of cold 5% trichloroacetic acid (TCA), whereas for orange tissue, 1 g of the powdered fresh weight was then homogenised in 5 mL of 5% TCA. For both apple and orange, the homogenates were shaken at 300 rpm for 1 min and then filtered to a new set of fresh tubes. An aliquot of the samples was then centrifuged at 12500 rpm for 15 min at 4 ºC. H_2O_2 amounts were determined using a PeroxiDetect™ Kit H_2O_2-560 colorimetric assay from Sigma-Aldrich (Saint Louis, USA). The sample preparation procedure was described previously, and the measurement was conducted as described in the manufacturer's manual. All H_2O_2 determination procedures were performed at 4 ºC in the dark. The assay was based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by H_2O_2 under acidic conditions. Ferric ions bound with the indicator dye xylenol orange to form a stable coloured complex, which can be measured at 560 nm. The H_2O_2 content was expressed as μmol/kg of fresh weight (FW), and each value was the mean of four fruit determinations.

2.8. H_2O_2 detection by the DAB-uptake method

In situ staining of H_2O_2 production in apple and orange peel tissues in response to wounding and inoculation with *P. digitatum* and *P. expansum* was achieved using 3,3´-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) following the methodology described by Macarisin *et al.* (2007). In the DAB-uptake method, H_2O_2 is visualised as a reddish-brown coloration (Thordal-Christensen *et al.* 1997).

At each harvest period, apple and orange fruits were washed and air-dried as described above. Three wounds were made at the equator of each fruit using a nail (3 mm wide and 3 mm deep). Wounds were inoculated with 10 μL of sterile water or with conidia suspension (pathogens). As a positive control for the uptake and reactivity of DAB, 100 μM H_2O_2 was inoculated into some wounds. For compatible interactions, orange-*P. digitatum* and apple-*P. expansum*, 10^5 conidia/mL of *P. digitatum* and *P. expansum* were inoculated. For non-host pathogen interactions, orange-*P. expansum* and apple-*P. digitatum*, the concentration in both cases was 10^7 conidia/mL. All fruits were incubated at 20 ºC and 85% RH in the darkness. Twenty-four hours after inoculation, 50 μL of a solution containing 1 mg/mL DAB was introduced into the wounds. The DAB was dissolved in water immediately before use. Fruits were then incubated overnight at 20 ºC and 85% RH in the darkness to allow DAB uptake and reaction with H_2O_2 (Macarisin *et al.* 2007). After the incubation period, fruit discs (16 mm in diameter and 5 mm deep) were removed from oranges and apples using a cork borer and placed into sterile Petri plates for further examination. Samples were examined using a stereoscope (Leica MZ16F), and images were captured using the Leica DFCTwain software. Three fruits constituted a single replicate, and each treatment was repeated three times for each harvest date.

2.9. Statistical analysis

Differences in the percentage of germination, infected wounds (incidence), lesion diameter (severity) and time-course H_2O_2 production for each harvest date were evaluated by the t-test using the statistical package SAS (Microsoft) and subjected to mean separation by the least significant difference (LSD) test. Differences between mean values were considered significant when $P \leq 0.05$.

3. Results

3.1. Response of pathogens to H_2O_2 treatments in *in vitro* conditions

The ecophysiological responses of *P. digitatum* after H_2O_2 treatments are shown in Fig. 1. Untreated conidia of *P. digitatum* reached the maximum germination percentage after 15 h of incubation (82.9%) at 25 ºC, whereas at 4 ºC, the maximum germination percentage was reached after 116 h of incubation (69.6%). At 25 ºC, 2 mM H_2O_2-treated conidia showed a similar pattern and percentage of spore germination than untreated conidia (75.8%), whereas 20, 50 and 200 mM H_2O_2-treated conidia required 37 h to reach the maximum germination percentage corresponding to 43.7%, 32.8% and 20.0%, respectively (Fig. 1A). At 4 ºC, 2 mM H_2O_2-treated conidia showed a similar pattern and percentage of spore germination than untreated conidia (61.0%), whereas 20 mM of H_2O_2 treatments caused an important reduction in the germination percentage, which reached the maximum (13.3%) after 378 hours of incubation. Conidia treated with 50 and 200 mM of H_2O_2 and stored at 4 ºC showed a sub-lethal (4.9%) and almost lethal (0.9%) effect, respectively, on the spore germination percentage of this pathogen (Fig. 1B).

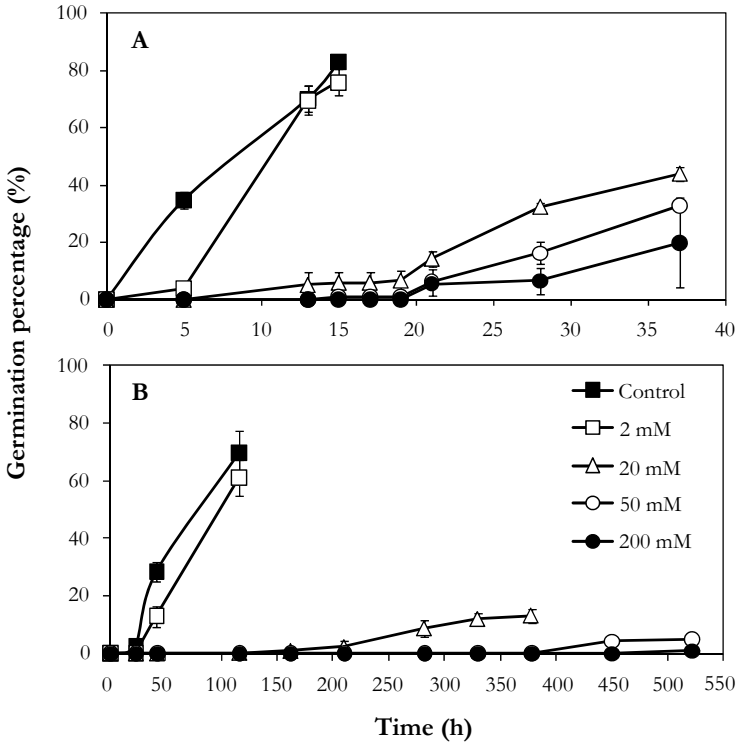

Figure 1. Effect of H_2O_2 treatment on the germination percentages of *P. digitatum*. *P. digitatum* conidia were treated with different concentrations of H_2O_2 (2, 20, 50 and 200 mM). The germination percentage of *P. digitatum* after oxidative treatment and incubation on PDA at 25 ºC (A) and at 4 ºC (B). Germination percentages were determined at the indicated times by microscopic examination. The values are the means of three replicates and 150 conidia per replicate. The vertical bars indicate the standard deviation.

The ecophysiological responses of *P. expansum* after H_2O_2 treatments are shown in Fig. 2. Untreated conidia of *P. expansum* reached the maximum germination percentage after 8 h of incubation (74.2%) at 25 ºC, whereas at 0 ºC, the maximum germination percentage was reached after 96 h of incubation (74.0%). At 25 ºC, 2 mM H_2O_2-treated conidia showed a similar pattern but with a lower germination percentage (48.3%) than untreated conidia, whereas at 0 ºC, the maximum germination percentage (70.2%) was similar to untreated conidia. Concentrations of 50 and 200 mM H_2O_2-treated conidia

showed a sub-lethal (7.8%) and almost lethal (1.8%) effect, respectively, on the spore germination percentage at 25 ºC (Fig. 2A). In contrast, 50 and 200 mM H_2O_2-treated conidia stored at 0 ºC showed an almost lethal (4.9%) and lethal (0.0%) effect, respectively, on the spore germination percentage of this pathogen (Fig. 2B). Therefore, only 200 mM of H_2O_2 treatment showed a lethal effect on the spore germination percentage of *P. expansum* at 0 ºC (Fig. 2).

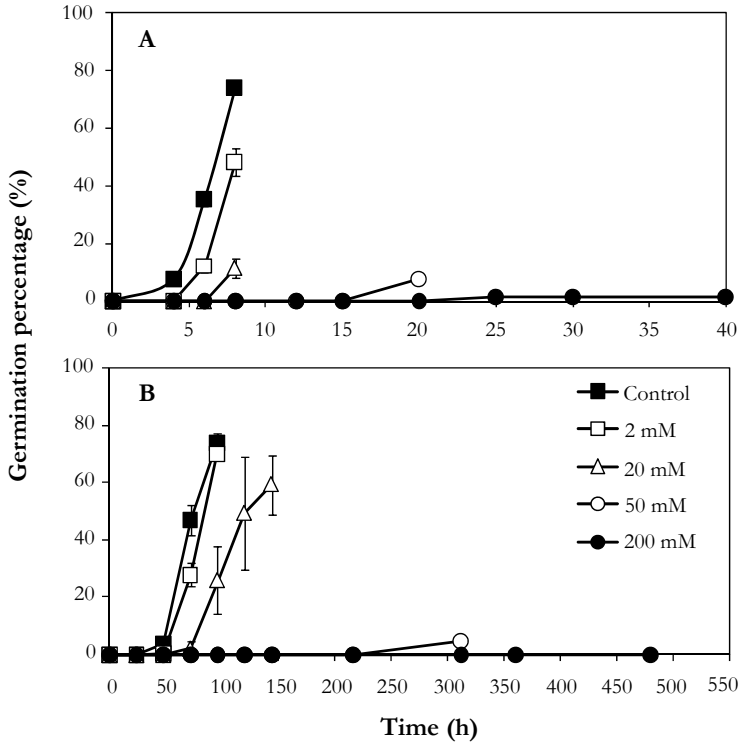

Figure 2. Effect of H_2O_2 treatment on the germination percentages of *P. expansum*. *P. expansum* conidia were treated with different concentrations of H_2O_2 (2, 20, 50 and 200 mM). The germination percentage of *P. expansum* after oxidative treatment and incubation on PDA at 25 ºC (A) and at 0 ºC (B). Germination percentages were determined at the indicated times by microscopic examination. The values are the means of three replicates and 150 conidia per replicate. The vertical bars indicate the standard deviation.

3.2. Changes in quality parameters

In 'Valencia' oranges, significant differences in some quality parameters were found among harvest dates (Supplemental Table 1). Although the total soluble solids (TSS) did not differ significantly among the harvest dates, the titratable acidity (TA) and colour index (CI) decreased as the harvest date progressed. Accordingly, the TSS/TA ratio was higher in the over-mature harvest when compared with the immature and commercial harvests. No significant differences were found for deformation among harvest dates ($P < 0.05$). Although the deformation parameter was not useful to define maturity stages, the acidity and colour index were good parameters to indicate the orange maturity stage.

'Golden Smoothee' apples quality parameters changed significantly with harvest date (Supplemental Table 2). As expected, immature harvested fruit had significantly higher flesh firmness values and a lower TSS and starch index ($P < 0.05$). The flesh firmness, TSS and starch index were the most useful quality parameters to define the apple maturity stage. Concerning acidity and colour, over-mature fruit showed significantly lower and higher values, respectively, compared with immature and commercial harvests. The acidity and colour parameters were not as useful to define the apple maturity stage as were the flesh firmness, TSS and starch index.

3.3. Pathogenicity studies

The influence of the harvest date on the decay incidence and on the lesion diameter in 'Valencia' oranges and in 'Golden Smoothee' apples inoculated with *P. digitatum* or *P. expansum* are shown in Fig. 3.
Concerning compatible interactions, a comparison among harvests for the orange-*P. digitatum* interaction showed no significant differences in the decay incidence (100%) (Fig. 3A), whereas significant differences in the lesion diameter were found among immature, commercial and over-mature harvests (9.9, 13.6 and 14.3 cm, respectively) (Fig. 3B). Similarly, for the apple-*P. expansum* interaction, no significant

differences in the decay incidence were found among immature, commercial and over-mature harvests (approximately 100%) (Fig. 3A). In contrast, significant differences were found in the lesion diameter among harvests. For example, immature apples showed a significantly smaller lesion diameter (1.2 cm) than over-mature harvest apples (2.7 cm) (Fig. 3B).

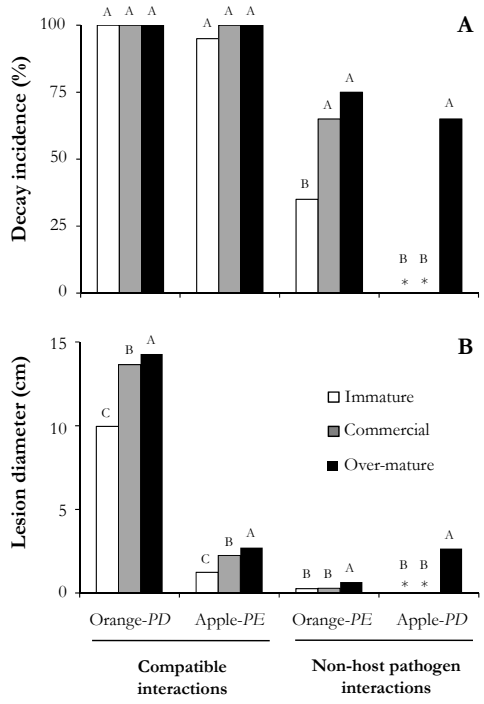

Figure 3. Influence of the harvest date on the disease incidence (A) and on the lesion diameter (B). 'Valencia' oranges and 'Golden Smoothee' apples were harvested at three maturity stages. Fruits were inoculated for host pathogen interactions, orange-*P. digitatum* and apple-*P. expansum*, with 10^5 conidia/mL of *P. digitatum* (PD) and 10^4 conidia/mL of *P. expansum* (PE), respectively. For non-host pathogen interactions, orange-*P. expansum* and apple-*P. digitatum*, fruits were inoculated with 10^7 conidia/mL in both cases. Fruits were stored at 20 ºC and 85% RH for 7 days. Each column represents the mean of 20 fruits. For each interaction, different letters indicate significance differences between means using the LSD test ($P < 0.05$) among harvest dates. * No decay development.

For non-host pathogen interactions, *P. expansum* was able to infect and develop rot in oranges at each harvest date assayed. The decay incidence was significantly lower in fruit from the immature harvest (35%) compared with fruit from the commercial and over-mature harvests (65 and 75%, respectively) (Fig. 3A). Moreover, significant differences in the lesion diameter were also obtained between immature and commercial harvests (approximately 0.3 cm) compared with the over-matured harvest (0.6 cm) (Fig. 3B). In contrast, for the apple-*P. digitatum* interaction, no decay symptoms were observed in the immature and commercial harvest, and only the over-mature harvest showed decayed fruit (65%), with a lesion diameter of 2.7 cm (Fig. 3).

3.4. H_2O_2 production in response to wounding and pathogen infection in apple

H_2O_2 production as a resistance response to abiotic (wounding) and biotic (*P. expansum* or *P. digitatum*) stresses in compatible and non-host pathogen interactions in apple at different maturity stage is shown in Fig. 4.

Fruit from the immature harvest showed two peaks of H_2O_2 production after wounding. The first peak was observed at 4 hours (82.2 μmol/kg FW) and, thereafter, levels of H_2O_2 declined for up to 16 hours after wounding. The second peak was characterised by the highest H_2O_2 production at 24 hours after wounding (117.7 μmol/kg FW). Significant differences were observed at 4 hours post inoculation (hpi), where H_2O_2 production was significantly higher in wounded compared with inoculated fruit with *P. expansum* (33.7 μmol/kg FW). Moreover, H_2O_2 production was significantly higher in wounded than in inoculated fruit 24 hpi, where H_2O_2 content for *P. digitatum* and *P. expansum* was 81.3 and 66.9 μmol/kg FW, respectively. After this significant increase, all treatments showed an important decrease in H_2O_2 level for up to 32 h (Fig. 4A).

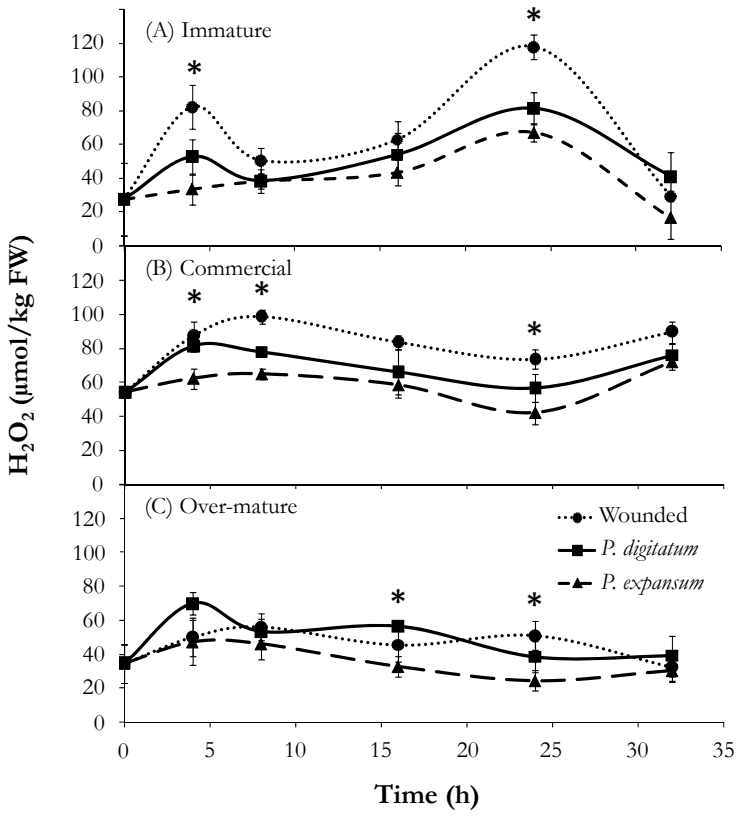

Figure 4. Effect of the harvest date on H_2O_2 levels in 'Golden Smoothee' apples. Time-course production of H_2O_2 (expressed as µmol/kg FW) in wounded and in inoculated fruit with *P. digitatum* at 10^7 conidia/mL and *P. expansum* at 10^5 conidia/mL. Immature (A), commercial (B) and over-mature (C) harvest dates were used. Data are the means ± SE of four replicates. * Significant differences between means using LSD test ($P < 0.05$).

All samples from commercial harvests (wounded and inoculated fruit) exhibited a similar pattern of H_2O_2 production among treatments. In this case, H_2O_2 levels of wounded fruit remained somehow slightly higher compared with inoculate fruit with *P. digitatum* and *P. expansum*. One slight peak in H_2O_2 production was observed 8 h after wounding, where significant differences in H_2O_2 production were found among wounded, *P. digitatum* and *P. expansum* fruit (98.6, 77.8 and 64.8 µmol/kg FW, respectively). After this significant increase, there was a

gradual decrease in the level of H_2O_2 for up to 24 h after wounding (73.6 μmol/kg FW). H_2O_2 production 24 hpi with *P. digitatum* and *P. expansum* followed the same pattern as wounded fruit, although lower H_2O_2 levels were found (56.8 and 41.9 μmol/kg FW, respectively). All treatments showed an increasing trend in H_2O_2 production at 32 h (Fig. 4B).

H_2O_2 production in the over-mature harvest remained constant (30-70 μmol/kg FW), and no significant H_2O_2 peaks were observed throughout the time-course studied. In this case, H_2O_2 production in inoculated fruit with *P. digitatum* remained somehow slightly higher compared with wounded and inoculated fruit with *P. expansum*. Nevertheless, slight differences were found at 16 and 24 h among wounded and inoculated treatments. Significant differences in H_2O_2 production were obtained between *P. digitatum* and *P. expansum* 16 hpi, where H_2O_2 production was significantly higher in inoculated fruit with *P. digitatum* (56.4 μmol/kg FW) compared with *P. expansum* (32.8 μmol/kg FW). Notably, 24 h after wounding, H_2O_2 production significantly increased above the levels observed in inoculated fruit. Significant differences were observed 24 hpi, where H_2O_2 production was significantly higher in wounded (51.0 μmol/kg FW) compared with inoculated fruit with *P. expansum* (24.5 μmol/kg FW) (Fig. 4C).

3.5. H_2O_2 production in response to wounding and pathogen infection in orange

H_2O_2 production as a resistance response to abiotic (wounding) and biotic (*P. digitatum* or *P. expansum*) stresses in compatible and non-host pathogen interactions in orange at different maturity stages is shown in Fig. 5.

Fruit from the immature harvest showed the maximum H_2O_2 production 8 h after wounding (290.7 μmol/kg FW), whereas no significant differences in H_2O_2 levels were found between wounded and inoculated fruit with *P. expansum* (263.3 μmol/kg FW) and *P. digitatum* (256.2 μmol/kg FW). In contrast, H_2O_2 levels significantly

decreased at 16 hpi with *P. digitatum* (170.6 μmol/kg FW), which is below the levels observed in wounded (245.6 μmol/kg FW) and in inoculated fruit with *P. expansum* (256.7 μmol/kg FW). After this period, all treatments showed a stable level of H_2O_2 production of approximately 230-260 μmol/kg FW (Fig. 5A).

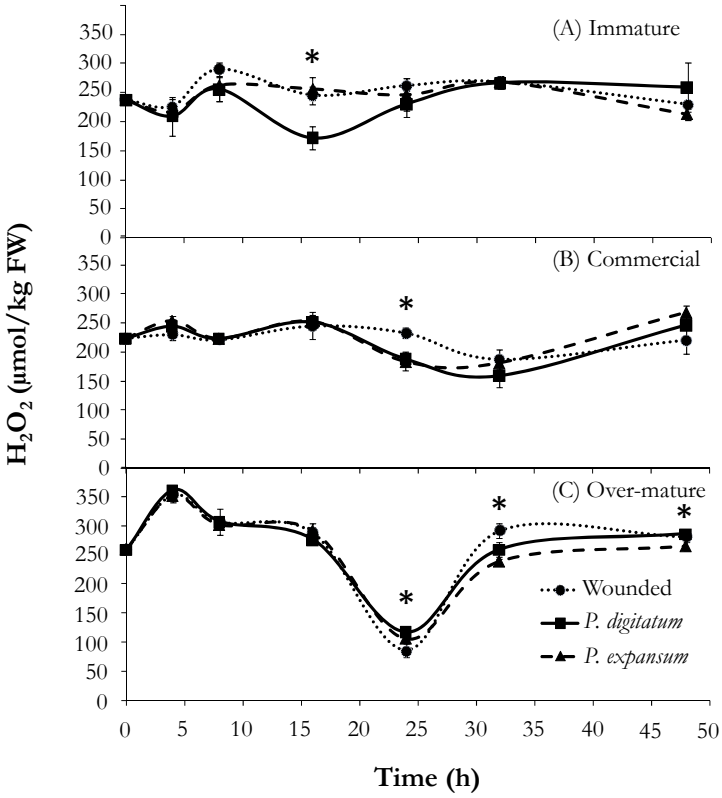

Figure 5. Effect of the harvest date on H_2O_2 levels in 'Valencia' oranges. Time-course production of H_2O_2 (expressed as μmol/kg FW) in wounded and in inoculated fruit with *P. digitatum* at 10^5 conidia/mL and *P. expansum* at 10^7 conidia/mL. Immature (A), commercial (B) and over-mature (C) harvest dates were used. The data presented are the means ± SE of four replicates. * Significant differences between means using LSD test ($P < 0.05$).

All samples from the commercial harvest (wounded and inoculated fruit) exhibited a similar pattern of H_2O_2 production among treatments. Only significant differences were found at 24 h among wounded and inoculated fruit, where H_2O_2 production was significantly higher in wounded compared with inoculated fruit with *P. digitatum* and *P. expansum* (233.0, 188.2 and 183.6 μmol/kg FW, respectively). After this significant decrease, there was an increasing trend in the level of H_2O_2 for up to 48 h after wounding and inoculation (Fig. 5B).

Fruit from the over-mature harvest reached the maximum peak of H_2O_2 accumulation at 4 h after wounding (353.8 μmol/kg FW) and inoculation with *P. digitatum* and *P. expansum* (361.5 and 351.6 μmol/kg FW, respectively). In contrast, a dramatic decrease, which was characterised by the lowest H_2O_2 production at 24 h after wounding (86.4 μmol/kg FW) and inoculation with *P. digitatum* and *P. expansum* (118.2 and 106.1 μmol/kg FW, respectively), was observed. Significant differences were observed 24 hpi, where H_2O_2 production was significantly lower in wounded compared with inoculated fruit with *P. digitatum*. Otherwise, significant differences were found at 32 h between treatments, where H_2O_2 levels of wounded fruit (291.7 μmol/kg FW) increased above the levels observed in inoculated fruit with *P. expansum* (238.8 μmol/kg FW). After this significant increase, all treatments showed a stable level of H_2O_2 production of approximately 260-280 μmol/kg FW (Fig. 5C).

3.6. Detection of H_2O_2 in fruits after wounding and pathogen inoculation

H_2O_2 accumulation in the immature apple harvest in response to wounding, inoculation with *P. expansum*, *P. digitatum* and treated with 100 μM H_2O_2 showed a similar brown-reddish coloration compared with that in the commercial harvest (data not shown).
Twenty-four hours after wounding and inoculation with *P. expansum*, exocarp tissue around wounds of apples from the commercial harvest were stained with DAB, which revealed a distinct brown reddish

coloration that indicated a high concentration of H_2O_2. A similar intensity of DAB staining was also detected around wounds treated with 100 μM H_2O_2 and around wounds inoculated with *P. digitatum*. Based on qualitative differences in the intensity of DAB staining, it appeared that *P. expansum* induced a significant accumulation of H_2O_2 at inoculated sites, whereas *P. digitatum* (non-host pathogen) showed weaker DAB polymerisation compared with wounds inoculated with either *P. expansum* or water (Fig. 6A-D). Regarding the staining, no remarkable H_2O_2 accumulation in the over-mature harvest was detected in all treatments (Fig. 6E-H). Therefore, the DAB staining noticeably declined with increasing maturity stages in apple.

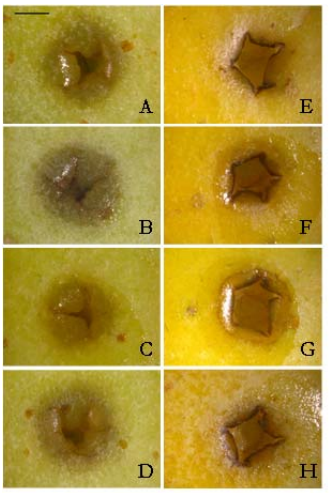

Figure 6. *In situ* visualisation of H_2O_2 in commercial (A-D) and over-mature (E-H) 'Golden Smoothee' apple fruits by DAB staining at 24 h after incubation. The appearance of brown DAB polymers indicates the accumulation of H_2O_2 in response to wounding (A and E), inoculation with *P. expansum* at 10^5 conidia/mL (B and F) or *P. digitatum* at 10^7 conidia/mL (C and G) and 100 μM H_2O_2 (D and H). Images were examined using a stereoscope (Leica MZ16F). Scale bar = 2 mm.

H_2O_2 accumulation in the immature orange harvest in response to wounding, inoculation with *P. digitatum*, *P. expansum* and treated with 100 μM H_2O_2 showed a similar brown-reddish coloration compared with that in the commercial harvest (data not shown).

Oranges from the commercial harvest showed the higher concentration of H_2O_2 24 h after wounding (inoculated with water) compared with wounds inoculated with *P. digitatum* and *P. expansum* or wounds treated with 100 μM H_2O_2. As described for apples, based on qualitative differences in the intensity of the DAB staining, wounding appeared to induce a significant accumulation of H_2O_2, whereas wounds inoculated with *P. expansum* (non-host pathogen) or wounds treated with 100 μM H_2O_2 accumulated lower levels of H_2O_2 (Fig. 7A-D). A weak but noticeable DAB polymerisation was also observed at wounded sites inoculated with water in the over-mature harvest. However, no remarkable H_2O_2 accumulation was detected in wounds inoculated with *P. digitatum* and *P. expansum* or in wounds treated with 100 μM H_2O_2 (Fig. 7E-H). Therefore, the DAB staining declined weakly with the maturity stage in orange.

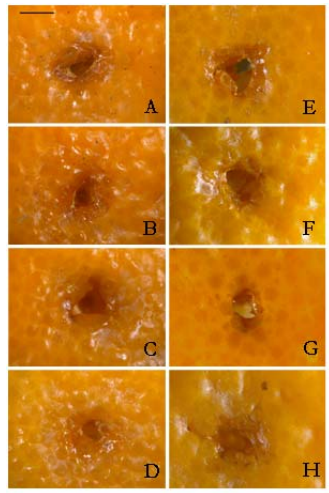

Figure 7. *In situ* visualisation of H_2O_2 in commercial (A-D) and over-mature (E-H) 'Valencia' orange fruits by DAB staining at 24 h after incubation. The appearance of brown DAB polymers indicates the accumulation of H_2O_2 in response to wounding (A and E), inoculation with *P. digitatum* at 10^7 conidia/mL (B and F) or *P. expansum* at 10^5 conidia/mL (C and G) and 100 μM H_2O_2 (D and H). Images were examined using a stereoscope (Leica MZ16F). Scale bar = 2 mm.

4. Discussion

In many plant-pathogen interactions, the production of ROS plays multifaceted signalling functions that mediate the establishment of multiple responses and that can act as local toxins (Torres, 2010); however, the role that ROS play in restricting fungal growth on fruit is not fully elucidated. In the current report, a study to increase the knowledge regarding ROS production in abiotic and biotic interactions between fruit and postharvest pathogens has been performed.

Many functions are postulated for ROS production in response to pathogens, and one direct function could be an antifungal effect. Thus, a higher concentration of H_2O_2 in a localised zone (such as around wounds) may be toxic and could affect the spore germination process of many fungal pathogens (Peng and Kuc, 1992; Cerioni *et al.* 2010; Cerioni *et al.* 2013). In this sense, the potential antifungal effect of H_2O_2, which is one of the main reactive species produced in fruit, was studied for both pathogens under *in vitro* conditions. Considering that *P. digitatum* and *P. expansum* are postharvest pathogens that produce decay on oranges and apples under cold storage conditions, respectively, the effect of the storage temperature on the germination process was also evaluated.

The current work demonstrated that the ecophysiological response of both pathogens depends on the temperature. In general, the maximum germination percentage of both strains reached similar values at both temperatures, although there was a delay in the percentage of germinated spores when the storage temperature decreased. Similar values for the percentage of germinated spores of both strains were reached for *P. expansum* faster than for *P. digitatum* at both temperatures. For instance, after 8 h of incubation on PDA at 25 ºC, the germination percentage for *P. expansum* was 74.2%, whereas *P. digitatum* reached a similar value (70%) after 13 h of incubation. A similar phenomenon was observed under cold storage conditions, where the germination percentage for *P. expansum* after 96 h was

74.0%. Whereas, the germination percentage for *P. digitatum* was 69.6% after 116 h of incubation. Interestingly, the cold storage conditions used were not identical for both pathogens; therefore, it might be expected that *P. digitatum* (4 ºC) could germinate faster than *P. expansum* (0ºC). In contrast, *P. expansum* germinated faster than *P. digitatum*, even at the cold storage temperature. It is not the first time that these pathogens showed this germination pattern under cold storage conditions. Our previous results indicated identical behaviour for other *P. digitatum* and *P. expansum* isolates at both temperatures (Buron-Moles *et al.* 2012).

Moreover, the effect of high H_2O_2 concentration on restricting the germination percentage of both pathogens was more intense in *P. expansum*. The current results under cold storage conditions demonstrated that the tolerance of *P. digitatum* at a high concentration of H_2O_2 (200 mM) decreased and that this treatment showed an almost lethal effect on spore germination in this strain. In contrast, *P. expansum* showed that 200 mM of H_2O_2 treatment prevented 100% of conidial germination at the cold storage temperature. Therefore, our *in vitro* pathogen-H_2O_2 survival experiment showed that *P. expansum* is more susceptible to higher levels of H_2O_2 than *P. digitatum* at both temperatures.

Our germination percentage values are higher and earlier than those values reported by Cerioni et al. (2010) for *P. digitatum*, who showed that, at 28 ºC, 100 mM of H_2O_2 treatment had a lethal effect on spore germination in this fungus. These differences in the germination percentage could be due to the different isolates used in both studies and to the differences in the shock time. Previous studies performed by Cerioni et al. (2013) have reported that *P. expansum* could be extremely susceptible to oxidative stress. This response is consistent with our results, where a direct relation between the increases in H_2O_2 concentration used in H_2O_2 treatments and decreases in conidial germination in *P. digitatum* and *P. expansum* were observed.

The fact that susceptibility to mechanical damage and/or infection increases with fruit maturity has been previously described (Spotts, 1985; Boonyakiat *et al.* 1987; Torres *et al.* 2003; Su *et al.* 2011; Vilanova *et al.* 2012a; Vilanova *et al.* 2012b). In agreement with these previous results, the current study showed that the maturity stage in orange and apple is an important factor in determining the resistance of fruit to mechanical damage and/or to infection during postharvest storage. In fact, these results demonstrated that fruit susceptibility to compatible pathogens increased with fruit ripening in 'Golden Smoothee' apples and in 'Valencia' oranges, where the lesion diameter increased significantly with fruit ripening in both fruits.

Because the accumulation of H_2O_2 in response to wounding and to pathogen attack may be a requirement in the establishment of the host defence (Mehdy, 1994; Wu *et al.* 1995; Orozco-Cardenas *et al.* 2001; Rea *et al.* 2002), in this study, we further analysed the apple and orange H_2O_2 production in the resistance response to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses at different maturity stages. In general, H_2O_2 production in the resistance response was quite different between apples and oranges. Moreover, the level of H_2O_2 accumulation in response to abiotic and biotic stresses depended on the harvest date.

In apples, the most significant differences in H_2O_2 production among treatments were observed in fruit from the immature harvest. The defence reaction elicited in response to infection with both pathogens showed lower levels of H_2O_2 than in wounding in immature fruit, where a markedly biphasic response was observed with an increase in the level of H_2O_2 production at 4 and 24 h. These biphasic oxidative bursts only in immature apple fruit induced by wounding and, to a lesser extent, by compatible and non-host pathogen inoculations confirm the important role of the enhanced production of H_2O_2 in fruit resistance reactions and are in agreement with those results that described the role of a second sustained phase that is usually associated with the establishment of the defences (Grant and Loake, 2000). Furthermore, these results demonstrated the ability of *P. expansum* to prevent the oxidative burst by suppressing H_2O_2

production in the host cells during the studied time-course, which appears to be strongly associated with its pathogenicity in apple at immature and commercial harvests but not at the over-mature harvest. In addition, *P. expansum* is a well-known patulin producer, an important mycotoxin involved in its own pathogenicity and virulence (Sanzani *et al.* 2012). The role that the hosts play in influencing the patulin production as pathogenesis-related factor, and consequently *P. expansum* aggressiveness, it has been recently described (Sanzani *et al.* 2013).

Oranges from immature and commercial harvests exhibited a similar pattern of H_2O_2 production among treatments. The most significant differences were observed in orange from the over-mature harvest. In general, treatments did not cause marked changes in the level of H_2O_2, except for the dramatic decrease found at 24 h after wounding and inoculation with *P. digitatum* and *P. expansum*, which was characterised by the lowest H_2O_2 production.

Overall, we demonstrated that the assayed pattern of H_2O_2 accumulation in response to abiotic and biotic stresses is quite different between fruits (apples and oranges). These differences found may be because apples and oranges are climacteric and non-climacteric fruits, respectively. This observation indicates that climacteric fruits are characterised by a dramatic increase in ethylene production, which is responsible for the typical respiratory burst during ripening and the activation of many biochemical steps, whereas non-climacteric fruits are ethylene-independent (Barry and Giovannoni, 2007).

The pathogenicity of *P. digitatum* is associated with the suppression of the defence-related H_2O_2 burst in citrus fruit (Macarisin *et al.* 2007) and with the imbalance between H_2O_2-generating and H_2O_2-scavenging activities (Ballester *et al.* 2006). In agreement with these previous results, the current study showed that, after 16 h, the defence-related H_2O_2 burst was markedly suppressed by *P. digitatum*, but not by *P. expansum*, at the immature harvest.

In general, apples and oranges showed a reduction in the ability to produce H_2O_2 in response to abiotic and biotic stresses with fruit ripening, which resulted in increased susceptibility to pathogen infection. It has been shown in this study that *P. digitatum* and *P. expansum* (postharvest pathogens) have developed strategies to suppress the defence-related H_2O_2 burst, whereas it has been reported that *Botrytis cinerea* and *Sclerotinia sclerotiorum* (necrotrophic fungi) induce ROS formation in plants, resulting in hypersensitive cell death that facilitates fungal colonisation, indicating that the oxidative burst is a weapon for this fungus (Govrin *et al.* 2006). Evidence that *P. digitatum* suppresses fruit defences was first provided by Ismail and Brown (1979), who reported that the elevation of phenylalanine ammonia-lyase (PAL) activity in citrus peel, induced by mechanical wounding, is inhibited around wounds inoculated with the fungus. Concerning wound healing, Vilanova et al. (2014) showed that, in apples, the lignin content was higher in wounded immature fruit than in over-mature fruit. Similar results were found in oranges, where the wound response at cold temperatures was insufficient or too slow to prevent infection by *P. digitatum* and *P. expansum*. In oranges, the data indicate that wound healing at 20 ºC can prevent infection by both pathogens (Vilanova *et al.* 2013). Therefore, we speculated that H_2O_2 accumulation in response to wounding is modulated by fruit maturity and is required for efficient wound healing.

The generation of H_2O_2 in response to wounding was required for induced resistance in tomato leaves (Orozco-Cardenas and Ryan, 1999) and in winter squash (Watanabe *et al.* 2001). As Castro-Mercado et al. (2009) described previously in the unripe avocado, the accumulation of H_2O_2 occurs at the wound sites, indicating that the process is regulated by a systemic signalling system. Studies performed in 'Golden Delicious' (Torres *et al.* 2003) and in 'Gala' apples (Su *et al.* 2011) demonstrated that the higher resistance of early harvested fruit to pathogen infection was correlated with an increase in H_2O_2 production. In agreement with these previous results, the current study showed that wound- and pathogen-induced H_2O_2 production, which plays an important role in wound- and pathogen-defence

processes, is also regulated by fruit ripening in apple and orange, where resistance to *P. digitatum* declined clearly with ripening in apple fruit, whereas the resistance to *P. expansum* is more maturity independent in orange. From *in vitro* studies at 25 ºC, the germination percentage for *P. expansum* was faster than for *P. digitatum*, and *P. expansum* was more susceptible to higher levels of H_2O_2 than *P. digitatum* (50-200 mM). Therefore, the infection in these non-host interactions appears to be more related to the quickness at which the pathogen germinates to avoid the role of H_2O_2 to mediate plant defence by wound healing. Thus, H_2O_2 might serve as a second messenger to activate the expression of fruit defence-related genes and to strengthen mechanical barriers (Orozco-Cardenas *et al.* 2001). These preformed barriers can prevent the ingress of the pathogen and the subsequent activation of inducible defence responses or the disease symptom development. In the powdery mildew-barley interaction, cross-linking of plant cell wall proteins, which is a process mediated by H_2O_2, also appears to be involved in restricting the fungal penetration of epidermal cells (Thordal-Christensen *et al.* 1997). Therefore, the role of H_2O_2 in mediating fruit defences by wound healing, thereby increasing the fruit resistance to postharvest pathogens, is not fully elucidated. Thus, there is a requirement to characterise H_2O_2 production to increase in our understanding of one of the possible defence mechanisms of apple and orange fruits against wounding, compatible and non-host pathogen interactions at different maturity stages.

In situ staining of H_2O_2 can be achieved using compounds such as DAB, which provides a qualitative indicator of H_2O_2 generation. DAB is an *in vivo* semi-quantitative staining method that polymerises instantly and that develops a localised dark colour immediately with contact with H_2O_2 in the presence of peroxidase. DAB oxidation relies on *in vivo* peroxidases, whose activity could be different between compartments or conditions (Thordal-Christensen *et al.* 1997). Because the turnover of H_2O_2 can be rapid and because its metabolism is particularly active in chloroplasts, peroxisomes, and mitochondria, the localisation of the H_2O_2 reported by the assays must

be established (Cheeseman, 2006). In this study, the wounding and inoculation of fruit with *P. digitatum* or *P. expansum* resulted in a similar staining with DAB, which was restricted to the wound sites. Staining has been called H_2O_2, although the assay does not distinguish between this form and other hydroperoxides. Therefore, we suggest the possibility of similar sources of H_2O_2 in these two important postharvest interactions. Although i*n situ* staining of H_2O_2 with DAB has been used in plants (Thordal-Christensen *et al.* 1997; Orozco-Cardenas *et al.* 2001; Borden and Higgins, 2002; Mellersh *et al.* 2002; Romero *et al.* 2008), there is only one study in lemon fruit reported by Macarisin et al. (2007), who demonstrated that *P. expansum* induced a significant accumulation of H_2O_2 at inoculated sites, whereas *P. digitatum* inhibited the production of H_2O_2 compared with wounds inoculated with either *P. expansum* or with water. While in tomato cotyledons, Borden and Higgins (2002) found that the lower rate at which DAB staining occurred in the compatible compared with the incompatible interactions also suggests the possibility of different sources of H_2O_2. In spite of numerous studies in plants, many unknown players in ROS signalling still exist. Because ROS are linked to many biotic and abiotic responses, deciphering ROS signalling is likely to have a significant impact on agriculture and biotechnology in the future (Mittler *et al.* 2011).

In summary, our results demonstrated that H_2O_2 production in oranges and apples because of wounding or because of pathogen infection is maturity dependent. Thus, the fruit effect of H_2O_2 in the defence response against pathogens (compatible and non-host) decreases as the fruit maturity increase. Fruit maturity stage may provide the opportunity for non-host pathogens to infect fruit due to the decrease in H_2O_2 production by over-mature fruit, following different patterns of H_2O_2 production depending on the fruit. Although additional research is required to prove the causal relation, the positive correlation implicates several other compounds as part of the complex mechanisms involved in the defence response in fruit against abiotic and biotic stresses. H_2O_2 metabolism was studied to acquire a better understanding of the oxidative response during host

and non-host pathogen interactions in apple and orange that will help in the development of future strategies to prevent green and blue mould progression by manipulating the host antioxidant defence machinery. Although many components of non-host resistance appear to be well known from host resistance, there is clearly a long way to go before we fully understand non-host resistance.

Acknowledgements

The authors are grateful to the Spanish Government for its financial support of the projects AGL2008-04828-C03/AGR and AGL2011-30519-C03/AGR (Plan Nacional de I+D+I, Ministerio de Ciencia e Innovación, Spain) and for the G. Buron-Moles PhD grant (BES-2009-027752). We also want to thank the excellent technical assistance of Cèlia Sánchez and Cristina Solsona. The authors have declared no conflict of interest.

Appendix I. Supplementary data

Supplementary data associated with this article can be found at Appendix I.

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Chapter 3

Analysis of changes in protein abundance after wounding in 'Golden Delicious' apples

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Postharvest Biology and Technology 87 (2014) 51-60

Postharvest Biology and Technology 87 (2014) 51–60

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Analysis of changes in protein abundance after wounding in 'Golden Delicious' apples

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ARTICLE INFO

Article history:
Received 14 May 2013
Accepted 27 July 2013

Keywords:
MALDI-TOF
Malus domestica
Phenol-based method
Protein extraction
Abiotic stress
Mechanical damage

ABSTRACT

The apple (*Malus* × *domestica* Borkh.) cultivar (cv) 'Golden Delicious' is one of the most important apple varieties worldwide, and is widely cultivated for export of fruit to Europe and other countries. However, if damaged, the fruit becomes susceptible to opportunistic infection by postharvest phytopathogens such as *Botrytis cinerea* (gray mould) and *Penicillium expansum* (blue mould), which annually lead to large economic losses. Therefore, the study of fruit responses to wounding at the proteome level can contribute to a better understanding of the physiological mechanisms underlying fruit stress responses. In this study we report the first systematic description of the changes in protein abundance following wounding of 'Golden Delicious' apples, using 2D-PAGE and MS. At the proteome level, the dominant biological process in wounding response was 'response to stress', whereas proteins without abundance changes were found to be mainly involved in 'metabolism', 'response to stress', and 'oxidation-reduction processes'. We speculate that fruit respond to wound stress by modulating the abundance of appropriate proteins and to react to mechanical damage by synthesizing a broad range of PR proteins. Therefore, increasing the information on apple fruit proteins after wounding will be a useful resource in developing strategies to minimize postharvest losses.

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1. Introduction

Plant survival, and consequently crop production, is highly dependent on the ability of plants to respond, adapt, and tolerate variable environmental conditions. As a result, plants have developed complex mechanisms to deal with a variety of biotic (pathogens, herbivores, parasitic plants) and abiotic (heat, drought, salinity, wounding, etc.) stresses.

The apple (*Malus* × *domestica* Borkh.) cultivar (cv) 'Golden Delicious' is one of the most important apple varieties in fruit growing areas of the world, and is widely cultivated for the export. However, if damaged, the fruit becomes susceptible to opportunistic infection by postharvest wound pathogens such as *Botrytis cinerea* (gray mould) and *Penicillium expansum* (blue mould), which annually lead to large economic losses. The primary infection route for these pathogens is via surface wounds where nutrients and volatiles stimulate conidial germination. This is then followed by colonization of the fruit tissue (Droby et al., 2008). Therefore, mechanical damage, which can occur during harvesting, packing-house operations, handling and transport, represents a serious threat to fruit quality and can significantly reduce the economic value of the product (Van Zeebroeck et al., 2007; Pedreschi et al., 2013).

Fruit tissues possess an entire arsenal of defence mechanisms to combat the effects of wounding. For example, Vilanova et al. (2012) demonstrated that lignin deposition is an important factor that helps to explain the lower susceptibility of immature apple fruit to wounding compared to mature and over-mature fruit (Sticher et al., 1997). Furthermore, Filonow (2005) showed that in 'Golden Delicious' apples, 1 and 3 day-old puncture wounds were less prone to infection by postharvest pathogens than 0 day-old puncture wounds. In addition to lignification, wounding also leads to the induction of a broad range of cellular defences, which help to prevent secondary infections with pathogens (Osbourn, 1996).

Acclimation to stress is mediated by changes in gene expression, resulting in changes to the composition of the plant proteome and metabolome (Kosová et al., 2011), but it is well recognized that these changes are not always well correlated with each other (Gygi et al., 1999). Therefore, characterization of the plant stress response proteome is important and can lead to the identification of novel proteins that are the direct effectors of plant stress

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http://dx.doi.org/10.1016/j.postharvbio.2013.07.039

response (Kosová et al., 2011). This process has been greatly aided by the availability of complete genome sequences for a number of important crop species, including apple (Velasco et al., 2010).

Although proteomic approaches have been used in the study of fruit ripening in tomato (Rocco et al., 2006), grape (Zhang et al., 2008), citrus (Muccilli et al., 2009; Perotti et al., 2011), prunus (Nilo et al., 2012), papaya (Huerta-Ocampo et al., 2012; Nogueira et al., 2012), mango (Andrade et al., 2012) and banana (Toledo et al., 2012), there are only a few reports dealing with the response of plants to wounding (Shen et al., 2003; Chaves et al., 2009; Dafoe et al., 2009; Valcu et al., 2009; Lewandowska-Gnatowska et al., 2011; Soares et al., 2011; Barkla et al., 2013). Regarding apple proteomic studies, Guarino et al. (2007) described the repertoire of proteins present in the pseudocarp tissues of three accessions of *Malus* × *domestica* Borkh. cv. 'Annurca', and more recently, changes in the protein profiles of 'Golden Delicious' during ripening and in response to exogenous ethylene treatment have also been reported (Zheng et al., 2013).

Even though the response of plant tissues to wounding has been studied for some time (Rees and Beevers, 1960; Bowles, 1990; Zhou and Thornburg, 1999), data on the specific responses of fruit to this stress is lacking. In this study, we report the first systematic description of the changes in protein abundance following wounding in 'Golden Delicious' apple, using 2D-PAGE and MS for protein identification against publicly available protein and EST databases.

2. Materials and methods

2.1. Plant material

Apples (*Malus* × *domestica* L. cv 'Golden Delicious') were harvested in 2010 from trees grown at the University's field stations in Aarschot, Belgium. A total of 15 fruit were washed thoroughly with tap water and dried at room temperature. Apples were artificially wounded with a sterilized nail (3 mm wide and 3 mm deep), inoculated with 10 μL of distilled water (0.01% (w/v) Tween-80), and then incubated at 20 °C and 85–90% RH. Samples were collected for analysis after 0 (W0), 24 (W24), and 48 (W48) h of wounding. Since this study aimed to analyze changes in protein abundance W24 and W48 h after wounding, W0 was used as the wounded control. For each sampling time point, a total of fifty cylinders of peel and flesh tissue (8 mm diameter and 3 mm deep) from five individual fruit (ten cylinders per fruit) were excised and removed using a cork borer. All cylinders were pooled per time point, immediately frozen with liquid nitrogen and stored at −80 °C until used.

2.2. Chemicals

All chemicals used in this study were of the highest grade available from Sigma–Aldrich (St. Louis, MO, USA) and GE Healthcare (Uppsala, Sweden). Milli-Q water with resistance greater than 18 MΩ was used throughout. Acrylamide and 2D SDS-PAGE standards were purchased from Bio-Rad (Nazareth, Belgium).

2.3. Determination of fruit quality parameters

Twenty fruit were used individually for the analysis of skin colour, flesh firmness, soluble solids content (SSC) and titratable acidity (TA). Fruit colour was measured on opposite sides of individual fruit with a CM-2500d colorimetric spectrophotometer (Konica Minolta, Tokyo, Japan) providing $L^*a^*b^*$ values. Individual fruit flesh firmness was determined on two opposite peeled sides of the fruit using a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip; results were expressed as N. SSC and TA were assessed in juice pressed from the whole fruit. SSC was determined using a digital refractometer (Atago, Tokyo, Japan) and expressed as %. TA was analyzed by titration of 10 mL of juice with 0.1 N NaOH to pH 8.1 with 1% (v/v) phenolphthalein as an indicator, and data expressed as g/L malic acid. Starch hydrolysis was rated visually using a 1–10 EUROFRU scale (1, full starch; 10, no starch), after dipping of cross-sectional fruit halves in 0.6% (w/v) I2–1.5% (w/v) KI solution for 30 s. Data on maturity indexes represent the means of analyses of 20 individual fruit.

2.4. Protein extraction from apple fruit

Total soluble protein was essentially extracted according to the phenol-based protocol of Hurkman and Tanaka (1986), with modifications. Fruit tissues from fifty discs of wounded apple tissue (approximately 10 g), from five individual fruit were powdered in liquid nitrogen in a pestle and mortar, pre-chilled with liquid nitrogen. Fresh weight between 250–300 mg of this powder was then homogenized in 500 μL of cold extraction buffer (700 mM sucrose, 500 mM Tris–HCl (pH 7.5), 50 mM EDTA, 100 mM KCl, 1 mM PMSF, 0.3% Triton X-100, 1% DTT, and 2% (w/v) insoluble PVPP). Unless otherwise stated, all procedures were carried out at 4 °C and centrifugation at 19,060 g. The homogenate was centrifuged for 10 min and the supernatant transferred to a fresh Eppendorf microcentrifuge tube. Three serial extractions were carried out and the supernatants combined together to give a total volume of 1500 μL. To the first 500 μL of the combined supernatant, 500 μL of Tris-buffered phenol (pH 8.0) was added, the sample vortexed for 5 min and then centrifuged for 10 min, the process was repeated two times. The upper phenol phase of the three microcentrifuge tubes were recovered together, and re-extracted twice more with cold extraction buffer as described above. Proteins in the final phenol phase were then precipitated overnight with five volumes of acetone containing 0.2% DTT, pre-chilled to −20 °C. The precipitated proteins were collected by centrifugation for 60 min, and after discarding the acetone/DTT supernatant, the pellet was washed twice with cold 100 mM ammonium acetate in methanol followed by once with cold 0.2% DTT/acetone. The precipitate was then briefly dried under vacuum for 3 min before being solubilized in 150 μL of R2D2 lysis buffer (2 M thiourea, 5 M urea, 2% (w/v) CHAPS, 2% (w/v) C7BzO, 20 mM DTT, 5 mM TCEP–HCl and 0.5% and 0.25% (v/v) of a mixture of carrier ampholytes of pH 4–7 and pH 3–10 (Bio-Rad), respectively (Mechin et al., 2003; Delaplace et al., 2006). The concentration of proteins in the final resolubilised extract was determined using the 2D Quant Kit (GE Healthcare), using Bovine Serum Albumin (BSA) as a standard.

2.5. Two dimensional gel electrophoresis

Proteins were first separated by isoelectric focussing (IEF) using a Protean IEF Cell (Bio-Rad) and Immobiline™ Drystrip gels (18 cm) (GE Healthcare) with a nonlinear pH gradient (3–10), according to the manufacturer's instructions.

For analytical gels IPG strips were loaded with 120 μg (350 μg for preparative gels) of total protein and passively rehydrated overnight with 340 μL IEF buffer (2 M thiourea, 5 M urea, 2% (w/v) CHAPS, 2% (w/v) C7BzO, 0.002% BPB, 40 mM DTT, 5 mM TCEP–HCl and 0.25% (v/v) of a mixture of carrier ampholytes of pH 4–7 and pH 3–10 (Bio-Rad), respectively). Rehydrated strips were focused using a Protean IEF Cell (Bio-Rad) at 22 °C with a current limit of 50 μA/strip. The strips were equilibrated for the second dimension separation under continuous shaking in SDS equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% SDS, 0.375 M Tris–HCl, pH 8.8, and 0.002% BPB). The proteins were first reduced by incubating the IPG strips with buffer containing 65 mM DTT and 5 mM TCEP–HCl for 20 min and afterwards alkylated with buffer containing 135 mM iodoacetamide for 20 min. The strips were washed briefly with

running buffer, then transferred to the top of 12.5% acrylamide SDS-PAGE gels (18 cm × 24 cm × 1 mm) with MM standards Precision Plus Protein (Bio-Rad) on electrode wicks (PROTEAN® IEF System, Bio-Rad), and covered with 0.5% (w/v) agarose (0.002% BPB). Proteins were separated using a Ettan Daltsix Electrophoresis System (GE Healthcare) in SDS electrophoresis buffer (0.25 M Tris pH 8.8, 1.92 M glycine and 1% SDS) at 80 V and then at 500 V applied for 1 h and 9 h, respectively until solvent front just exited the bottom of the gel. The resulting gels were fixed for 90 min in a fixing solution of 34% methanol, 17% ammonium sulphate, 3% o-phosphoric acid, and then rinsed overnight in a wash solution (50% methanol and 10% acetic acid, v/v). Gels were then stained for 2 days in a staining solution (18%, v/v methanol; 2%, v/v o-phosphoric acid; 8%, w/v ammonium sulphate; and 0.1%, w/v Coomassie brilliant blue (CBB) G-250). Stained gels were washed briefly in deionised water and subsequently neutralized using a solution of 1.2% Tris, pH 6.5 for 5 min, before finally rinsing with wash solution for 1 min. Gels were stabilized in a stabilizing solution (20% ammonium sulphate). At each time point, three separate protein extracts were performed, and a minimum of three 2D-PAGE gels were run.

2.6. Image acquisition and analysis

Stained gels were scanned immediately after stabilizing and digitized using a GS-800 Calibrated Densitometer (Bio-Rad) with 300 dpi resolution. Data were saved in TIF format (*.tif). Image analysis was performed using PDQuest 2D analysis software (Version 8.0, Bio-Rad Laboratories) for spot quantification, normalization and matching. A total of nine gels, resulting from three technical replicates per time point (W0, W24 and W48), were analyzed. Spot detection and matching between gels were performed automatically, followed by manual verification and adjustment. Gels were compared within a 'match set' created from the three replicates gels from three separate protein extracts for each time point. A standard gel (master) was generated from the image displaying the highest spot number without streaking or other gel distortions.

Quantitative analysis was carried out after normalizing the spots quantities (as spot optical density, OD) in all gels, in order to compensate for non-expression related variations. Quantitative comparisons between the three sample sets were carried out taking into account only statistically significant spots (t-test with a $P < 0.05$). Differential abundance between the groups was assessed using a 2-fold increase between average gels.

2.7. In-gel protein digestion and MS analysis

Selected spots were manually excised from gels, digested with trypsin using 96-well perforated plates and a MultiScreen™ HTS Vacuum Manifold (Millipore). Each gel piece with protein was minced, washed twice with deionised water and dehydrated with 50% ethanol in 50 mM NH_4HCO_3 for 10 min and then with 100% ethanol for 10 min. Gel pieces were then reduced with 10 mM DTT in 50 mM NH_4HCO_3 for 1 h at 56 °C and alkylated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min at room temperature in the dark. After this, the gel pieces were washed twice in 50 mM NH_4HCO_3 for 15 min and dehydrated with 5% acetonitrile (ACN) in 25 mM NH_4HCO_3 for 15 min, 50% ACN in 25 mM NH_4HCO_3 for 15 min twice and finally with 100% ACN for 10 min. After total evaporation of the ACN, 15 μL of 20 ng μL^{-1} trypsin in 25 mM NH_4HCO_3 was added and left at 4 °C for 45 min in order to allow full rehydration of the gel pieces with trypsin solution. The gel pieces were then covered with 25 mM NH_4HCO_3 and incubated at 37 °C overnight for proteolysis. Following digestion, eluted peptides were transferred to a new eppendorf. One μL of this protein digest was used for a first peptide mass fingerprints (PMFs) analysis. If necessary, the minced gel was washed three times more with 0.25% trifluoroacetic acid (TFA) in 50% (v/v) ACN, twice with 100% ACN, evaporated in a SpeedVac (Savant) and then resuspended in 5 μL of 70% ACN–0.1% TFA to collect the remaining peptides.

On a MALDI target, 1 μL of peptide solution was spotted per well, and allowed to evaporate at room temperature before being covered with 1 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 50% (v/v) ACN containing 1% TFA. Mass calibrations were carried out using a standard peptide mixture. Mass spectra were acquired using Autoflex Speed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics).

2.8. Database search and protein identification

The identification of the vast majority of the proteins was performed using MALDI-TOF mass fingerprint (PMF). For proteins not directly identified with this approach, MALDI-TOF/TOF (MS/MS) was also carried out. PMF and MS/MS spectra were compared against SwissProt, *Malus* and Plants EST, and NCBI nr *viridiplantae* databases, using the search engine MASCOT algorithm (Version 2.3, Matrix Science, London, UK). The following parameters were used for PMF database searches: monoisotopic peptide masses; allowed modifications, cysteine carbamidomethyl (fixed), oxidation of methionine (variable); one trypsin missed cleavage and a maximum of ±60 ppm mass accuracy. Search parameters used for MS/MS searches were also the same as for PMF with a maximum MS/MS tolerance peptide of ±0.4 Da. The charge (Z) of the precursor ion was +1. Only the hit with the highest Mowse score ($P < 0.05$) was considered as significant hit or the most probable identification.

The full list of identified protein sequences was then functionally annotated using the Blast2GO software v2.6.4 (http://www.blast2go.org, Conesa et al., 2005), with default parameters. Furthermore, InterProScan homology search (as implemented in the Blast2GO software) was performed to find functional domains and their related GO terms with the default parameters. The 'Merge InterProScan GOs to Annotation' function was used to refine all annotations. Functional classification was performed according to the primary biological function of the protein and was based on Blast2GO information and on available data in the literature.

3. Results and discussion

3.1. Characterization of fruit quality

Characterization of the fruit was carried out through the measurements of skin colour, flesh firmness, SSC and TA. Our results for ($a^* + b^*$) parameter on 'Golden Delicious' apples were 58.8. This parameter indicates that skin colour was changing from green to yellow. A mean value of 23.4 N of flesh firmness was found. Our results for SSC and TA were 11.7% and 1.4 g/L malic acid, respectively. Starch index is the most useful quality parameter to define the maturity stage in apples and in this case the measured value was 10. Relative to a previous study on 'Golden Smoothee' (Vilanova et al., 2012), our quality results indicate that 'Golden Delicious' apples were approaching the stage of 'over-mature', which may also have influenced the defence response of these apples, since it is known that apple fruit stress responses are maturity dependent (Torres et al., 2003).

3.2. 2D-PAGE of apple fruit

A comparison of several extraction procedures showed that phenol extraction followed by DTT/Acetone precipitation gave the best results, generating a sufficiently high protein concentration and purity for IEF (data not shown). Fresh weight of frozen peel and flesh tissue were used for protein extraction, followed by two

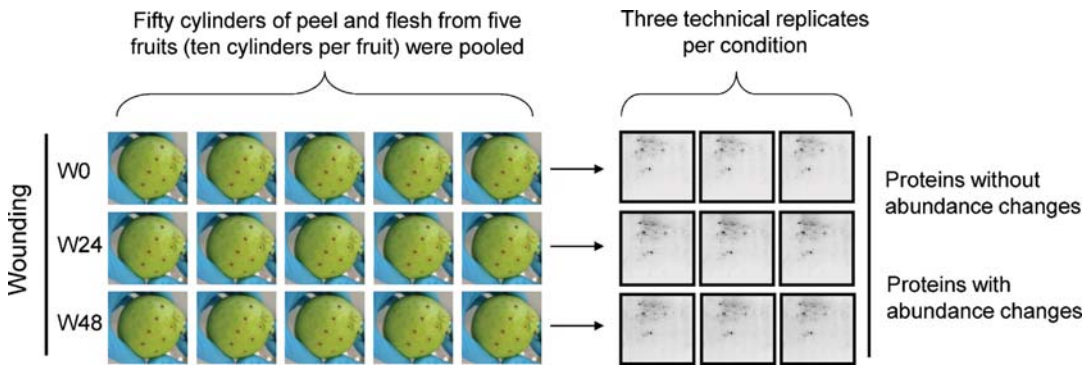

Fig. 1. Experimental design to describe 'Golden Delicious' protein abundance changes after wounding by means of two-dimensional gel electrophoresis. Samples from five individual fruits were pooled and three separate protein extracts were performed. These proteins were used to generate three technical replicates for each of the three conditions analyzed. W0, W24 and W48 indicate the hours after wounding.

dimensional gel electrophoresis (2D gel) (Fig. 1). The yield upon extraction was ~0.1 mg protein/g of fresh tissue.

3.3. Proteins without abundance changes after wounding

In this study we identified both wound-responsive and non-responsive proteins of 'Golden Delicious' peel and flesh tissues. Representative 2D gels of total protein extracts from peel and flesh (W48) are shown in Fig. 2. On average 250 protein spots were identified on each 2D-PAGE gel at each time point, and of these, 64 of the 250 best-resolved spots were excised, digested with trypsin and subjected to MS analysis. In total 58 (91%) were successfully identified (Table 1). Detailed information about the sequences of the matched peptides can be found in Supplementary Table 1. Fifty-three out of the 58 identified spots were unambiguously identified as proteins from plants belonging to the Rosaceae family, and 51 to *Malus* genera. Several of these spots have the same protein description, e.g. Mal d 1 (spots 1, 2, 63 and 64), suggesting either that post-translational modifications are present, or that they are members of a large protein family. For example it is known that there are at least 15 Mal d 1 genes in apple (Beuning et al., 2004).

Sequenced and identified proteins whose abundance did not change after wounding were classified into functional categories using the Blast2GO software (Conesa et al., 2005) and according to the three main vocabularies of the gene ontology (GO): cellular component, molecular function and biological process (Fig. 3). Concerning the cellular component, the most well-represented categories were 'cell part' and 'membrane-bounded organelle', with more than 55% of the total annotations, followed by 'organelle part' (18.6%), 'apoplast' (12.4%), 'non-membrane-bounded organelle' (4.6%) and 'protein complex' (3.1%) (Fig. 3A). As far as molecular function is concerned, the most represented categories are 'small molecule binding' (21.4%), 'oxidoreductase activity' (16.8%), 'ion binding' (13.7%) and 'transferase activity' (11.5%), whereas other molecular functions were represented at a much lower level (Fig. 3B). Over 45 categories were found for the Biological Process vocabulary, including 'response to stress' (9.1%), 'response to chemical stimulus' (8.7%), 'cellular metabolic process' (7.5%),

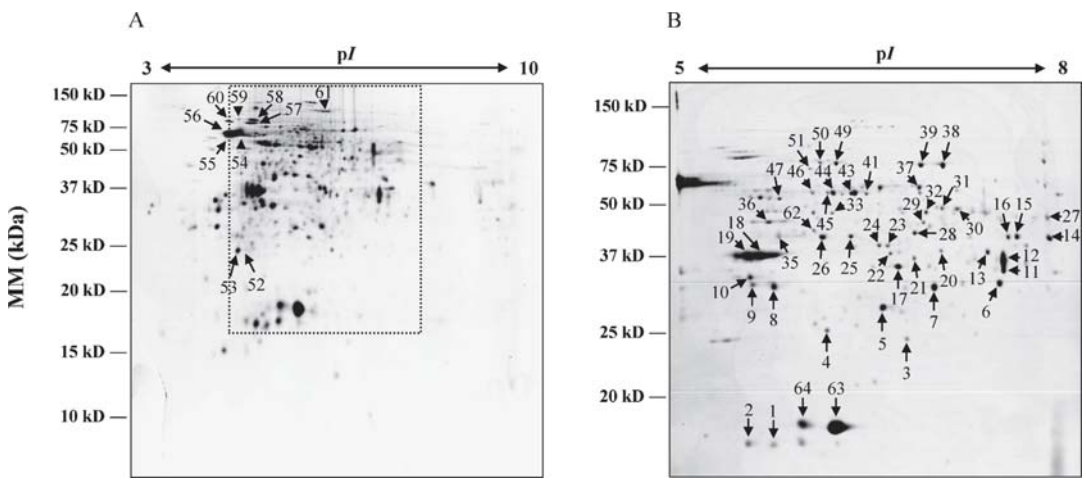

Fig. 2. Representative 2D gels of total peel and flesh proteins from of 'Golden Delicious' at W48. Separation of apple fruit proteins was realized on 18 cm strips p*I* 3–10 (A) and p*I* 5–8 (B). The gel region that show particularly distribution of protein spots are highlighted by a rectangular box. Gels were stained with colloidal CBB G-250. Protein spots that do not change their abundance after wounding are numbered in gels A and B. Spot numbering refers to Table 1 and Supplemental Table 1 shows protein identification obtained by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS) analysis.

Table 1
Proteins without abundance changes after wounding. Identification of the 'Golden Delicious' proteins without abundance changes after wounding. Proteins were excised from the 2D PAGE gel and digested with trypsin. The peptides were extracted and their masses were measured by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS). Spot number, protein description, accession number, organism, database, method of identification, MASCOT score and finally the theoretical and experimental molecular mass (MM) and isoelectric point (p*I*) are listed.

Spot[a]	Protein description[b]	Accession number[c]	Organisms	Database	Identification method	MASCOT score	MM (kDa)[d] Th./Exp.	p*I*[d] Th./Exp.
Metabolism process								
6	Xyloglucan endotransglucosylase/hydrolase 4	ACD03228	*Malus domestica*	nrNCBI	PMF	121	34.1/34.6	7.0/7.0
7	Endo-xyloglucan transferase	CV091856	*Malus domestica*	Malus EST	PMF	115	33.0/33.7	6.8/6.4
10	Plasma membrane intrinsic polypeptide	CV630815	*Malus domestica*	Malus EST	PMF	88	27.5/35.6	4.9/5.2
13	NADP-dependent D-sorbitol-6-phosphate dehydrogenase	P28475	*Malus domestica*	nrNCBI	PMF	149	35.2/41.2	6.8/6.9
14	Putative glyceraldehyde 3-phosphate dehydrogenase	DT041019	*Malus domesticax Malus sieversii*	Malus EST	MS/MS	73	22.9/44.7	8.7/7.5
15	Glyceraldehyde 3-phosphate dehydrogenase	CN930456	*Malus domestica*	Malus EST	MS/MS	186	34.1/45.0	8.6/7.2
16	Cytosolic aldolase	DR994863	*Malus domestica*	Plants EST	PMF	119	24.0/45.0	6.0/7.1
20	Probable fructose-bisphosphate aldolase chloroplastic-like	CN860303	*Malus domestica*	Malus EST	MS/MS	174	24.3/41.5	5.1/6.5
23	Cytosolic malate dehydrogenase	EB134567	*Malus domestica*	Malus EST	PMF	132	24.5/42.7	7.4/6.0
24	Cytoplasmic malate dehydrogenase	CN496973	*Malus domestica*	Malus EST	PMF	97	20.0/42.8	6.5/5.9
25	Glutamine synthetase	DT041154	*Malus domestica* × *Malus sieversii*	Malus EST	MS/MS	155	26.4/45.1	6.3/5.8
26	Glutamine synthetase	DT041154	*Malus domestica*	Malus EST	MS/MS	141	26.4/44.8	6.3/5.6
28	Phosphoglycerate kinase, cytosolic	CV151429	*Malus domestica*	Malus EST	MS/MS	112	36.3/46.0	9.4/6.2
29	Formate dehydrogenase	EB146460	*Malus domestica*	Plants EST	MS/MS	73	22.0/48.9	6.1/6.3
30	Citrate synthase	ADL62695	*Malus xiaojinensis*	nrNCBI	MS/MS	52	52.7/53.0	8.5/6.6
32[e]	Nadp-specific isocitrate dehydrogenase + isocitrate dehydrogenase	DR998291 + EB135844	*Malus domestica*	Malus EST	PMF	242	23.0/51.9 18.4/51.9	5.7/6.3 9.3/6.3
33	S-adenosylmethionine synthetase	EB151311	*Malus domestica*	Malus EST	PMF	137	24.4/51.7	5.4/5.7
37	ATP synthase subunit alpha, mitochondrial	P05492	*Oenothera biennis*	nrNCBI	MS/MS	297	55.8/59.9	6.2/6.3
38	Malic enzyme	1803524A	*Populus trichocarpa* × *Populus deltoides*	nrNCBI	MS/MS	133	65.3/68.3	6.4/6.5
39	Nadp-dependent malic enzyme	DT042932	*Malus domesticax Malus sieversii*	Malus EST	MS/MS	126	25.4/68.1	8.5/6.3
43	UDP-glucose pyrophosphorylase	EG631379	*Malus domestica*	Malus EST	PMF	83	60.7/57.9	6.8/5.8
45	UDP-glucose pyrophosphorylase	EG631379	*Malus domestica*	Malus EST	PMF	103	60.7/58.2	6.8/5.7
46	Enolase	EB127345	*Malus domestica*	Malus EST	PMF	80	17.9/58.3	8.8/5.6
49[e]	Nadp-dependent malic enzyme-like + Nadp-dependent malic enzyme	EB137788 + EB118041	*Malus domestica*	Malus EST	PMF	181	24.8/68.8 11.5/68.8	8.4/5.7 9.5/5.7
50	Enolase	P42896	*Ricinus communis*	nrNCBI	MS/MS	99	48.1/69.0	5.6/5.6
51	Apgm	CAA06215	*Malus domestica*	nrNCBI	PMF	98	60.9/66.8	5.4/5.6
52	Adenine phosphoribosyltransferase 1	CN851062	*Malus domestica*	Malus EST	PMF	117	23.5/27.1	5.1/5.2
53	Adenine phosphoribosyltransferase chloroplastic-like	CN848920	*Malus domestica*	Plants EST	MS/MS	338	21.2/27.1	4.7/5.2
61	Cytosolic aconitase	ADM34980	*Pyrus pyrifolia*	nrNCBI	MS/MS	113	98.5/86.1	5.9/6.2
62	Phosphoglycerate kinase, chloroplastic isoform 1	XP002263796	*Vitis vinifera*	nrNCBI	MS/MS	161	50.2/46.7	8.3/5.6
Response to stress								
1	Mal d 1-like	AAS00049	*Malus domestica*	nrNCBI	PMF	89	17.6/17.3	5.5/5.4
2	Mal d 1-like	AAS00044	*Malus domestica*	nrNCBI	PMF	155	17.4/17.4	5.2/5.2
4	Abscisic stress ripening-like protein	DT000286	*Malus domestica*	Malus EST	MS/MS	48	25.6/28.1	5.8/5.7
5	Abscisic acid response protein	CO417849	*Malus domestica*	Malus EST	MS/MS	322	19.5/31.0	8.9/5.9
8	Dehydrin	EB130640	*Malus domestica*	Malus EST	PMF	80	23.8/33.8	9.2/5.4
9	Dehydrin	ABG56268	*Malus domestica*	nrNCBI	PMF	106	24.2/34.1	6.6/5.2
11	Desiccation-related protein, putative	DT002512	*Malus domestica*	Malus EST	PMF	223	26.4/36.9	8.6/7.1

Table 1 (*Continued*)

Spot[a]	Protein description[b]	Accession number[c]	Organisms	Database	Identification method	MASCOT score	MM (kDa)[d] Th./Exp.	pI[d] Th./Exp.
12	Desiccation-related protein, putative	DT002512	*Malus domestica*	Malus EST	PMF	199	26.4/39.6	8.6/7.1
18	ACC oxidase	ABA03057	*Malus domestica*	nrNCBI	PMF	83	35.5/40.2	5.2/5.3
19	ACC oxidase	ABA03057	*Malus domestica*	nrNCBI	PMF	99	35.5/40.1	5.2/5.2
27	Polygalacturonase	EE663891	*Malus sieboldii*	Malus EST	MS/MS	67	20.5/50.3	9.5/7.5
54	Dehydrin 9	EB116657	*Malus domestica*	Malus EST	MS/MS	131	4.1/66.2	4.6/5.1
55	Dehydrin	EB116099	*Malus domestica*	Malus EST	PMF	141	12.3/66.2	5.1/5.1
56	Dehydrin 2	ACL01289	*Eriobotrya japonica*	nrNCBI	MS/MS	45	31.6/66.2	5.3/5.1
57	High molecular weight heat shock protein	AAF34134	*Malus domestica*	nrNCBI	PMF	167	71.6/75.3	5.2/5.2
59	Heat shock protein 81-2	GO506274	*Malus domestica*	Plants EST	PMF	141	26.2/78.3	5.1/6.2
60	cphsc 70-1	XP002869727	*Arabidopsis lyrata*	nrNCBI	MS/MS	342	76.6/76.6	5.1/5.1
63	Major allergen d 1	CAD32318	*Malus domestica*	nrNCBI	PMF	122	17.5/18.5	5.3/5.7
64	Major allergen Mal d 1	Q40280	*Malus domestica*	nrNCBI	PMF	119	17.5/18.9	5.6/5.5
Oxidation-reduction process								
3	Glutathione-S-transferase	EB117932	*Malus domestica*	Malus EST	MS/MS	249	21.3/27.1	5.4/6.2
22	Probable aldo-keto reductase 2-like	DY255277	*Malus hybrid rootstock*	Malus EST	MS/MS	323	21.1/40.8	8.6/6.0
31	Monodehydroascorbate reductase	ACN88682	*Malus domestica*	nrNCBI	PMF	175	47.0/53.1	6.5/6.5
35	Beta-cyanoalanine synthase 1	ABF13209	*Malus domestica*	nrNCBI	PMF	115	41.1/45.3	7.6/5.4
41	Glutathione reductase	DR999957	*Malus domestica*	Malus EST	PMF	138	21.3/57.7	5.3/5.8
Other proteins								
17	Annexin	CO723326	*Malus domestica*	Malus EST	MS/MS	548	32.9/37.8	8.7/6.1
21	Rossmann-fold nad-binding domain-containing protein	CN895090	*Malus domestica*	Plants EST	MS/MS	252	25.3/39.7	5.6/6.2
36	Actin, partial	BAL14271	*Malus domestica*	nrNCBI	PMF	227	40.4/49.0	5.7/5.4
58	Luminal binding protein 4 precursor	GO542220	*Malus domestica*	Plants EST	PMF	111	24.1/77.5	8.48/5.3

[a] Spot identification number as assigned in Fig. 2.
[b] Description of proteins identified from the Plants and *Malus* EST database were inferred using the Blast2GO software.
[c] Accession numbers according to *Viridiplantae* SwisProt database, nrNCBI database, Plants and *Malus* EST Database of NCBI.
[d] Theoretical (th.) and experimental (exp.) molecular mass (MM) and isoelectric point (p*I*).
[e] Spot with more than one protein identified.

'response to abiotic stimulus' (7.5%), 'primary metabolic process' (6.8%), 'oxidation-reduction process' (5.2%) and 'small molecule metabolic process' (4.3%) (Fig. 3C).

Due to the hierarchical nature of gene ontology, is very difficult to establish separate categories for further classification of the proteins. Therefore, these proteins without abundance changes are reported in Table 1 according to their biological process, covering 'metabolism', 'response to stress' and 'oxidation-reduction processes'.

The proteins assigned to the 'response to stress' group deserve particular attention. These results showed that, perhaps surprisingly in many cases, their abundance did not change in response to wounding. For example, although the major allergen Mal d 1 belongs to the PR10 class of pathogenesis-related (PR) proteins and has been reported to be both stress- and pathogen-inducible (Puhringer et al., 2000; Beuning et al., 2004; Gao et al., 2005), only two Mal d 1 spots (67 and 68) changed in abundance following wounding. Mal d 1 is considered to be the major allergen of apple, and is encoded for members of a large gene family showing a high degree of sequence identity (Pagliarani et al., 2012). This suggests that there is a functional specialization of the different gene family members and could explain why only a portion of the Mal d 1 spots responded to wounding. Other stress-related proteins identified include heat shock proteins (HSP), such as high molecular weight HSP (spot 57), Hsp81-2 (spot 59) and chloroplast HSP 70-1 (cpHsc70-1) (spot 60), dehydrins (DHNs) in spots 8, 9, 54, 55 and 56, as well as desiccation-related proteins in spots 11 and 12. However all of these are described as cold stress responsive proteins (Cui et al., 2005; Garcia-Bañuelos et al., 2009), and have been observed to change during fruit ripening (Guarino et al., 2007; Muccilli et al., 2009); none showed a specific wound response. Additional interesting classes of stress-related proteins include proteins related to oxidation-reduction, and the 'Abscisic acid stress ripening' (ASR spot 4) protein which is associated with ripening and salt and water stress in tomato (*Solanum lycopersicum*) (Goldgur et al., 2007).

3.4. Proteins with abundance changes after wounding

Because mechanical damage in apple provides an optimal infection site for wound pathogens, which require an injury to germinate and colonize the tissue, it is very important to examine the fruit defence mechanisms after wounding. In order to identify proteins specifically related to the wound response in apple fruit, changes in protein abundance were measured at W24 and W48, using W0 as a wounded control. Representative gel scans of the apple wounded tissue at W0, W24 and W48 are shown in Fig. 4A and the amount of each spot was determined according to the protein volume, and changes in abundance expressed relative to the highest volume (100%) at W0, W24 or W48, respectively (Fig. 5). Visual inspection of the gels and mean spot volumes indicated that wounding did not lead to dramatic changes in the overall protein profile. These protein changes were highly localized around the site of the wound and this could explain the relatively few changes observed. In fact, only eight spots displayed an altered abundance (2.0-fold) after wounding (Fig. 4A; Supplementary Table 3). Five of these eight spots were successfully identified (Table 2) and detailed information on the peptide sequences and homology matches can be found in Supplementary Table 2. Fig. 4B shows a summary of the response dynamics of these proteins in response to wounding. For instance, three proteins decreased (spots 65, 70, 72), while one protein increased (spot 66) its abundance at W24. Similarly, at W48, four proteins increased their abundance (spots 67, 68, 69, 71) (Fig. 5 and Supplementary Table 3). Five of these eight protein spots were functionally annotated (Table 2 and Supplementary Table 2), and GO analysis

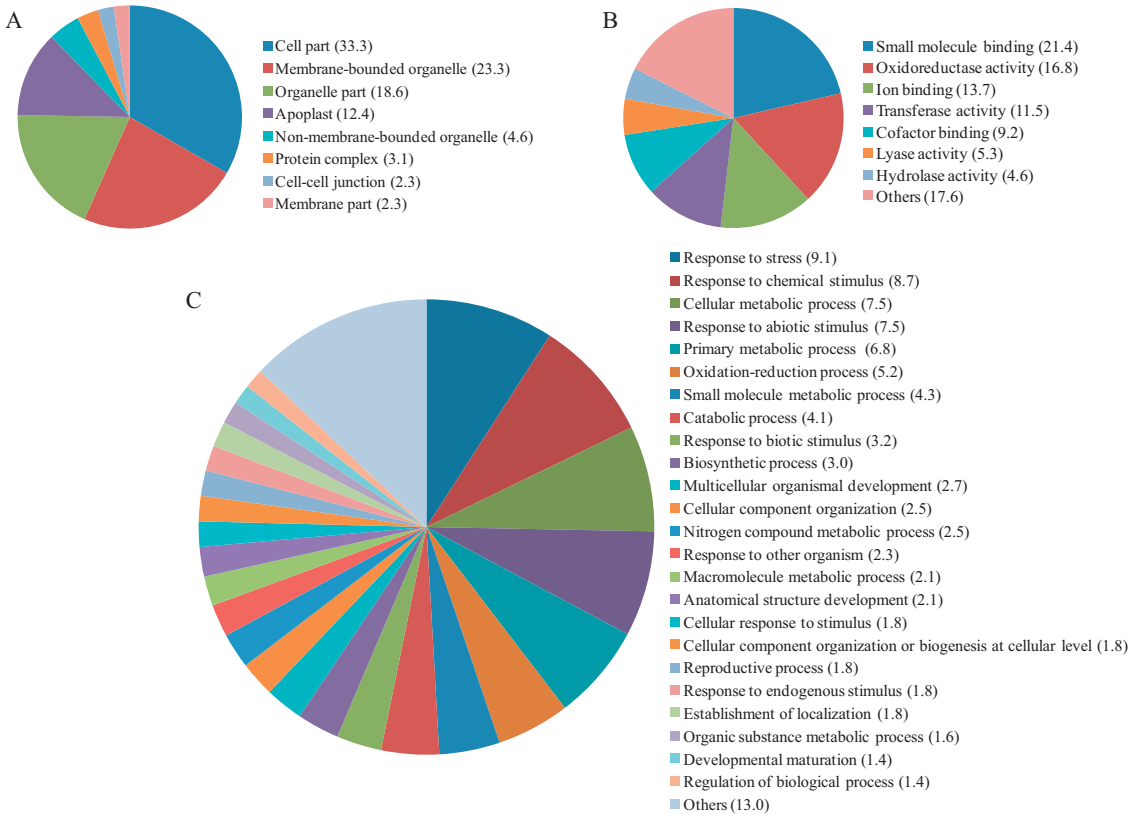

Fig. 3. GO terms distribution of the proteins without abundance changes after wounding 'Golden Delicious' apple. GO terms distribution according to the Blast2GO software were grouped, at the level 3, by cellular components (A), molecular functions (B), and biological processes (C) with circle graphs. B and C were filtered by number of Seqs (cutoff = 5). In (A) 'cell part' was the most represented cellular compartment. In (B) the most represented categories were 'small molecule binding proteins', followed by proteins with 'oxidoreductase activities'. In (C) 'metabolism related processes', 'response to stress' and 'oxidation-reduction processes' were the most represented. The numbers on each fraction indicate the percentage of detected proteins belonging to each category.

indicates that the most abundant classes of the identified proteins are represented by proteins involved in 'response to stress'. Beta glucanase (spot 65), thaumatin-like protein 1-a (spot 66), Mal d 1 like (spot 67), Mal d 1.03G (spot 68) and flavoprotein WrbA-like (spot 69) proteins were all identified.

Our results showed an increase in abundance of thaumatin-like protein 1-a (PR-5) at W24 (2.19-fold) (Fig. 4), suggesting that it may also be involved in the initial apple response to wounding. In fact, Dafoe et al. (2009) reported that a thaumatin-like protein in poplar phloem exudates was wound-inducible, as it was present at

Table 2
Proteins with abundance changes after wounding. Identification of the 'Golden Delicious' proteins with abundance changes after wounding. Proteins were excised from the 2D PAGE gel and digested with trypsin. The peptides were extracted and their masses were measured by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS). Spot number, protein description, accession number, organism, database, method of identification, MASCOT score and finally the theoretical and experimental molecular mass (MM) and isoelectric point (p*I*) are listed.

Spot[a]	Protein description[b]	Accession number[c]	Organisms	Database	Identification method	MASCOT score	MM (kDa)[d] Th./Exp.	p*I*[d] Th./Exp.
65	Beta glucanase	EG025934	*Malus domestica*	Plants EST	MS/MS	155	12.3/34.5	5.6/4.7
66	Thaumatin-like protein 1a	Q9FSG7	*Malus domestica*	SWP	MS/MS	39	26.6/35.1	5.0/5.3
67	Mal d 1-like	AAS00049	*Malus domestica*	nrNCBI	PMF	106	17.6/17.6	5.5/5.5
68	Major allergen Mal d 1.03G	AAX18324	*Malus domestica*	nrNCBI	PMF	81	17.8/17.2	5.2/5.8
69	Flavoprotein WrbA-like	DT000454	*Malus domestica*	Malus EST	MS/MS	127	24.4/25.9	7.9/6.4
70[e]	N.i						/34.5	/4.6
71[e]	N.i						/42.7	/5.2
72[e]	N.i						/50.0	/6.3

[a] Spot identification number as assigned in Fig. 4.
[b] Description of proteins identified from the Plants and *Malus* EST database were inferred using the Blast2GO software.
[c] Accession numbers according to *Viridiplantae* SwisProt database, nrNCBI database, Plants and *Malus* EST Database of NCBI.
[d] Theoretical (th.) and experimental (exp.) molecular mass (MM) and isoelectric point (p*I*).
[e] Not identified.

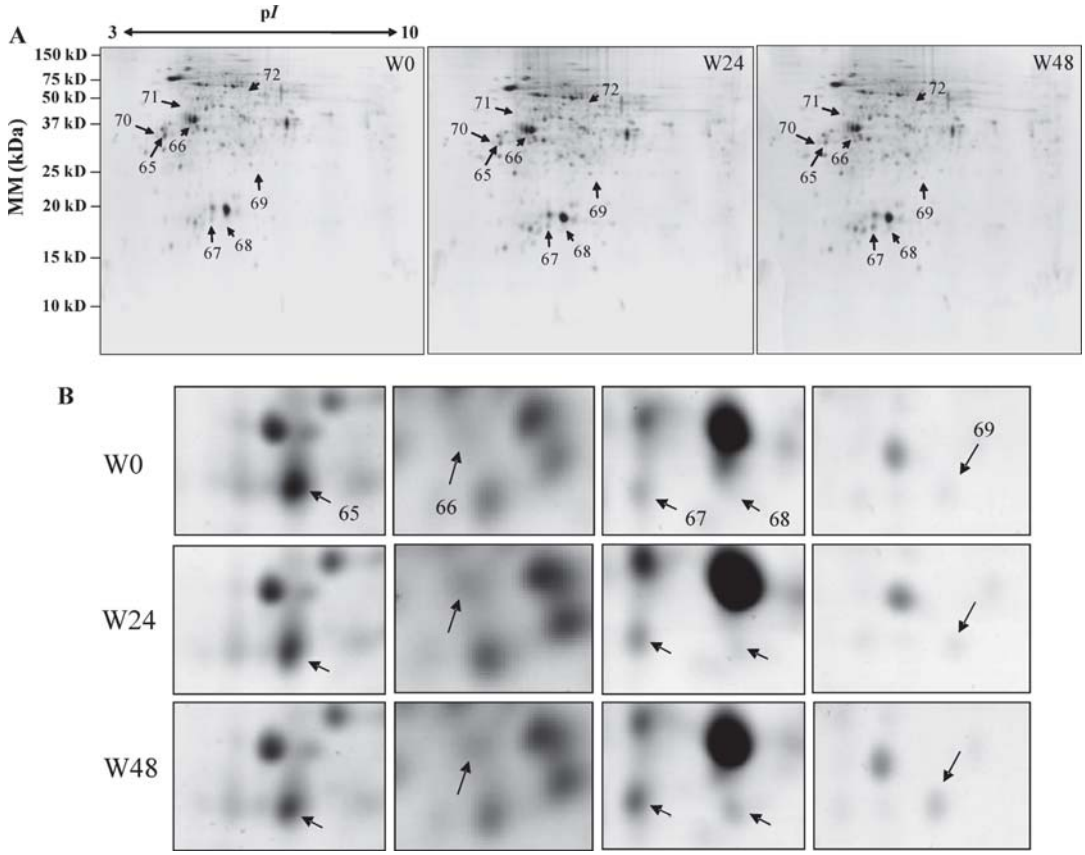

Fig. 4. Protein spots with abundance changes after wounding. (A) The graph represents one example from at least three different gels used for the differential analysis. W0, W24 and W48 indicate the hours after wounding. Proteins were separated over the p*I* range 3–10 in the first dimension and on 12.5% (w/v) SDS-polyacrylamide gels in the second dimension. Gels were stained with colloidal CBB G-250. Spots identified by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS) were labelled with the same numbers in Table 2 and Supplemental Table 2. (B) Representative areas of 2D gels. These proteins are Beta glucanase (spot 65), Thaumatin-like protein 1-a (spot 66), Mal d 1 like (spot 67), Mal d 1.03G (spot 68) and Flavoprotein WrbA-like (spot 69).

higher levels in phloem exudates of plants whose leaves had been wounded 24 h prior to collection. Other authors have reported that thaumatin-like protein 1 precursor (and perhaps thaumatin-like protein 2 precursor) might also be involved in protecting against chilling injury in peach (Dagar et al., 2010).

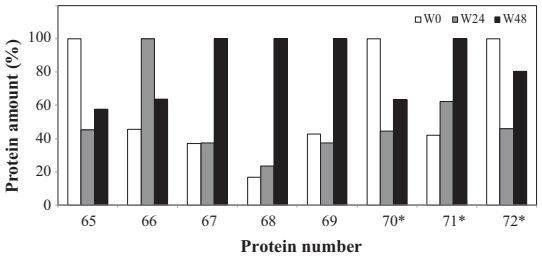

Fig. 5. The histograms show the changes in amount proteins over time. The protein amount was determined according to the protein volume, and the decreased or increased in protein abundance was referred as the highest 100% at W0, W24 and W48, respectively. * Not identified.

Interestingly, levels of Mal d 1 like (2.70-fold) and Mal d 1.03G (5.98-fold) proteins increased in abundance at W48 (Fig. 4). As described above, Mal d 1 proteins (PR-10) are stress inducible and others have reported that levels of these proteins may be related to disease resistance (Hsieh et al., 1995). It is well known that disease resistance in harvested fruit is associated with an induction of PR proteins, which typically accumulate to high levels following pathogen challenge and help to retard fungal growth (Droby et al., 2002; El Ghaouth et al., 2003). Here, our findings suggest that in 'Golden Delicious', Mal d 1 like and Mal d 1.03G are specifically induced in response to wounding suggesting that they may have an important role in fruit defence.

The beta-1,3-glucanases are abundant, highly regulated enzymes and widely distributed in seed-plant species (Simmons, 1994). Although the major interest in beta-1,3-glucanases (PR-2) stems from their possible role in the fruit defence mechanisms against fungal infection (El Ghaouth et al., 2003), these enzymes are also implicated in diverse physiological and developmental processes in plant including cell division, fruit ripening, seed germination, and responses to wounding, cold, ozone and UV B. McCollum et al. (1997) the presence of both basic and acidic forms of beta 1,3-glucanase in grapefruit flavedo, where the basic isoforms of beta-1,3-glucanases represent the greatest amount

of the total activity. These basic glucanases are localized to the vacuole while the acidic forms of the enzymes are localized in the apoplast. Based on the acidic p*I* of the isoforms identified here, it may be assumed that these are probably apoplast beta-glucanases. In general, the activity of beta-1,3-glucanase in fruit tissues have received limited study but here we see that levels of beta-1,3-glucanase decreased at W24 (0.45-fold) (Fig. 4). In peaches and tomatoes the glucanase activity increased during ripening whereas in orange flavedo the amount of the beta-1,3-glucanase do not change during fruit development (McCollum et al., 1997). Our results suggest that a decreased in abundance of this PR-2 protein may be related to the accelerated ripening of the fruit over the time course of the experiment.

Our results also showed that the flavoprotein WrbA-like protein increased in abundance more than 2-fold at W48 (Fig. 4). Recently this protein has been implicated in grass salinity tolerance (Liu et al., 2012), but the functions in apple responses to wounding are not yet clear and warrants further analysis in future studies.

It is known that different tissues respond differently to wounding (Valcu et al., 2009). For example, while root wounding produced local changes related to protein synthesis and processing, in leaf, wounding mostly induced changes involved in energy and primary metabolism. Whereas in root, wounding induced flavonoid and alkaloid synthesis pathways, in leaf there was an upregulation of mechanisms involving reactive oxygen species (ROS) (Valcu et al., 2009). Our results show that fruit wounding induced mainly defence related functions (e.g. PR proteins).

4. Conclusions

Global proteomic analysis using 2D-PAGE and MS is the used method to identify protein abundance changes following wounding of 'Golden Delicious' apples. Five proteins (one at W24 and four at W48) increased in abundance following wounding and were found to be mainly involved in 'response to stress' and could indicate a specific response of the fruit to mechanical damage, whereas, protein species without abundance changes after wounding, classified according to their primary biological function were mainly involved in 'metabolism', 'response to stress', and 'oxidation-reduction processes'. Our findings showed that Mal d 1 like and Mal d 1.03G were wound-inducible at W48, suggesting a specific induced response in 'Golden Delicious' fruit. This study represents an important contribution towards a better understanding of the defence reactions and resistance against possible postharvest pathogen infection in 'Golden Delicious'. Therefore, studies on fruit protein abundance changes after wounding will be useful to provide biomarkers and stimulate innate defence mechanisms of the fruit to help minimize postharvest losses.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

Authors are grateful to the Spanish Government for its financial support with the project AGL2011-30519-CO3-03, and for the scholarship BES-2009-027752 (G. Buron-Moles). The authors are indebted to Pablo Librado from 'Departament de Genètica. UB' for the bioinformatician support and to Cèlia Sánchez from 'IRTA' for her excellent technical assistance. We also would like to thank Dra. Isabel Sánchez and M. Alba Sorolla from the 'Servei de Genòmica i Proteomica. UdL' for helping on peptide mass fingerprinting and MS–MS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2013.07.039.

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Chapter 4

Characterising the proteome and oxi-proteome of apple in response to a compatible (*Penicillium expansum*) and a non-host (*P. digitatum*) pathogen

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Submitted to: Journal of Proteomics

Characterising the proteome and oxi-proteome of apple in response to a compatible (*Penicillium expansum*) and a non-host (*P. digitatum*) pathogen

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Research highlights

1. Apple recognizes and reacts to *P. expansum* in a similar manner to wounding.
2. Oxidative changes in response to both pathogens were mainly involved in 'metabolism processes'.
3. ACO and two GS were the most oxidised proteins in response to *P. digitatum* inoculation.
4. Changes of selected responsive proteins did not correlate with the transcribed genes.

Abstract

We monitored changes at the proteome and oxi-proteome level in 'Golden Smoothee' apple fruits in response to *P. expansum* (compatible) and *P. digitatum* (non-host) pathogens with select transcriptional studies. To examine the biological relevance of the results we described the proteins with quantitative and oxidative changes into the gene ontology functional categories, as well as into de KEGG pathways.

Results revealed that quantitative changes in response to a non-host pathogen belonged to the functional category response to stress. The oxidative changes in response to either the compatible or the non-host pathogen were categorized as being involved in metabolism processes. On the one hand, changes in protein abundance showed some similarities between the apple responses to biotic and abiotic stresses, Mal d 1.03A case, whereas some quantitative changes were specific only to non-host pathogen, Mal d 1.03E and EF-Tu case. On the other hand, highly oxidation were observed for ACC oxidase and two glutamine synthetases in response to *P. digitatum* compared with the level of carbonylation in response to *P. expansum*.

Documenting changes in the proteome and, specifically in oxi-proteome of apple can provide information that can be used to better understand how impaired protein functions may affect apple defence mechanisms.

Keywords:

Malus domestica; Green mold; Blue mold; protein carbonylation; RT-qPCR

1 Introduction

Agriculture is one of the most important economic activities in Spain partially due to its favourable climatic conditions. Pome fruits are one of main products grown in Spain and exported to the rest of Europe. Worldwide production of apples was over 76 million tons in 2011 [1]. In addition to fresh market consumption, apples can also be stored for some months in controlled-atmosphere chambers where ethylene-induced ripening is delayed, enabling a steady year-round supply of high-quality fruit. During this postharvest period, however, apples are subjected to both abiotic and biotic stresses.

During harvest and postharvest handling, apples are frequently subjected to mechanical injury which makes the fruit more susceptible to opportunistic infection by postharvest wound pathogens, such as *Penicillium expansum*, the causal agent of blue mould [2]. Among the various postharvest pathogens of apple, *P. expansum* is the most devastating, being responsible for significant economic losses wherever apples are produced and stored. Whereas some postharvest pathogens of fruits, such as *P. digitatum* are highly specialized, affecting only one or two types of fruit such as citrus fruits, *P. expansum*, has the ability to infect a broader range of fruit species, including apples, pears, strawberries and tomatoes. *P. expansum* may be categorized as a compatible or host-pathogen of apple because is able to overcome innate apple defence barriers and establish disease symptoms. In contrast, *P. digitatum* may be considered a non-host pathogen because it has a very limited ability to cause infection in apples and can only do so in overripe fruit [3-5].

Presently, the use of synthetic fungicides is the main method used to control postharvest diseases in apples. The development of resistance in fungal pathogens to chemical fungicides [6] and public concern over the health and environmental hazards associated with high levels of pesticide use in fruits and other crops have fostered a significant interest in the development of alternative methods to manage postharvest diseases. Promising alternative strategies being developed include the use of UV, heat (both hot air and hot water dipping),

simple salts such as sodium bicarbonate, and biological control with antagonistic microorganisms [7-10].

Despite the use of commercial fungicides and the implementation of new alternative strategies, blue mould remains a critical disease of pome and other stored fruits worldwide. Therefore, studies designed to characterise host-pathogen interactions and the nature of virulence are essential for understanding host resistance in apple and for the development of novel, safe, and more effective control strategies. Furthermore, such studies can form the basis for identifying genes that can serve as molecular markers for breeders developing genotypes with greater resistance to postharvest diseases. Few detailed studies, however, have been published on apple fruit response to wounding and inoculation with *P. expansum* [5]. High-throughput sequencing and genomic approaches have gained increased popularity as tools to study a plethora of biological processes. The availability of the complete genome sequence of *Malus x domestica* [11] has greatly aided the development of '-omic' approaches such as genomics, transcriptomics and proteomics in *Malus x domestica.*

Recently, a transcriptional approach of apple gene expression in response to compatible (*P. expansum*) and non-host (*P. digitatum*) pathogens was conducted to characterise disease resistance mechanisms in apples [12]. Although transcriptomic analyses have provided insights into host resistance responses in apple, a number of defence-associated proteins, such as kinases, may not be responsive to defence-related signals at the transcriptional level, and thus the potential role of these proteins\genes cannot be fully characterised in transcriptomic studies. A complementary approach to transcriptome-based defence gene discovery is the use of proteomics to identify pathogen-induced proteins [13]. This method allows one to gain a functional view of the proteins present during spatial and temporal host-pathogen interactions which is extremely important since levels of transcription and proteins usually do not correspond [14, 15]. Proteomic studies also can provide insight into mechanisms that involve the post-translational modification (PTM) of proteins.

While changes in the proteome of the vegetative portions of a plant in response to a broad range of abiotic stress factors, such as cold, heat,

drought, salinity, flooding, ozone treatment and mechanical wounding, have been extensively described [16, 17], the response of fruit to abiotic stresses is in need of further research. In this regard, changes in the apple fruit proteome in response to wounding have been recently published [18]. Proteomic approaches have been widely used to study the postharvest physiology of fruit and vegetables during ripening or in response to methods of storage [19]. An extensive list of the proteomic studies of fruits ripening was provided by Palma *et al.* [20].

Proteomic approaches have also been successfully used to study plant-pathogen interactions (biotic stress) [21]. Despite the economic importance of some postharvest diseases, however, proteomics has only been applied in a few cases to study fruit-pathogen interactions. This include studies in peach [22], sweet cherry [23], jujube [24] and tomatoes [25], designed to characterise the biochemical interactions between fruits and pathogens subjected to different environmental conditions [26].

One of the earliest and most rapid defence responses of fruit is the oxidative burst, which involves the production of reactive oxygen species (ROS) [27]. The association of ROS production with the induction of host defence responses has been demonstrated in many plant-pathogen interactions [28], where hydrogen peroxide (H_2O_2) produced in response to wounding and/or pathogen attack was shown to be integral to the induction of defence mechanisms in fruit [29-34]. Additionally, ROS, originating from oxidative metabolism, are responsible for oxidative damage to proteins (carbonylation), a deleterious PTM that alters protein function [35, 36]. The role of PTMs in the adaptation of plants to different abiotic and biotic stimuli is considered to play an important role in the operation and functioning of many host-pathogen interactions [37, 38].

The level of protein carbonylation has been used to assess protein oxidative damage in peach [39] and in sweet cherry fruit [40, 41] using two dimensional gel electrophoresis coupled with immunoblotting. Different methods for the detection and quantification of oxidised (carbonylated) proteins, including the use of fluorescent probes, such as BodipyFL-Hz have also been developed [42]. The use of fluorescent probes has the advantage of being able to detect a lower

level of carbonylated proteins. Additionally, the method also provides the timely detection and excision of the protein spot after two-dimensional gel electrophoresis for further identification [35].
In the present study, a proteomic analysis was conducted to examine temporal changes in the total proteome and oxi-proteome of 'Golden Smoothee' apples in response to a compatible (*P. expansum*) and a non-host (*P. digitatum*) pathogen, and whether specific proteomic changes are correlated with related changes in transcription with the objective of developing a better understanding of disease resistance in apple fruit. Additionally, changes in the total proteome and oxi-proteome were integrated into functional networks in order to identify their potential biological significance in the compatible and in the non-host pathogen interaction.

2 Materials and methods

2.1 Fruit source and quality parameters

Apples (*Malus* x *domestica* L. cv 'Golden Smoothee') were harvested on September 7, 2011 from a commercial orchard in Mollerussa (Catalonia, Spain) and used immediately after harvest. Fruit were selected for uniform size, absence of physical defects or apparent infections, washed thoroughly with tap water, and dried at room temperature. Twenty fruit were used to evaluate and record skin colour, flesh firmness, soluble solids, acidity, and starch.

Fruit colour was measured on opposite equatorial sides of each apple with a CR-200 colorimetric spectrophotometer (Konica Minolta, Tokyo, Japan) providing L*a*b* values. Flesh firmness was also determined on opposite peeled sides of each fruit using a penetrometer (Effegi, Milan, Italy) equipped with an 11-mm diameter plunger tip and results were expressed in Newton (N). Total soluble solids (TSS) and titratable acidity (TA) were assessed in juice pressed from whole fruit. TSS was determined using a digital refractometer (Atago, Tokyo, Japan) and expressed as a percentage. TA was analysed by titration of 10 mL of juice with 0.1N NaOH up to pH 8.1 with 1%

(v/v) phenolphthalein as an indicator, and results calculated as g/L malic acid. Starch hydrolysis was rated visually, using a 1–10 EUROFRU scale (1, full starch; 10, no starch), after dipping the cut surface of fruit halves in 0.6% (w/v) I2–1.5% (w/v) KI solution for 30 s. Data on the maturity indexes represent the mean of 20 individual fruit.

2.2 Fungal cultures

P. digitatum (strain PDM-1) and *P. expansum* Link (strain CMP-1) were isolated from decayed citrus and pome fruits, respectively. These isolates were the most aggressive in our fungal collection (Pathology Laboratory, IRTA, Lleida) in regards to their ability to infect citrus and pome fruits, respectively. *P. digitatum* and *P. expansum*, were grown on Petri dishes containing Potato Dextrose Agar medium (PDA: 200 mL/L boiled potato extract; 20 g/L dextrose, 20 g/L agar, pH 5.5) in the dark at 25 ºC for 7-10 days to achieve conidia production. Conidial suspensions were prepared by adding 10 mL of sterile distilled water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. The final conidia concentration was adjusted using a haemacytometer and diluted to different concentrations depending on each assay.

2.3 Pathogenicity study

Pathogenicity was assessed for both the host (*P. expansum*-apple) and non-host (*P. digitatum*-apple) fungi. Each fruit was artificially wounded once with a nail (3 mm wide and 3 mm deep) and inoculated with 15 μL of either sterile distilled water (control) or a conidial suspension. *P. expansum* (10^4 conidia mL^{-1}) and *P. digitatum* (10^7 conidia mL^{-1}) were used to study host and non-host pathogenicity, respectively. Fruit were incubated at 20 ºC and 85% relative humidity (RH) for seven days after being inoculated. The percentage of infected wounds (incidence) and the lesion diameters (severity) were measured at the end of the seven day period of incubation. Five fruits constituted a single

replicate and each treatment was repeated four times. The average of five fruits was used as a single replicate and data from all four experiments were pooled for the analysis of the data (n = 4).

2.4 Fruit inoculation and sample preparation

Prior to inoculation, fruit were randomized, washed with tap water, and allowed to air-dry at room temperature. Wounds were inoculated with 10 μL of sterile water (mock inoculation) or a conidial suspension (pathogens).
P. expansum (compatible pathogen) or *P. digitatum* (non-host pathogen) were inoculated at 10^5 and 10^7 conidia mL^{-1}, respectively. Fruit were then stored at 20 ºC and 85% RH. Fifteen cylinders of peel and pulp tissue (8 mm diameter and 3 mm deep) encompassing the wounds from five individual fruits were removed using a cork borer. Wounded tissue (mock inoculation) was removed at 24 h (W24), 48 h (W48) and 7 days (W7). Tissue from unwounded (UW) and only wounded (W0) apples were removed at time 0h. Wounded tissue, inoculated with *P. digitatum*, was removed at 24 h (PD24), 48 h (PD48) and 7 days (PD7). Likewise, wounded tissue, inoculated with *P. expansum* was removed at 24 h (PE24) and 48 h (PE48). Samples were stored at - 80 ºC before processing. The experiment was repeated to obtain three biological replicates. For the proteomic analysis, tissue was removed at 24 and 48h, whereas for the quantification of relative mRNA levels, tissue was removed at 24 h, 48 h, and 7 days. Cylinders used in the Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR) assay were lyophilized for 72 hours prior to RNA extraction.

2.5 Protein extraction and solubilisation

The three biological replicates of each sample were pooled and ground in liquid nitrogen. Total protein was extracted according to the phenol-based protocol described by Buron-Moles *et al.* (2014) but in the presence of a protease inhibitor cocktail (1x Complete Tablet, Roche Applied Science, Mannheim, Germany) in the extraction buffer. The final protein precipitate was dried under vacuum for 3 min

before being solubilised in 150 µl of Tris-HCl 20 mM pH 7.5 and 2% SDS. Protein concentrations were determined with a 2D Quant Kit (GE Healthcare), using Bovine Serum Albumin (BSA) as a standard.

2.6 Protein carbonylation analysis

Protein carbonylation (oxidation) in a total protein extract was determined using an ascorbate/iron system. The inactivation of the enzyme was performed in opened 1.5 ml polypropylene tubes in a Thermomixer Comfort (Eppendorf) for 30 min at 20 °C and 1000 rpm. The reaction mixture (20 µl) contained 9 pmol of pyruvate kinase, 50 µM $FeCl_3$ and 10mM ascorbate in 50mM MES–KOH buffer pH 6.2.

The fluorescent probe Bodipy-FL-hydrazide (Bodipy-Hz) was used to derivatize protein carbonyls, as described by Tamarit *et al.* [42]. Briefly, the Bodipy-Hz was reconstituted with DMSO to a 50 mM stock solution and stored at - 80 °C. For protein carbonyl derivatization, the stock solution of the hydrazide was diluted 1:10 in 0.1 M sodium acetate pH 5, 1 mM EDTA and 1% SDS. Then 83 µl of this solution was added to 83 µl of a protein sample containing 120 µg of protein. The mixture was incubated at 25 °C at 500 rpm in a Thermomixer Comfort (Eppendorf). After 30 min incubation time, 45.7 µl of 2 M Tris and 12.5 µl of 0.2 M $NaCNBH_4$ were added to the reaction mixture and then incubated at 25 °C for 15 min. This treatment stops the derivatization reaction and stabilizes the formed hydrazone. The derivatized proteins were precipitated by adding 25 µl of 100% TCA to each sample tube and the tubes were then kept for 30 min at room temperature. Proteins were concentrated by centrifugation at 14.500 rpm for 5 min and the pellets washed in 3 cycles of ethanol/ethyl acetate (50% v/v), and then air dried. For two-dimensional (2D)-gel electrophoresis, pellets were resuspended in Protein Extraction Reagent type 4 (C0356, Sigma Chemical Co., Madrid, Spain), which consists of 40 mM Trizma Base, 7.0 M urea, 2.0 M thiourea and 1% C7BzO, adding 20 mM DTT and 5 mM TCEP-HCl. In order to eliminate insoluble protein, extracts were incubated for 3 h at 22 ºC with mild agitation and then centrifuged at 14500 rpm for 5 min.

2.7 Two dimensional gel electrophoresis

Total protein extracts were first separated by isoelectric focussing (IEF) using a Protean IEF Cell (Bio-Rad) and ReadyStrip™ IPG strip gels (18 cm) (BioRad) with a linear pH gradient 4-7, according to the manufacturer's instructions.

For analytical gels, IPG strips were loaded with 120 μg of total protein and passively rehydrated overnight in a final volume of 340 μl of Protein Extraction Reagent type 4, with the addition of 20 mM DTT, 5 mM TCEP-HCl and 1% IPG buffer, containing carrier ampholytes pH 3–10 (Bio-Rad). Rehydrated strips were focused using a Protean IEF Cell (Bio-Rad) at 22 ºC with a current limit of 50 μA/strip. IEF was conducted using a sequential gradient procedure of 150V for 2 h, 300V for 30 min, 600V for 1 h, 6.000V for 30 min and 8.000V until a total of 40.000 VoltH had been achieved. The focused strips were equilibrated prior second dimension separation under continuous shaking in SDS equilibration buffer (6 M urea, 20% w/v glycerol, 2% SDS, 0.375 M Tris-HCl, pH 8.8, and 0.002% Bromophenol Blue (BPB)) containing 130 mM DTT for 15 min, and afterwards alkylated with equilibration buffer containing 135 mM iodoacetamide for 15 min. The strips were washed briefly with running buffer, then transferred to the top of 11.5% acrylamide SDS-PAGE gels (21 cm x 20 cm x 1 mm) with MW Precision Plus Protein™ Standards (Bio-Rad), and covered with 0.5% (w/v) agarose (0.002% BPB). Second dimension was performed in an Ettan Dalt-6 electrophoresis system (GE Healthcare), in SDS electrophoresis buffer (0.25 M Tris pH 8.8, 1.92 M glycine and 1% SDS), applying a constant voltage intensity of 11 mA overnight.

Gel images of carbonylated proteins were acquired in a VersaDoc MP4000 imager (BioRad), with an excitation wavelength of 468 nm and an emission filter of 530BP for Bodipy-Hz. After obtaining these images, gels were fixed for 2 h in a fixing solution containing 40 % ethanol, 10% acetic acid, (v/v) and total protein staining was performed with Flamingo (BioRad). Images of total protein were acquired using the same imager system, setting the excitation

wavelength to 468 nm and using an emission filter of 605BP for Flamingo.

2.8 Image analysis

Image analysis was performed using PDQuest analysis software (version 8.0, Bio-Rad) for spot quantification, normalization, and matching. A total of twenty-one gels, resulting from three technical replicates per each condition, were analysed. Image analysis was first conducted separately on Flamingo stained gels. Spot detection and matching between gels were performed automatically, and then manually, in order to verify the accuracy of automated gel matching and to correct for any errors or missing spots that occurred in the automatic procedure, prior to final data analysis. A standard gel (master) was generated from the image displaying the highest spot number without streaking or other gel distortions.

Quantitative analyses were carried out after normalizing the spot volume, in order to compensate for non-expression related variations. The normalized spot volumes (individual spot intensity/normalization factor), calculated for each gel based on the total quantity of valid spots, were determined and used for statistical calculation of protein expression.

Total proteome analysis of Flamingo gels was performed using spots that were reproducible and showed a quantitative volume variation by a factor of at least twofold increase/decrease, as well as being statistically significant (Student's *t* test, $P < 0.05$).

Protein oxidative image analysis or oxi-proteome analysis of Bodipy gels was performed according to published method [42], with some modification. Bodipy gel images were used for automatic spot detection and matching (analysis was checked manually), to select the oxidised protein spots in each condition. Then, Flamingo images were added to the analysis and their spots matched to the selected spots in their corresponding Bodipy images. For each spot, Bodipy signal was normalized to that of the protein signal. Spots displaying a Bodipy-fluorescence/protein signal ratio greater than 1.5 were selected for further analysis [43]. The theoretical mass (MM) and p*I* of the

identified proteins were calculated from sequence data with the Expasy Compute p*I*/MM tool (http://web.expasy.org/compute_pi/).

2.9 *In-gel* digestion and protein identification by mass spectrometry

Protein spots were excised from gels and subjected to in-gel digestion with trypsin on a perforated microtiter plate (Proxeon Biosystems). Gel pieces were minced and washed with MilliQ water and dehydrated with 50% ethanol in 50 mM NH_4HCO_3 for 10 min, and then with 100% ethanol for 10 min. Gel pieces were then reduced with 10 mM DTT in 50 mM NH_4HCO_3 for 1 h at 56 ºC and alkylated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min at room temperature in the dark. After this, the gel pieces were washed twice in 50 mM NH_4HCO_3 for 15 min and dehydrated with 5% acetonitrile (ACN) in 25 mM NH_4HCO_3 for 15 min, 50% ACN in 25 mM NH_4HCO_3 for 15 min twice and finally with 100% ACN for 10 min. After total evaporation of the ACN, 15 µl of 20 $ng \cdot \mu l^{-1}$ trypsin in 25 mM NH_4HCO_3 was added and left at 4 ºC for 45 min in order to allow full rehydration of the gel pieces with the trypsin solution. The gel pieces were then covered with 25 mM NH_4HCO_3 and incubated at 37 ºC overnight for proteolysis. One µl of this tryptic peptide solution was applied on a MALDI plate. The rest of the tryptic peptides were collected, evaporated in a SpeedVac (Savant) and resuspended in 5 µl of 70% ACN/0.1% TFA. One µl of this concentrated solution was also applied on a MALDI plate. The spotted samples were dried at room temperature and covered with 1 µl of saturated α-cyano-4-hydroxycinnamic acid prepared in 50% v/v ACN containing 0.1% TFA.

Protein identification was performed by peptide mass fingerprinting (PMF) or MS/MS mass spectrometry in an AutoflexSpeed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bruker Española, S.A., Madrid, Spain). Mass spectra (mode reflectron, MH +) were acquired by Flex Control version 3.0 software (Bruker Daltonics), recording in the range 800–4500 Da, and the MS/MS information was obtained in LIFT (laser-induced forward transfer) mode. MS spectra were externally calibrated using Peptide Calibration Standard II

(Bruker Daltonics). MS spectra were post processed with FlexAnalysis version 3.3 software (Bruker Daltonics) and, when possible, internal calibration with peptides from the autodigestion of trypsin was also applied. BioTools version 3.1 protein analysis package (Bruker Daltonics) was use for protein database searching, including MS and MS/MS combined spectra. The peak lists obtained were compared against the Swiss-Prot and non-redundant NCBI protein databases, and also the Plant EST database of the NCBI, using the MASCOT version 2.3 software package (Matrix Sciences, UK; www.matrixscience.com). The search parameters were set as monoisotopic peptide masses, carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modifications, respectively, one trypsin missed cleavage and a maximum of ±100 ppm for PMF peptide tolerance and ±0.4 Da for MS/MS tolerance, where the charge (Z) of the precursor ion was +1. MASCOT protein scores and peptide ion scores greater than 52 were considered statistically significant ($P < 0.05$) and indicate identity or extensive homology ($P < 0.05$).

2.10 Bioinformatic analysis

The proteome and oxi-proteome list of identified protein sequences was then functionally annotated using Blast2GO software v2.7.0 (http://www.blast2go.org, [44]). Furthermore, an InterProScan homology search (implemented within the Blast2GO software) was performed to find functional domains and related GO terms using the default parameters. The 'Merge InterProScan GOs to Annotation' function was used to refine all annotations.

To examine whether some specific GO terms are over-represented in our experimental conditions, we used the Ontologizer software v2.0 [45]. In particular, the reference GO annotation set for all the *Malus* x *domestica* proteins was downloaded from the GDR website (http://www.rosaceae.org/). To determine the over-representation of GO terms, a Fisher's exact test was performed. The corresponding P-values were corrected for multiple-testing via the Benjamini-Hochberg

approach. GO categories with an adjust P-value less than 0.05 were considered significantly over-represented.
Likewise, a test for the over-representation of particular metabolic pathways was conducted using the Kyoto Encyclopaedia of Genes and Genomes (KEGG - http://www.genome.jp/kegg/ [46]. The reference KEGG annotation set for all the *Malus x domestica* proteins was also downloaded from the GDR website. The hypergeometric test ($P < 0.05$) was used to determine the significantly over-representation of the KEGG pathways.

2.11 RNA extraction

Total RNA was extracted as described by Vilanova *et al.* (2014), with modification. In brief, 1 g of lyophilized apple tissue (pulp and peel) powder was added to a preheated mixture of 5 mL of extraction buffer (100 mM Tris-HCl, pH 8.0, 2 M NaCl, 25 mM EDTA, pH 8.0, 2 % (w/v) cetyltrimethylammonium (CTAB), 2 % (w/v), polyvinyl-pyrrolidone 40, 0.5 g L^{-1} spermidine). 2 % β-mercaptoethanol and 2 % sarkosyl were added just before use. After homogenization with a Polytron PT 10/55 (Kinematica AG, Lucerne, Switzerland) for 30 s, the extract was incubated at 70 ºC for 15 min and 3.75 mL of chloroform were added. The homogenate was centrifuged at 10,000 g for 15 min at 4 ºC and the supernatant was transferred to different eppendorf tube. A half volume of 95% ethanol was added and the homogenate was transferred onto a Qiagen RNeasy Mini spin column. RNA was purified according to the manufacturer's instructions. RNA was eluted in 100 μL of RNase-free water. RNA concentration was quantified using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, DE, USA) and the RNA integrity was verified by agarose gel electrophoresis stained with ethidium bromide. Extracted RNA was treated and purified with turbo DNA-free kit (Ambion, TX, USA) according to the manufacturer's instructions.

2.12 Quantification of gene expression by Reverse Transcriptase-Quantitative Polymerase Chain Reaction Assay (RT-qPCR)

Total RNA was isolated from pulp and peel apple tissue using RNeasy Minikit (Qiagen), treated with DNase (Turbo DNA-free Kit; Ambion, Austin, TX, USA) as described above, and diluted to 6.25 ng μl^{-1}. Transcript levels of six apple genes encoding ACO, APX, Mald1.03E, HSP, NAC and EF-Tu were determined using RT-qPCR analysis, which was performed using Power SYBR Green RNA-to-C_T™ 1-step kit (Applied Biosystems, CA, USA). Gene-specific primers, listed in Table 1, were used at an optimal concentration of 2.0 pmol of each primer per reaction. The ABI 7900 (Applied Biosystems, CA, USA) was set to cycle as follows: cDNA synthesis at 48.0 °C for 30 min; 95.0 °C denaturation for 10 min; 40 cycles of 95.0 °C for 15 s followed by 57-58 °C (depending on primers used, Table 1) for 1 min; followed by ABI-specified hold and melt curve stages.

Table 1. Primer sequences used to analyze expression of the *M. domestica* selected genes: ACC oxidase 1 (ACO), ascorbate peroxidase (APX), major allergen Mal d 1.03E (MalD1.03E), class I heat shock protein (HSP), translation elongation factor EF-TU precursor, chloroplast (EF-TU) and NAC domain class transcription factor (NAC). *EF1a* as reference gene.

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
ACO	GTGTGCTTAGAGGGGAAAT	TTGTTTTCCGACCCTCTT
APX	GACGAGGGTTGAAGTTCT	CCATCCCAAACCAAACCA
Mal d 1.03E	CCAGCCACTACCATACCA	CCAATTACAAGCCAAAACAC
HSP	GTGCCGAAAGAGGTGAAGA	AAACCCAAAAGTAGCCGA
EF-Tu	CAGAGAAGGAGGGAAGAC	GGCACCAAATTGCAAAAAC
NAC	CCATATCAACTGCCTGGAA	ATCCTCTATCATCCCCCAA
EF1a	GACATTGCCCTGTGGAAGTT	GGTCTGACCATCCTTGGAAA

Primers were verified for specificity by using apple genomic DNA template and assessing the resulting amplicon by agarose gel electrophoresis and qPCR with genomic DNA on the ABI 7900; all primers had a single band and single peak. Non-amplification of cDNA derived from *P. expansum* and *P. digitatum* DNA was also verified. Primer efficiency was also determined for all primer sets by qPCR analysis of a standard curve, constructed by serially diluting RNAs from the sample set starting at some concentration above what was used in unknown samples and ending at a concentration well below it.

To investigate whether gene expression is correlated with protein expression, the relative quantitation in RT-qPCR for 6 genes selected was employed based on the results obtained in the proteomic data. Three technical repetitions were used for each biological replicate for both the test and reference genes. The standard curve method was used to calculate transcript abundance relative to EF1α (AJ223969.1) and an apple fumarase which were used as reference gene (user bulletin no. 2; Applied Biosystems). The EF and fumarase genes, along with other endogenous reference genes (actin, tubuline and 26S rRNA), were assessed for their stability within a tissue and across time points. EF and fumarase were deemed the best overall reference gene using NormFinder software [47].

The ANOVA test was used to assess significant differences in gene expression between the experimental conditions.

3 Results

3.1 Apple quality parameters and effect of maturity stage on decay development

Fruit quality was characterised by measurements of skin colour, flesh firmness, TSS, TA, and starch hydrolysis. The result for the (a* + b*) parameter on 'Golden Smoothee' apples was 24.3, whereas a mean value of 66.6 N was recorded for flesh firmness. Results for TSS and TA were 13.4% and 4.9 g/L malic acid, respectively. The most relevant quality parameter for defining the stage of apple maturity was

the starch index and in this case the measured value was 6.9. The quality results obtained in this study, relative to a previous study on 'Golden Smoothee' apples [4], indicate that the apples were at a commercial maturity stage, which may also have influenced the defence response of these apples, since that the response of apple fruit to biotic and abiotic stress is maturity dependent [34].

No decay symptoms were observed in wounded apples at 24 and 48 h when wound healing was observed in the peel (exocarp) and pulp (mesocarp) around the wound site. However, at 24 and 48 h after wounding and inoculation with *P. expansum* the initiation of infection was observed. *P. expansum* was able to infect and develop rot in 100% of the inoculated apples at this maturity stage and lesion diameter was 2.7 cm by 7 days after inoculation. In contrast, growth of *P. digitatum* was confined to the wound site and no signs of infection or decay were evident in the surrounding tissues. A yellow (degreened) circle in the peel tissue surrounding the infection site was observed in fruit inoculated with *P. digitatum* (10^7 conidia mL^{-1}) at 7 days post inoculation. No reaction was evident at 24 and 48 h after *P. digitatum* inoculation. These observations are similar to and in agreement with observations made on commercial fruit in a previous study [4].

3.2 Changes in the proteome and oxi-proteome of apple in response to abiotic and biotic stress

An effort was made to examine temporal changes in the proteome and oxi-proteome of 'Golden Smoothee' apple in response to abiotic and biotic stress. Apples wounded (mock inoculation) or wounded/inoculated with either a compatible (*P. expansum*) or a non-host (*P. digitatum*) pathogen were sampled at different time-points and used to identify changes in protein abundance and the level of protein oxidative damage (carbonylation).

Representative 2D gels displaying total proteins (Flamingo staining) and proteins identified by carbonyl detection (BODIPY staining) from unwounded, wounded, and wounded/inoculated apple tissue at 24 h are shown in Fig. 1.

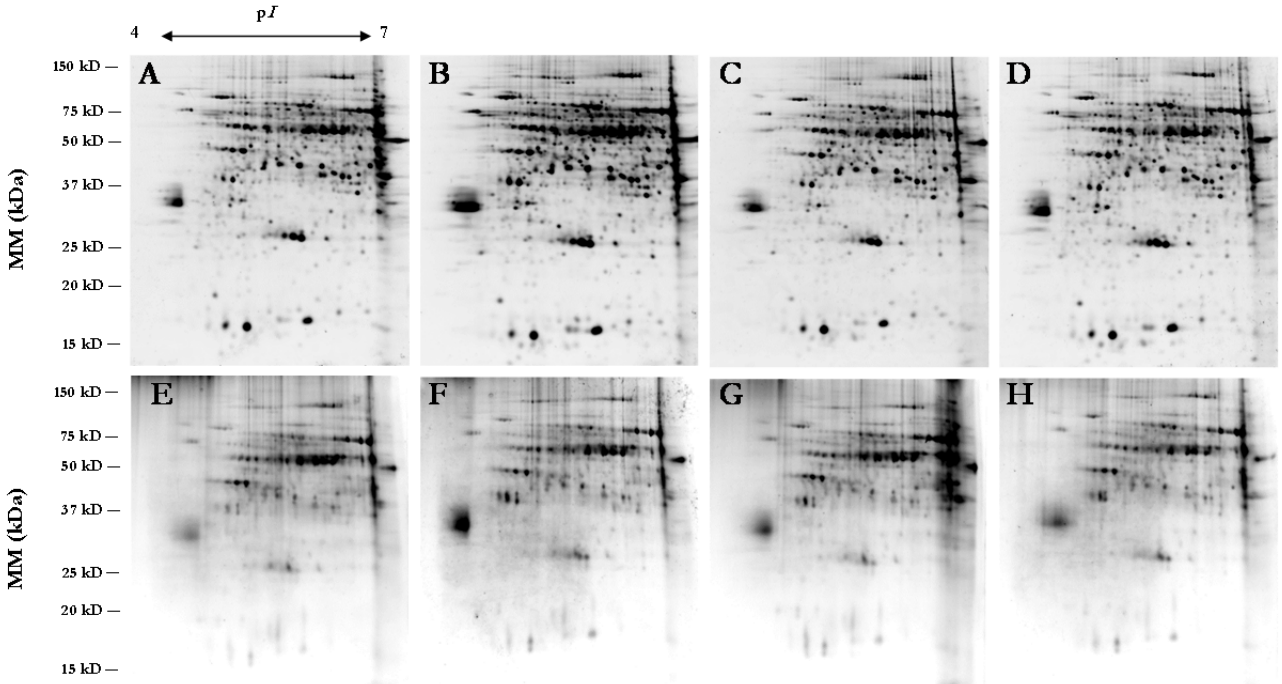

Figure 1. Protein extracts obtained from unwounded, wounded and inoculated with *P. digitatum* and *P. expansum* 'Golden Smoothee' apples were derivatized with Bodipy-Hz and analyzed by 2D-gel electrophoresis. The images shown correspond to total protein stain by Flamingo (BioRad) (A-D) and Bodipy signal (E-H) in unwounded, wounded and inoculated samples at 24 hours.

Images corresponding to 48 h are shown in Supplemental Fig. 1. Immobilized pH gradient (IPG) strips (18 cm, pH 4-7) with 15-150 kDa relative molecular masses were used to separate proteins and Flamingo (Fig. 1A-D) and Bodipy (Fig. 1E-H) fluorescent dyes were used to stain and visualise the separated proteins. Gels were run three times on each sample and a high level of reproducibility was observed. Approximately 350 protein spots were detected on each gel stained with Flamingo (Fig. 1A-D), whereas approximately 87 protein spots were detected on each gel stained with Bodipy (Fig. 1E-H). To match protein spots identified by carbonyl detection with spots on the total protein gels, the gels stained with Flamingo and Bodipy dyes were superimposed. Protein spots coincided with the Bodipy-derivatized spots. Images were analysed using PDQuest 8.0.1 software and the ratio between the Bodipy signal and the Flamingo signal was calculated for each spot. This ratio was considered a relative measure of the carbonyl content of each spot.

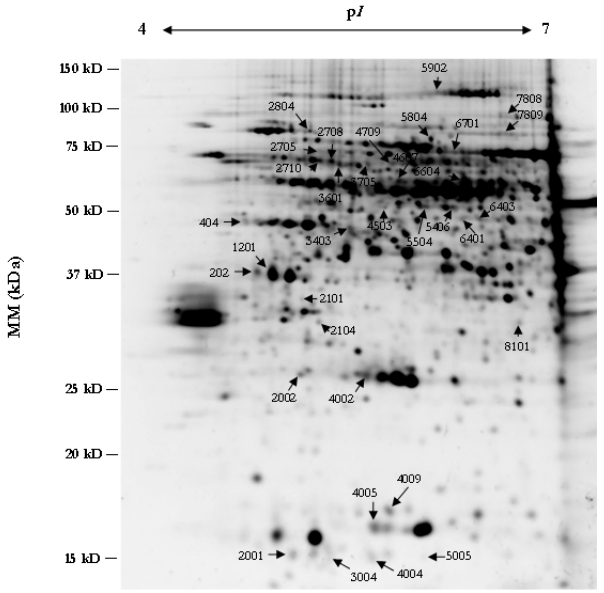

Figure 2. Representative 2D gel of total protein stain by Flamingo (BioRad) from 'Golden Smoothee' wounded sample at 24 hours. Protein spots differentially expressed (2-fold and $P < 0.05$) are numbered. Spot numbering refers to Table 2 and Sup. Table 2 shows protein identification obtained by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS) analysis.

Thirty-four out of the 350 detected proteins detected in total protein gels showed significant changes (2.0-fold and $P < 0.05$) in different samples, compared with their respective control. Eight spots, however, could not be identified by MALDI-TOF/TOF. The location of the 26 spots that exhibited significant changes in relative intensity among the treatments is shown in the image of a representative 2D gel (Fig. 2). The identified proteins are reported in Table 2. Detailed information on each of the peptide sequences and their homology to matching proteins can be found in Supplementary Table 2.

Twenty-six out of the 87 spots detected in the oxi-proteome gels displayed a significant increase in oxidation (carbonylation) among treatments (Fig. 3). All were successfully identified and are listed in Table 3. Detailed information on each of the peptide sequences and their homology to matching proteins can be found in Supplementary Table 3.

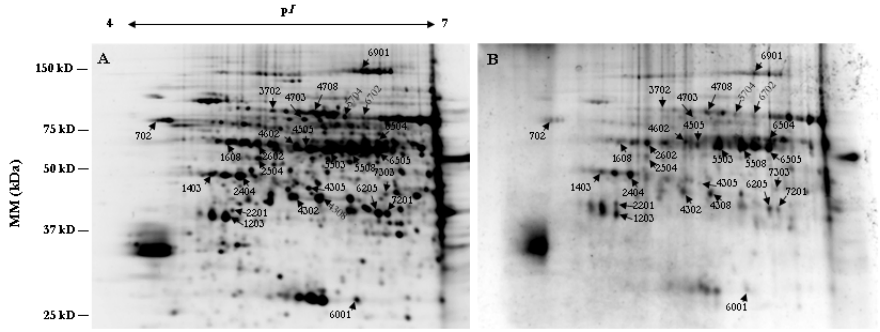

Figure 3. Protein extracts obtained from 'Golden Smoothee' apples were derivatized with Bodipy-Hz and analyzed by 2D-gel electrophoresis. The images shown correspond to total protein stain by Flamingo (BioRad) (A) and Bodipy signal (B) in wounded sample at 24 hours. Spot numbering refers to Table 3 and Sup. Table 3 shows protein identification obtained by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS) analysis.

Table 2. Proteins with significant abundance changes after wounding and pathogen inoculation. Proteins were excised from the 2D PAGE gel and digested with trypsin. The peptides were extracted and their masses were measured by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS). Spot number, protein description, accession number, database, method of identification, MASCOT score, theoretical molecular mass (MM) and isoelectric point (p*I*) are listed.

Spot [a]	Protein description [b]	Accession number [c]	Database	Identification method	Mascot Score	MM (kDa)	p*I*	Responsive to	Functional category [d]
202	ACC oxidase 1	O24063	SWP	PMF	142	35.6	5.2	Wound	Metabolic process/Response to stress/Oxidation-reduction process
1201	ACC oxidase 1	O24063	SWP	PMF	164	35.6	5.2	Wound	Metabolic process/Response to stress/Oxidation-reduction process
2001	Major allergen Mal d 1.06C	gi|60280825	nrNCBI	PMF	167	17.4	5.5	Wound	Response to stress
2101	Short-chain dehydrogenase/reductase 2b-like	EB123506	Plants EST	MS/MS	194	20.0	8.9	PE	Metabolic process
2104	Thiazole biosynthetic enzyme	AT000365	Plants EST	MS/MS	85	7.3	8.1	PE	Metabolic process/Response to stress
2708	Polyphenol oxidase 2 precursor	gi|14194273	nrNCBI	PMF	224	65.8	6.1	Wound/PD/Int.	Metabolic process/Oxidation-reduction process
2710	Mitochondrial HSO70 2 isoform 1	CN908186	Plants EST	MS/MS	71	12.4	4.5	Wound	Response to stress
2804	High molecular weight heat shock protein	gi|6969976	nrNCBI	PMF	242	71.6	5.2	Wound	Response to stress/Oxidation-reduction process
3004	Major allergen Mal d 1.03E	gi|60280855	nrNCBI	PMF	234	17.6	5.4	PD	Response to stress
3403	Probable sarcosine oxidase-like	CN849922	Plants EST	MS/MS	72	19.6	5.8	PE	Metabolic process/Oxidation-reduction process
3601	Polyphenol oxidase 2 precursor	gi|14194273	nrNCBI	PMF	222	65.8	6.1	Wound/Int.	Metabolic process/Oxidation-reduction process
3705	Polyphenol oxidase 2 precursor	gi|14194273	nrNCBI	PMF	258	65.8	6.1	Wound	Metabolic process/Oxidation-reduction process
4004	Major allergen Mal d 1.03D	gi|60280849	nrNCBI	PMF	143	17.6	5.5	PD/PE	Response to stress
4005	Major allergen mal d 1	gi|4590378	nrNCBI	PMF	174	17.7	5.7	Wound	Response to stress

Table 2. (Continued)

Spot [a]	Protein description [b]	Accession number [c]	Database	Identification method	Mascot Score	MM (kDa)	p*I*	Responsive to	Functional category [d]
4009	Class I Heat shock protein	CO723224	Plants EST	PMF	143	18.6	6.1	Wound	Response to stress/Oxidation-reduction process
4503	4-hydroxyphenylpyruvate dioxygenase-like	EB136504	Plants EST	PMF	118	16.4	9.1	PD/PE	Metabolic process/Oxidation-reduction process
4607	Enolase	EB131141	Plants EST	MS/MS	162	21.5	7.7	Wound	Metabolic process
4709	Apgm	DY256418	Plants EST	MS/MS	167	18.5	5.9	Wound	Metabolic process/Response to stress
5005	Major allergen Mal d 1.03A	gi|60280841	nrNCBI	PMF	152	17.6	5.7	Wound/PD/PE	Response to stress
5504	Aminoacylase, putative	DT001087	Plants EST	MS/MS	101	24.0	9.0	Wound	Metabolic process
5804	Heat shock protein STI-like	EB135855	Plants EST	MS/MS	251	21.9	4.8	PE	Metabolic process/Response to stress
5902	Cytosolic aconitase	CN900040	Plants EST	PMF	111	21.5	6.5	Wound	Metabolic process
6401	Translation elongation factor EF-Tu precursor, chloroplast	DT000463	Plants EST	MS/MS	71	20.6	9.8	PD/Int.	Metabolic process
6604	ATP1 (mitochondrion)	gi|404481694	nrNCBI	PMF	188	55.6	6.2	Wound	Metabolic process
6701	NADP-dependent malic enzyme-like	EB136452	Plants EST	PMF	121	23.8	8.9	Wound	Metabolic process/Oxidation-reduction process
8101	Pyridoxine biosynthesis protein - like	GO510968	Plants EST	PMF	187	23.5	6.0	PD	Metabolic process/Response to stress

[a] Spot identification number as assigned in Fig. 2A;
[b] Description of proteins identified from the Plants EST, nrNCBI or SwisProt database;
[c] Accession numbers according to *Viridiplantae* SwisProt, nrNCBI or Plants EST Database of NCBI;
[d] Functional categories were inferred using the Blast2GO software.

Table 3. Proteins oxidatively modified after wounding and pathogen inoculation. Proteins were excised from the 2D PAGE gel and digested with trypsin. The peptides were extracted and their masses were measured by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS). Spot number, protein description, accession number, database, method of identification, MASCOT score, theoretical molecular mass (MM) and isoelectric point (p*I*) are listed.

Spot [a]	Protein description [b]	Accession number [c]	Database	Identification method	Mascot Score	MM (kDa)	p*I*	Responsive to	Functional category [d]
702	Putative cytosolic NADP-malic enzyme	EB117833	Plants EST	MS/MS	146	19.6	6.4	Wound	Metabolic process/Oxidation-reduction process
1203	ACC oxidase 1	O24063	SWP	PMF	189	35.6	5.2	PD/Int.	Metabolic process/Response to stress/Oxidation-reduction process
1403	Actin 7	CN896143	Plants EST	PMF	140	57.5	5.8	Wound/PE	Metabolic process/Response to stress
1608	ATP synthase subunit beta, mitochondrial-like	CN898384	Plants EST	PMF	218	22.1	6.2	PE/Int.	Metabolic process/Response to stress
2201	Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial-like	ES790079	Plants EST	PMF	202	62.3	9.0	Wound	Metabolic process/Oxidation-reduction process
2404	Actin, partial	gi|355329944	nrNCBI	PMF	202	40.4	5.7	Wound/PE	Metabolic process/Response to stress
2504	Regulatory particle triple-A ATPase 3 isoform 2, partial	CN888682	Plants EST	PMF	223	24.4	6.5	PD	Metabolic process/Response to stress
2602	ATP synthase beta chain, mitochondrial precursor	DR991435	Plants EST	MS/MS	279	17.8	4.7	Int.	Metabolic process
3702	Vacuolar H+-ATPase	gi|131573315	nrNCBI	PMF	408	69.2	5.4	PD	Metabolic process
4302	Glutamine synthetase cytosolic isozyme 1	DT003241	Plants EST	MS/MS	120	27.7	9.5	Int.	Metabolic process
4305	Phosphoglycerate kinase, chloroplastic-like	CN893843	Plants EST	PMF	149	21.1	8.8	Wound	Metabolic process/Response to stress
4308	Glutamine synthetase nodule isozyme-like	CN894859	Plants EST	PMF	113	16.7	5.8	Int.	Metabolic process
4505	Enolase	GO520405	Plants EST	PMF	94	22.1	8.9	Int.	Metabolic process/Response to stress
4602	Enolase	CV081922	Plants EST	PMF	107	18.7	7.8	PD	Metabolic process

Table 3. (Continued)

Spot [a]	Protein description [b]	Accession number [c]	Database	Identification method	Mascot Score	MM (kDa)	p*I*	Responsive to	Functional category [d]
4703	Apgm	DY256418	Plants EST	MS/MS	134	18.5	5.9	PE	Metabolic process/Response to stress
4708	Polyphenol oxidase IIa	gi|384563679	nrNCBI	PMF	217	67.8	6.2	PD/Int.	Metabolic process/Oxidation-reduction process
5503	Enolase	GO520405	Plants EST	PMF	106	22.1	8.9	PD/Int.	Metabolic process/Response to stress
5508	Enolase-like	EB151863	Plants EST	PMF	154	22.8	5.3	Int.	Metabolic process
5704	Phosphoglucomutase, cytoplasmic	DT041983	Plants EST	MS/MS	121	26.8	8.8	Wound	Metabolic process
6001	Triosephosphate isomerase, cytosolic-like	CN908475	Plants EST	PMF	147	24.0	6.4	PD/PE	Metabolic process/Response to stress
6205	Cytosolic malate dehydrogenase	gi|78216493	nrNCBI	PMF	256	36.0	6.0	Wound	Metabolic process
6504	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit, partial (chloroplast)	CN872362	Plants EST	PMF	141	22.5	8.8	PE	Metabolic process
6505	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit, partial (chloroplast)	CN872362	Plants EST	PMF	223	22.5	8.8	Wound	Metabolic process
6702	Bifunctional 3-dehydroquinate dehydratase /shikimate dehydrogenase, chloroplastic-like	gi|462403009	nrNCBI	MS/MS	46	54.5	6.2	PE	Metabolic process/Oxidation-reduction process
6901	Cytosolic aconitase	EB122770	Plants EST	MS/MS	131	17.9	8.9	PE	Metabolic process
7201	Cytosolic malate dehydrogenase	gi|78216493	nrNCBI	PMF	187	36.0	6.0	Wound/PD/Int.	Metabolic process
7303	Phosphoglycerate kinase, cytosolic-like	CN938321	Plants EST	PMF	200	22.1	9.2	Wound	Metabolic process

[a] Spot identification number as assigned in Fig. 3;

[b] Description of proteins identified from the Plants EST, nrNCBI or SwisProt database;

[c] Accession numbers according to *Viridiplantae* SwisProt, nrNCBI or Plants EST Database of NCBI;

[d] Functional categories were inferred using the Blast2GO software.

Identified proteins, whose abundance or oxidation change after wounding or pathogen inoculation, were classified into functional categories using Blast2GO software (Conesa *et al.* 2005). Proteins were categorized according to their biological process, encompassing mainly 'metabolism', 'response to stress' and 'oxidation-reduction processes' (Table 2 and 3).

3.3 Abiotic stress: Identification of wound-responsive proteins

Our previous studies analysed changes in protein abundance in 'Golden Delicious' apple, after wounding, using wounded tissue at 0 h as a control [18]. In order to analyse wound-responsive proteins in 'Golden Smoothee' apple in the present study, unwounded tissue at time 0h served as a control in the time-course study. Representative 2D gels of total protein extracts from peel (exocarp) and flesh (mesocarp) tissues of unwounded and wounded apple tissue at 24 h are shown in Fig. 1A-B.

Eighteen spots displayed a significant difference (2.0-fold and $P < 0.05$) in abundance after wounding, when compared with the unwounded control. Sixteen of the 18 spots were successfully identified and grouped according to their biological process using Blast2GO software (Table 2, Supplementary Table 2). In general, most of the proteins whose abundance increased are involved in response to stress, whereas the proteins whose abundance decreased are involved in metabolism process.

Two (spots 202, 4607) out of the 16 identified proteins increased, whereas one (spot 2804) decreased in abundance at time 0 (W0) relative to the unwounded control. At 24 h after wounding (W24), four (spots 202, 1201, 4005, 4009) increased in abundance, while three (spots 3601, 3705, 6701) decreased in abundance relative to the unwounded sample. Five proteins (spots 2001, 4009, 4709, 5005, 5504) increased in abundance at 48 h after wounding (W48) relative to the unwounded control, whereas another four proteins (spots 2710, 3601, 5902, 6604) decreased in abundance (Supplementary Fig. 4A). For example, Mal d 1.06C (spot 2001), Mal d 1 (spot 4005) and Mal d 1.03A (spot 5005) proteins were identified. Levels of Mal d 1 protein

increased in abundance 2.1 fold at W24, whereas Mal d 1.06C and Mal d 1.03A increased in abundance 3.0 and 4.5 fold, respectively at W48 (Supplementary Fig. 4A).

Ten protein spots exhibited a significant 1.5 fold increase in carbonylation after wounding; all were successfully identified and grouped according to their biological process using Blast2GO software (Table 3, Supplementary Table 3). Many proteins were already highly carbonylated at W0, compared with the unwounded control, whereas only a few carbonylated proteins were observed at W24 and W48. Most of the carbonylated proteins induced after wounding are grouped in the category 'metabolism processes' (Table 3). Eight out of the ten oxidatively modified proteins identified in W0 samples were spots 1403, 2201, 2404, 4305, 5704, 6205, 6505, and 7201 (Supplementary Fig. 4B). Two of them were glycolytic enzymes, pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial-like (spot 2201) and phosphoglucomutase, cytoplasmic (spot 5704), and two were chloroplastic, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (spot 6505) and phosphoglycerate kinase (spot 4305), involved in the Calvin cycle. Two isoforms of cytosolic malate dehydrogenase (spots 6205 and 7201) are involved in glyoxylate metabolism. Oxidation of these proteins indicates that apple defence response may be impaired due to decreased metabolism. Actin 7 (spot 1403) and Actin, partial (spot 2404), carbonylated only at W0, is a cytoskeletal protein involved in cell vesicle formation and movement, cell motility and signalling. Actin, partial showed the highest increase in oxidation (5.1-fold) at W0 (Supplementary Fig. 4B).

3.4 Biotic stress: Identification of pathogen (*P. expansum*) and non-host pathogen (*P. digitatum*) responsive proteins

Mechanical damage to apples provides an optimal infection site for wound pathogens, which require damaged tissue to germinate and an avenue of ingress to colonize the tissue. Therefore, it is important to examine defence mechanisms in fruit that occur after wounding and are directly associated with the presence of the pathogen. In this

regard, temporal changes in apple protein expression in response to wounding/inoculation with *P. expansum* (compatible pathogen) and *P. digitatum* (non-host pathogen) was analysed, with wounded tissue serving as a control. In particular, special attention was placed on a comparison of temporal changes in protein expression that were specific to the non-host pathogen, *P. digitatum*, and those that were specific to the compatible pathogen, *P. expansum*. Representative 2D gels of total protein extracts from peel and flesh from wounded and wounded/inoculated apple tissue at 24 h are shown in Fig. 1B-D.

3.4.1 Apple proteome and oxi-proteome changes in response to *P. digitatum* inoculation

Apple protein changes in response to *P. digitatum* inoculation at 24 h (PD24) and 48 h (PD48) were analysed using wounded samples from the same time periods as a control.

Seven proteins displayed a significant difference in abundance (2.0-fold and $P < 0.05$) during the time-course of the study and were successfully identified (Table 2, Supplementary Table 2). The proteins were grouped according to their biological process using Blast2GO software. Results indicated that the category with considerable enrichment was response to stress, suggesting that this process plays a leading role in apple fruit defence response against the non-host pathogen, *P. digitatum*.

Few differences in total proteins were observed at 24 h, whereas six of the seven identified proteins were markedly more abundant at 48 h. The six proteins were spots 2708, 3004, 4004, 4503, 5005 and 6401 (Fig. 4A). For example Mal d 1.03E (spot 3004), Mal d 1.03D (spot 4004) and Mal d 1.03A (spot 5005) proteins were identified. Whereas Mal d 1.03D (4.0-fold) and Mal d 1.03A (7.3-fold), exhibited increased abundance at 24 h, relative to the wounded control, all three Mal d 1 proteins remained high (4.3, 3.2, 4.5-fold, respectively) at 48 h (Fig. 4A). Other proteins also displayed increased abundance in *P. digitatum*-inoculated tissue at 48 h, compared with the wounded control, indicating that they might also be involved in the response of apple fruit to a non-host pathogen. These proteins included a polyphenol

oxidase 2 precursor (spot 2708), 4-hydroxyphenylpyruvate dioxygenase-like (spot 4503), and a translation elongation factor EF-Tu precursor (spot 6401) (Table 2, Supplementary Table 2). No proteins were observed that had a significant (2.0-fold and $P < 0.05$) decrease in abundance in response to the non-host pathogen.

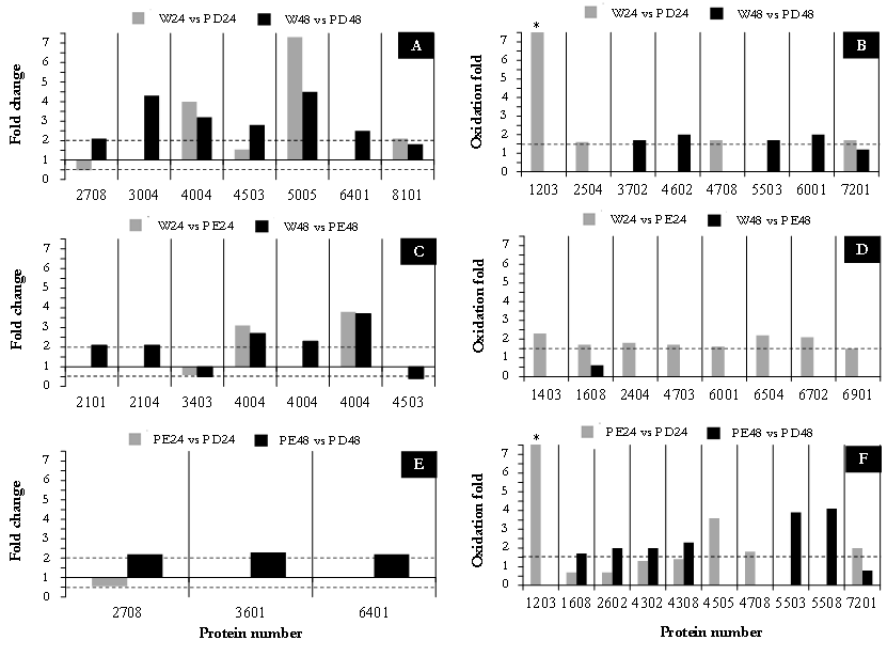

Figure 4. Temporal changes in the proteome and oxi-proteome of 'Golden Smoothee' apples in response to a compatible (*P. expansum*) and a non-host (*P. digitatum*) pathogen inoculation. In A, C and E, columns represent the fold changes (2-fold) of protein abundance between inoculated and control conditions. Values ≥ 2 or ≤ 0.5 (--- threshold) are considered an increase or a decrease in protein abundance. In B, D and F, columns represent the oxidation fold (1.5-fold) ratio [Bodipy signal/protein signal] of each spot between inoculated and control conditions. Values ≥ 1.5 (--- threshold) are considered oxidised. * Protein with an oxidation fold greater than 7. Data are the mean of three independent gels. W = wounded control (mock inoculated), PD = *P.digitatum*, PE = *P. expansum*. Column absence indicates a spot absence at the specific condition.

Eight protein spots displayed marked changes in oxidation and had a significant (1.5-fold) increase in carbonylation in response to *P. digitatum* inoculation during the time-course of the study. The carbonylated proteins were successfully identified and grouped according to their biological process using Blast2GO software (Table 3, Supplementary Table 3). Most of the carbonylated proteins were categorized as being involved in metabolism processes (Table 3).

Four of the eight modified proteins exhibited increased carbonylation at 24 h. These four spots were identified as ACC oxidase 1 (spot 1203), regulatory particle triple-A ATPase 3 isoform 2, partial (spot 2504), polyphenol oxidase IIa (4708) and cytosolic malate dehydrogenase (7201). Two of the proteins, ACC oxidase 1 (16.8-fold) and polyphenol oxidase IIa (1.7 fold) are involved in the biosynthesis of secondary metabolites, whereas a cytosolic protein, malate dehydrogenase (1.7-fold), is involved in the tricarboxylic acid cycle. Regulatory particle triple-A ATPase 3 isoform 2, partial or 26S protease regulatory subunit 6B homolog (1.6-fold) is involved in the ATP-dependent degradation of ubiquitinated proteins.
Lastly, for instance, a Vacuolar H+-ATPase (spot 3702), that exhibited increased carbonylation at 48 h (1.7-fold), is implicated in the transport of protons against an electrochemical gradient using energy from ATP hydrolysis (Fig. 4B).

3.4.2 Apple proteome and oxi-proteome changes in response to *P. expansum* inoculation

Temporal changes in the abundance of proteins in response to *P. expansum* inoculation at 24 (PE24) and 48 h (PE48), relative to a wounded control were also analysed.
A total of eleven proteins displayed significant (2.0-fold and $P < 0.05$) changes in abundance during the time-course of the study. Seven of the eleven differentially expressed proteins were successfully identified and grouped according to their biological process using Blast2GO software (Table 2, Supplementary Table 2). Only a few changes in abundance were observed at 24 h, and these proteins were associated

with response to stress in the Blast2GO analysis. The proteins that increased in abundance at 48 h are involved in response to stress, whereas proteins that decreased in abundance are involved in metabolism process. Since the categories with considerable enrichment were response to stress and metabolism process, this suggests that these processes play an important role in apple fruit defence response against the compatible pathogen, *P. expansum*.

Two of the seven identified proteins in the analysis of total proteins exhibited increased abundance at 24 h (spots 4004, 5005). The other five proteins (spots 2101, 2104, 4004, 4503, 5005) exhibited increased abundance at 48 h after inoculation, relative to the wounded control, while two proteins (spots 3403, 5804) exhibited decreased abundance at 48 h.

Mal d 1.03D (spot 4004) and Mal d 1.03A (spot 5005) proteins were among the identified proteins. High levels of these two Mal d 1 proteins, relative to the wounded control, were observed during the entire time-course analysed. At 24 h, the abundance of Mal d 1.03D and Mal d 1.03A increased 3.1-fold and 3.8-fold, respectively. Similarly, at 48 h, the abundance of Mal d 1.03D and Mal d 1.03A were 2.7-fold and 3.7-fold, respectively. Other proteins also exhibited increased abundance in *P. expansum*-inoculated tissue at 48 h, indicating that they may be involved in the response of apple fruit to a compatible pathogen, *P. expansum*. These proteins included, a short-chain dehydrogenase/reductase 2b-like (spot 2101), a thiazole biosynthetic enzyme (spot 2104), and a 4-hydroxyphenylpyruvate dioxygenase-like (4503) protein (Table2, Supplementary Table 2). Proteins with significantly reduced abundance (0.5-fold and $P < 0.05$) were also found in this compatible interaction at 48 h, where probable sarcosine oxidase-like (spot 3403) and heat shock protein STI-like (spot 5804) decreased 0.5-fold and 0.4-fold, respectively (Fig. 4C).

In regards to the oxi-proteome, eight protein spots displayed a significant (1.5-fold) increase in carbonylation at 24 h in response to *P. expansum* inoculation. These proteins were successfully identified and grouped according to their biological process using Blast2GO software (Table 3, Supplementary Table 3). Most of the proteins that exhibited increased levels of carbonylation in response to *P. expansum*

inoculation are involved in metabolism processes (Table 3). The eight proteins that were highly carbonylated at 24 h were an actin 7 (spot 1403), an ATP synthase subunit beta, mitochondrial-like (spot 1608), an actin, partial (spot 2404), an apple phosphoglyceromutase (apgm) (spot 4703), a triosephosphate isomerase, cytosolic-like (spot 6001), a ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) (spot 6504), a bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic-like (spot 6702), and a cytosolic aconitase (spot 6901). The actin 7 (2.3-fold), RUBISCO (2.2-fold), and the bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic-like (2.1-fold) were the proteins with the highest level of carbonylation in response to *P. expansum* at 24 h after inoculation (Fig. 4D).

3.4.3 Differences in the changes in the apple proteome and oxi-proteome in response to *P. expansum* and *P. digitatum* inoculation

To compare the non-host pathogen interaction with the compatible pathogen interaction, changes in protein abundance of apple fruit in response to *P. digitatum*- and *P. expansum*-inoculation at 24 and 48 h were analysed using *P. expansum*-inoculated tissue as the control at time point.

Among total proteins, seven exhibited significant differences (2.0-fold and $P < 0.05$) in abundance during the time-course of the study. Three of the seven proteins were successfully identified and grouped according to their biological process using Blast2GO software (Table 2, Supplementary Table 2). Results indicated that these three proteins displayed considerable enrichment in the categories of metabolism and oxidation-reduction processes, suggesting that these processes play a significant role in apple defence response against the non-host pathogen *P. digitatum*. The three proteins that increased in abundance at 48h were polyphenol oxidase 2 precursor (spot 2708 and 3601) and a translation elongation factor EF-Tu precursor (6401). The two spots identified as the same protein most likely indicates the presence of multiple isoforms. The abundance of these three proteins at 48 h increased by 2.2, 2.3 and 2.2-fold, respectively (Fig. 4E).

Ten protein spots displayed a significant increase (1.5-fold) in carbonylation in response to *P. digitatum* inoculation compared with inoculation with *P. expansum* during the time-course of this study. These proteins were successfully identified and grouped according to their biological process using Blast2GO software (Table 3, Supplementary Table 3). Most of the carbonylated proteins identified in this interaction were associated with the category metabolism processes (Table 3).

At 24 h, four proteins exhibited an increased level of carbonylation, whereas six of the ten identified proteins exhibited increased carbonylation at 48 h. The four carbonylated proteins identified at 24 h were ACC oxidase 1 (spot 1203), enolase (spot 4505), polyphenol oxidase IIa (spot 4708), and cytosolic malate dehydrogenase (spot 7201). ACC oxidase 1 (28.2-fold) and polyphenol oxidase IIa (1.8 fold) are involved in the biosynthesis of secondary metabolites. Enolase (3.6-fold) is involved in glycolysis, and malate dehydrogenase (2.0-fold) is involved in the tricarboxylic acid cycle (Fig. 4F). Both of the latter proteins are cytosolic proteins.

The six carbonylated proteins identified at 48 h were ATP synthase subunit beta, mitochondrial-like (spot 1608), ATP synthase beta chain, mitochondrial precursor (spot 2602), glutamine synthetase cytosolic isozyme 1 (spot 4302), glutamine synthetase nodule isozyme-like (spot 4308), enolase (spot 5503) and enolase-like (spot 5508). The enolase (3.9-fold), enolase-like (4.1-fold) and glutamine synthetase nodule isozyme-like (2.3-fold) proteins were exhibited the highest level of carbonylation in response to *P. digitatum* inoculation compared with *P. expansum* inoculation at 48h after inoculation (Fig. 4F).

3.5 GO functional analysis, enrichment, and integration of proteomic data into biological interaction networks

Proteins with quantitative changes in abundance and levels of oxidation were classified into functional categories, according to the three main vocabularies of the gene ontology (GO): biological process, molecular function and cellular component, using Blast2GO software (Conesa *et al.* 2005). Over 26 categories were identified within the

biological process vocabulary for the proteins whose abundance changes after wounding and inoculation (Fig. 5A). In relation to molecular function the most represented categories were 'ion binding' (20.0%), 'oxidoreductase activity' (18.2%), and 'small molecule binding' (12.7%), whereas the most well-represented 'cellular component' categories, with more than 83% of the total annotations, were 'cell part', 'membrane-bounded organelle' and 'organelle part' (Supplementary Fig. 5A-B).

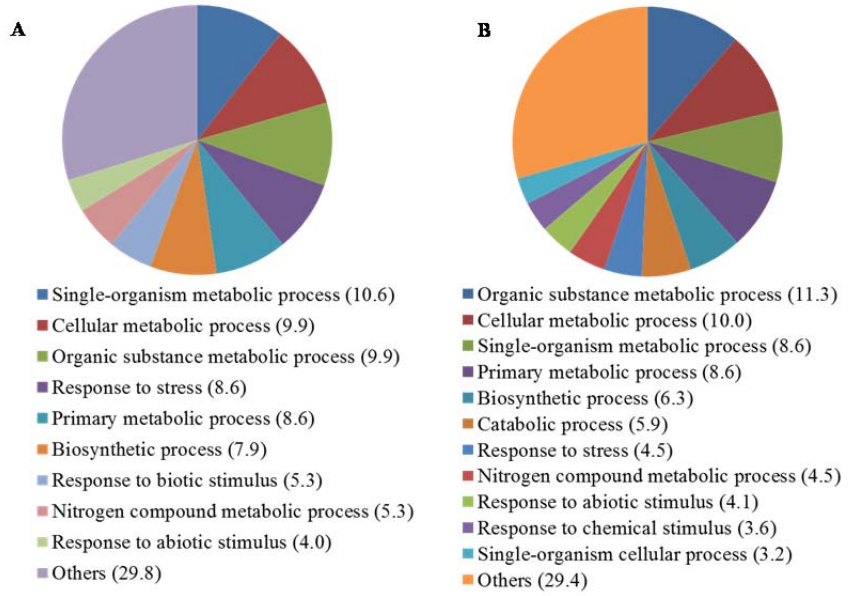

Figure 5. Proteome (A) and oxi-proteome (B) GO terms distribution after abiotic and biotic stresses in 'Golden Smoothee' apple. GO terms distributions according to the Blast2GO software were grouped by biological processes, at the level 3 and filtered by number of Seqs (cutoff = 5) with circle graphs. The numbers on each fraction indicate the percentage of detected proteins belonging to each category.

Over 36 categories were found within the biological process vocabulary for the proteins that exhibited a significant change in their level of oxidation after wounding and inoculation (Fig. 5B). In relation to molecular function, the most highly represented categories were 'ion binding' (20.4%), 'organic cyclic' and 'compound binding' (13.3%), whereas the most represented categories in the cellular component category, with more than 75% of the total annotations, were 'cell part', 'membrane-bounded organelle', 'organelle part' and 'protein complex' (Supplementary Fig. 5C-D).

Due to the hierarchical nature of gene ontology, it is very difficult to establish separate categories for further classification of the proteins identified in the current study. Therefore, proteins with significant changes in abundance or their level of oxidation are listed in Table 2 and Table 3, respectively, according to their principal biological process, encompassed by 'metabolism', 'response to stress' and 'oxidation-reduction processes'.

Ontologizer software was used to gain further insight into the GO distribution of the identified proteins. In this regard, a Benjamini-Hochberg test was performed to examine whether some specific GO terms are over-represented in our experimental conditions. This test enabled the identification of GO categories that were significantly over-represented in wounded and inoculated apples in relation to their respective controls. The total proteomic and oxidative changes in apple, as well as the specific response of individual identified proteins following wounding, and *P. expansum* and *P. digitatum* inoculation, were examined.

GO categories that were over-represented for wound-responsive proteins were 'response to biotic stimulus' (GO:0009607), 'aminoacylase activity' (GO:0004046), 'phosphoglycerate mutase activity' (GO:0004619) and 'glucose catabolic process' (GO:0006007).

GO categories over-represented for proteins responding to *P. digitatum* were 'response to biotic stimulus' (GO:0009607), 'defence response' (GO:0006952) and '4-hydroxyphenylpyruvate dioxygenase activity' (GO:0003868). Whereas, GO categories over-represented for proteins responding to *P. expansum* were 'response to biotic stimulus' (GO:0009607), 'sarcosine oxidase activity' (GO:0008115),

'tetrahydrofolate metabolic process' (GO:0046653), '4-hydroxyphenylpyruvate dioxygenase activity' (GO:0003868) and 'thiamine biosynthetic process' (GO:0009228). Results of the analysis did not indicate any significant over-representation of any GO categories between the proteins that were responsive to *P. expansum* and *P. digitatum*.

The same statistical analysis was applied to the GO terms over-represented in changes in the apple oxi-proteome. Related to the wound-responsive proteins whose oxidation level change, GO categories related to wound-responsive proteins that were over-represented included 'phosphoglycerate kinase activity' (GO:0004618), 'malate metabolic process' (GO:0006108), 'glycolysis' (GO:0006096), 'ribulose-bisphosphate carboxylase activity' (GO:0016984), 'malate dehydrogenase (decarboxylating) (NAD+) activity' (GO:0004471), 'L-malate dehydrogenase activity' (GO:0030060), 'carbon fixation' (GO:0015977), and 'intramolecular transferase activity, phosphotransferases' (GO:0016868). Results of the analysis did not reveal a significant over-representation of any GO categories in response to *P. digitatum* inoculation, whereas GO categories over-represented in proteins response to *P. expansum* inoculation were 'ribulose-bisphosphate carboxylase activity' (GO:0016984), 'hydrogen-exporting ATPase activity, phosphorylative mechanism' (GO:0008553), 'phosphoglycerate mutase activity' (GO:0004619), 'mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)' (GO:0000275) and 'shikimate 3-dehydrogenase (NADP+) activity' (GO:0004764). GO categories that were over-represented in the comparison of proteins that respond to *P. expansum* vs. *P. digitatum* were 'glutamate-ammonia ligase activity' (GO:0004356), 'glutamine biosynthetic process' (GO:0006542), 'hydrogen-exporting ATPase activity, phosphorylative mechanism' (GO:0008553) and 'mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)' (GO:0000275).

To gain further insight into which metabolic pathways are over-represented in our experimental conditions, a hypergeometric test was used. This test enabled the identification of KEGG pathways that were significantly over-represented in wounded and

wounded\inoculated apples. The KEGG metabolic pathways over-represented in global changes in the apple proteome in response to the experimental treatments were 'RNA degradation' (ko03018), 'methane metabolism' (ko00680), 'vitamin B6 metabolism' (ko00750) and 'glycolysis / gluconeogenesis' (ko00010).
The same statistical analysis was used to identify KEGG metabolic pathways over-represented in global changes in the apple oxi-proteome in response to experimental treatments. The results indicated an over-representation of the KEGG metabolic pathways 'carbon fixation in photosynthetic organisms' (ko00710), 'glycolysis / gluconeogenesis' (ko00010), 'glyoxylate and dicarboxylate metabolism' (ko00630), 'citrate cycle (TCA cycle)' (ko00020), 'pyruvate metabolism' (ko00620) and 'proximal tubule bicarbonate reclamation' (ko04964).

3.6 Correlation between gene transcription and protein expression

RT-qPCR was performed to examine the transcript levels of a select number of proteins identified in the proteomic studies (Table 2 and Table 3). The transcriptional levels of six *M. domestica* genes-corresponding to the identified *M. domestica* proteins ACC oxidase 1 (ACO), ascorbate peroxidase (APX), major allergen Mal d 1.03E (MalD1.03E), class I heat shock protein (HSP), translation elongation factor EF-TU precursor, chloroplast (EF-TU) and NAC domain class transcription factor (NAC) were assessed at 0, 24, 48 h and 7 d in wounded, wounded/inoculated and unwounded tissue (Fig. 6). The correlation between mRNA and protein expression was also examined for proteins, such as APX and NAC, whose abundance or oxidation level did not change in response to the experimental treatments. The EF gene was used as a reference gene and for normalization. In general, changes in mRNA abundance at the three time points were not well correlated with changes in the abundance of their corresponding proteins, except for MalD1.03E, where the calculated Pearson correlation value was 0.93 (P-value < 0.001).

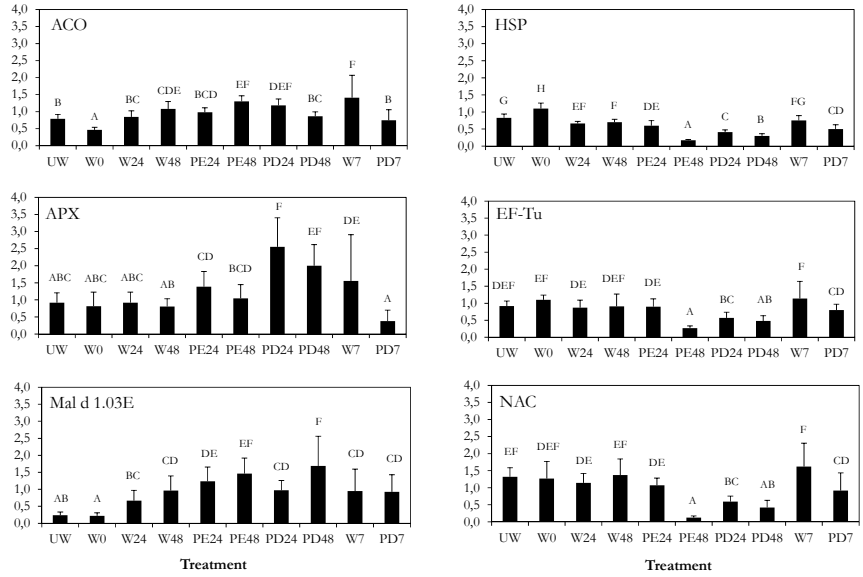

Figure 6. RT-qPCR analysis of genes encoding proteins in spots: 1203 (ACO), 3009 (APX), 3004 (MalD1), 4009 (HSP), 6401 (EF-TU) and 7305 (NAC), in unwounded (UW), wounded (W) and pathogen (PE for *P. expansum* and PD for *P. digitatum*) inoculated samples. Gene expression data were normalized against expression of EF. Values were analyzed by Student's t-test. Different letters indicates statistically significant difference ($P < 0.05$).

4 Discussion

A greater understanding of how apple fruit respond to fungal pathogens is essential for developing and implementing effective strategies for postharvest disease control that do not lie on synthetic chemical fungicides. Therefore, to gain deeper insight into how mature apple fruit respond to a compatible and a non-host pathogen, a proteomic analysis was performed along with select transcriptional studies. In our research, we have focused on identifying resistance mechanisms in apple fruit against a non-host pathogen, such as *P. digitatum* whose infection is suppressed or limited in apples [5]. Since

P. expansum and *P. digitatum* are both wound pathogens, requiring injured tissues as an avenue of ingress, wounded tissue was used as a control at each time point to examine changes in apple (cv. 'Golden Smoothee') proteins in response to *P. expansum* (compatible pathogen) and *P. digitatum* (non-host pathogen). Changes in protein abundance and oxidation specific to the non-host pathogen, *P. digitatum*, were also examined using the data obtained in response to *P. expansum* as a control. Additionally, a major objective of the present study was to identify wound-responsive proteins in 'Golden Smoothee' apple by examining the effect of wounding on temporal changes in protein abundance and levels of oxidation.

The results of the study revealed slightly different changes in the abundance of proteins that responded to the compatible and to the non-host pathogen, suggesting that both apple-pathogen interactions results from fungal-specific communication between the fruit and the pathogen. Changes in protein abundance included a variety of proteins involved in different biological processes, mainly associated with 'metabolism', 'response to stress,' and 'oxidation-reduction processes' (Table 2, Supplementary Table 2). Results on oxidative damage to proteins indicate that important cell functions are compromised in apple after wounding, and in response to compatible and non-host pathogens (Table 3, Supplementary Table 3).

An oxidative burst is one of the earliest and most rapid defence responses that occur in many plant–pathogen interactions [27, 28] and so a significant attention has been placed on characterising oxidative metabolism. Superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) are among the most important proteins responsible for ameliorating oxidative stress [48]. It is noteworthy that none of these proteins showed significant changes in abundance or levels of oxidation in our experimental conditions. However, due to the importance of these proteins in the modulation of ROS levels in fruit, the *APX* gene was examined at transcriptional level. mRNA levels of *APX* were evaluated using RT-qPCR. *APX* transcript abundance was observed at any of the time points after wounding. Transcript abundance in response to the non-host pathogen was significantly higher than in response to the compatible pathogen at 24

and 48 h. Although changes in transcript abundance are good indicators of fruit response to abiotic and biotic stresses, they may not always correlate with protein abundance, as demonstrated in the results of our study.

The current study also provided evidence on the induction of several defence-related proteins, such as pathogenesis-related (PR) proteins. The major allergen Mal d 1 belongs to the PR-10 class of PR proteins [49], and are induced under a range of abiotic and biotic stresses [49]. Three Mal d 1 proteins increased in abundance in response to wounding, predominantly at 48 h, which were Mal d 1 (MDP0000103627), Mal d 1.03A (MDP0000295540) and Mal d 1.06C (MDP0000216907). The wound-induced Mal d 1.03A (4.5-fold), was also induced in response to the compatible (3.7-fold) and non-host pathogen (4.5-fold) at 48 h. However, the highest level of Mal d 1.03A was exhibited in response to *P. digitatum* at 24 h (7.3-fold). Overall suggests that there are some similarities between the apple defence responses to pathogens and abiotic stresses such as wounding. Mal d 1.03D (MDP0000827820) increased in abundance specifically in response to *P. digitatum* and *P. expansum* inoculation. This result indicated that some Mal d 1 proteins are induced commonly by both pathogens suggesting that this protein may be associated with an innate resistance response. Interestingly, Mal d 1.03E (MDP0000295543) was specifically induced in response to *P. digitatum* at 48 h after inoculation, relative to the wounded control. This indicates that Mal d 1.03E may be specifically associated with response of apple to a non-host pathogen and in some measure confer enhanced resistance to *P. digitatum*. Interestingly, none of the Mal d 1 proteins exhibited oxidative modifications in response to the experimental treatments. The expression of the *Mal d 1.03E* gene was characterised with RT-qPCR. Results indicated that the *Mal d 1.03E* gene was induced after wounding and in response to *P. expansum* and *P. digitatum* inoculation. Although, significant differences were found between transcript levels of *P. expansum* and *P. digitatum* with their respective wounded controls at 48 h, *P. digitatum* exhibit the high transcript level. The corresponding Mal d 1.03E protein increased in abundance specifically in response to *P. digitatum*. Transcript and

protein levels of the Mal d 1.03E protein correlated in our experimental conditions. In our study, wounding and inoculation with either pathogen could have induced or increased *Mal d 1.03E* gene expression that, otherwise, would have been expressed at very low levels as indicated by our results for unwounded and wounded tissue at 0 h. These results are in agreement with those obtained by Pagliarani *et al.* [50], where no transcript expression for *Mal d 1.03E* was observed by qPCR in apple fruit cv. 'Florina'. Mal d 1 has been described by other authors as an apple defence-associated protein that is induced in response to abiotic and biotic stresses [18, 51-54]. In particular, Beuning *et al.* [51] and Paris *et al.* [55] reported an induction in *Mal d 1* gene expression in apple leaves in response to *Venturia inaequalis* inoculation. Buron-Moles *et al.* [18] reported that only a portion of the Mal d 1 proteins (such as Mal d 1.03G) increase in abundance in over-mature 'Golden delicious' apple fruit after wounding. Those results suggest that there is a functional specialization present in the different Mal d 1 protein family members and that this may explain why only a portion of the Mal d 1 proteins are wound-induced and others are specifically induced in response to a compatible or a non-host pathogen.

In apple, Mal d 1 is considered a complex, multigene family, which includes at least 20 different genes, mainly clustered on two linkage groups (LGs) 13 and 16 [50, 52, 53]. *Mal d 1.03G* has been recently mapped to LG 16 [56]. Mal d 1 genes have been classified into four subfamilies based on the presence or absence of introns and their length. Our results encompass members from two out four of the groups, specifically *Mal d 1.06*C a member of subfamily III, which contains introns, and *Mal d 1.03A*, *Mal d 1.03D* and *Mal d 1.03E*, members of subfamily IV, which is intronless. *Mal d 1.06*C is located on LG 16, whereas *Mal d 1.03A*, *Mal d 1.03D* and *Mal d 1.03E* are located on LG 13, suggesting that the *Mal d 1* clusters on these two LGs evolved differently [52]. These data support the hypothesis that the presence of many homologous Mal d 1 genes in the apple genome has been conserved during evolution due to their role in signal transduction or in pathogen mediated-selection, considering that the most rapidly evolving families have been associated with pathogen

defence [57]. The role of each Mal d 1 protein in apple response to abiotic and biotic stresses deserves further studies in order to understand their involvement in apple disease resistance and the molecular mechanisms by which Mal d 1 regulates host defence responses.

Two glutamine synthetases were found to be significantly carbonylated at 48 h in response to *P. digitatum* compared with the level of carbonylation in response to *P. expansum*. The oxidised proteins were identified as glutamine synthetase cytosolic isozyme 1 (MDP0000196909) and glutamine synthetase nodule isozyme-like (MDP0000151581). Glutamine synthetases (GS) are enzymes involved in the primary assimilation of inorganic nitrogen, as well as in the reassimilation of free photorespiratory ammonium. Oxidation of both these GS proteins would presumably inhibit their activity and increase their susceptibility to proteolytic degradation [36, 42]. We hypothesize that apple GS might be oxidised to avoid the ability of *P. digitatum* to utilize fruit nitrate as a nitrogen source.

The genome sequences of two Spanish strains of *P. digitatum* have recently become available [58]. Comparative genomics studies by same authors revealed that *P. digitatum* has a remarkable reduction in gene content compared with its close relative but non-phytopathogenic, *P. chrysogenum*. Functional classes of the genes lost in *P. digitatum* have important physiological implications including a loss in the ability to use nitrate as a nitrogen source. Therefore, taking into account that *P. digitatum* has lost its ability to use nitrate as a nitrogen source, we hypothesize that apple fruit might have evolved a defence response that takes advantage of this lack and has develop the ability to oxidise both GS proteins involved in nitrogen assimilation to avoid utilization of nitrate by the fungus. This hypothesis requires further studies to verify that *P. digitatum* has lost homologues of these GS genes and to demonstrate the involvement of apple GS genes in defence mechanisms associated with resistance to this non-host pathogen.

Heat shock proteins (HSPs) play important roles in maintaining plants function when they are exposed to a variety of abiotic and biotic stresses [59]. Data in the present study indicated that four HSPs exhibited significant changes in abundance in response to the

experimental treatments, although none of HSPs exhibited increase levels of carbonylation (evidence of oxidation). Mitochondrial HSO70 2 isoform 1 (MDP0000416692) and a high molecular weight heat shock protein (MDP0000322220) decreased in abundance, while a Class I Heat shock protein (MDP0000323296) increased in abundance after wounding. It is noteworthy that Heat shock protein STI-like (MDP0000422652) was the specific HSP protein which decreased in abundance in response to *P. expansum* at 48 h after inoculation, relative to the wounded control at 48 h. The results also demonstrated that only Class I Heat shock protein increased in abundance after wounding, suggesting that it may be involved in wound response in apple, however, its role in abiotic stress-related responses has not been clearly elucidated. The expression level of the *Class I HSP* gene was characterised using RT-qPCR to further validate its association with apple response to abiotic stress. The resulting data demonstrated that the *Class I HSP* gene was up-regulated after wounding but down-regulated in response to both *P. digitatum* and *P. expansum*. In contrast, the proteomic data indicated a functional specificity for the corresponding Class I HSP protein since it was induced specifically in response to wounding. A recent report documenting apple gene expression in response to *P. expansum* and *P. digitatum* showed that an *HSP101* gene was up-regulated in response to *P. expansum* and an *HSP70* gene was induced by both *P. expansum* and *P. digitatum* [12]. The role of HSPs in response to wounding and pathogens, however, remains to be elucidated.

Elongation Factor-Tu (EF-Tu) plays a pivotal role in protein biosynthesis and response to both abiotic and biotic stresses in plants. Furthermore, EF-Tu proteins possess chaperone activity and play an important role in plant cells in inducing innate immunity [60]. The data revealed a 2.5-fold increase in the abundance of EF-Tu (MDP0000291397) in response to *P. digitatum* at 48 h after inoculation, relative to the abundance in the wounded control at 48 h. More interestingly, this protein also increased 2.2-fold in abundance in response to *P. digitatum* at 48 h after inoculation, relative to the abundance observed in response to *P. expansum* at 48 h. Transcript expression of the *EF-Tu* gene was similar at all-time points after

wounding and was not significantly different to transcript levels in unwounded tissue. In contrast, a significant decrease in transcript abundance was observed in response to *P. expansum* at 48 h after inoculation, relative to transcript levels in wounded controls at 48h. The reduction in the mRNA level of *EF-Tu* in response to compatible pathogen has not been previously reported. A significant, but less marked, decrease in transcript levels of *EF-Tu* was found in response to *P. digitatum* at 24 and 48 h after inoculation, relative to transcript levels in wounded controls at 24 and 48 h. Although *EF-Tu* expression in response to different environmental stresses has been studied in several plant species, in the present study in apple, no evidence was found of abiotic stress-related EF-Tu expression at the proteomic level.

Since transcription factors play an important role both abiotic and biotic stress response in plants [61], additional attempts were made to analyse their response to wounding and wounding/inoculation. Another transcription factor, specifically a NAC domain class protein (MDP0000868419) -without significant changes in abundance or levels of oxidation in our experimental conditions- was also evaluated at the transcriptomic level using RT-qPCR. *NAC* gene exhibited a pattern of expression very similar to *EF-Tu*. Although *NAC* genes have been reported to be induced in response to several postharvest abiotic stresses, including wounding, anoxia, low temperature, and ethylene [62], their role in the regulation of plant defence response against pathogens is less understood [63].

An oxidative burst and ethylene production are part of the innate immunity response of plant cells and which forms the basis for resistance to infection [60]. Two proteins (spots 202 and 1201), both encoding ACO (MDP0000195885), were among the wound-responsive proteins identified to increase in abundance at 24 h. Wound-induced ethylene production, regulated by ACC synthase, has been previously reported to be involved in the generation of ROS [64]. ACC oxidase (ACO) catalyses the last step of ethylene biosynthesis. In this regard, apple ACO genes are part of a multigene family [65], and are regulated by a complex network of developmental and environmental signals responding to both internal and external stimuli

[66]. In the present study, ACO (spot 1203) was observed to be highly oxidised (16.8 and 28.2-fold), and thus presumably inactivated, in response to *P. digitatum* at 24 h after inoculation, relative to the levels evident in either wounded tissue or apple tissue that had been wounded and then inoculated with *P. expansum*. Carbonylation was absent after either wounding or *P. expansum* inoculation. RT-qPCR results indicated slight but statistically significant changes in the abundance of the ACO transcript. *ACO* transcripts increased in response to wounding and in response to *P. expansum* at 48 h after inoculation, but decreased in response to *P. digitatum*.

The results obtained on the oxi-proteome indicated that, in regards to ethylene biosynthesis, ACO was strongly oxidised in response to *P. digitatum* at 24 h after inoculation. This result suggests that oxidation of ACO may reduce ethylene production and respiration rates in apple fruit and consequently decrease metabolic activities, such as protein synthesis. Two other metabolic proteins were found to be oxidised in response to *P. digitatum* at 48 h after inoculation, relative to levels of oxidation observed in response to *P. expansum*. Both were identified as ATP synthase. One of the ATP synthases exhibited increased levels of oxidation in response to *P. expansum* at 24 h after inoculation, relative to levels of oxidation observed in wounded at 24 h. These results suggest that the oxidation of ATP synthase could inhibit proton movement more severely in response to the non-host pathogen than in response to the compatible pathogen. Modulation of plasma membrane H^+-ATPase activity has been previously suggested as a possible molecular switch in several plant resistance responses [67]. In this regard, we hypothesize that the inactivation of apple proteins related to energy metabolism due to their oxidation, may contribute to an energy deficiency that it will differentially impact the ability of *P. expansum* or *P. digitatum* to acquire nitrogen, carbon, and proteins from injured apple tissue. It appears that an important apple defence mechanism against both compatible and non-host pathogens involves the prevention of free nutrient movement, thus starving the pathogen and contributing to resistance by either by stopping or slowing pathogen ingress. Slowing down the penetration process, would provide additional time for the activation of further defence

mechanisms. Additionally, the role of the oxidation of specific proteins as part of an innate immunity response, pathogenicity, and host resistance needs to be further explored.

5 Concluding remarks

To the best of our knowledge, this is the first study conducted in apple fruit that has examined global changes in the apple proteome and oxi-proteome in response to wounding and compatible (*P. expansum*) and non-host (*P. digitatum*) pathogens. The results of the proteomic study indicate a strong interaction between the non-host pathogen and apple relation to both defence responses and metabolic pathways, such as nitrogen, carbon and protein metabolism. The relationship between some of the identified proteins and their transcripts was low. Only the Mal d 1.03E protein was correlated with the level of transcripts of its corresponding gene in our experimental conditions, supporting the general observation that levels of transcription and protein expression usually do not correspond. However, it must be kept in mind that the transcript levels of only a small number of genes were examined.

The present study provided a functional view of the proteins present during temporal host- and non-host pathogen interactions in apple. Additional information was provided on protein oxidation in apples harvested at a commercial stage of maturity in response to compatible and non-host pathogens. Results indicate that oxidation of apple proteins related to energy metabolism may contribute to an energy deficiency that may differentially impact the ability of *P. expansum* and *P. digitatum* to acquire nitrogen, carbon and proteins from infected apple tissues. We hypothesize that an important apple defence mechanism involves the prevention of free nutrient movement which may help to starve the pathogen. This would contribute to resistance, either by stopping pathogen ingress directly or by slowing down the penetration process, thus allowing the fruit to activate further defence mechanisms, such as the induction of PR proteins.

Documenting changes in the oxi-proteome of apple can provide information that can be used to better understand how impaired protein functions may affect apple defence mechanisms. It also

provides new biomarkers for oxidative damage mainly caused by the oxidative response occurring in fruit tissue in response to compatible and non-host pathogens. The molecular and bioinformatics data presented represent the first step towards both a functional genomics and systems biology approach to understanding gene function and the regulatory networks involved in apple innate immunity responses.

Acknowledgments

Authors are grateful to the Spanish Government for its financial support with the project AGL2011-30519-CO3-03, and for the scholarship BES-2009-027752 (G. Buron-Moles). The authors are indebted to Pablo Librado from 'Departament de Genètica. UB' for the bioinformatician support and to Cèlia Sánchez from 'IRTA' for her excellent technical assistance. We also would like to thank Dra. Isabel Sánchez and M. Alba Sorolla from the 'Servei de Genòmica i Proteomica. UdL' for helping on peptide mass fingerprinting and MS-MS. Erik Burchard is acknowledged for his assistance in conducting the RT-qPCR experiments. The authors have declared no conflict of interest.

Appendix II. Supplementary data

Supplementary data associated with this article can be found at Appendix II.

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Discussion

DISCUSIÓN GENERAL

A pesar de la utilización de fungicidas sintéticos, los patógenos *P. digitatum* y *P. expansum* continúan siendo responsables de importantes pérdidas económicas en todo el mundo durante la postcosecha de frutos cítricos y de pepita, respectivamente. Para poder diseñar nuevos métodos de control más efectivos, a la par que menos contaminantes, debemos entender estos patosistemas, teniendo en cuenta, tanto los mecanismos de patogenicidad del hongo como las respuestas de defensa del huésped.

En la presente tesis doctoral nos hemos propuesto abordar este objetivo desde una nueva perspectiva basada en el conocimiento de los factores de virulencia y especificidad de los patógenos, así como de las respuestas de defensa de los frutos, centrándonos principalmente en la explosión oxidativa. Para ello, proponemos una aproximación multidisciplinar que incluye aspectos moleculares, bioquímicos y patológicos.

1. Uso de cepas de *P. digitatum* y *P. expansum* marcadas con la proteína verde fluorescente para el estudio de la interacción huésped-patógeno

El uso de la proteína verde fluorescente o GFP (del inglés Green Fluorescent Protein) ha facilitado el estudio de las interacciones planta-patógeno, habiéndose utilizado tanto como marcador para visualizar el proceso de infección, como para estudiar el patrón espacial de expresión génica y de localización proteica [1]. Existen múltiples ejemplos del uso de la GFP en la localización de hongos fitopatógenos, incluyendo *Fusarium oxysporum* en tomate [2] o *Rosellinia necatrix* en aguacate [3]. Sin embargo, de los pocos ejemplos que existen del uso de la GFP para visualizar el proceso de infección en patógenos de postcosecha destacamos los de *Alternaria citri* en cítricos [4], *Botrytis cinerea* en fresa [5] y *Aspergillus carbonarius* en uva [6].

Para introducir ADN extracelular en cualquier especie fúngica es imprescindible disponer en primer lugar de sistemas de transformación genética. Por tanto, mientras que para *P. digitatum* ya se habría descrito la transformación genética mediada por *Agrobacterium tumefaciens* o ATMT (del inglés *A. tumefaciens*-Mediated Transformation) [7], aún no se habría descrito para *P. expansum*. Fue el grupo del Dr. Luís González Candelas, del Instituto de Agroquímica y Tecnología de Alimentos (IATA) de Valencia, quien por primera vez puso a punto el sistema de transformación ATMT para *P. expansum*.

Por tanto, una vez obtenido un sistema de transformación genética efectivo se procedió a transformar las cepas de *P. digitatum* y *P. expansum* con el gen que codifica la *gfp*, usando la resistencia a la higromicina B como marcador de selección. Sin embargo, el gen original *gfp* no mostró fluorescencia alguna en nuestros transformantes, posiblemente por la diferencia en el uso de codones [8]. Por ello para la transformación de ambos patógenos hubo que utilizar tanto la versión adaptada a plantas (sGFP) como la de mamíferos (eGFP). La variante sGFP posee una mutación (S65T) que permite alcanzar un alto nivel de concentración de proteína GFP y, por tanto, de fluorescencia en los transformantes. La otra variante eGFP es similar a sGFP pero difiere en el aminoácido 64, donde cambia una Phe por una Leu [9]. Ambas versiones han sido utilizadas con éxito en diferentes especies fúngicas [8, 10, 11].

Finalmente y por primera vez, se obtuvieron cepas de *P. digitatum* y *P. expansum* transformadas con las dos variantes de GFP. Entre ellas, y tras el estudio microscópico, se seleccionaron para posteriores estudios los transformantes eGFP-*P. digitatum* y sGFP-*P. expansum* (Figura 1).

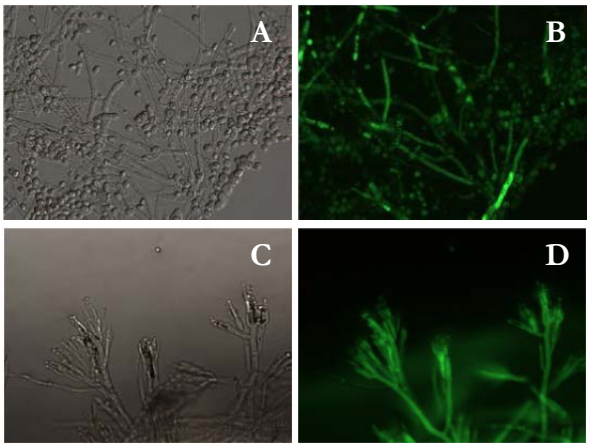

Figura 1. Visualización microscópica de las cepas de *P. digitatum* y *P. expansum* transformadas con eGFP y sGFP, respectivamente. Los paneles A y C corresponden a microscopia de contraste de interferencia diferencial, mientras que los paneles B y D corresponden a imágenes de fluorescencia de micelio, conidios y conidióforos. Aumento x400.

Una vez obtenidos los transformantes (eGFP-*P. digitatum* y sGFP-*P. expansum*) se quiso determinar su utilidad para el estudio de la interacción compatible e incompatible en naranjas y manzanas. Sin embargo, para poder realizar estos estudios, es importante evaluar previamente las características de patogenicidad, esporulación, capacidad de germinación y/o crecimiento de los transformantes que expresan constitutivamente la GFP. En estos estudios es esencial que el comportamiento de las cepas marcadas con GFP sea igual al de las cepas parentales para poder extrapolar los resultados.

Respecto a los resultados de patogenicidad, tanto de *P. digitatum* vs. eGFP-*P. digitatum* en naranjas, como de *P. expansum* vs. sGFP-*P. expansum* en manzanas, fueron similares en incidencia, severidad e índice de esporulación (Figura 2).

Figura 2. Las cepas parentales (A y C) y marcadas con GFP (B y D) de *P. digitatum* y *P. expansum* inoculadas en naranjas y manzanas a los 7 días de incubación a 20 ºC.

Además, nuestros estudios *in vitro* demostraron que la transformación con GFP no modificó ni la capacidad de germinación ni la de crecimiento, tanto en PDA (Figura 3), como en los medios de cultivo OSA y AM.

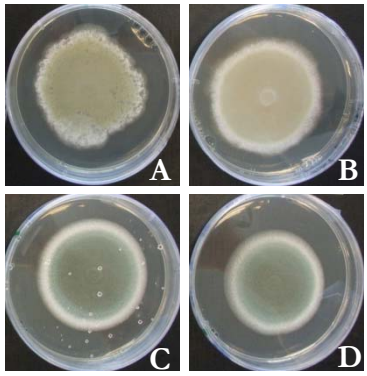

Figura 3. Cepas parentales (A y C) y marcadas con GFP (B y D) de *P. digitatum* y *P. expansum* cultivadas durante 7 días a 25 ºC en PDA.

Los medios de cultivo OSA y AM se utilizaron por tener una composición química bastante similar al de naranja y manzana, respectivamente. Por tanto, la ecofisiología de las cepas transformadas no mostró estar influenciada por el medio de cultivo utilizado, tanto PDA, como OSA o AM.

La temperatura es uno de los factores abióticos más determinantes de la germinación y el crecimiento miceliar. Por tanto, conocer las condiciones óptimas de germinación y crecimiento de las diferentes

especies fúngicas nos permitirá inferir qué especie germinará y crecerá antes y, por consiguiente, cuáles serán las podredumbres que dominarán en postcosecha. En este estudio, además de la temperatura ambiental (25 ºC), hemos evaluado las temperaturas típicas de frigoconservación de naranjas (4 ºC) y manzanas (0 ºC).

Tras 12 horas de incubación en PDA a temperatura óptima (25 ºC), nuestra cepa parental de *P. digitatum* germinó un 85%. Sin embargo, en condiciones de frigoconservación (4 ºC) necesitó 96 horas para alcanzar un porcentaje similar del 77%. En otros estudios se ha observado que las temperaturas de incubación en frío (0 - 4 ºC) también retrasan o inhiben la germinación de *P. digitatum* en OSA [12, 13]. No obstante, el grado de inhibición también depende de la cepa analizada. Por ejemplo, tras 96 horas de incubación en OSA, nuestra cepa de *P. digitatum* mostró valores de germinación hasta tres veces superiores a los mostrados por Plaza *et al.* [12]. Prevalece entonces la idea de que es necesario caracterizar la ecofisiología de cada cepa ya que existe una variación considerable en lo que a capacidad de germinación y/o crecimiento se refiere. Por tanto, no sólo la germinación es importante, sino que la posterior elongación del tubo germinativo y crecimiento miceliar son especialmente relevantes en el proceso infectivo. Por eso, también es interesante conocer las velocidades de crecimiento de los patógenos [14]. En condiciones óptimas de temperatura, *P. digitatum* crece a una velocidad superior que *P. expansum* tanto en PDA (8.3 y 8.0 mm/d, respectivamente), como en OSA (8.8 mm/d) y AM (7.9 mm/d). Sin embargo, a temperaturas de frigoconservación, *P. expansum* crece a una velocidad superior que *P. digitatum* en PDA (0.5 y 0.4 mm/d, respectivamente), pero muy similar en OSA y AM (aproximadamente 0.6 mm/d) (Tablas 1 y 2; Capítulo 1).

En conjunto, nuestros estudios ecofisiológicos indican que *P. digitatum* germina antes y crece más rápido a temperatura ambiente, mientras que *P. expansum* aunque empieza a germinar un poco después, alcanzó valores de germinación del 100% antes que *P. digitatum*. Además, *P. expansum* presentó un crecimiento mayor en PDA a temperatura de

frigoconservación (0 ºC), aunque dicha temperatura sea inferior a la utilizada en el estudio con *P. digitatum* (4 ºC), corroborando que *P. expansum* es un patógeno adaptado a temperaturas de conservación en frío [15].

Existen modelos matemáticos basados en estudios *in vitro* que permiten predecir el crecimiento de *P. expansum* en función de la temperatura y el tiempo de almacenamiento. Sin embargo, los resultados obtenidos *in vitro* son difícilmente extrapolables *in vivo*, puesto que obvian el efecto barrera de la piel del fruto, entre otros aspectos. Aun así, estos modelos son muy útiles ya que ofrecen información sobre el comportamiento de los patógenos [16-18].

Una vez caracterizada la ecofisiología de ambos patógenos, y comprobado que las cepas marcadas con GFP son equivalentes a las cepas parentales, estas se utilizaron para visualizar y comparar el proceso de infección en las interacciones compatibles e incompatibles en naranjas y manzanas. Hasta la fecha, las interacciones naranja-*P. digitatum* y manzana-*P. expansum* se han considerado compatibles porque los patógenos son capaces de superar las barreras de defensa del fruto y provocar infección. Al contrario, las interacciones manzana-*P. digitatum* y naranja-*P. expansum* se han considerado incompatibles porque los patógenos no son capaces de provocar infección. Previamente, otros autores han descrito que *P. expansum* tiene la capacidad de infectar naranjas bajo condiciones de madurez del fruto específicas, así como también que *P. digitatum* sólo es capaz de causar infección limitada alrededor de la zona herida en manzanas [19, 20]. Gracias al uso de nuestras cepas marcadas con GFP, hemos comprobado que tanto *P. expansum* como *P. digitatum* necesitan una vía de entrada, como la herida, para iniciar el proceso infectivo en sus interacciones compatibles. Además, hemos podido corroborar y visualizar *in situ* como *P. expansum* puede causar infección en naranja a madurez comercial (Figura 4), mientras que *P. digitatum* en manzana sólo limita su crecimiento alrededor de la herida (Figura 5).

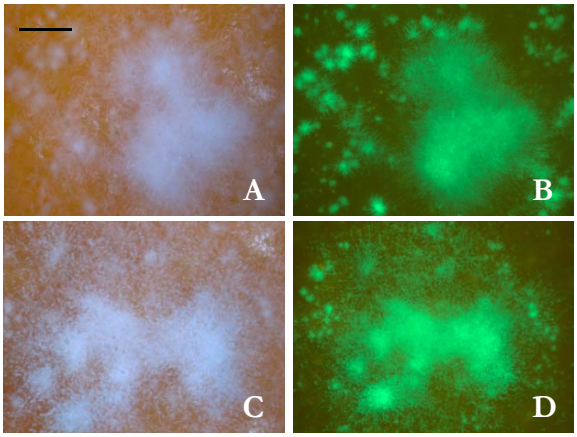

Figura 4. Visualización *in situ* de las cepas de *P. digitatum* y *P. expansum* transformadas con eGFP y sGFP, respectivamente, colonizando heridas en naranja 'Lanelate' tras 96 horas de incubación a 20 ºC. Los paneles A y C corresponden a microscopía de contraste de interferencia diferencial. Los paneles B y D corresponden a imágenes de fluorescencia de micelio, conidios y conidióforos. *P. digitatum* (A), eGFP-*P. digitatum* (B), *P. expansum* (C) y sGFP-*P. expansum* (D). Barra de escala = 2 mm.

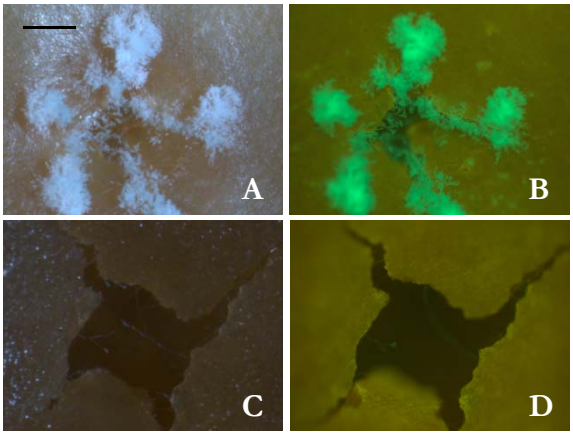

Figura 5. Visualización *in situ* de las cepas de *P. digitatum* y *P. expansum* transformadas con eGFP y sGFP, respectivamente, colonizando heridas en manzana 'Golden Delicious' tras 96 horas de incubación a 20 ºC. Los paneles A y C corresponden a microscopía de contraste de interferencia diferencial. Los paneles B y D corresponden a imágenes de fluorescencia de micelio, conidios y conidióforos. *P. expansum* (A), sGFP-*P. expansum* (B), *P. digitatum* (C) y eGFP-*P. digitatum* (D). Barra de escala = 1 mm.

Por tanto, de aquí en adelante las interacciones manzana-*P. digitatum* y naranja-*P. expansum* las consideraremos interacciones no compatibles, debido a que de algún modo los frutos son capaces de prevenir o limitar significativamente el crecimiento de los patógenos en función de su estado de madurez. Ya que la madurez del fruto influye en estas interacciones, sería importante caracterizar los mecanismos de defensa que subyacen a la resistencia natural de estos frutos contra ambos patógenos.

2. Papel del peróxido de hidrógeno en el proceso de resistencia

En el capítulo 1, hemos visto como *P. digitatum* y *P. expansum* requieren de vías de entrada, como las heridas, para provocar infección en sus respectivos huéspedes. Por tanto, las lesiones causadas durante la cosecha y el manejo en postcosecha deberían reducirse al máximo, ya que estos patógenos las utilizan para superar parte de las defensas del fruto. Por su parte, el fruto utiliza como mecanismo de defensa la explosión oxidativa contra los patógenos. Tradicionalmente, las interacciones compatible y no compatible se han investigado a través del sistema ERO o ROS (del inglés Reactive Oxygen Species). La producción de ROS, en muchas interacciones planta-patógeno, además de tener un efecto tóxico a nivel local, también desempeña múltiples funciones de señalización, induciendo diversas respuestas celulares. Sin embargo, el papel que juegan las ROS restringiendo el crecimiento de ciertos hongos en la fruta no está completamente esclarecido [21].

Debido a la dificultad en la cuantificación directa de ROS en material vegetal intacto, los estudios que monitorizan la producción de ROS a lo largo del tiempo se han limitado a células de *Arabidopsis* (u otros materiales vegetales) en suspensión. Diversos autores han estudiado el papel del peróxido de hidrógeno (H_2O_2) en cultivos celulares vegetales como defensa en respuesta a patógenos [22, 23]. En el caso de cultivos celulares de manzana, se ha descrito un aumento brusco en la cantidad de H_2O_2 inmediatamente después de añadir el patógeno (*P. expansum*),

un aumento que recordaría una respuesta típica de fase I [24]. A pesar de las ventajas de utilizar un sistema de suspensión celular - cultivo/patógeno- como modelo de interacción planta-hongo, éstos no pueden sustituir a los estudios de sistemas biológicos intactos.

En esta tesis nos hemos centrado en los cambios en los niveles de H_2O_2 tanto *in vitro*, como *in vivo*. En concreto, para evaluar el papel del H_2O_2 en la resistencia del fruto se estudió: (i) su efecto *in vitro* en la ecofisiología de *P. digitatum* y *P. expansum*, y (ii) su producción *in vivo* en manzana y naranja en respuesta a un estrés abiótico (herida) y biótico (patógeno compatible y no compatible), a diferentes estados de madurez (inmadura, comercial y sobremadura).

2.1 Efecto del estrés oxidativo -H_2O_2 como tratamiento- sobre los patógenos

En ensayos i*n vitro*, hemos analizado el efecto antifúngico en el proceso de germinación tras aplicar concentraciones crecientes de H_2O_2, algunas de ellas similares a las encontradas en fruta. Además, ya que *P. digitatum* y *P. expansum* son patógenos de postcosecha, y pueden producir podredumbre en naranjas y manzanas, respectivamente, bajo condiciones de almacenamiento en frío, también estudiamos el efecto de la frigoconservación.

En concordancia con lo observado en otras cepas de *P. digitatum* y *P. expansum* (Capítulo 1), los resultados obtenidos en esporas sin tratar (control) muestran que el porcentaje máximo de germinación se alcanza antes en *P. expansum* que en *P. digitatum*, para ambas temperaturas (ambiental y frigoconservación). Por ejemplo, tras 8 horas de incubación en PDA a temperatura ambiental (25 ºC), *P. expansum* germinó un 74%, mientras que -para alcanzar ese porcentaje- *P. digitatum* necesitó más de 13 horas. Este mismo fenómeno se observó en condiciones de frigoconservación, donde *P. expansum* germinó un 74% tras 96 horas de incubación a 0 ºC, mientras que *P. digitatum* necesitó 116 horas de incubación a 4 ºC para germinar aproximadamente un 70%. Una vez más, *P. expansum*

muestra mayor tolerancia al frío, lo que junto a su rápida germinación lo convierten en un patógeno de difícil control, en las cámaras de conservación de fruta.

En cuanto a los tratamientos *in vitro* de concentraciones crecientes de H_2O_2, observamos una relación directa entre el aumento de H_2O_2 y la disminución en la germinación de ambos patógenos tanto a temperatura ambiental, como de frigoconservación. En concreto, *P. expansum* se mostró más susceptible que *P. digitatum* a concentraciones elevadas de H_2O_2 (200 mM), especialmente a 25 ºC. Nuestros valores de germinación de *P. digitatum*, tras el tratamiento con H_2O_2 (200 mM), son mayores que los previamente descritos por otros autores, los cuales muestran como una concentración menor de H_2O_2 (100 mM) tuvo un efecto letal sobre la germinación de este hongo a 28 ºC [25]. Estas diferencias en el porcentaje de germinación podrían deberse, por ejemplo, al uso de cepas distintas en ambos estudios, a los tres grados de diferencia en la temperatura de incubación, y/o al tiempo en que el patógeno está sometido a estrés oxidativo. En cualquier caso, estudios recientes han descrito que *P. expansum* puede ser extremadamente susceptible al estrés oxidativo [26], corroborando nuestros resultados.

2.2 Caracterización de la producción de H_2O_2 como respuesta de defensa a un patógeno compatible y a uno no compatible, en manzanas y naranjas, a diferentes estados de madurez

La primera vez que se observó que las ROS intervenían en la defensa contra hongos fue en 1983, concretamente en patata infectada por *Phytophthora infestans* [27]. Más tarde, la acumulación de H_2O_2 en respuesta a herida o ataques fúngicos se ha estudiado en diversos frutos, como aguacate [28], manzana [29-31], naranja [32] y limón [33]. De hecho, la posible participación del H_2O_2 en la resistencia de manzanas a *P. expansum* fue demostrado por Torres *et al.* [31].

Los frutos pueden activar diferentes mecanismos de defensa en respuesta a herida o a un ataque patógeno. Sin embargo, dichos mecanismos son dependientes del estado de madurez del fruto, lo que va a determinar su susceptibilidad a sufrir daños mecánicos y/o infecciones [19, 20, 30, 31, 34, 35]. Posiblemente, ésto es debido a que las resistencias pasivas o constitutivas del fruto (cutícula, piel, *etc.*) se van degradando durante la madurez, además de reducir la capacidad de resistir a un ataque patógeno (resistencias activas o inducidas). Precisamente por este motivo nuestro estudio se realizó a diferentes estados de madurez.

En general, nuestros resultados indican que el estado de madurez en manzanas 'Golden Smoothee' y naranjas 'Valencia' aumentó la susceptibilidad a sufrir infecciones por patógenos compatibles, y por patógenos no compatibles; hay que destacar la reducción de la resistencia de manzana sobremadura en respuesta a *P. digitatum* (Figura 6).

A pesar de las evidencias que vinculan la producción de H_2O_2 y la defensa del fruto, todavía no se conoce con claridad esta relación. Por tanto, para discernir el papel del H_2O_2 en respuesta a estrés abiótico (herida) y biótico (patógeno compatible y no compatible), en el presente trabajo nos propusimos caracterizar la producción de H_2O_2 a diferentes estados de madurez en manzanas y naranjas, con el fin de establecer su posible relación con la defensa de estos frutos.

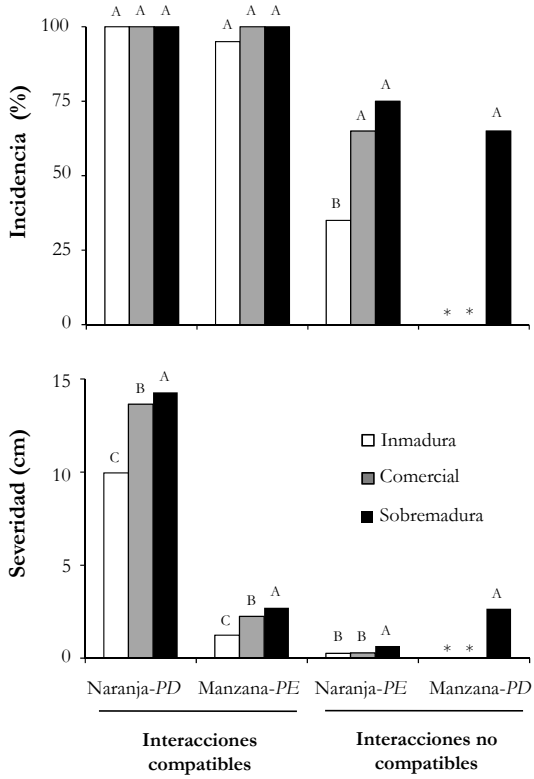

Figura 6. Influencia del estado de madurez de los frutos en la incidencia (panel superior) y severidad (panel inferior) a los 7 días, tanto en las interacciones compatibles como en las no compatibles. Cada columna representa la media de 20 frutos. Letras diferentes entre cosechas indican diferencias significativas entre las medias usando el test LSD (del inglés Least Significant Difference) ($P < 0.05$). * No hubo desarrollo de podredumbre.

Cuantificación de H_2O_2 en manzanas

La metodología utilizada en la cuantificación de H_2O_2 en manzana se adaptó de Torres *et al.* [31]. Los valores de H_2O_2 cuantificados en manzana en los tres estados de madurez oscilan entre un mínimo de 30 y un máximo de 118 μmol/kg Peso Fresco (PF).

Sólo en manzanas inmaduras, se observó una producción de H_2O_2 bifásica inducida por daño mecánico y, en menor medida, por las inoculaciones con *P. expansum* y *P. digitatum*. Esto confirma el hecho de

que la producción de H_2O_2 tiene un papel importante en las reacciones de resistencia de la manzana, y los resultados están de acuerdo con otros trabajos que describen una segunda fase sostenida que se suele asociar con el establecimiento de las defensas [36]. En nuestro estudio comparamos la producción de H_2O_2 en tejido inoculado con *P. expansum* y *P. digitatum*, con el tejido herido e inoculado con agua (control). Cabe destacar que en este trabajo no utilizamos tejido sano como control. Por un lado porque los patógenos utilizados en este estudio necesitan heridas para provocar infección. Por otro lado, resultados previos del grupo demostraron como la producción de H_2O_2 del tejido sano a lo largo del tiempo varía muy poco [31].

Nuestros resultados muestran valores de H_2O_2 inferiores en fruta inoculada con el patógeno compatible (*P. expansum*) y con el no compatible (*P. digitatum*), en comparación al control (tejido herido). Estos resultados observados, tanto a cosecha inmadura como en comercial, sugieren que ambos patógenos deben poseer mecanismos implicados en la supresión del H_2O_2. Sin embargo, aún no se han descrito los mecanismos a través de los cuales, *P. expansum* y *P. digitatum*, podrían detoxificar el H_2O_2 en manzana. La posible presencia de enzimas detoxificantes como la catalasa (CAT) -enzima degradadora de H_2O_2-, no se ha descrito para ninguno de los dos patógenos. Además, hemos visto como en cosecha sobremadura la inoculación con *P. digitatum* mostró valores de H_2O_2 superiores al control, siendo la única cosecha en la que el patógeno no compatible (*P. digitatum*) desarrolló podredumbre (Figura 6). Por tanto, es posible que *P. digitatum* no sólo posea mecanismos para degradar el H_2O_2 sino que a su vez sea capaz de producirlo. La presencia de ambos patógenos en un mismo huésped les obliga a competir entre ellos por los mismos recursos. Por tanto, aquel que supere antes las respuestas de defensa del fruto colonizará el tejido más rápido.

Recientemente, en un estudio en manzanas infectadas con *P. expansum* se determinó que los ácidos orgánicos mayoritarios fueron el glucónico seguido del fumárico [37]. El ácido glucónico lo produce *P. expansum*, y se ha obervado que un mutante deficiente en este ácido

mostró ser menos virulento en las variedades de manzana 'Golden Delicious' y 'Granny Smith' [38]. Así como otros ácidos orgánicos han mostrado cierta capacidad para suprimir el H_2O_2 en cítricos, como los ácidos oxálico, cítrico y ascórbico [33], el mecanismo por el cual el ácido glucónico suprime la producción de H_2O_2 en manzana no está claro. Debido a que ambos patógenos han detoxificado el H_2O_2 producido en la explosión oxidativa del huésped -manzanas a cosecha inmadura y comercial-, podemos otorgarle al H_2O_2 un papel fundamental en la defensa del fruto. Sin embargo, el papel que este pueda tener en la defensa del fruto deberá establecerse en futuros estudios, ya que parece desempeñar una función señalizadora, más que antifúngica.

Cuantificación de H_2O_2 en naranjas

La metodología utilizada en la cuantificación de H_2O_2 en naranja fue la descrita por Torres *et al.* [32], pero con modificaciones. Los valores de H_2O_2 cuantificados en naranja en los tres estados de madurez oscilan entre un mínimo de 86 y un máximo de 362 μmol/kg PF, observando muy pocas diferencias entre fruta herida e inoculada con los patógenos compatible (*P. digitatum*) y no compatible (*P. expansum*). Es decir, la respuesta de la naranja en lo que a producción de H_2O_2 se refiere fue bastante similar tanto en respuesta a un estrés abiótico como biótico. Acorde con nuestros resultados, se ha descrito un comportamiento similar en naranjas 'Valencia Late' a madurez comercial. Es decir, el nivel de H_2O_2 no mostró grandes cambios bajo diferentes tipos de estreses [32]. Por tanto, parece que las diferencias observadas en nuestro estudio respecto al nivel de H_2O_2 acumulado tuvieron más que ver con el estado de madurez del fruto que con el tipo de estrés sufrido. Sin embargo, otros autores obtuvieron resultados contradictorios en cítricos. Estas diferencias pueden deberse a que las metodologías de detección del H_2O_2 utilizadas en ambos estudios fueron distintas. Estos autores mostraron, mediante microscopía confocal de fluorescencia, como *P. digitatum* fue capaz de suprimir la producción de H_2O_2, mientras que *P. expansum* desencadenó su acumulación masiva [33]. Se han descrito algunos compuestos que

juegan un papel importante en la patogenicidad de *P. digitatum* y parecen ser los responsables de la supresión de la producción del H_2O_2 en cítricos [33, 39]. Por un lado, se ha observado como el ácido cítrico suprime la producción de H_2O_2 en el huésped [33]. Por otro lado, la aplicación exógena de CAT en el lugar de la herida es suficiente para alterar las defensas del huésped, haciendo al fruto susceptible al patógeno no compatible, *P. expansum* [39]. Otro ácido orgánico, como el ácido oxálico producido por *P. expansum* en condiciones *in vitro* [40], también se ha descrito como un inhibidor de la producción de H_2O_2, y en consecuencia aumenta su virulencia e infecta cítricos [33].

Por último, destacar que en naranjas sobremaduras encontramos los valores máximos de producción de H_2O_2 (350-360 μmol/kg PF), concretamente a las 4 horas y tras sufrir ambos estreses (abiótico y biótico). Curiosamente, también en cosecha sobremadura observamos a las 24 horas una brusca reducción en la producción de H_2O_2 (86-120 μmol/kg PF). En conjunto, estos resultados parecen atribuirle al H_2O_2 un papel menos relevante en naranja, en lo que a respuesta de defensa se refiere, en comparación con lo descrito en manzanas.

Detección de H_2O_2 por tinción en manzanas y naranjas

La tinción *in situ* del H_2O_2 se puede conseguir utilizando compuestos como el DAB (3,3´-diaminobenzidine tetrahydrochloride), considerándolo un indicador cualitativo de la generación de H_2O_2. En este método, cuando el DAB entra en contacto con el H_2O_2, polimeriza dando lugar a una coloración marrón rojiza [41]. Aunque la tinción *in situ* del H_2O_2 mediante DAB se ha utilizado ampliamente en plantas [41-45], prácticamente no existen estudios realizados en frutos. El trabajo de Macarisin *et al.* [33], es uno de los pocos ejemplos en frutos, donde describen en limón que *P. expansum* provoca una acumulación significativa de H_2O_2 en el punto de inoculación, mientras que *P. digitatum* inhibe la producción de H_2O_2 respecto a las heridas inoculadas con *P. expansum* o agua.

Precisamente, en nuestro estudio hemos utilizado la misma tinción *in situ* del H_2O_2 con DAB descrita por Macarisin *et al.* [33].

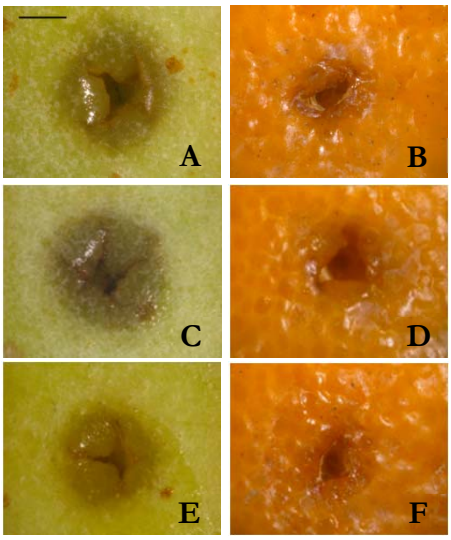

Figura 7. Visualización *in situ* de la producción de H_2O_2 mediante la tinción por DAB en manzanas 'Golden Smoothee' y naranjas 'Valencia' comerciales. La tinción marrón rojiza indica acumulación de H_2O_2 en respuesta a herida (A y B), e inoculación con *P. expansum* (C y D) o *P. digitatum* (E y F). Lupa Leica MZ16F. Barra de escala = 2 mm.

En general, nuestros resultados mostraron que el H_2O_2 se produce localmente en respuesta a un estrés abiótico (herida) y biótico (patógeno compatible y no compatible) tanto en manzana como en naranja (Figura 7). La visualización de la tinción con DAB fue más evidente (más intensa) en frutos inmaduros y comerciales, y disminuyó con el estado de madurez en ambos frutos.

En definitiva, por un lado, hemos visto como *P. digitatum* siendo más resistente al H_2O_2 *in vitro*, es incapaz de infectar manzanas inmaduras y a madurez comercial, a pesar de presentar niveles más bajos de H_2O_2. Por otro lado, también hemos visto como *P. expansum* siendo más susceptible al H_2O_2 *in vitro*, es capaz de producir infección en naranjas en los tres estados de madurez estudiados, especialmente en sobremadura.

En conjunto, los resultados de nuestro trabajo sugieren que el H_2O_2 tiene un papel fundamental en la defensa del fruto climatérico (manzana), mientras que parece ser menos importante en el fruto no climatérico (naranja). El papel que éste pueda tener en la defensa del fruto deberá establecerse en futuros estudios, ya que parece desempeñar una función señalizadora, más que antifúngica.

Por tanto, una vez caracterizado el efecto directo del H_2O_2 tanto *in vitro* como *in vivo*, nos propusimos abordar el estudio de uno de nuestros patosistemas (manzana-*P. expansum* y manzana-*P. digitatum*) mediante una aproximación molecular.

3. Proteómica como aproximación para estudiar la respuesta del fruto a un estrés abiótico y biótico

Actualmente existe una enorme cantidad de información genómica como consecuencia del abaratamiento de costes a la hora de secuenciar cualquier organismo. Para manejar esta información se requieren aproximaciones que incluyan estudios de cambios globales en la expresión génica (transcriptómica), proteica (proteómica) y metabólica (metabolómica). Estas aproximaciones proporcionan información básica que al integrarse permitirá conocer el funcionamiento de las plantas y su interacción con el medio [1]. Hasta la fecha, se ha estudiado el nivel de expresión del ácido ribonucleico mensajero (ARNm), mediante técnicas como los *microarrays* o la reacción en cadena de la polimerasa con transcriptasa inversa o RT-PCR (del inglés Reverse Transcription Polymerase Chain Reaction). Sin embargo, entre el ARNm y los niveles de expresión de proteínas existe una correlación moderada, debido a los mecanismos de regulación y procesado que se dan con posterioridad a la transcripción [46-48]. Además, el análisis a nivel de transcrito (o ARNm) no considera las modificaciones postraduccionales o PTMs (del inglés Post-translational Modifications), como tampoco la interacción entre proteínas, lo que realmente determinará al final su actividad [49]. Por tanto, las técnicas proteómicas, presentan una gran ventaja respecto a

otras técnicas de análisis masivo, puesto que trabajan directamente con el efector final, la proteína.

Optimización de la extracción proteica en manzana 'Golden Delicious'

Aunque la proteómica ha avanzado mucho en los últimos años, existen todavía limitaciones técnicas. Por ejemplo, la extracción proteica continúa siendo una etapa clave en cualquier estudio proteómico. Las células vegetales presentan ciertos inconvenientes específicos en el momento de extraer sus proteínas. Por un lado, cuentan con una baja concentración proteica, y por otro lado, contienen proteasas y otros compuestos (polifenoles, sales, polisacáridos, lípidos y ácidos nucleicos) que interfieren en la extracción de las proteínas. Estos compuestos si no se eliminan correctamente pueden llegar a interferir en las etapas posteriores de la separación proteica. Actualmente, no existe un método de extracción universal que permita obtener todas las proteínas con buena calidad y cantidad. En el caso de material vegetal, el más utilizado es la precipitación con ácido tricloroacético (TCA)-acetona [50]. Pero también existen otros métodos de extracción por precipitación en los que se utiliza el fenol como solubilizante proteico para posteriormente precipitar con acetato amónico y metanol. La combinación de extracción con fenol y precipitación con TCA-acetona se ha utilizado en tejidos recalcitrantes, como las hojas de diversas plantas dando buenos resultados [51, 52].

Debido a la ausencia en la literatura -en el momento de realización de esta tesis- de un protocolo de extracción proteica específico para manzana nos propusimos optimizar el método, a fin de obtener una concentración suficiente y libre de compuestos que pudieran afectar a la posterior separación de las proteínas. Para ello, utilizando muestras de manzana 'Golden Delicious' comparamos dos métodos de precipitación proteica: (i) acetato amónico en metanol y (ii) DTT/Acetona. Asimismo, determinamos las condiciones idóneas para la separación de las proteínas mediante electroforesis bidimensional, el

rango de pH, y la concentración proteica a cargar en los geles, entre otros parámetros.

Para obtener las imágenes de los geles bidimensionales de la Figura 8 se cargaron tiras de 18 cm (pH 4-7) con 200 μg de proteína. Cabe destacar que la extracción fenólica seguida de la precipitación con DTT/Acetona fue el método con el que se obtuvieron mejores resultados a nivel de concentración y pureza proteica (Figura 8).

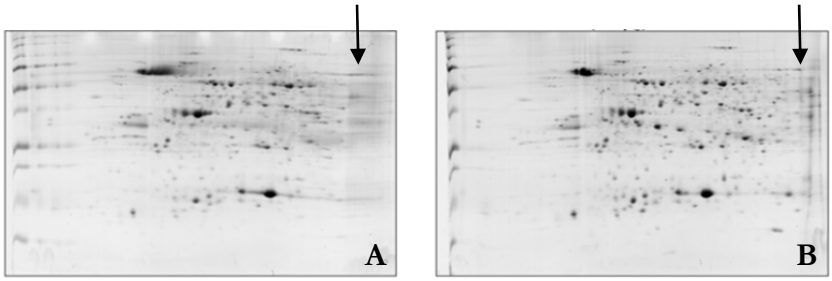

Figura 8. Geles 2D representativos que muestran las proteínas totales extraídas de piel y pulpa de manzana 'Golden Delicious' usando acetato amónico en metanol (A) y DTT/Acetona (B), como métodos de precipitación. Tiras de pH 4-7 con 200 μg de proteína.

Debido a que más allá del límite del rango de pH utilizado (4-7) todavía quedaban proteínas por separar -tal y como indican las flechas de la Figura 8- se decidió aumentar el rango de pH a 3-10. Además, con la intención de mejorar la separación y evitar agrupaciones proteicas en los geles, ya que podrían complicar el posterior proceso de identificación, se redujo la concentración a cargar a 120 μg (Figura 9). En ambos casos, el método de tinción utilizado fue el de azul de coomassie G-250 (Figuras 8 y 9).

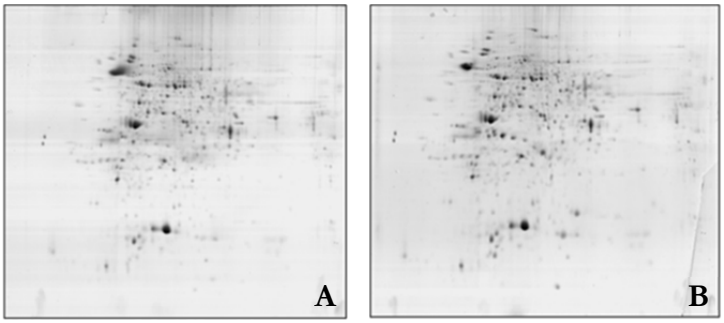

Figura 9. Geles 2D representativos que muestran las proteínas totales extraídas de piel y pulpa de manzana 'Golden Delicious' usando acetato amónico en metanol (A) y DTT/Acetona (B), como métodos de precipitación. Tiras de pH 3-10 con 120 μg de proteína.

El protocolo optimizado para 'Golden Delicious' con el que obtuvimos los mejores resultados -en lo que a calidad y cantidad proteica se refiere- fue la extracción fenólica seguida de la precipitación con DTT/Acetona, usando tiras de pH 3-10 cargadas con 120 μg de proteína (Figura 9). Por tanto, este protocolo de extracción proteica optimizado para manzana solventará este paso crítico en cualquier estudio proteómico futuro.

4. Cambios en la abundancia proteica en manzanas 'Golden Delicious' en respuesta a herida

Gran parte de los estudios de proteómica vegetal se han descrito en *Arabidopsis thaliana* [53] o *Oryza sativa* [54] y, en menor medida en especies de cultivo con importancia agronómica como *Zea mays* (maíz), *Nicotiana tabacum* (tabaco), *Pisum sativum* (guisante), *Triticum aestivum* (trigo), *Hordeum vulgare* (cebada), *Glycine max* (soja), *Solanum lycopersicum* (tomate) y *S. tuberosum* (patata). Muchas veces el éxito de estos estudios depende de la disponibilidad de la información genómica en las bases de datos del organismo a estudiar [55]. Por lo que respecta a los frutos, los pocos trabajos que utilizan aproximaciones proteómicas se han centrado en estudiar el proceso de la maduración. Una revisión actual de Palma *et al.* [56] nos da idea de los múltiples trabajos publicados en

este tema por ejemplo en tomate [57], uva [58], cítricos [59, 60], papaya [61, 62], mango [63], banana [64], además del recientemente publicado en manzana [65]. Por tanto, aparte de los estudios proteómicos de maduración y de postcosecha, como los centrados en daños causados por frío [66], no existen hasta la fecha estudios relacionados con la respuesta específica de los frutos a un estrés abiótico como la herida. Es por ello que, una vez optimizado el protocolo de extracción proteica, nos planteamos hacer la primera descripción de los cambios en el proteoma de 'Golden Delicious' en respuesta a herida.

Básicamente, la aproximación proteómica utilizada en nuestro estudio consistió en separar las proteínas por electroforesis bidimensional y analizarlas mediante espectrometría de masas [67]. El rango de pH utilizado fue de 3-10 en tiras de pH de 18 cm. La electroforesis desnaturalizante se realizó en geles de poliacrilamida al 12% (SDS-PAGE). Una vez separadas las proteínas, los geles se tiñeron con azul de coomassie G-250. El análisis comparativo o diferencial de las imágenes de los geles bidimensionales mediante el *software* específico PDQuest 2D indicó que existían diferencias significativas en los perfiles proteicos entre fruta herida a 24 y 48 horas, respecto al control. Se marcaron para su posterior digestión con tripsina e identificación por espectrometría de masas o MS (del inglés Mass Spectrometry), tanto las proteínas que no cambiaron su abundancia como las que mostraron cambios cuantitativos en respuesta a herida en los tiempos estudiados. La espectrometría de masas tipo MALDI-TOF (del inglés Matrix Assisted Laser Desorption Ionization-Time of Flight) nos proporciona la 'huella peptídica' o PMF (del inglés Peptide Mass Fingerprinting) de las proteínas. Sin embargo, muchas veces la identificación de las proteínas por PMF se hace muy difícil, debido básicamente a la falta de información en las bases de datos o a la mezcla de varias proteínas en la mancha seleccionada para la identificación. Por tanto, aquellas proteínas que no se pudieron identificar por PMF se identificaron mediante fragmentación de los péptidos obteniendo la secuencia de aminoácidos correspondiente. Para la segunda fragmentación o secuenciación *de novo* se utilizó la

ionización tipo MALDI (MALDI TOF-TOF o MALDI MS/MS). Identificamos tanto las proteínas que no modificaron su abundancia como las específicas de respuesta a herida en manzana 'Golden Delicious'.

Proteínas que no modificaron su abundancia en respuesta a herida

Debido a la escasa información en referencia al proteoma de 'Golden Delicious' nos propusimos identificar también aquellas proteínas comunes a los tres tiempos de estudio (0, 24 y 48 horas), las cuales no modificaron su abundancia significativamente en respuesta a herida. Tras el análisis de imagen de los geles bidimensionales, se detectaron aproximadamente 250 manchas proteicas (en inglés, y a partir de ahora, spots) presentes en cada uno de los geles, de las cuales 64 -las mejor resueltas- se recortaron para su posterior identificación por MS. En la Figura 10 se pueden observar dos geles representativos donde se aprecia la resolución de los mismos.

Las proteínas de interés se identificaron mediante comparación de los patrones de huella peptídica o secuencia aminoacídica obtenidos mediante MS con los anotados en las bases de datos de ADN genómico y de ESTs. De las 64 proteínas comunes a los tres tiempos de estudio, 58 de ellas se pudieron identificar bioinformáticamante (Tabla 1; Capítulo 3). Como ya hemos comentado anteriormente, el éxito de cualquier estudio proteómico depende en gran medida de la disponibilidad de información genómica sobre el organismo a estudiar. Y es gracias a que el genoma de la manzana está actualmente secuenciado y anotado [68, 69], por lo que se han logrado identificar, en este trabajo, un número bastante elevado de proteínas pertenecientes al género *Malus* (Tabla 1; Capítulo 3).

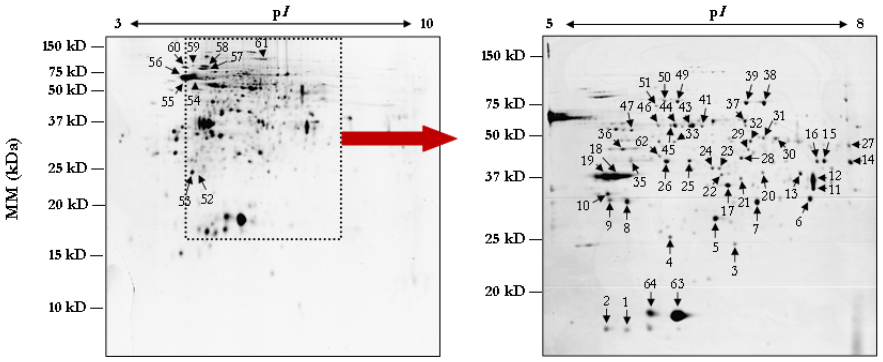

Figura 10. Geles 2D representativos que muestran las proteínas totales extraídas de piel y pulpa de manzana 'Golden Delicious' 48 horas después de la herida. Aparecen marcadas con una flecha aquellas proteínas comunes -que no variaron su abundancia- a los tres tiempos de estudio (0, 24 y 48 horas) para su identificación posterior por PMF o MS/MS. Números referentes a la Tabla 1 y Tabla suplementaria 1 del capítulo 3.

Con el fin de agrupar las proteínas identificadas, utilizamos los tres principales vocabularios de la ontología génica o GO (en inglés Gene Ontology): componente celular, función molecular y proceso biológico, con la ayuda del *software* Blast2GO [70]. Estas proteínas que no modificaron su abundancia en respuesta a herida se clasificaron de acuerdo al proceso biológico en el que mayoritariamente están implicadas: 'procesos metabólicos', 'respuesta a estrés' y 'procesos de oxidación-reducción'.

Las proteínas asignadas al grupo de 'respuesta a estrés' merecen especial atención porque aunque muchas de ellas han sido descritas en otros estudios como proteínas de respuesta a diferentes tipos de estrés, en nuestro estudio no han variado su abundancia.

Por ejemplo, las Mal d, son proteínas relacionadas con patogénesis que pertenecen a la clase PR-10 (en inglés Pathogenesis-Related). Aunque se han descrito previamente como proteínas inducidas tanto por estrés como por patógeno [71-73], podemos ver en la Tabla 1 del capítulo 3 como los spots 1, 2, 63 y 64, que corresponden a proteínas Mal d 1, no variaron su abundancia. Las variaciones que se dieron en las masas moleculares (MM) y puntos isoeléctricos (p*I*) experimentales de cada una de estas Mal d 1, sugieren que son diferentes isoformas y esto podría deberse a las modificaciones postraduccionales que se dan durante su síntesis.

Otros ejemplos de proteínas previamente relacionadas con el estrés incluyen: las proteínas de choque térmico, más conocidas por su denominación inglesa 'heat shock proteins' (HSP), como la HSP de alto peso molecular (spot 57), Hsp81-2 (spot 59) y HSP 70-1 cloroplástica (cpHsc70-1) (spot 60), las dehidrinas (DHNs) (spots 8, 9, 54, 55 y 56), así como también las proteínas relacionadas con la desecación (spots 11 y 12). Aunque todas ellas se han descrito como proteínas de respuesta a un estrés abiótico como el frío [74, 75], y que varían en función de la madurez del fruto [59, 76], en nuestro estudio ninguna de ellas mostró cambios en su abundancia en respuesta a herida a lo largo del tiempo estudiado. Esto podría deberse o bien al estado de madurez del fruto (demasiado maduro), donde las resistencias constitutivas son menos efectivas debido al propio proceso de maduración, o bien a que la respuesta de estas proteínas a la herida sea a corto plazo.

Proteínas que modificaron su abundancia en respuesta a herida

Las buenas prácticas del manejo de fruta tanto en campo como en central recomiendan evitar al máximo los daños mecánicos, como las heridas, para reducir al máximo las vías de entrada de los principales mohos responsables de enfermedades de postcosecha. Sin embargo, existe muy poca información sobre los procesos metabólicos que se activan/reprimen en la fruta en respuesta a herida. Por ello, nos propusimos examinar los cambios en abundancia de las proteínas de

manzana específicamente relacionadas con la respuesta a herida tras 24 y 48 horas, utilizando tejido herido a 0 horas como control. Puesto que muchos de los mohos responsables de importantes enfermedades de postcosecha necesitan de vías de entrada para producir infección, decidimos utilizar un control con herida en vez de tejido sano.

Observando detenidamente las imágenes de la Figura 11 vemos como la respuesta de la manzana a la herida tras 24 y 48 horas no dio lugar a cambios dramáticos en el perfil proteico global. Parece lógico esperar pocos cambios en el perfil proteico puesto que estamos estudiando una respuesta muy localizada (lugar de la herida). De hecho, entre los 250 spots citados en el punto anterior, tan sólo 8 presentaron cambios en abundancia o intensidad relativa (ratio) (Figura 11). La cuantificación relativa determina el aumento (≥ 2) o la disminución (≤ 0.5) de la expresión proteica.

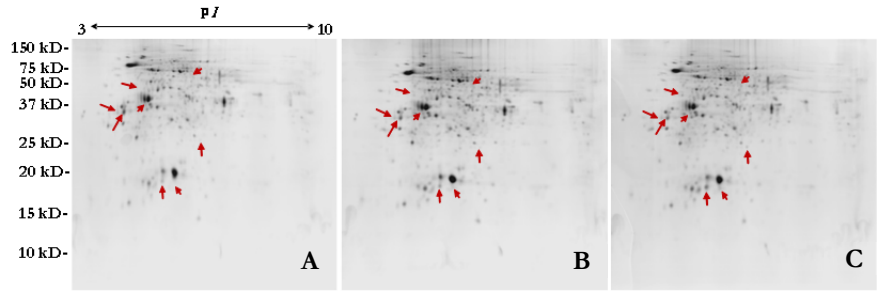

Figura 11. Geles 2D representativos que muestran las proteínas de respuesta a herida en manzana 'Golden Delicious'. Aparecen marcadas con flechas las proteínas que significativamente cambiaron su abundancia a 0 (A), 24 (B) y 48 (C) horas después de llevar a cabo la herida.

Las ocho proteínas que modificaron su abundancia en respuesta a herida mostraron cambios en función del tiempo. A 24 horas, 3 proteínas estaban reprimidas y 1 inducida; mientras que a 48 horas, 4 fueron las inducidas (Figura 11). Tan sólo 5 de las 8 se pudieron anotar funcionalmente mediante la combinación de técnicas PMF y MS/MS.

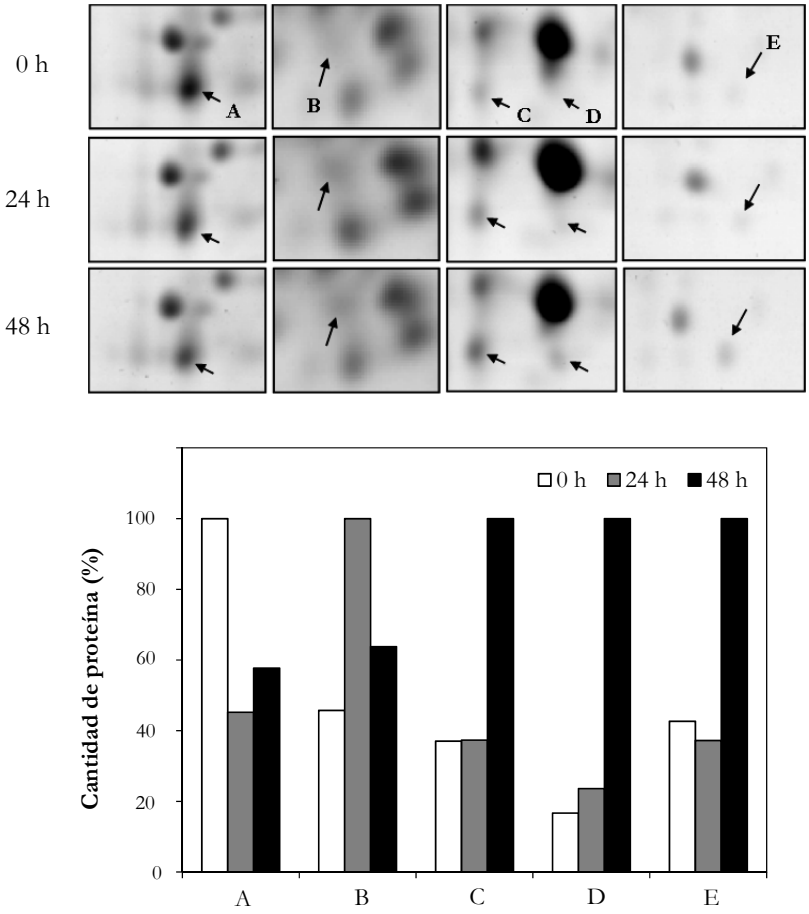

Figura 12. En la parte superior de la figura encontramos las áreas representativas de los geles 2D con las proteínas identificadas que cambiaron su abundancia a 0, 24 y 48 horas después de herir al fruto. En la parte inferior encontramos el histograma que muestra los cambios en abundancia en función del volumen más alto (100%) a 0, 24 y 48 horas, respectivamente. Las proteínas fueron: *β*-glucanase (A), thaumatin-like protein 1-a (B), Mal d 1 like (C), Mal d 1.03G (D) y flavoprotein WrbA-like (E).

Las 5 proteínas identificadas de respuesta a herida fueron: una *β*-glucanasa, una taumatina, dos Mal d 1 (Mal d 1 like y Mal d 1.03G) y una flavoproteina. Mientras que a 24 horas, la *β*-glucanasa (A) estaba reprimida y la taumatina (B) inducida; a 48 horas, observamos sólo inducción de la expresión de tres proteínas: Mal d 1 like (C), Mal d 1.03G (D), y flavoproteína (E) (Figura 12).

Función de las proteínas de respuesta a herida

Entre las proteínas identificadas en respuesta a herida a 24 y 48 horas, no encontramos ninguna relacionada con el sistema de detoxificación celular como la superóxido dismutasa (SOD), la catalasa (CAT) o la ascorbato peroxidasa (APX). Esto podría explicarse o bien por el estado de madurez del fruto (demasiado maduro), o bien por el método de extracción utilizado. Por un lado, los datos de calidad de las manzanas utilizadas en este estudio indicaron que el estado de madurez de los frutos era cercano a sobremaduro, hecho que pudo influenciar en la repuesta de defensa del fruto. Por otro lado, el método de extracción proteica utilizado, como tantos otros, presenta limitaciones. Es decir, no todas las proteínas solubilizan fácilmente. Por ejemplo, las proteínas hidrofóbicas y de membrana son de difícil solubilización, así como proteínas con rangos extremos de pH. Motivos por los cuales el proceso de extracción y solubilización de las proteínas continúa siendo un paso crítico dentro de la electroforesis bidimensional. Por último, también podría deberse a que la respuesta de estas proteínas a herida sea a corto plazo.

A pesar de no haber identificado ninguna proteína relacionada con el sistema de detoxificación celular, hemos visto como la mayoría de las proteínas identificadas en respuesta a herida, según su proceso biológico, están involucradas en 'respuesta a estrés'. Por ejemplo, entre las proteínas identificadas, la taumatina (PR-5) fue la única proteína inducida a 24 horas (ratio de 2.2) (Figura 12), sugiriendo que podría estar involucrada en la respuesta inicial del fruto a la herida. En plantas se ha descrito un comportamiento similar en el que la taumatina se induce como respuesta a herida [77], mientras que se ha observado en melocotón que podría estar implicada en evitar el daño por frío [78]. Por todo ello, la taumatina podría jugar un papel en la defensa inicial del fruto contra el daño mecánico. Cabe destacar también que, la *β*-glucanasa (PR-2) fue la única proteína reprimida significativamente a 24 horas (ratio de 0.45) (Figura 12). Así como se ha descrito la función de defensa de la *β* glucanasa en el fruto contra infecciones fúngicas [79], su papel de defensa en respuesta a herida no está claro. Se ha

descrito, en melocotón y tomate, que la actividad glucanasa aumenta durante la madurez de estos frutos, mientras que en flavedo de naranja no sufre cambios durante la maduración [80]. Nuestros resultados en manzana muestran como esta proteína disminuye su abundancia en respuesta a herida, probablemente debido al estado de madurez del fruto más que a cualquier otro papel relacionado con defensa.

Por lo que respecta la respuesta a herida a 48 horas, sólo mostró aumento de abundancia proteica. Por ejemplo, la Mal d 1.03G presentó el mayor aumento (ratio de 6.0) seguido de la Mal d 1 like (ratio de 2.70) (Figura 12). Las Mal d 1 están codificadas por miembros de una familia multigénica con un alto grado de identidad de secuencia [81]. Por tanto, es de esperar que encontremos múltiples isoformas de estas proteínas. Previamente, hemos visto como cuatro Mal d 1 no variaron su abundancia después de la herida (Tabla 1; Capítulo 3), mientras que acabamos de ver como otras dos Mal d 1 distintas sí variaron su abundancia, incrementándola en respuesta a herida (Tabla 2; Capítulo 3). Esto sugiere que podría haber una especialización funcional de los diferentes miembros de la familia de genes y podría explicar por qué sólo una parte de las proteínas Mal d 1 responden a herida. ¿Podrían tener entonces, algunas de estas proteínas Mal d 1, un papel importante en la defensa de 'Golden Delicious'?

Por último, cabe destacar la inducción de una flavoproteína, que junto con las Mal d 1, indujo su abundancia a 48 horas en respuesta a herida (ratio de 2.34). Las flavoproteínas están involucradas en un amplio rango de procesos biológicos que incluyen: la eliminación de los radicales que contribuyen al estrés oxidativo, la fotosíntesis, la reparación del ADN, y la apoptosis. En nuestro estudio, la flavoproteína identificada ha sido únicamente descrita en césped, y relacionada con la tolerancia a salinidad [82]. Aunque las funciones que pueda tener esta flavoproteína en la respuesta de 'Golden Delicious' a herida no están claras, ya que es la primera vez que se identifica en fruta, parece tener una contribución significativa. Es decir, en manzana sobremadura la producción de etileno es mayor [30], y en

consecuencia la tasa de respiración también aumenta, lo que conllevará a una mayor deshidratación del fruto. Por tanto, nuestros resultados sugieren que la 'flavoprotein WrbA-like' debe tener una función esencial y relacionada con el estado hídrico del fruto.

5. Cambios en la abundancia proteica en manzanas 'Golden Smoothee' en respuesta a un patógeno compatible (*P. expansum*) y uno no compatible (*P. digitatum*)

Actualmente existen diversos trabajos que han utilizado la proteómica comparativa para estudiar las interacciones planta-patógeno, por ejemplo: arroz-*Magnaporthe grisea* [54], maíz-*Fusarium verticillioides* [83], trigo-*Puccinia triticina* [84] y trigo-*Fusarium graminearum* [85]. Sin embargo, son muy pocos los que se centran en el estudio de las interacciones fruta-patógeno, a pesar de la importancia económica de algunas de las enfermedades de postcosecha [86-88]. Concretamente, los estudios proteómicos realizados para caracterizar las interacciones bioquímicas entre frutas y patógenos, sometidos a diferentes condiciones ambientales, se han llevado a cabo en melocotón [86], cereza [87], y tomate [88].

En este estudio, se utilizaron manzanas 'Golden Smoothee' a madurez comercial y se comprobó que el protocolo de extracción proteica optimizado fuera igual de efectivo en presencia de ambos patógenos. La posterior aproximación proteómica utilizada en este estudio fue la descrita en el capítulo 3, con algunas modificaciones. Las proteínas se separaron por electroforesis bidimensional en tiras de pH 4-7 (18 cm) y en geles SDS-PAGE al 11.5%. Una vez separadas las proteínas, los geles se tiñeron con Flamingo. El análisis de imagen de los geles bidimensionales indicó que existían diferencias significativas en los perfiles proteicos entre los diferentes tratamientos a lo largo del tiempo de estudio.

Por un lado, en el capítulo 3, analizamos los cambios en abundancia proteica de la respuesta a herida a lo largo del tiempo utilizando tejido herido a 0 horas como control. Sin embargo, en este apartado que corresponde al capítulo 4, para poder determinar las proteínas diferenciales de respuesta a herida, se utilizó como control tejido sano. Por otro lado, se estudió la respuesta del fruto al patógeno compatible (*P. expansum*) y al no compatible (*P. digitatum*). Debido a que ambos patógenos requieren de heridas para penetrar en el tejido y producir infección, se utilizó para describir las proteínas diferenciales de respuesta a *P. expansum* y *P. digitatum*, tejido herido como control para cada tiempo de estudio (24 y 48 horas). Por último, con el fin de obtener más información sobre las proteínas diferenciales entre *P. expansum* y *P. digitatum*, se compararon los perfiles proteicos de ambos patógenos, utilizando el tejido herido e inoculado con *P. expansum* como control.

Tras el análisis de imagen se detectaron aproximadamente 350 spots que estaban presentes en cada uno de los geles, de los cuales 34 presentaron diferencias significativas según el test t Student ($P < 0.05$), además de un cambio de intensidad igual o mayor que 0.5 o 2, respecto a sus controles (Figura 13). De los 34 spots que presentaron diferencias significativas pudieron identificarse 26 (Tabla 2; Capítulo 4).

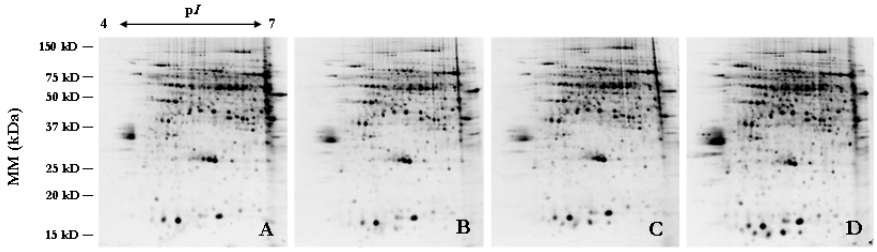

Figura 13. Geles 2D representativos que muestran las proteínas totales de manzana 'Golden Smoothee' teñidas con Flamingo en tejido sano (A), herido (B), e inoculado con *P. digitatum* (C) y *P. expansum* (D), tras 48 horas de incubación a 20 ºC.

Las proteínas que modificaron su abundancia en respuesta a estrés (abiótico o biótico) se clasificaron en función del proceso biológico, en el que mayoritariamente están implicadas: 'procesos metabólicos', 'respuesta a estrés' y 'procesos de oxidación-reducción'.

Proteínas diferenciales de respuesta a herida

Dieciocho proteínas modificaron su abundancia en respuesta a herida mostrando cambios en función del tiempo (0, 24 y 48 horas) respecto al tejido sano como control. Entre ellas, 16 se pudieron anotar funcionalmente mediante la combinación de técnicas PMF y MS/MS.

Se observaron pocos cambios en abundancia proteica comparando tejido herido a 0 horas respecto al tejido sano. Sin embargo, tanto a 24 como a 48 horas el número de proteínas que varió su abundancia aumentó progresivamente. Mientras que a 24 horas, 3 proteínas estaban reprimidas (spots 3601, 3705, 6701) y 4 inducidas (spots 202, 1201, 4005, 4009); a 48 horas, 4 proteínas estaban reprimidas (spots 2710, 3601, 5902, 6604) y 5 inducidas (spots 2001, 4009, 4709, 5005, 5504). En general, la mayoría de las proteínas que aumentaron su abundancia están involucradas, según su proceso biológico, en 'respuesta a estrés', mientras que las proteínas cuya abundancia disminuyó están involucradas mayoritariamente en 'procesos metabólicos'.

Por ejemplo, tres de las proteínas identificadas relacionadas con la 'respuesta a estrés' fueron: Mal d 1.06C (spot 2001), Mal d 1 (spot 4005) y Mal d 1.03A (spot 5005). Mientras que la Mal d 1 aumentó su abundancia a 24 horas (ratio de 2.1); la Mal d 1.06C y la Mal d 1.03A (ratios de 3.0 y 4.5, respectivamente) lo hicieron a 48 horas.

A pesar de usar manzanas 'Golden Smoothee' a madurez comercial, tejido sano como control, y modificar algunos aspectos de la electroforesis bidimensional, tampoco hemos identificado proteínas de respuesta a herida relacionadas con el sistema de detoxificación celular, por ejemplo: SOD, CAT o APX.

En este caso, identificamos una proteína relacionada con el metabolismo del etileno, la ACO, que aumentó su abundancia en respuesta a herida tanto a 0 como a 24 horas. Por tanto, nuestros resultados sugieren que la herida, a tiempos cortos, induce la producción de etileno de forma importante. La inducción de ACO (enzima formadora del etileno) podría traducirse en una mayor concentración de etileno. Esta relación debería establecerse en futuros estudios, además de determinar los valores de producción de etileno para cada tiempo de estudio.

Proteínas diferenciales de respuesta al patógeno compatible (*P. expansum*)

Once proteínas modificaron su abundancia en respuesta a *P. expansum* mostrando cambios en función del tiempo, respecto al tejido herido como control, tanto a 24 como a 48 horas. Entre ellas, 7 se pudieron anotar funcionalmente por PMF o MS/MS.

Mientras que a 24 horas hubo muy pocos cambios, a 48 horas, 2 proteínas estaban reprimidas (spots 3403 y 5804) y 5 inducidas (spots 2101, 2104, 4004, 4503 y 5005). Las proteínas que aumentaron su abundancia están involucradas mayoritariamente en 'respuesta a estrés', mientras que las proteínas cuya abundancia disminuyó están involucradas en 'procesos metabólicos'.

Dos de las proteínas que aumentaron su abundancia tanto a 24 como a 48 horas fueron: la Mal d 1.03D y la Mal d 1.03A. Otras proteínas que indujeron o reprimieron su abundancia en respuesta a *P. expansum* a 48 horas mostraron una sobrerrepresentación significativa de algunas categorías de GO. Por ejemplo, las categorías sobrerrepresentadas de las proteínas inducidas fueron: 'respuesta a estímulos bióticos' (GO:0009607), 'actividad 4-hidroxifenilpiruvato dioxigenasa' (GO:0003868) y 'procesos biosintéticos de la tiamina' (GO:0009228), mientras que en las proteínas reprimidas fueron: 'actividad sarcosina oxidasa' (GO:0008115) y 'metabolismo del tetrahidrofolato' (GO:0046653).

Proteínas diferenciales de respuesta al patógeno no compatible (*P. digitatum*)

Hubo siete proteínas que modificaron su abundancia en respuesta a *P. digitatum*, mostrando cambios en función del tiempo, respecto al tejido herido como control tanto a 24 como a 48 horas. Todas ellas se anotaron funcionalmente.

Cabe destacar que todas la proteínas identificadas en respuesta a *P. digitatum* incrementaron su abundancia. Hubo muy pocos cambios a 24 horas, por ejemplo, la Mal d 1.03D (spot 4004) y la Mal d 1.03A (spot 5005). A 48 horas hubo 6 proteínas que indujeron su expresión (spots 2708, 3004, 4004, 4503, 5005 y 6401); la mayoría de ellas implicadas en 'respuesta a estrés'. En este caso, las categorías GO sobrerrepresentadas de las proteínas inducidas fueron: 'respuesta a estímulos bióticos' (GO:0009607), 'respuesta de defensa' (GO:0006952) y 'actividad 4-hidroxifenilpiruvato dioxigenasa' (GO:0003868).

Mientras dos de las proteínas inducidas a 24 y 48 horas ya se identificaron en respuesta a *P. expansum*, la Mal d 1.03D y la Mal d 1.03A, una nueva Mal d 1 fue identificada específicamente en respuesta a *P. digitatum*, concretamente la Mal d 1.03E. Entre otras proteínas inducidas en respuesta a *P. digitatum* destacamos al precursor del factor de elongación de la traducción EF-Tu (spot 6401). Por tanto, la inducción específica de la Mal d 1.03E y del EF-Tu en respuesta a *P. digitatum* sugiere que ambas proteínas podrían tener un papel importante en la defensa de 'Golden Smoothee' contra este patógeno no compatible.

Proteínas diferenciales entre *P. expansum* y *P. digitatum*

Puesto que la manzana a madurez comercial es capaz de suprimir o limitar la infección del patógeno no compatible (*P. digitatum*), es muy importante identificar los mecanismos de resistencia que lo hacen posible. Para ello, comparamos los perfiles proteicos de *P. expansum*

con los de *P. digitatum*. Es decir, utilizamos en este caso al tejido herido e inoculado con *P. expansum* como control. Así obtuvimos información específica de la respuesta del fruto a *P. digitatum*.

Tras el análisis de imagen detectamos 7 proteínas que mostraron cambios en su abundancia a lo largo del tiempo de estudio (24 y 48 horas), de las cuales solo 3 se pudieron identificar. Las 3 proteínas identificadas aumentaron su abundancia a 48 horas, y corresponden a dos precursores de la polifenol oxidasa (spots 2708 y 3601) y al EF-Tu (spot 6401).

Las proteínas que aumentaron su abundancia en respuesta a *P. digitatum*, en comparación con *P. expansum*, están involucradas mayoritariamente en 'procesos metabólicos' y 'procesos de oxidación-reducción'. Estos resultados vuelven a señalar un posible efecto específico del EF-Tu en la defensa de 'Golden Smoothee' en respuesta a *P. digitatum*.

Función de las proteínas diferenciales identificadas en respuesta a estrés (abiótico y biótico)

El presente trabajo proporciona datos sobre la variación en abundancia de algunas proteínas relacionadas con la defensa de 'Golden Smoothee'. Nuestros resultados sugieren que el fruto además de respuestas específicas, activa mecanismos de defensa similares en respuesta a estreses distintos.

Con las proteínas Mal d 1, hemos visto como por un lado, la Mal d 1.03A se ha inducido de forma común, tanto en respuesta a herida como en respuesta a ambos patógenos (*P. expansum* y *P. digitatum*). Por otro lado, la Mal d 1.03D se ha inducido tanto en respuesta a *P. expansum* como a *P. digitatum*, usando como control tejido herido a 24 y 48 horas. Lo realmente interesante ha sido detectar la inducción específica en respuesta a *P. digitatum* de la Mal d 1.03E (MDP0000295543). Esto sugiere que la Mal d 1.03E -implicada en procesos de defensa- podría asociarse específicamente con la respuesta

de 'Golden Smoothee' a un patógeno no compatible (*P. digitatum*), y de alguna forma concederle una mayor resistencia al fruto frente a este patógeno. Por tanto, estos datos corroborarían los resultados obtenidos en el capítulo 3 donde también vimos que los diferentes miembros de la familia Mal d 1 poseen una especialización funcional. Las Mal d 1 se consideran en manzana una familia multigénica compleja, que incluye al menos 20 genes diferentes, agrupados principalmente en dos grupos de ligamiento (GLs) 13 y 16 [72, 89, 90]. Los genes Mal d 1 se han clasificado en cuatro subfamilias basándose en la presencia o ausencia de intrones y su longitud. Nuestros resultados incluyen miembros de dos de los cuatro grupos. Específicamente *Mal d 1.06*C miembro de la subfamilia III, que contiene intrones, mientras que *Mal d 1.03A*, *Mal d 1.03D* y *Mal d 1.03E*, miembros de la subfamilia IV, no contienen intrones. La *Mal d 1.06*C está situada en el GL 16, mientras que *Mal d 1.03A*, *Mal d 1.03D* y *Mal d 1.03E* están situadas en el GL 13 [72]. Cabe destacar que las familias génicas que evolucionan más rápido han sido asociadas con la defensa de patógenos [91]. Estos datos corroborarían la hipótesis de que la presencia de tantos genes homólogos Mal d 1, en el genoma de manzana, se han conservado durante la evolución, debido básicamente a su papel en la transducción de señal o en la selección mediada por patógeno. Sin embargo, el papel que pueden tener las proteínas Mal d 1 en la defensa de 'Golden Smoothee', tanto contra un estrés abiótico como biótico, aún no está claro.

En plantas, se ha visto como el EF-Tu juega un papel importante en la biosíntesis de proteínas, la respuesta a ambos estreses, abiótico y biótico, además de poseer actividad chaperona e inducir la inmunidad innata de las células vegetales [92]. Aunque en planta se ha estudiado la expresión del gen *EF-Tu* en respuesta a diferentes estreses ambientales, por ejemplo: temperatura, salinidad o sequía, en este estudio no hemos encontrado evidencias de su expresión diferencial a nivel proteómico en respuesta a herida. Sin embargo, hemos visto como 'Golden Smoothee' ha aumentado la abundancia proteica de EF-Tu (MDP0000291397) en respuesta a *P. digitatum* a 48 horas, utilizando tejido herido a 48 horas como control. Pero más interesante

ha sido observar como esta proteína también ha aumentado su abundancia en respuesta a *P. digitatum* a 48 horas, utilizando tejido inoculado con *P. expansum* como control. En ambos casos, usando controles distintos, la respuesta de 'Golden Smoothee' contra *P. digitatum* ha sido la de inducir la abundancia de EF-Tu. ¿Podría tener entonces el EF-Tu un papel en la defensa de 'Golden Smoothee' contra *P. digitatum*? ¿O es simplemente el efecto secundario de otros procesos metabólicos debidos a esta interacción no-compatible?

6. Cambios en la oxidación proteica en manzanas 'Golden Smoothee' en respuesta a un patógeno compatible (*P. expansum*) y uno no compatible (*P. digitatum*)

Cualquier microorganismo que intente colonizar heridas recientes probablemente tendrá que hacer frente al estrés oxidativo provocado como consecuencia de la herida. Este estrés oxidativo provocado por la producción de ROS, y entre ellas el H_2O_2, probablemente es percibido por las células a través de las PTMs, provocando daño oxidativo en las proteínas. La carbonilación u oxidación proteica es una PTM que puede afectar a la actividad enzimática, la localización celular, las interacciones proteína-proteína y la estabilidad proteica [93, 94]. El estudio de las PTMs debe abordarse mediante estrategias proteómicas, ya que la información que proporcionan es directa y va más allá de las predicciones deducidas a partir de la secuencia genómica. Por tanto, es importante tener información sobre cuáles son las proteínas afectadas (oxidadas) y en qué metabolismos están implicados.

Existen pocos ejemplos de los efectos que causa el daño oxidativo en plantas [94], y aún menos focalizados en fruta. Sólo tenemos constancia de tres estudios en fruta donde se haya descrito el nivel de oxidación de las proteínas (daño oxidativo) provocado por las ROS, concretamente en melocotón [95] y cereza [96, 97]. Estos autores utilizarón la electroforesis bidimensional acoplada a 'immunoblotting'. Sin embargo, se han desarrollado recientemente otros métodos para detectar y cuantificar -de forma más rápida y precisa- la oxidación

proteica, incluyendo el uso de sondas fluorescentes, como el Bodipy-Hz [98]. El uso del Bodipy-Hz presenta múltiples ventajas. Entre ellas, ahorra tiempo porque evita el uso de técnicas Western-blot para las que se necesitan anticuerpos específicos para las proteínas de estudio, además de detectar niveles más bajos de oxidación proteica.

El método utilizado en este trabajo para detectar la oxidación proteica fue el descrito por Tamarit *et al.* [98] en *Saccharomyces cerevisiae*. Al tratarse de un método desarrollado para un microorganismo hubo que optimizar la metodología de derivatización (marcaje de carbonilación u oxidación proteica con Bodipy-Hz) para muestras de manzana 'Golden Smoothee'. Una vez optimizado el método se corrieron los geles 2D donde cada gel contenía tanto proteína total (teñida con Flamingo) como proteína oxidada (teñida con Bodipy-Hz). Dependiendo del filtro de emisión utilizado, 605BP para Flamingo o 530BP para Bodipy-Hz, se obtuvieron imágenes de proteína total (Figura 13) o de proteína oxidada (Figura 14), respectivamente. Las proteínas se separaron por electroforesis bidimensional en tiras de pH 4-7 (18 cm) y en geles SDS-PAGE al 11.5%. Los cambios metodológicos en la electroforesis bidimensional del capítulo 4 respecto al capítulo 3 se debieron a la optimización del protocolo desarrollado para el estudio del oxi-proteoma.

Las imágenes de la Figura 14 muestran la oxidación proteica en 'Golden Smoothee', en tejido sano, herido e inoculado con los patógenos, tras 48 horas de incubación a 20 ºC. El cambio más remarcable se observó en respuesta a la inoculación con *P. expansum*, donde se observa una oxidación menor (Figura 14 D). En lo que a número de proteínas se refiere se detectaron aproximadamente 87 spots que estaban presentes en cada uno de los geles. Para cada spot la señal Bodipy fue normalizada respecto a la señal de proteína total. Aquellos spots que presentaron un ratio de oxidación entre tratamientos igual o mayor a 1.5, se marcaron para su posterior identificación [99]. Veintiséis de los 87 spots aumentaron su oxidación en respuesta a estrés (abiótico y biótico), comparados con sus respectivos controles (Figura 14). Además de identificar las 26

proteínas, también se clasificaron en función del proceso biológico en el que están implicadas (Tabla 3; Capítulo 4).

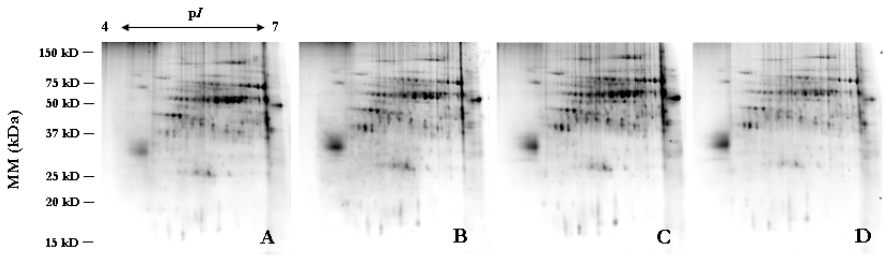

Figura 14. Geles 2D representativos que muestran las proteínas totales de manzana 'Golden Smoothee' teñidas con Bodipy en tejido sano (A), herido (B), e inoculado con *P. digitatum* (C) y *P. expansum* (D) tras 48 horas de incubación a 20 ºC.

Proteínas diferencialmente oxidadas en respuesta a herida

Diez proteínas fueron las que se oxidaron en respuesta a herida mostrando cambios en función del tiempo (0, 24 y 48 horas) respecto al tejido sano como control.

Cabe destacar que los mayores cambios en oxidación proteica se dieron a 0 horas respecto al tejido sano, mientras que a 24 y 48 horas se observaron muy pocos cambios. A 0 horas, fueron 8 las proteínas identificadas que mostraron un índice de oxidación igual o superior a 1.5 (spots 1403, 2201, 2404, 4305, 5704, 6205, 6505, y 7201). Las proteínas oxidadas en respuesta a herida están mayoritariamente implicadas en 'procesos metabólicos'.

Entre las proteínas oxidadas e identificadas hay: enzimas glucolíticas (spots 2201 y 5704), del ciclo de Calvin (spots 6505 y 4305) y del metabolismo del glioxilato (spots 6205 y 7201). La proteína que mostró una oxidación mayor en respuesta a herida a 0 horas fue la Actina (spot 2404) con un ratio de 5.1.

Proteínas diferencialmente oxidadas en respuesta al patógeno compatible (*P. expansum*)

Ocho proteínas presentaron ratios de oxidación iguales o superiores a 1.5 en respuesta a *P. expansum* mostrando cambios en función del tiempo, respecto al tejido herido como control, tanto a 24 como a 48 horas.

Estos cambios en oxidación proteica se dieron específicamente a 24 horas, mientras que no se detectaron cambios a 48 horas. Las proteínas identificadas con mayores ratios de oxidación fueron: Actina 7 (spot 1403), RUBISCO (spot 6504), y la bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic-like (spot 6702). La mayoría de proteínas oxidadas en respuesta a *P. expansum* están involucradas en 'procesos metabólicos' (Tabla 3; Capítulo 4). Por tanto, vemos como la defensa de la manzana en respuesta a *P. expansum* también pasa por disminuir su metabolismo primario a través de la oxidación de proteínas, como ya hemos visto que ocurría en respuesta a herida.

Proteínas diferencialmente oxidadas en respuesta al patógeno no compatible (*P. digitatum*)

Fueron ocho las proteínas que presentaron ratios de oxidación iguales o superiores a 1.5 en respuesta a *P. digitatum* mostrando cambios en función del tiempo, respecto al tejido herido como control tanto a 24 como a 48 horas.

Entre las ocho proteínas oxidadas, 4 se detectaron a 24 horas y otras 4 distintas a 48 horas. Por un lado, las 4 proteínas oxidadas a 24 horas son enzimas involucradas en: la biosíntesis de metabolitos secundarios (spots 1203 y 4708), el ciclo de los ácidos tricarboxílicos (spot 7201) y en la degradación ATP-dependiente de proteínas ubiquitinizadas (spot 2504). Es interesante destacar que la proteína que mostró el mayor ratio de oxidación en respuesta a *P. digitatum* a 24 horas fue la ACC oxidasa 1 (ACO) con un ratio de 16.8. Por otro lado, las 4 proteínas

oxidadas a 48 horas son enzimas involucradas en glicólisis (spots 4602, 5503 y 6001) y en el transporte de protones (spot 3702).

Por tanto, aunque la mayoría de las proteínas oxidadas en respuesta a *P. digitatum*, respecto al tejido herido como control, están involucradas en 'procesos metabólicos', hemos visto como existen diferencias respecto a la defensa de la manzana en respuesta a herida y a *P. expansum*. Nuestros resultados sugieren que la respuesta de defensa del fruto en respuesta a *P. digitatum* pasa por disminuir su metabolismo primario, además del secundario, a través de la oxidación proteica, afectando además al metabolismo del etileno, y al transporte de protones.

Proteínas diferencialmente oxidadas entre *P. expansum* y *P. digitatum*

Una vez comparados los perfiles proteicos de oxidación de *P. expansum* con *P. digitatum*, utilizando como control al tejido herido e inoculado con *P. expansum*, obtuvimos información respecto a la respuesta específica del fruto, en lo que a oxidación se refiere, contra el patógeno no compatible (*P. digitatum*).

En este caso fueron 10 las proteínas que mostraron ratios de oxidación igual o superiores a 1.5 a lo largo del tiempo de estudio (24 y 48 horas), pudiéndose identificar todas ellas. Entre las 10 proteínas oxidadas, 4 se detectaron a 24 horas y 6 distintas a 48 horas.

Tras 24 horas, las 4 proteínas con un incremento significativo del ratio de oxidación están involucradas en los procesos biológicos de: biosíntesis de metabolitos secundarios (spots 1203 y 4708), glicólisis (spot 4505) y ciclo de los ácidos tricarboxílicos (spot 7201). En concreto, la ACC oxidasa 1 mostró el mayor ratio de oxidación en respuesta a *P. digitatum* (ratio de 28.2). Tras 48 horas, las 6 proteínas con un incremento significativo del ratio de oxidación fueron: dos ATP sintasas subunidad beta (spots 1608 y 2602), dos glutaminas sintetasas (spots 4302 y 4308), y dos enolasas (spots 5503 y 5508).

Las proteínas oxidadas en respuesta a *P. digitatum* están involucradas mayoritariamente en 'procesos metabólicos'. Por un lado, hemos visto como la defensa del fruto, en respuesta a *P. digitatum*, está relacionada con la oxidación de la enzima formadora del etileno (ACC oxidasa 1). Por otro lado, detectamos oxidación de dos glutaminas sintetasas relacionadas con el metabolismo del nitrógeno.

En conjunto, nuestros resultados muestran como la defensa de 'Golden Smoothee' en respuesta a *P. digitatum* consiste en oxidar la ACO y, en consecuencia, disminuir o inhibir la producción de etileno. Posiblemente, la reducción del etileno disminuye la respiración del fruto, dejando disponibles menos recursos para *P. digitatum*. Remarcablemente, la oxidación de la ACO no se detecta en respuesta a *P. expansum* en manzana, lo cual indica que el etileno podría ser clave en el proceso de infección de este importante patógeno de postcosecha.

Función de las proteínas diferencialmente oxidadas en respuesta a estrés (abiótico y biótico)

Nuestros resultados sugieren que la oxidación proteica causada en parte por el H_2O_2 generado durante la explosión oxidativa, pone en marcha múltiples funciones de señalización celular que finalmente podrían regular ciertos genes de defensa en 'Golden Smoothee'.

Básicamente, hemos observado que la oxidación del fruto afecta a procesos metabólicos, especialmente el del etileno. La producción de etileno, junto con la explosión oxidativa, son parte de la respuesta innata de inmunidad de las células vegetales, una respuesta que forma la base de la resistencia a la infección [92]. El etileno se ha asociado a distintos procesos de desarrollo, senescencia y respuesta a estrés en plantas, incluida la respuesta frente a la infección por patógenos [100, 101]. Sin embargo, existen resultados contradictorios en lo que se refiere a la implicación del etileno en la activación de procesos relacionados con la defensa, en función de la interacción planta-patógeno que se estudie.

En nuestros resultados, hemos observado como la enzima formadora del etileno (la ACO; MDP0000195885) sólo se oxidó -y de forma muy importante- en respuesta a *P. digitatum* tras 24 horas de incubación, tanto respecto al tejido herido como tejido herido e inoculado con *P. expansum*. La ACO forma parte de una familia multigénica [102], y está regulada por una compleja red de señales del desarrollo y ambientales [101]. Ésto explicaría porque dos ACO han variado su abundancia en respuesta a herida a 0 y 24 horas, y otra en respuesta a *P. digitatum* a 24 horas. Por tanto, nuestros resultados sugieren que la oxidación de la ACO en respuesta a *P. digitatum* podría reducir o inhibir la producción de etileno y la tasa de respiración del fruto y, en consecuencia, disminuir las actividades metabólicas, como la síntesis de proteínas, la disponibilidad de sustratos para el patógeno, e incluso podría disminuir la degradación de la pared celular.

Aunque en manzana no se dispone de información que relacione *P. digitatum* y etileno, esta relación ha sido objeto de estudio en cítricos durante bastante tiempo por el grupo del Dr. Luís González Candelas del IATA de Valencia.

El etileno desencadena la maduración en frutos climatéricos (p. ej. manzana), mientras que en frutos no climatéricos (p. ej. naranja) acelera el cambio de color del flavedo en cítricos, un proceso conocido como desverdización comercial en postcosecha. Durante las primeras etapas de la infección de cítricos por *P. digitatum*, la producción de etileno proviene del fruto, mientras que en etapas tardías del hongo [103]. Para estudiar la implicación de esta hormona vegetal en la infección de *P. digitatum* en naranjas 'Navelate', se trataron con 1-metil ciclopropeno (1-MCP), un bloqueador de los receptores de etileno. Estos autores observaron un incremento en la susceptibilidad a la infección por *P. digitatum*, además de cambios en la transcripción de genes implicados en la síntesis del etileno como la *ACS* y la *ACO* [104]. Existen estudios en naranjas sobre la regulación de la síntesis de etileno en respuesta a la infección por *P. digitatum*, donde se ha observado que la percepción del etileno induce respuestas de defensa.

Sin embargo, la implicación en defensa de esta hormona en respuesta a un ataque patógeno aún no está clara [9, 105, 106].

Otro metabolismo afectado que no habríamos detectado si no hubiéramos analizado el oxi-proteoma es el del nitrógeno. Identificamos dos glutaminas sintetasas (MDP0000196909 y MDP0000151581) oxidadas a 48 horas en respuesta a *P. digitatum* comparado con el nivel de oxidación en respuesta a *P. expansum*. Las glutaminas sintetasas (GS) son enzimas involucradas en la asimilación primaria de nitrógeno, así como también en la reasimilación de amonio liberado durante la fotorespiración. La oxidación de estas dos GSs podría reducir o inhibir su actividad e incluso incrementar su susceptibilidad a la degradación proteolítica [98, 107].

Es importante destacar que la información genómica de dos cepas españolas de *P. digitatum* está disponible [108]. Gracias a esta información genómica, sabemos que *P. digitatum* ha experimentado una reducción importante en el contenido génico comparado con *P. chrysogenum*, una especie filogenéticamente cercana pero no-fitopatogénica. Los mismos autores han descrito que las clases funcionales a las que pertenecen los genes perdidos en *P. digitatum* tienen importantes implicaciones fisiológicas, incluyendo la incapacidad de utilizar nitrato como fuente de nitrógeno. Por tanto, de acuerdo con nuestros resultados, la hipótesis que se plantearía es entender con qué objetivo 'Golden Smoothee' oxida estas dos GSs. ¿Quizá el fruto intenta evitar que *P. digitatum* utilice su nitrato como fuente de nitrógeno?

Otras dos proteínas relacionadas con el metabolismo, identificadas como ATP sintasas (MDP0000565338), se oxidaron en respuesta a *P. digitatum*, comparado con el nivel de oxidación en respuesta a *P. expansum* a 48 horas. Una de ellas también se oxidó en respuesta a *P. expansum*, respecto al tejido herido a 24 horas. Por tanto, de las dos ATP sintasas identificadas, tan sólo una de ellas, el precursor, fue específica de respuesta a *P. digitatum*. La modulación de la actividad de la membrana plasmática -H^+-ATPasa- se ha descrito como un posible

interruptor molecular en varias respuestas de resistencia de la planta [109]. En este sentido, nuestros resultados muestran como la ATP sintasa, dos en respuesta a *P. digitatum* y una en respuesta a *P. expansum*, se ha oxidado. Este resultado sugiere que el movimiento de protones podría verse reducido o inhibido de forma más importante en respuesta al patógeno no compatible (*P. digitatum*).

La integración de todos nuestros resultados sugiere una modificación del metabolismo primario (p. ej. la asimilación de nitrógeno) y secundario (p. ej. la biosíntesis de etileno). Aunque se han realizado considerables progresos para entender las respuestas de defensa de las plantas, se sabe muy poco sobre el papel de las rutas metabólicas primarias, necesarias para el crecimiento y desarrollo, en la regulación de las respuestas de defensa. Algunas respuestas de defensa en plantas consisten en reducir la fotosíntesis y otros procesos asociados con el metabolismo primario, que es esencial para el crecimiento de la planta. Se ha sugerido que la energía ahorrada en disminuir el metabolismo primario se desvía para potenciar las respuestas defensivas. Sin embargo, varios estudios han demostrado lo contrario, que la inducción del metabolismo primario también se produce durante las interacciones planta-patógeno [110].

En conjunto, nuestros resultados sugieren que la oxidación podría tener un impacto diferencial en la capacidad de *P. expansum* o de *P. digitatum* a la hora de adquirir recursos del fruto. Todo parece indicar que un importante mecanismo de defensa en 'Golden Smoothee' en respuesta a ambos patógenos (compatible y no compatible) implica evitar la libre circulación de nutrientes, reduciendo los procesos asociados con el metabolismo primario y secundario. En consecuencia, y contribuyendo a la resistencia, el fruto intenta detener o bien retrasar la penetración del patógeno. El hecho de ralentizar la penetración del patógeno proporcionaría un tiempo de ventaja al fruto para activar otros mecanismos de defensa, por ejemplo el de las proteínas PR, como las Mal d 1.

Por todo ello, la búsqueda de proteínas o procesos metabólicos sobre los que diseñar futuras estrategias efectivas para el control de enfermedades de postcosecha, hacen de la proteómica una de las aproximaciones moleculares más útiles hoy en día.

7. Correlación entre la abundacia de transcrito y proteína

Los cambios en abundancia proteica pueden estar regulados a diferentes niveles, incluyendo variaciones transcripcionales y postraduccionales. Para evaluar si los cambios de abundancia proteica resultan de modificaciones transcripcionales, analizamos 6 genes seleccionados a partir de su interés, revelado por nuestros resultados previos.

Por un lado, algunas de las proteínas que variaron su abundancia u oxidación en respuesta a herida o a patógeno se seleccionaron por su posible implicación en defensa, como: la ACC oxidasa 1 (ACO), la Mal d 1.03E (MalD1.03E), la heat shock protein clase I (HSP) y el precursor del factor de elongación de la traducción EF-TU (EF-TU). Por otro lado, a pesar de no haber detectado cambios en la abundancia u oxidación proteica bajo nuestras condiciones experimentales, nos propusimos analizar también el nivel de expresión de los genes: ascorbato peroxidasa (APX) y factor de transcripción dominio NAC (NAC).

El estudio de estos seis genes se realizó a nivel de transcripción en: i) tejido sano; ii) tejido herido a 0, 24, 48 horas y 7 días; iii) tejido inoculado con *P. expansum* a 24 y 48 horas y iv) tejido inoculado con *P. digitatum* a 24, 48 horas y 7 días. En general, a nivel transcripcional, no hubo grandes cambios transcripcionales que puedan explicar los cambios en abundancia proteica (Figura 15).

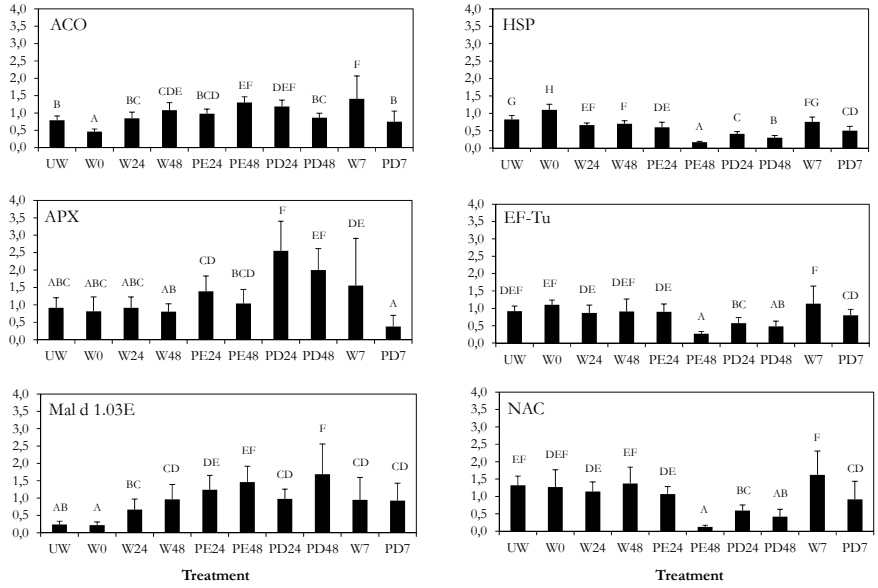

Figura 15. Expresión relativa mediante RT-qPCR (del inglés Real Time-quantitative Polymerase Chain Reaction) de los genes que codifican las proteínas identificadas en tejido sano (UW), herido (W), e inoculado con *P. expansum* (PE) y *P. digitatum* (PD). Datos de expresión génica normalizados contra la expresión del factor de elongación o EF (del inglés Elongation Factor). Letras diferentes indican diferencias significativas entre las medias usando el test *t* Student ($P < 0.05$).

Aunque la transcripción de la *ACO* muestra pocos cambios entre tratamientos, éstos son estadísticamente significativos. Los transcritos de la *ACO* aumentaron tanto en respuesta a herida como en respuesta a *P. expansum* tras 48 horas de incubación. Transcurrido este tiempo, la transcripción de la *ACO* en respuesta a *P. digitatum* disminuyó, manteniéndose al mismo nivel tras 7 días. Esta reducción del nivel de transcrito a 48 horas en respuesta a *P. digitatum* no se vio reflejada en una reducción de abundancia proteica de la ACO. A nivel de abundancia proteica, sólo se observó un aumento en la expresión de la ACO en respuesta a herida (0 y 24 horas). Por tanto, podemos decir que la correlación de la ACO entre los niveles de transcrito y proteína es baja. Estos resultados muestran como las modificaciones postraduccionales que se dan en la síntesis de la ACO son de vital

importancia en la función final que pueda desempeñar en respuesta a un estrés u otro.

Respecto a los dos factores de transcripción (*EF-Tu* y *NAC*) y a la 'heat shock protein' clase I (*HSP*), observamos un patrón transcripcional similar entre tratamientos, siendo destacable la reducción en respuesta a *P. expansum* tras 48 horas. Aunque *EF-Tu* y *NAC* presenten patrones transcripcionales similares, bajo nuestras condiciones experimentales no detectamos cambios en la abundancia proteica de NAC, pero sí en la abundancia proteica de EF-Tu (en respuesta a *P. digitatum* tras 48 horas). Por lo que respecta a la Clase I HSP, sólo aumentó la abundancia proteica en respuesta a herida, especialmente a 24 horas. Una vez más, estos resultados muestran como las modificaciones postraduccionales son de vital importancia para la función que desempeñan estas proteínas en respuesta a estrés. Curiosamente, el gen correspondiente a la *APX* -una de las proteínas que no varió su abundancia u oxidación bajo nuestras condiciones experimentales- mostró los mayores niveles de transcripción en respuesta a *P. digitatum*.

La correlación entre la abundancia de transcrito y proteína es solo significativa para la Mal d 1.03E (correlación de Pearson; $r = 0.93$; $P < 0.001$). En este caso, una mayor transcripción sí se vio reflejada en un aumento de la abundancia proteica. Bajo nuestras condiciones experimentales, la trasncripción de *Mal d 1.03E* se vio aumentada tanto en respuesta a herida como en respuesta a ambos patógenos, mientras que en tejido sano y herido a 0 horas se transcribió a niveles muy bajos. Este resultado está en consonancia con los obtenidos por Pagliarani *et al.* [90], donde no se detectó transcripción de *Mal d 1.03E* por qPCR en manzanas cv. 'Florina'.

Múltiples trabajos describen la baja correlación entre la abundancia de transcrito y proteína [46, 48]. Estos casos donde la correlación es baja pueden deberse, o bien al desfase temporal entre transcripción y traducción, o bien a regulaciones postranscripcionales.

En resumen, en esta tesis, mediante el uso de cepas marcadas con GFP, hemos visualizado la infección, confirmando que estas cepas son una buena herramienta para llevar a cabo estudios de interacción y colonización de los patógenos (Capítulo 1). Una vez establecidas las relaciones de compatibilidad, hemos caracterizado el efecto del H_2O_2 en los patógenos y los huéspedes. En conjunto, nuestros resultados sugieren que el H_2O_2 producido por la fruta, en respuesta al estrés abiótico y biótico, podría estar implicado en la señalización de procesos de resistencia del huésped, además de inhibir la germinación de *P. digitatum* y *P. expansum* (Capítulo 2).

Con el fin de descifrar los principales mecanismos de defensa del fruto, usamos una aproximación proteómica (Capítulos 3 y 4). Tras estudiar los cambios proteicos en manzana (ambos, abundancia y oxidación) en respuesta al estrés abiótico (herida) y biótico (*P. expansum* y *P. digitatum*), destacamos los resultados relacionados con los cambios proteicos específicos en respuesta a *P. digitatum*. Entre los cambios de abundancia, hemos identificado a la Mal d 1.03E y al EF-Tu, así como la oxidación de la ACC oxidasa (biosíntesis del etileno) y las glutaminas sintetasas (asimilación de nitrógeno). En general, estos cambios de abundancia proteica en respuesta a herida y a patógeno no resultan de variaciones transcripcionales. Por tanto, futuros estudios globales (donde la transcriptómica, la proteómica y la metabolómica se complementen) son necesarios para proporcionar información completa acerca de las respuestas de defensa del fruto.

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Conclusions/Conclusiones

Use of GFP-tagged strains to study fruit-pathogen interaction

1. This study represents the first report on the characterisation of GFP-tagged strains of *P. expansum* and *P. digitatum*. In fact, the GFP transformation did not significantly affect the pathogenicity and the sporulation index of these two important postharvest pathogens on oranges and apples in *in vivo* studies.

2. The GFP transformation did not modify the ecophysiology (germination pattern and growth rate) of *P. digitatum* and *P. expansum* on different growth media and temperatures (ambient and cold storage) in *in vitro* studies.

3. The use of GFP-tagged strains of *P. digitatum* and *P. expansum* allowed us to confirm that both are wound pathogens, as well as to visualise the infection process of the host and the non-host pathogen. In mature non-host fruits, *P. expansum* infected 'Lanelate' oranges, while *P. digitatum* only caused a limited infection around the wound on 'Golden Delicious' apples.

H_2O_2 characterisation to study fruit-pathogen interaction at different maturity stages

4. Ecophysiological *in vitro* studies of *P. digitatum* and *P. expansum* showed that the maximum spore germination values were reached faster for *P. expansum* than for *P. digitatum* at both temperatures (ambient and cold-storage). However, cold-storage delayed the germination values of both pathogens by 10-fold.

5. The effect of H_2O_2 treatment on *P. digitatum* and *P. expansum* spore germination showed that high concentrations of H_2O_2 (200 mM) reduced the germination values of both pathogens at ambient (25 ºC) and cold-storage (4 and 0 ºC, respectively) temperatures. However, *P. expansum* was more susceptible to high concentrations of H_2O_2 treatment than *P. digitatum*, especially at 25 ºC.

6. An advanced maturity stage of 'Golden Smoothee' apples and 'Valencia' oranges increased the susceptibility to be infected by compatible pathogens. On-tree maturation also increased fruit susceptibility to non-host pathogen interactions, especially reducing apple resistance to *P. digitatum* in the over-mature maturity stage.

7. Production of H_2O_2 by apples and oranges in response to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses was maturity-dependent. In immature apples, there was a remarkable biphasic H_2O_2 production (at 4 and 24 hours), especially after wounding. In contrast, in over-mature oranges, there was a sharply reduction in the H_2O_2 production at 24 hours.

8. The *in situ* staining of H_2O_2 with DAB in apple and orange fruits showed that H_2O_2 is locally produced in response to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses. The visualisation of DAB staining was more evident at immature and commercial ripening stage, and declined as maturity progressed in both fruits.

Apple proteome changes in response to wounding

9. The phenol-based protein extraction followed by DTT/Acetone precipitation was the best method for obtaining 'Golden Delicious' apple tissue proteins at high concentrations and without interfering compounds, which is essential for further proteomics studies.

10. The proteomic analysis of changes in protein abundance after wounding (at 24 and 48 hours) in 'Golden Delicious' apples allowed the identification of fifty-eight non-responsive and five wound-responsive proteins. According to their biological function the non-responsive proteins were involved in 'metabolism', 'response to stress' and 'oxidation-reduction', while the wound-responsive proteins mainly were categorized in 'response to stress'. Specifically the identified

wound-responsive proteins were: *β*-glucanase, thaumatin-like protein 1-a, Mal d 1 like, Mal d 1.03G and flavoprotein WrbA-like.

Apple proteome and oxi-proteome changes in response to *P. expansum* and *P. digitatum*

11. Changes at the proteome and oxi-proteome level in 'Golden Smoothee' apple fruits in response to *P. expansum* (compatible) and *P. digitatum* (non-host) pathogens (at 24 and 48 hours) were detected. While twenty-six spots exhibited significant changes in abundance (2.0-fold), another twenty-six spots showed oxidation changes (1.5-fold) among treatments. The fifty-two spots were successfully identified.

12. The proteomic studies revealed that the proteins changing its abundance after a non-host pathogen inoculation belong to the functional category 'response to stress', whereas after a compatible pathogen inoculation to both 'response to stress' and 'metabolism processes'.

13. Protein abundance changes showed some similarities between the apple responses to abiotic (wounding) and biotic (pathogen and non-host pathogen) stresses. For example, the PR-protein Mal d 1.03A increased its abundance in response to both stresses, especially at 48 hours after inoculation. Conversely, some apple protein changes were specifically induced after a non-host pathogen inoculation, such as the identified Mal d 1.03E and elongation transcription factor (EF-Tu) at 48 hours.

14. Oxi-proteomic studies revealed that oxidative changes in response to either the compatible or the non-host pathogen were mainly involved in 'metabolism processes'.

15. Protein oxidation changes showed that the ACC oxidase at 24 hours and two glutamine synthetases at 48 hours were more oxidised in response to *P. digitatum* than in response to *P. expansum* inoculation.

16. The transcriptional levels of six *M. domestica* genes (coding for the identified proteins: ACC oxidase, ascorbate peroxidase, Mal d 1.03E, class I heat shock protein (HSP), translation elongation factor EF-Tu precursor and NAC domain class transcription) showed different patterns of response to abiotic and biotic stresses.

17. Transcript levels of HSP, EF-Tu and NAC genes were significantly lower following *P. expansum* inoculation than in control wounded apples, at 48 hours. The greatest transcriptional change was for APX, at 24 hours after *P. digitatum* inoculation.

18. Only one of the six studied genes showed a significant correlation at the transcript and protein level: Mal d 1.03E.

Uso de cepas marcadas con GFP para el estudio de la interacción fruta-patógeno

1. Este estudio representa el primer trabajo que caracteriza las cepas de *P. expansum* y *P. digitatum* marcadas con GFP. De hecho, la transformación con GFP de estos dos importantes patógenos de postcosecha no afectó significativamente ni su patogenicidad, ni su índice de esporulación en los estudios *in vivo*, en manzanas y naranjas.

2. La tranformación con GFP no modificó la ecofisiología (patrón de germinación y tasa de crecimiento) en los estudios *in vitro* de *P. digitatum* y *P. expansum* en diferentes medios de cultivo y temperaturas (ambiental y de frigoconservación).

3. El uso de cepas de *P. digitatum* y *P. expansum* marcadas con GFP ha permitido confirmar que ambos patógenos son de herida, así como también visualizar el proceso de infección del patógeno compatible y del no compatible. En el caso de los patógenos no compatibles, *P. expansum* infectó naranjas 'Lanelate', mientras que *P. digitatum* solo causó infección limitada alrededor de la herida en manzanas 'Golden Delicious'.

Caracterización del H_2O_2 para estudiar la interacción fruta-patógeno a diferentes estados de madurez

4. Los estudios ecofisiológicos *in vitro* de *P. digitatum* y *P. expansum* mostraron que los valores máximos de germinación se alcanzaron antes por *P. expansum* que por *P. digitatum*, para ambas temperaturas estudiadas (ambiental y frigoconservación). Sin embargo, el almacenamiento en frío retrasó 10 veces los valores de germinación de ambos patógenos.

5. El efecto del H_2O_2 como tratamiento sobre la germinación de *P. digitatum* y *P. expansum* mostró que, a concentraciones elevadas de H_2O_2 (200 mM), se redujeron los valores de germinación de ambos patógenos tanto a temperatura ambiente (25 ºC) como de

frigoconservación (4 y 0 ºC, respectivamente). Sin embargo, *P. expansum* fue más susceptible a altas concentraciones del tratamiento con H_2O_2 que *P. digitatum*, especialmente a 25 ºC.

6. Un estado avanzado de madurez aumentó la susceptibilidad a la infección por los patógenos compatibles, tanto en manzanas 'Golden Smoothee' como en naranjas 'Valencia'. El estado de madurez también aumentó la susceptibilidad del fruto a los patógenos no compatibles, destacando la reducción de la resistencia a *P. digitatum* en manzana sobremadura.

7. En manzanas y naranjas, la producción de H_2O_2 en respuesta a estrés abiótico (herida) y biótico (patógeno compatible y no compatible) fue dependiente de la madurez. En manzanas inmaduras, hubo una notable producción bifásica de H_2O_2 (a 4 y 24 horas), especialmente después de la herida. Por el contrario, en naranjas sobremaduras, hubo una brusca reducción en la producción de H_2O_2 a 24 horas.

8. La tinción *in situ* del H_2O_2 con DAB en manzana y naranja mostró que el H_2O_2 se produce localmente tanto en respuesta a estrés abiótico (herida) como biótico (patógeno compatible y no compatible). La visualización de la tinción con DAB fue más evidente en los estados de madurez inmaduro y comercial, y disminuyó a medida que aumentó la madurez de los frutos.

Cambios en el proteoma de manzana en respuesta a herida

9. En manzana 'Golden Delicious', el método que obtuvo una elevada concentración de proteínas sin compuestos que interfieran en los pasos posteriores, fue la extracción proteica con fenol seguida de la precipitación con DTT/Acetona, lo cual es esencial para futuros estudios proteómicos.

10. El análisis proteómico de los cambios en abundancia proteica en manzanas 'Golden Delicious' después de realizar una herida (a 24 y

48 horas) permitió la identificación de cincuenta y ocho proteínas comunes y cinco de respuesta a herida. De acuerdo con su función biológica, las proteínas comunes están implicadas en 'metabolismo', 'respuesta a estrés' y 'oxidación-reducción', mientras que las proteínas de respuesta a herida se clasificaron principalmente en 'respuesta a estrés'. Específicamente las proteínas identificadas de respuesta a herida fueron: *β*-glucanase, thaumatin-like protein 1-a, Mal d 1 like, Mal d 1.03G y flavoprotein WrbA-like.

Cambios en el proteoma y oxi-proteoma de manzana en respuesta a *P. expansum* y *P. digitatum*

11. En manzanas 'Golden Smoothee', se detectaron cambios a nivel proteómico y oxi-proteómico en respuesta a los patógenos *P. expansum* (compatible) y *P. digitatum* (no compatible) a 24 y 48 horas. Mientras veintiséis proteínas mostraron cambios significativos en abundancia (criterio 2.0), otras veintiséis proteínas presentaron cambios en oxidación (criterio 1.5) entre tratamientos. Las cincuenta y dos proteínas se identificaron con éxito.

12. Los estudios proteómicos revelaron que las proteínas que cambiaron su abundancia tras la inoculación con el patógeno no compatible pertenecen a la categoría funcional 'respuesta a estrés', mientras que tras la inoculación con el patógeno compatible se clasifican en 'respuesta a estrés' y 'procesos metabólicos'.

13. Los cambios en abundancia proteica mostraron similitudes entre las respuestas de manzana a estrés abiótico (herida) y biótico (patógeno compatible y no compatible). Por ejemplo, la PR-proteína Mal d 1.03A aumentó su abundancia en respuesta a ambos estreses, especialmente 48 horas después de la inoculación. Por el contrario, algunos cambios de abundancia proteica en manzana se indujeron específicamente después de la inoculación con el patógeno no compatible, como la Mal d 1.03E y el factor de elongación de la traducción (EF-Tu) a 48 horas.

14. Los estudios oxi-proteómicos revelaron que los cambios de oxidación en respuesta tanto al patógeno compatible como al no compatible estaban principalmente implicados en 'procesos metabólicos'.

15. Los cambios en oxidación proteica mostraron que la ACC oxidasa a 24 horas y dos glutaminas sintetasas a 48 horas se oxidaron de forma más importante en respuesta a *P. digitatum* que en respuesta a *P. expansum*.

16. Los niveles de transcripción de seis genes de *M. domestica* (que codifican para las proteínas: ACC oxidasa, ascorbato peroxidasa, Mal d 1.03E, proteína de choque térmico clase I (HSP), precursor del factor de elongación de la traducción EF-Tu y dominio clase NAC de la transcripción) mostraron diferentes patrones de expresión en respuesta a estrés abiótico y biótico.

17. Los niveles de transcripción de los genes HSP, EF-Tu y NAC fueron significativamente menores 48 horas después de la inoculación con *P. expansum*, en comparación con el control de manzana herida. El mayor cambio transcripcional fue observado en APX, a 24 horas después de la inoculación con *P. digitatum*.

18. Tan solo uno de los seis genes seleccionados mostró correlación significativa a nivel de transcrito y proteína: la Mal d 1.03E.

Concluding remarks/ Consideraciones finales

What does the *P. digitatum* and *P. expansum* GFP labeling represent for the study of fruit-pathogen interactions?

Once demonstrated that the GFP transformation does not affect the ecophysiology, pathogenicity and sporulation index of the pathogens, these strains of *P. digitatum* and *P. expansum* became an extremely versatile tool. In particular, these strains have allowed us the *in situ* monitorization of the compatible and the non-compatible interactions in oranges and apples. The importance of obtaining these GFP-tagged strains also relies on the possibility of further studying other interactions, and other colonization environments.

What is the role of ROS, specifically of H_2O_2, in the fruit defence response against postharvest pathogens?

The H_2O_2 role in defence of both fruits (climateric and non climateric) should be further explored, since it appears to be involved in triggering signal transduction cascades, in addition to have a direct antifungal effect. Future studies should focus on immature fruit, where H_2O_2 appears to play a major role in inducing defence response mechanisms.

Can we obtain the global protein profile of fruit (apple) in response to different stresses (abiotic and biotic)?

Considering that this thesis provides an optimized protein extraction protocol for apple, a crucial step in proteomics, we can study the apple response against other abiotic stresses, as well as other apple-pathogen interactions.

Proteomics to study apple-pathogen interactions…

This is the first time that information of the apple protein profile in response to wounding, compatible pathogen (*P. expansum*) and non host pathogen (*P. digitatum*) is provided. The specific responses after the *P. digitatum* inoculation may help us to unravel a set of proteins that have an important role in defence against the non-host pathogen.

What does the protein oxidation profile provide to the apple-pathogen interaction studies?

On the one hand, the oxi-proteome has provided information about affected proteins (oxidised). On the other hand, this methodology optimized for apple can establish the basis for future oxi-proteomic studies.

Finally…

The molecular, biochemical and pathological approaches realised in this thesis establish the basis for additional analyses of fruit-pathogen interactions on the field of postharvest pathology. Our results should be the starting point for further studies that will provide valuable insights into the infection process in fruit, especially into the determinants of the fruit-pathogen compatibility. Numerous questions have raised and still awaiting response, such as: What genes are ultimately responsible to start the infection? What is their role in this process? What metabolic pathways are activated as a defence response in immature fruit? To what extent are they weakened with ripening? Are the defence mechanisms differentially deployed in climateric (apple) and non climateric (orange) fruits? What is the relationship between a primary metabolism, as nitrogen, and a secondary one, as ethylene, in apple defence responses?

¿Qué supone el marcaje de *P. digitatum* y *P. expansum* con GFP en el estudio de la interacción fruta-patógeno?

Una vez se ha demostrado que la transformación con GFP no afecta a la ecofisiología, patogenicidad e índice de esporulación de los patógenos, estas cepas de *P. digitatum* y *P. expansum* se han convertido en una herramienta extremadamente versátil. En particular, estas cepas nos han permitido monitorizar *in situ* las interacciones compatible y no compatible en naranjas y manzanas. La importancia en la obtención de estas cepas marcadas con GFP radica en que se podrán utilizar en el futuro, para monitorizar otras interacciones y otros ambientes de colonización.

¿Qué papel juegan las ROS, concretamente el H_2O_2, en la defensa de la fruta en respuesta a patógenos de postcosecha?

El papel que pueda tener el H_2O_2 en la defensa de ambos frutos (climatéricos y no climatéricos) deberá establecerse en futuros estudios, ya que parece desempeñar una función señalizadora, además de antifúngica. En el futuro, los estudios deberían centrarse en fruta inmadura donde el H_2O_2 parece ejercer una función importante en la inducción de respuestas defensivas.

¿Es posible obtener el perfil proteico de respuesta global del fruto (manzana) a diferentes estreses (abióticos y bióticos)?

Considerando que hemos optimizado un protocolo de extracción proteica para manzana, en cual es un paso crítico en proteómica, podremos estudiar la respuesta a otros estreses abióticos, y otras interacciones manzana-patógeno.

La proteómica para estudiar las interacciones manzana-patógeno…

Por primera vez, se aporta información del perfil proteico de manzana en respuesta a: herida, al patógeno compatible (*P. expansum*) y al no

compatible (*P. digitatum*). La respuesta diferencial del fruto tras la inoculación con *P. digitatum*, puede ayudar a identificar aquellas proteínas con un papel importante en la defensa en respuesta al patógeno no compatible.

¿Qué aporta la oxidación proteica a los estudios de interacción manzana-patógeno?

Por un lado, el oxi-proteoma nos ha aportado información sobre las proteínas afectadas (oxidadas). Por otro lado, la optimización de esta metodología en manzana posibilita futuros estudios oxi-proteómicos en otras interacciones fruta-patógeno.

En definitiva…

Las aproximaciones moleculares, bioquímicas y patológicas realizadas en esta tesis establecen las bases para realizar futuros estudios de las interacciones fruta-patógeno en el ámbito de la patología de la postcosecha. Nuestros resultados son el punto de partida de futuros estudios para identificar los mecanismos moleculares que subyacen a los procesos infecciosos en fruta, especialmente frente a interacciones no compatibles. Numerosas son las preguntas generadas que todavía esperan respuesta. Por poner algunos ejemplos: ¿Qué genes utiliza el patógeno para iniciar la infección? ¿Cuál es su papel en este proceso? ¿Qué metabolismos se activan como resistencia en fruta inmadura? ¿Cuáles de éstos se van atenuando conforme el fruto madura? ¿Son distintos los mecanismos de defensa de un fruto climatérico (manzana) y de uno no climatérico (naranja)? ¿Qué relación tiene un metabolismo primario, como el del nitrógeno, y otro secundario, como el del etileno, en las respuestas de defensa de la manzana?

Future perspectives/ Perspectivas de futuro

The results obtained in this thesis allowed us to place some pieces of the complex puzzle which is the fruit-pathogen interaction, opening a new range of possibilities, such as:

1. The transformation protocol and the successful GFP expression will allow studying the expression and function of various genes in the pathogen.

2. Using GFP-tagged strains, under controlled conditions that prevent their release into the environment, will allow us: (i) to evaluate the survival of *P. digitatum* and *P. expansum* on postharvest epidemiology studies, as well as (ii) to know the time that the fungus can stay on the surface or atmosphere of the chamber (on environmental contamination studies, or after disinfectant treatments in fruit storage chambers). This information may be essential for designing new control strategies in infections caused by fungi of the genus *Penicillium*.

3. To study the mechanisms by which *P. digitatum* and *P. expansum* are able to degrade H_2O_2. For instance, to determine whether fungi produce detoxifying enzymes as CAT, or organic acids as oxalic acid, since they appear to be responsible for suppressing the H_2O_2 fruit production. In the future, obtaining fungi mutants unable to produce CAT or oxalic acid will provide valuable insights into their importance in virulence.

4. To analyze the role of the Mal d 1 and EF-Tu proteins in the apple fruit resistance against a non compatible pathogen, focusing on the molecular mechanisms by which they regulate host defence responses. The Mal d 1 gene family members, which experienced a gene functional diversification, are excellent candidates to investigate their role in different defence response mechanisms.

5. To evaluate the biological processes identified by the oxi-proteome as major determinants of the 'Golden Smoothee' resistance to *P. digitatum*: the nitrogen and ethylene metabolisms.

6. To determine the possible involvement of Glutamine Synthetase (GS) in the 'Golden Smoothee' resistance to *P. digitatum*.

7. To characterise the ethylene biosynthesis regulation in different apple varieties, in response to injury and infection by *P. digitatum* and *P. expansum*.

Los resultados obtenidos en esta tesis nos han permitido situar algunas de las piezas de este complejo puzle que es la interacción huésped-patógeno en fruta, abriendo un nuevo abanico de posibilidades, como:

1. El protocolo de transformación y la exitosa expresión de la GFP permitirán estudiar la expresión de varios genes y evaluar su función en el patógeno.

2. Utilizar las cepas marcadas con GFP, siempre en condiciones controladas que eviten su dispersión en el ambiente, para evaluar (i) la supervivencia de *P. digitatum* y *P. expansum* en estudios de epidemiología de postcosecha, así como (ii) en estudios de contaminación ambiental, o tras tratamientos desinfectantes en cámaras de conservación de fruta, nos permitirá saber si el hongo es capaz de mantenerse en la superficie o ambiente de la cámara y durante cuánto tiempo. Esta información podría ser esencial para diseñar nuevas estrategias de control en las infecciones causadas por los hongos del género *Penicillium*.

3. Estudiar los mecanismos a través de los cuales *P. digitatum* y *P. expansum* son capaces de degradar el H_2O_2. Por ejemplo, determinar si los hongos producen enzimas detoxificantes como la CAT, o ácidos orgánicos como el ácido oxálico, ya que éstos parecen ser los responsables de suprimir la producción de H_2O_2 en la fruta. En el futuro, la obtención de mutantes de *P. digitatum* y *P. expansum*, deficientes en la producción de CAT o ácido oxálico, ayudaría a entender la participación de estas moléculas en virulencia.

4. Analizar las proteínas Mal d 1 y EF-Tu para entender su implicación en la resistencia de manzana en respuesta a un patógeno no compatible, focalizando en los mecanismos moleculares que subyacen a la respuesta defensiva del huésped. Concretamente, los miembros de la familia multigénica Mal d 1 han experimentado una diversificación funcional, siendo excelentes candidatos para investigar su función en diferentes mecanismos de respuesta defensiva.

5. Evaluar los dos procesos metabólicos identificados por el estudio oxi-proteómico como posibles causantes de la resistencia de 'Golden Smoothee' a *P. digitatum*: el del nitrógeno y el del etileno.

6. Determinar la posible participación de la Glutamina Sintetasa (GS) en los mecanismos de resistencia de 'Golden Smoothee' a *P. digitatum*.

7. Caracterizar la regulación de la síntesis de etileno en diferentes variedades de manzana, como respuesta a herida y a la infección por *P. digitatum* y *P. expansum*.

Appendix I

Supplementary Table 1. Effect of harvest date on fruit quality parameters of 'Valencia' oranges.

Harvest	Date	Deformation (mm)	Titratable acidity (TA in % citric acid)	Total soluble solids (TSS in %)	Ratio TSS/TA	CI (colour index)
Immature	17/03/2011	2.3 A	2.0 A	10.7 A	5.3 C	4.5 A
Commercial	29/04/2011	2.3 A	1.4 B	10.9 A	8.0 B	3.6 B
Over-mature	23/06/2011	2.1 A	0.8 C	10.7 A	12.7 A	2.2 C

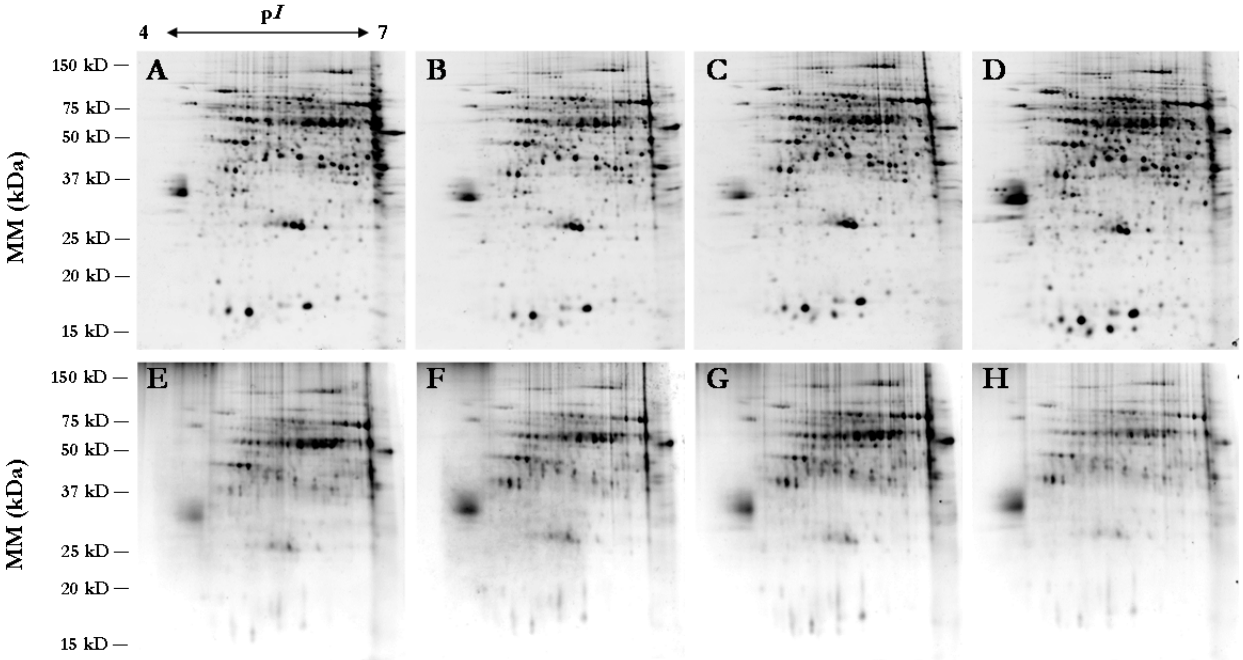
Mean of 20 individual fruits. Different letters in the same column indicate differences among means using the LSD test ($P < 0.05$).

Supplementary Table 2. Effect of harvest date on fruit quality parameters of 'Golden Smoothee' apples.

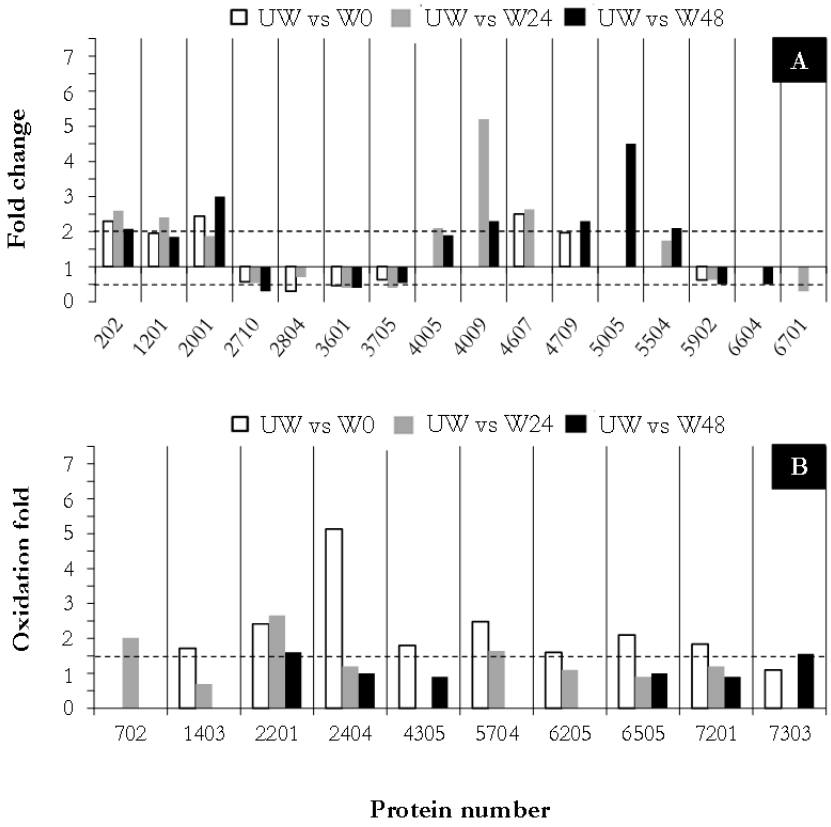
Harvest	Date	Flesh firmness (N)	Titratable acidity (TA in g/L malic acid)	Total soluble solids (TSS in %)	Starch index	(a*+ b*)
Immature	12/08/2010	77.9 A	6.3 A	10.2 C	1.1 C	23.1 B
Commercial	16/09/2010	65.9 B	6.0 A	12.1 B	6.0 B	24.4 B
Over-mature	21/10/2010	42.8 C	3.9 B	14.2 A	9.8 A	39.9 A

Mean of 20 individual fruits. Different letters in the same column indicate differences among means using the LSD test ($P < 0.05$).

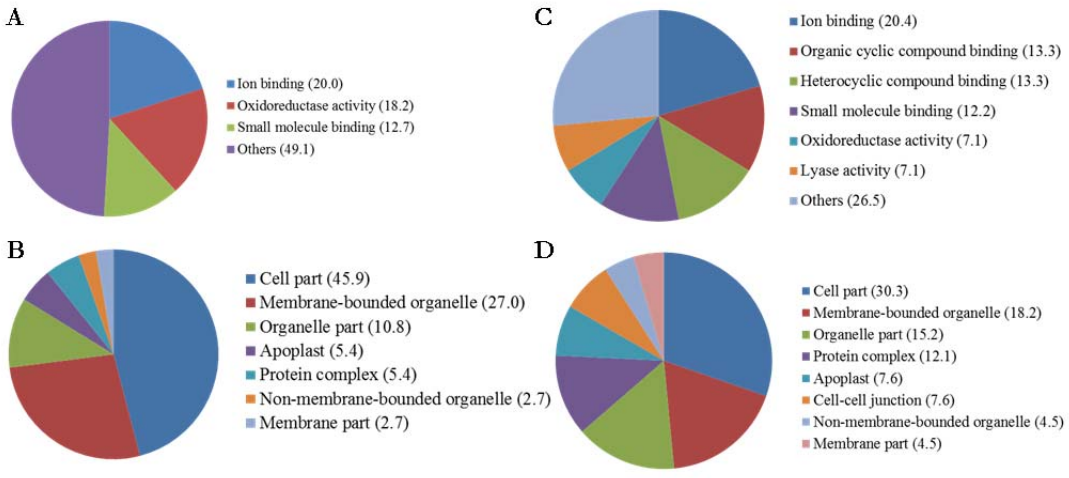
Appendix II



Supplementary Figure 1. Protein extracts obtained from unwounded, wounded and inoculated with *P. digitatum* and *P. expansum* 'Golden Smoothee' apples were derivatized with Bodipy-Hz and analyzed by 2D-gel electrophoresis. The images shown correspond to total protein stain by Flamingo (BioRad) (A-D) and Bodipy signal (E-H) in unwounded, wounded and inoculated samples at 48 hours.



Supplementary Figure 4. Temporal changes in the proteome and oxi-proteome of 'Golden Smoothee' apples in response to wounding. In A, columns represent the fold changes (2-fold) of protein abundance between wounded (mock inoculated) and unwounded tissue. Values ≥ 2 (--- threshold) are considered an increase in the abundance. Values ≤ 0.5 (--- threshold) are considered a decrease in the abundance. In B, columns represent the oxidation fold (1.5-fold) ratio [Bodipy signal/protein signal] of each spot between wounded (mock inoculated) and unwounded tissue. Values ≥ 1.5 (--- threshold) are considered oxidised. Data are the mean of three independent gels. UW = unwounded, W = wounded control (mock inoculated). Column absence indicates a spot absence at the specific condition.



Supplementary Figure 5. Proteome (A-B) and oxi-proteome (C-D) GO terms distribution after abiotic and biotic stresses in 'Golden Smoothee' apple. GO terms distributions according to the Blast2GO software were grouped, at the level 3, by molecular functions (A-C) and cellular components (B-D) with circle graphs. Molecular functions were filtered by number of Seqs (cutoff = 5). The numbers on each fraction indicate the percentage of detected proteins belonging to each category.

Supplementary Table 2A. Proteins with significant abundance changes after wounding and pathogen inoculation, and their identification by peptide mass fingerprint (PMF)

Spot [a]	Protein description [b]	Accession number [c]	Organism	Database	Identification method	MM (kDa) [d]	p*I* [d]	Mascot score	Coverage %	Peptides macthed/ total peptides	Matched peaks	Corresponding sequence
202	ACC oxidase 1	O24063	*M. domestica*	SWP	PMF	35.6	5.2	142	31	11/17	939.50	FKEMVAAK
											2128.01	HLPSSNISEIPDLEEEYR
											2256.11	HLPSSNISEIPDLEEEYRK
											1340.66	TMKEFAVELEK
											964.50	EFAVELEK
											1529.82	LLDLLCENLGLEK
											1627.85	VSNYPPCPKPDLIK
											1699.87	AHSDAGGIILLFQDDK
											946.50	VIAQSDGTR
											1064.47	FVFDDYMK
											1080.47	FVFDDYMK
1201	ACC oxidase 1	O24063	*M. domestica*	SWP	PMF	35.6	5.2	164	40	13/22	2522.16	GLDDVQSEIHDLDWESTFFLR
											2127.97	HLPSSNISEIPDLEEEYR
											2256.08	HLPSSNISEIPDLEEEYRK
											1340.65	TMKEFAVELEK
											964.49	EFAVELEK
											1529.79	LLDLLCENLGLEK
											720.38	GPNFGTK
											1627.81	VSNYPPCPKPDLIK
											1699.82	AHSDAGGIILLFQDDK
											946.48	VIAQSDGTR
											1149.55	KTEDAPTYPK
											1064.47	FVFDDYMK
											1080.46	FVFDDYMK
2001	Major allergen Mal d 1.06C	gi|60280825	*M. domestica*	nrNCBI	PMF	17.4	5.5	167	58	9/13	1866.02	GVLTYETEYASVIPPAR
											1671.99	LYNALVLDADNLIPK
											726.45	IAPQAVK
											1487.84	TVEILEGDGGVGTIK
											1615.93	TVEILEGDGGVGTIKK
											1579.82	KVSFGEGSEYSYVK
											1451.73	VSFGEGSEYSYVK
											1282.65	GDVEIKEEHVK
											1582.72	LIENHLVANPDAYN
2708	Polyphenol oxidase 2 precursor	gi|14194273	*M. domestica*	nrNCBI	PMF	65.8	6.1	224	28	17/23	1587.76	ATKGDNDNLDQGLAR
											923.54	LPDRGPLR
											1572.90	IAAQDVAKNPAYLAK
											776.40	NPAYLAK
											748.39	AIELMR
											898.45	ALPDDDPR
											1652.82	ALPDDDPRSLVQQAK
											892.45	NTAHQPPK
											1967.86	LLDLNYGGTDDDVDDATR
											1588.71	ENLTTMYQQMVSK
											1586.73	DPLFYSHHSNVDR
											1086.55	MWSIYKDK
											1114.58	KLGYVYDEK

											986.49	LGYVYDEK
											2784.33	GIEFAGNEPVKFDVYVNDDAESLAGK
											1684.85	DKSEFAGSFVHVPHK
											1441.72	SEFAGSFVHVPHK
2804	High molecular weight heat shock protein	gi|6969976	*M. domestica*	nrNCBI	PMF	71.6	5.2	242	39	27/43	1228.63	VEIIANDQGNR
											1487.70	TTPSYVAFTDTER
											1663.80	NQVAMNPVNTVFDAK
											1567.79	QFAAEEISSMVLVK
											1583.79	MREIAEAYLGSSIK
											1280.68	EIAEAYLGSSIK
											1680.83	NAVVTVPAYFNDSQR
											1197.68	DAGVIAGLNVLR
											1675.73	ATAGDTHLGGEDFDNR
											1278.64	MVNHFVQEFK
											1294.63	MVNHFVQEFK
											761.38	DISSSPR
											905.47	LRTACER
											1540.74	ARFEELNMDLFR
											1556.75	ARFEELNMDLFR
											1313.63	FEELNMDLFR
											908.38	CMEPVEK
											762.39	CLRDAK
											1426.76	STVHDVVLVGGSTR
											2658.29	EQVFSTYSDNQPGVLIQVYEGER
											1030.56	TRDNNLLGK
											1183.63	FELSGIPPAPR
											1017.56	ITTTNDKGR
											1358.61	NALENYAYNMR
											1374.61	NALENYAYNMR
											1031.56	IASKLDAADK
											1386.74	ELESICNPIIAK
3004	Major allergen Mal d 1.03E	gi|60280855	*M. domestica*	nrNCBI	PMF	17.6	5.4	234	80	14/25	1913.94	GVFTYESEFTSIIPPAR
											1689.92	LFNAFVLDADNLIPK
											726.45	IAPQAVK
											1445.74	SAEILEGDGGVGTIK
											1573.84	SAEILEGDGGVGTIKK
											1592.80	KINFGEGSTYSYVK
											1464.69	INFGEGSTYSYVK
											1412.70	IDGVDKDNFVYK
											1653.81	YSVIEGDAISETIEK
											740.40	ISYETK
											960.46	STSHYHTK
											1282.67	GDVEIKEEHVK
											959.53	EKASHLFK
											702.37	ASHLFK
3601	Polyphenol oxidase 2 precursor	gi|14194273	*M. domestica*	nrNCBI	PMF	65.8	6.1	222	23	17/20	829.43	SQTPLQR
											1587.77	ATKGDNDNLDQGLAR
											923.53	LPDRGPLR
											776.42	NPAYLAK
											732.42	AIELMR
											748.39	AIELMR
											898.43	ALPDDDPR
											892.46	NTAHQPPK

											1967.86	LLDLNYGGTDDDVDDATR
											1588.73	ENLTTMYQQMVSK
											1586.72	DPLFYSHHSNVDR
											827.41	MWSIYK
											1086.52	MWSIYKDK
											1114.56	KLGYVYDEK
											986.48	LGYVYDEK
											1684.85	DKSEFAGSFVHVPHK
											1441.72	SEFAGSFVHVPHK
3705	Polyphenol oxidase 2 precursor	gi|14194273	*M. domestica*	nrNCBI	PMF	65.8	6.1	258	24	18/20	1057.60	KPKQCLAGR
											1587.76	ATKGDNDNLDQGLAR
											923.53	LPDRGPLR
											776.45	NPAYLAK
											1067.58	NPAYLAKYK
											748.37	AIELMR.A
											898.45	ALPDDDPR
											1652.86	ALPDDDPRSLVQQAK
											892.44	NTAHQPPK
											1967.76	LLDLNYGGTDDDVDDATR
											1588.74	ENLTTMYQQMVSK
											1586.65	DPLFYSHHSNVDR
											827.38	MWSIYK
											1086.53	MWSIYKDK
											1114.55	KLGYVYDEK
											986.48	LGYVYDEK
											1684.83	DKSEFAGSFVHVPHK
											1441.73	SEFAGSFVHVPHK
4004	Major allergen Mal d 1.03D	gi|60280849	*M. domestica*	nrNCBI	PMF	17.6	5.5	143	65	9/22	1689.91	LFNAFVLDADNLIPK
											1445.75	SAEILEGDGGVGTIK
											1592.80	KINFGEGSTYSYVK
											1464.71	INFGEGSTYSYVK
											1412.71	IDGVDKDNFVYK
											1653.82	YSVIEGDAISETIEK
											960.48	STSHYHTK
											1282.68	GDVEIKEEHVK
											1734.84	LIENYLLEHQDAYN
4005	Major allergen mal d 1	gi|4590378	*M. domestica*	nrNCBI	PMF	17.7	5.7	174	72	12/32	1969.88	GVCTFENEFTSEIPPSR
											1315.71	AFVLDADNLIPK
											740.47	IAPQAIK
											1611.85	QAEILEGNGGPGTIKK
											1576.76	KITFGEGSQYGYVK
											1448.69	ITFGEGSQYGYVK
											740.38	ISYETK
											1092.57	LVACGSGSTIK
											1295.69	GNIEIKEEHVK
											929.51	EKAHGLFK
											865.49	LIESYLK
											1677.79	LIESYLKDHPDAYN
4009	Class I Heat shock protein	CO723224	*M. domestica*	Plants EST	PMF	18.6	6.1	143	45	9/12	2111.11	SKNPTQTHHLMALSLFGGR
											1071.54	ETPEAHIFK
											713.37	ADLPGIK
											1288.63	EEVKVEVEDGR

											803.39	VEVEDGR
											901.50	VLQISGER
											855.43	NDKWHR
											828.39	SSGKFMR
											974.53	FRLPENAK
4503	4-hydroxyphenylpyruvate dioxygenase-like	EB136504	*M. domestica*	Plants EST	PMF	16.4	9.1	118	68	6/9	2081.04	GQENDTHFNLVGFSNFVR
											2075.02	FHHVEFWCTDATNAALR
											1762.91	SDLSTGNQTHASYLLR
											2000.07	SGDLNFLFTAPYSPTLTR
											2386.06	TADPARPSNSSASIPTFDHTASR
											1115.62	AFSATHGLGVR
5005	Major allergen Mal d 1.03A	gi|60280841	*M. domestica*	nrNCBI	PMF	17.6	5.7	152	53	8/12	1689.92	LFNAFVLDADNLIPK
											1445.75	SAEILEGDGGVGTIK
											1592.79	KINFGEGSTYSYVK
											1464.70	INFGEGSTYSYVK
											1426.72	IDGVDKENFVYK
											1653.81	YSVIEGDAISETIEK
											1271.71	LIENYLLEHK
											1734.87	LIENYLLEHKDAYN
5902	Cytosolic aconitase	CN900040	*M. domestica*	Plants EST	PMF	21.5	6.5	111	59	11/49	1158.59	AGSINKDSPAAK
											945.40	DFNSYGSR
											1063.58	RGNDEVMAR
											778.41	GTFANIR
											1039.54	LFVFDAATR
											2254.07	YKADGHDTIVIAGAEYGSGSSR
											1962.94	ADGHDTIVIAGAEYGSGSSR
											1955.05	IHRSNLVGMGIIPLCFK
											1541.72	AGEDADTLGLTGHER
											870.40	SFTCTVR
											2257.21	SFTCTVRFDTEVELEYFN
6604	ATP1 (mitochondrion)	gi|404481694	*M. domestica*	nrNCBI	PMF	55.6	6.2	188	33	23/63	786.35	MEFSTR
											1971.00	MEFSTRAAELTTLLESR
											1203.75	AAELTTLLESR
											972.53	VVSVGDGIAR
											816.40	EGDLVKR
											1199.65	RTGSIVDVPAGK
											1196.69	VVDGLGVPIDGR
											755.46	APGIIER
											1354.70	KSVHEPMQTGLK
											1370.68	KSVHEPMQTGLK
											1012.58	AVDSLVPVGR
											1353.68	AVDSLVPVGRGQR
											1156.73	GQRELIIGDR
											815.42	ELIIGDR
											1714.83	QTGKTAIAIDTILNQK
											1537.77	EAFPGDVFYLHSR
											1153.58	IMKQVCGSSK
											765.38	QVCGSSK
											892.51	LELAQYR
											970.58	GARLTEVPK
											795.43	ISQYER
											1204.73	ISQYERAIPK

											996.55	MEPDTFLK
6701	NADP-dependent malic enzyme-like	EB136452	*M. domestica*	Plants EST	PMF	23.8	8.9	121	35	11/29	891.49	KLMNTIR
											763.42	LMNTIR
											1253.60	YMALTELQER
											1269.60	YMALTELQER
											726.38	FGSIFR
											1174.67	RPQGLYISLK
											1315.71	TVQVIVVTDGER
											1352.71	LLDNEFYIGLR
											1608.92	LLDNEFYIGLRQK
											1903.91	GKEYEDLLEEFMSAVK
											1718.78	EYEDLLEEFMSAVK
8101	Pyridoxine biosynthesis protein-like	GO510968	*M. domestica*	Plants EST	PMF	23.5	6.0	187	49	15/23	887.51	VGLAQMLR
											903.49	VGLAQMLR
											2313.20	VGLAQMLRGGVIMDVVNADQAR
											1444.72	GGVIMDVVNADQAR
											1460.71	GGVIMDVVNADQAR
											1576.75	VAEEAGACAVMALER
											1592.73	VAEEAGACAVMALER
											1309.74	VPADIRAQGGVAR
											947.49	MSDPQLIK
											994.45	VPFVCGCR
											772.43	NLGEALR
											763.37	EGAAMIR
											1515.81	TKGEAGTGNIIEAVR
											1286.67	GEAGTGNIIEAVR
											1447.61	NMDDDEVFTFAK

[a] Spot identification number as assigned in Fig. 2;

[b] Description of proteins identified from the Plants EST, nrNCBI or SwisProt database;

[c] Accession numbers according to Viridiplantae SwisProt, nrNCBI or Plants EST Database of NCBI;

[d] Molecular mass (MM) and isoelectric point (p*I*).

Supplementary Table 2B. Proteins with significant abundance changes after wounding and pathogen inoculation, and their identification with MALDI-TOF-TOF MS/MS

Spot [a]	Protein description [b]	Accession number [c]	Organism	Database	Identification method	MM (kDa) [d]	p*I* [d]	Mascot score	Coverage %	Matched peptides	Matched peaks	Peptide sequence (ion score) [e]
2101	Short-chain dehydrogenase/reductase 2b-like	EB123506	*M. domestica*	Plants EST	MS/MS	20.0	8.9	194	15	2	1208.67	LAIGPDDLIGPK (77)
											1882.99	QLTEALIPLLQQSDSAR (117)
2104	Thiazole biosynthetic enzyme	AT000365	*M. domestica*	Plants EST	MS/MS	7.3	8.1	85	17	1	1290.70	FDPIKESIVSR (85)
2710	Mitochondrial HSO70 2 isoform 1	CN908186	*M. domestica*	Plants EST	MS/MS	12.4	4.5	71	12	1	1564.82	AVITVPAYFNDAQR (71)
3403	Probable sarcosine oxidase-like	CN849922	*M. domestica*	Plants EST	MS/MS	19.6	5.8	72	5	1	1216.58	DQLHQEFSGR (72)
4607	Enolase	EB131141	*M. domestica*	Plants EST	MS/MS	21.5	7.7	162	11	2	1601.82	VNQIGSVTESIEAVR (122)
											806.44	YNQLLR (40)
4709	Apgm	DY256418	*M. domestica*	Plants EST	MS/MS	18.5	5.9	167	13	2	1489.67	ALEYADFDKFDR (82)
											1230.62	YLVEPPEIDR (85)
5504	Aminoacylase, putative	DT001087	*M. domestica*	Plants EST	MS/MS	24.0	9.0	101	11	2	1159.57	IAEEWAPTSR (51)
											1501.80	LGKPEIFPASTDAR (50)
5804	Heat shock protein STI-like	EB135855	*M. domestica*	Plants EST	MS/MS	21.9	4.8	251	21	3	1192.64	AFLQQPDFVK (65)
											1755.79	GGTGAEDEEVPESPPER (82)
											1761.93	AIELNDEDISFILNR (104)
6401	Elongation factor TU, chloroplast precursor (EF-TU)	DT000463	*M. domestica*	Plants EST	MS/MS	20.6	9.8	71	6	1	1435.67	KYDEIDAAPEER (71)

[a] Spot identification number as assigned in Fig. 2;

[b] Description of proteins identified from the Plants EST, nrNCBI or SwisProt database;

[c] Accession numbers according to Viridiplantae SwisProt, nrNCBI or Plants EST Database of NCBI;

[d] Molecular mass (MM) and isoelectric point (p*I*);

[e] The ion score is indicated in parentheses.

Supplementary Table 3A. Proteins with significant oxidation changes after wounding and pathogen inoculation, and their identification by peptide mass fingerprint (PMF)

Spot [a]	Protein description [b]	Accession number [c]	Organism	Database	Identification method	MM (kDa) [d]	pI [d]	Mascot score	Coverage %	Peptides macthed/ total peptides	Matched peaks	Corresponding sequence
1203	ACC oxidase 1	O24063	*M. domestica*	SWP	PMF	35.6	5.2	189	57	16/28	3284.52	INDACENWGFFELVNHGMSTELLDTVEK
											2522.09	GLDDVQSEIHDLDWESTFFLR
											2127.92	HLPSSNISEIPDLEEEYR
											2256.15	HLPSSNISEIPDLEEEYRK
											1324.65	TMKEFAVELEK
											1340.71	TMKEFAVELEK
											964.49	EFAVELEK
											1529.76	LLDLLCENLGLEK
											720.38	GPNFGTK
											1627.77	VSNYPPCPKPDLIK
											1699.78	AHSDAGGIILLFQDDK
											946.46	VIAQSDGTR
											2571.14	MSIASFYNPGNDSFISPAPAVLEK
											1149.55	KTEDAPTYPK
											1064.44	FVFDDYMK
											1080.43	FVFDDYMK
1403	Actin 7	CN896143	*M. domestica*	Plants EST	PMF	57.5	5.8	140	32	14/27	976.39	AGFAGDDAPR
											1198.65	AVFPSIVGRPR
											1176.49	HTGVMVGMGQK
											1932.80	YPIEHGIVSNWDDMEK
											1948.79	YPIEHGIVSNWDDMEK
											1515.69	IWHHTFYNELR
											1953.98	VAPEEHPVLLTEAPLNPK
											1547.68	LDLAGRDLTDSLMK
											1146.50	GYTFTTTAER
											1855.85	LAYVALDYEQELETAK
											1747.82	SYELPDGQVITIGAER
											1163.55	EITALAPSSMK
											895.48	VVAPPERK
											1475.63	SEYDESGPSIVHR
1608	ATP synthase subunit beta, mitochondrial-like	CN898384	*M. domestica*	Plants EST	PMF	22.1	6.2	218	79	15/27	1278.69	TIAMDGTEGLVR
											1750.95	GQRVLNTGSPITVPVGR
											1409.85	VLNTGSPITVPVGR
											1507.82	GDISTDHFLPIHR
											2172.21	EAPAFVEQATEQQILVTGIK
											1173.70	VVDLLAPYQR
											975.60	IGLFGGAGVGK
											1473.80	TVLIMELINNVAK
											1390.73	AHGGFSVFAGVGER
											1123.51	TREGNDLYR
											866.41	EGNDLYR
											1662.83	CALVYGQMNEPPGAR
											1678.82	CALVYGQMNEPPGAR
											1399.82	VGLTGLTVAEHFR
											1865.00	DAEGQDVLLFIDNIFR
2201	Pyruvate dehydrogenase E1 component subunit beta-1, mitocondrial-like	ES790079	*M. domestica*	Plants EST	PMF	62.3	9.0	202	24	17/21	1705.76	DALNSALDEEMSADPK
											1721.74	DALNSALDEEMSADPK
											1835.83	VFLMGEEVGEYQGAYK
											1133.60	GLLDKYGPDR
											1237.63	VLTPYSSEDAR
											1295.65	EGKDVTITAFSK
											1509.80	MVGYSLQAAEILAK
											1269.72	EGIRAEVINLR
											814.48	AEVINLR
											856.51	SIRPLDR
											984.60	SIRPLDRK
											804.42	TINDSVR

											932.53	TINDSVRK
											1687.83	IAGADVPMPYAANLER
											1703.82	IAGADVPMPYAANLER
											1238.71	LAVPQVEDIVR
											725.33	RACYR
2404	Actin, partial	gi|355329944	*M. domestica*	nrNCBI	PMF	40.4	5.7	202	56	18/33	976.46	AGFAGDDAPR
											1198.72	AVFPSIVGRPR
											1176.55	HTGVMVGMGQK
											1932.85	YPIEHGIVSNWDDMEK
											1948.85	YPIEHGIVSNWDDMEK
											1515.75	IWHHTFYNELR
											1954.04	VAPEEHPVLLTEAPLNPK
											3151.75	TTGIVLDSGDGVSHTVPIYEGYALPHAILR
											1547.73	LDLAGRDLTDSLMK
											1192.54	GYMFITTAER
											1855.91	LAYVALDYEQELETAK
											777.38	CDVDIR
											2183.06	DLYGNIVLSGGSTMFPGIADR
											2199.05	DLYGNIVLSGGSTMFPGIADR
											1163.61	EITALAPSSMK
											767.45	VVAPPER
											895.55	VVAPPERK
											1445.67	GEYDESGPSIVHR
2504	Regulatory particle triple-A ATPase 3 isoform 2, partial	CN888682	*M. domestica*	Plants EST	PMF	24.4	6.5	223	71	15/26	1678.89	EAVELPLTHHELYK
											895.52	QIGIDPPR
											1158.66	GVLLYGPPGTGK
											1271.72	AVANHTTAAFIR
											992.53	VVGSEFVQK
											791.42	YLGEGPR
											2029.06	ENAPAIIFIDEVDAIATAR
											980.48	FDAQTGADR
											1492.73	FDAQTGADREVQR
											1394.80	ADTLDPALLRPGR
											1114.65	KIEFPLPDR
											986.52	IEFPLPDR
											1142.65	IEFPLPDRR
											2140.98	MNLSDEVDLEDYVSRPDK
											2142.08	ISAAEISAICQEAGMHAVRK
3702	Vacuolar H+-ATPase	gi|131573315	*M. domestica*	nrNCBI	PMF	69.2	5.4	408	46	32/38	864.43	MPAVYGAR
											2052.92	LTTFEDSEKESEYGYVR
											1335.77	VGHDNLIGEIIR
											2736.34	LEGDSATIQVYEETAGLMVNDPVLR
											1062.55	RSGDVYIPR
											906.45	SGDVYIPR
											885.49	GVSVPALDK
											1175.62	DILWEFQPK
											1303.71	DILWEFQPKK
											1014.53	VALPPDAMGK
											1406.73	DTVLELEFQGQK
											1255.74	LAADTPLLTGQR
											1705.75	YSNSDTVVYVGCGER
											1561.80	TTLVANTSNMPVAAR
											745.42	WAEALR
											1795.88	LAEMPADSGYPAYLAAR
											1811.86	LAEMPADSGYPAYLAAR
											1015.53	VKCLGGPER
											788.36	CLGGPER
											2097.10	EVLQREDDLNEIVQLVGK
											1471.77	EDDLNEIVQLVGK
											775.45	FILETAK
											2057.02	LLREDYLAQNAFTPYDK

											809.37	SVWMMR
											777.38	AAGMDGQK
											1176.62	HRLGDLFYR
											883.51	LGDLFYR
											2060.05	LVSQKFEDPAEGEEALVAK
											1504.72	FEDPAEGEEALVAK
											1302.69	KLHEDLTSGFR
											1174.63	LHEDLTSGFR
											833.40	ALEDETR
4305	Phosphoglycerate kinase, chloroplastic-like	CN893843	*M. domestica*	Plants EST	PMF	21.1	8.8	149	48	8/16	2001.81	ADLNVPLDDSQNITDDTR
											1206.69	VILSSHLGRPK
											999.58	FSLAPLVPR
											1778.93	LVASLPEGGVLLLENVR
											2045.94	KLAALADLYVNDAFGTAHR
											1917.86	LAALADLYVNDAFGTAHR
											1000.51	AHASTEGVTK
											1102.61	RPFAAIVGGSK
4308	Glutamine synthetase nodule isozyme-like	CN894859	*M. domestica*	Plants EST	PMF	16.7	5.8	113	69	9/28	1922.02	KIIAEYIWIGGSGMDIR
											1938.01	KIIAEYIWIGGSGMDIR
											1793.91	IIAEYIWIGGSGMDIR
											1809.92	IIAEYIWIGGSGMDIR
											1435.82	TLPGPVSDPSKLPK
											2972.39	WNYDGSSTGQAPGEDSEVILYPQAIFK
											2373.17	GNNILVICDTYTPGGEPIPTNK
											2529.25	GNNILVICDTYTPGGEPIPTNKR
											2961.39	IFSHPDVVAEVPWYGIEQEYTLLQK
4505	Enolase	GO520405	*M. domestica*	Plants EST	PMF	22.1	8.9	94	52	8/27	1549.77	GEHYAKMTAEVGEK
											1510.85	VQIVGDDLLVTNPK
											1601.84	VNQIGSVTESIEAVR
											1087.50	AGWGVMASHR
											2252.10	SGETEDTFIADLSVGLATGQIK
											806.45	YNQLLR
											1565.77	IEEELGSEAVYAGAK
											1006.55	FRVPVEPY
4602	Enolase	CV081922	*M. domestica*	Plants EST	PMF	18.7	7.8	107	32	6/8	1601.82	VNQIGSVTESIEAVR
											1071.50	AGWGVMASHR
											1118.63	LAKYNQLLR
											806.44	YNQLLR
											1006.53	FRVPVEPY
											1424.66	IMKCFQSFCGM
4708	Polyphenol oxidase IIa	gi|384563679	*M. domestica*	nrNCBI	PMF	67.8	6.2	217	32	17/22	977.57	IKTFTPDR
											736.36	TFTPDR
											1684.93	TRPAAHLVTDEYLAK
											912.42	ALPEDDPR
											1684.79	ALPEDDPRSMVQQAK
											1184.59	MYLYFYER
											1200.59	MYLYFYER
											2842.27	NADHQPPVLVDLSYGGTDDDVDDQTR
											1403.73	TPHLFFGHEYR
											1643.77	DPLFYAHHCNVDR
											1200.66	DSLKPEKDLR
											2155.12	YTYEPVSVPWLFTKPTAR
											1253.71	TTVEVARPKPR
											2889.35	DIEFESNEAVKFDVFINDDAESLSR
											1627.79	FDVFINDDAESLSR
											2668.45	LGTTDLLEDLGVEDDSSVLVTLVPR
											1219.63	VSNSPTTGGFK
5503	Enolase	GO520405	*M. domestica*	Plants EST	PMF	22.1	8.9	106	58	11/42	1549.76	GEHYAKMTAEVGEK
											1510.83	VQIVGDDLLVTNPK
											1601.85	VNQIGSVTESIEAVR
											1215.58	KAGWGVMASHR

											1071.51	AGWGVMASHR
											1087.49	AGWGVMASHR
											2252.13	SGETEDTFIADLSVGLATGQIK
											1118.64	LAKYNQLLR
											806.43	YNQLLR
											1565.76	IEEELGSEAVYAGAK
											1789.91	QYTGNFRVSLFDSQK
5508	Enolase-like	EB151863	*M. domestica*	Plants EST	PMF	22.8	5.3	154	58	15/35	1189.58	MGVEVYHNLK
											1205.56	MGVEVYHNLK
											2480.09	KYGQDATNVGDEGGFAPNIQENR
											2351.98	YGQDATNVGDEGGFAPNIQENR
											1787.82	VVIGMDVAASEFYGSDK
											1803.79	VVIGMDVAASEFYGSDK
											1286.57	TYDLNFKEEK
											702.39	ISGNALK
											864.41	MTAEVGEK
											1524.80	IQIVGDDLLVTNPK
											1601.79	VNQIGSVTESIEAVR
											1245.60	KAGWGVMTSHR
											1101.52	AGWGVMTSHR
											1117.49	AGWGVMTSHR
											1603.74	AGWGVMTSHRSGETK
6001	Triosephosphate isomerase, cytosolic-like	CN908475	*M. domestica*	Plants EST	PMF	24.0	6.4	147	42	8/19	954.50	FFVGGNWK
											2029.03	SSLRPEFHVAAQNCWVK
											1590.86	RLILGESNEFVADK
											1049.62	VAYALSQGLK
											1374.73	VIACVGETLEQR
											1621.85	VASPAQAQEVHSELR
											1749.95	VASPAQAQEVHSELRK
											1618.84	WLQANVSSEVAASTR
6205	Cytosolic malate dehydrogenase	gi|78216493	*M. domestica*	nrNCBI	PMF	36.0	6.0	256	63	19/37	2881.54	GIMLGADQPVILHLLDIPPAAEALNGVK
											1346.74	MELVDAAFPLLK
											833.43	SQASALEK
											797.39	HAAPNCK
											1650.01	VLVVANPANTNALILK
											2648.52	VLVVANPANTNALILKEFAPSIPEK
											1017.54	EFAPSIPEK
											1498.72	NVTCLTRLDHNR
											859.48	ALGQVSER
											1001.55	LNVQVSDVK
											2466.23	NVIIWGNHSSSQYPDVNHATVK
											1033.53	TPSGEKCVR
											2291.14	ELVADDAWLNGEFISTVQQR
											799.49	GAAIIKAR
											1773.90	KLSSALSAASSACDHIR
											1645.81	LSSALSAASSACDHIR
											1363.74	IVQGLSIDEFSR
											1462.75	KLDATAEELSEEK
											1334.66	LDATAEELSEEK
6504	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	CN872362	*M. domestica*	Plants EST	PMF	22.5	8.8	141	59	17/38	1502.86	YGRPLLGCTIKPK
											910.47	AVYECLR
											2169.98	GGLDFTKDDENVNSQPFMR
											2186.03	GGLDFTKDDENVNSQPFMR
											1451.62	DDENVNSQPFMR
											1467.60	DDENVNSQPFMR
											1261.63	FLFCAEAIYK
											1830.74	GHYLNATAGTCEDMMK
											1187.69	DNGLLLHHR
											912.47	AMHAVIDR
											928.50	AMHAVIDR
											1170.56	QKNHGMHFR

											898.43	NHGMHFR
											914.44	NHGMHFR
											1465.77	MSGGDHHAGTVVGK
											1481.69	MSGGDHHAGTVVGK
											1275.76	EITLGFVDLLR
6505	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	CN872362	*M. domestica*	Plants EST	PMF	22.5	8.8	223	83	21/43	1502.85	YGRPLLGCTIKPK
											910.42	AVYECLR
											2169.99	GGLDFTKDDENVNSQPFMR
											2186.00	GGLDFTKDDENVNSQPFMR
											1451.62	DDENVNSQPFMR
											1261.61	FLFCAEAIYK
											946.49	AQAETGEIK
											1798.72	GHYLNATAGTCEDMMK
											1970.83	GHYLNATAGTCEDMMKR
											1986.84	GHYLNATAGTCEDMMKR
											3036.56	ELGVPIVMHDYLTGGFTANTTLAHYCR
											1187.65	DNGLLLHIHR
											928.45	AMHAVIDR
											1170.55	QKNHGMHFR
											898.39	NHGMHFR
											914.39	NHGMHFR
											1325.72	NHGMHFRVLAK
											1465.76	MSGGDHHAGTVVGK
											1481.73	MSGGDHHAGTVVGK
											2050.04	MSGGDHHAGTVVGKLEGER
											1275.72	EITLGFVDLLR
7201	Cytosolic malate dehydrogenase	gi|78216493	*M. domestica*	nrNCBI	PMF	36.0	6.0	187	50	12/18	2016.07	VLVTGAAGQIGYALVPMIAR
											2635.27	GVVATTDVVEACTGVNIAVMVGGFPR
											1649.94	VLVVANPANTNALILK
											1017.52	EFAPSIPEK
											863.45	NVTCLTR
											859.46	ALGQVSER
											1001.55	LNVQVSDVK
											2466.15	NVIIWGNHSSSQYPDVNHATVK
											2291.07	ELVADDAWLNGEFISTVQQR
											1773.83	KLSSALSAASSACDHIR
											1645.75	LSSALSAASSACDHIR
											1363.68	IVQGLSIDEFSR
7303	Phosphoglycerate kinase, cytosolic-like	CN938321	*M. domestica*	Plants EST	PMF	22.1	9.2	200	64	11/14	2015.89	VDLNVPLDDNSNITDDTR
											858.48	YLLGHGAK
											1072.63	YSLKPLVPR
											1100.68	LSELLGLEVK
											1376.59	IANDCIGEEVEK
											1820.00	LVAQLPEGGVLLLENVR
											2048.06	KLASLADVYVNDAFGTAHR
											1919.90	LASLADVYVNDAFGTAHR
											1497.80	YLKPSVAGFLMQK
											1404.76	ELDYLVGAVSNPK
											1102.62	RPFAAIVGGSK

[a] Spot identification number as assigned in Fig. 3;

[b] Description of proteins identified from the Plants EST, nrNCBI or SwisProt database;

[c] Accession numbers according to Viridiplantae SwisProt, nrNCBI or Plants EST Database of NCBI;

[d] Molecular mass (MM) and isoelectric point (p *I*).

Supplementary Table 3B. Proteins with significant oxidation changes after wounding and pathogen inoculation, and their identification with MALDI-TOF-TOF MS/MS

Spot [a]	Protein description [b]	Accession number [c]	Organism	Database	Identification method	MM (kDa) [d]	pI [d]	Mascot score	Coverage %	Matched peptides	Matched peaks	Peptide sequence (ion score) [e]
702	Putative cytosolic NADP-malic enzyme	EB117833	*M. domestica*	Plants EST	MS/MS	19.6	6.4	146	12	2	1315.74	TVQVIVVTDGER (62)
											1352.73	LLDNEFYIGLR (83)
2602	ATP synthase beta chain, mitochondrial precursor	DR991435	*M. domestica*	Plants EST	MS/MS	17.8	4.7	279	34	4	1662.80	CALVFGQMNEPPGAR (51)
											1399.78	VGLTGLTVAEHFR (68)
											1864.95	DAEGQDVLLFIDNIFR (76)
											1492.79	FTQANSEVSALLGR (84)
4302	Glutamine synthetase cytosolic isozyme 1	DT003241	*M. domestica*	Plants EST	MS/MS	27,7	9,5	120	8	2	1610.75	HKEHIAAYGEGNER (94)
											786.32	GYFEDR (26)
4703	Apgm	DY256418	*M. domestica*	Plants EST	MS/MS	18,5	5,9	134	13	2	1489.67	ALEYADFDKFDR (75)
											1230.62	YLVEPPEIDR (59)
5704	Phosphoglucomutase, cytoplasmic	DT041983	*M. domestica* x *M. sieversii*	Plants EST	MS/MS	26.8	8.8	121	8	2	986.43	YLFEDGSR (67)
											1512.70	LYIEQYEKDPSK (53)
6702	Bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic-like	gi|462403908	*Prunus persica*	nrNCBI	MS/MS	54.5	6.2	46	1	1	1168.60	DLLDLYNFR (46)
6901	Cytosolic aconitase	EB122770	*M. domestica*	Plants EST	MS/MS	17.9	8.9	131	22	3	945.58	DFNSYGSR (52)
											1039.53	LFVFDAATR (45)
											1962.88	ADGHDTIVIAGAEYGSGSSR (34)

[a] Spot identification number as assigned in Fig. 3;

[b] Description of proteins identified from the Plants EST, nrNCBI or SwisProt database;

[c] Accession numbers according to Viridiplantae SwisProt, nrNCBI or Plants EST Database of NCBI;

[d] Molecular mass (MM) and isoelectric point (p*I*);

[e] The ion score is indicated in parentheses.