UNIVERSIDAD D CÓRDOBA

Departamento de Bioquímica y Biología Molecular

Molecular Mechanisms of Strawberry Plant Defence against *Colletotrichum acutatum*

Francisco Amil Ruiz

TESIS DOCTORAL

Córdoba 2013

TITULO: *Molecular Mechanisms os Strawberry Plant Defence against Colletotrichum acutatum*

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UNIVERSIDAD DE CÓRDOBA
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR

Tesis Doctoral

"MOLECULAR MECHANISMS OF STRAWBERRY PLANT DEFENCE AGAINST *COLLETOTRICHUM ACUTATUM*"

por

Francisco Amil Ruiz

Memoria de Tesis Doctoral presentada por **Francisco Amil Ruiz**, Licenciado en Bioquímica, para optar al grado de Doctor por la Universidad de Córdoba con la mención de *Doctorado Internacional*.

Córdoba, 2013.

UNIVERSIDAD DE CÓRDOBA
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR

JOSÉ LUIS CABALLERO REPULLO, y JUAN MUÑOZ BLANCO, Catedráticos de Bioquímica y Biología Molecular de la Universidad de Córdoba

Certificamos

Que **FRANCISCO AMIL RUIZ**, Licenciado en Bioquímica por la Universidad de Córdoba, ha realizado bajo nuestra dirección el tragajo de investigación correspondiente a su Tesis Doctoral titulada "Molecular Mechanisms of Strawberry Plant Defence against *Colletotrichum acutatum*".

Revisado el presente trabajo, estimamos que reúne todos los requisitos exigidos por la Normativa vigente para optar al grado de Doctor y que puede ser presentado al Tribunal que ha de evaluarlo, por ello autorizamos la defensa de esta Tesis en la Universidad de Córdoba.

Córdoba, 7 de Junio de 2013

Firma de los directores

Fdo.: Dr: José Luis Caballero Repullo

Fdo.: Dr. Juan Muñoz Blanco

TÍTULO DE LA TESIS:

"Molecular Mechanisms of Strawberry Plant Defence against *Colletotrichum acutatum*"

DOCTORANDO/A: FRANCISCO AMIL RUIZ

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El Ldo. FRANCISCO AMIL RUIZ ha desarrollado el trabajo de investigación denominado **"Molecular Mechanisms of Strawberry Plant Defence against *Colletotrichum acutatum*"**, que constituye el tema central de su Tesis para optar al grado de Doctor con Mencion Internacional, en el seno del Grupo de investigación BIO-278 del Departamento de Bioquímica y Biología Molecular de esta Universidad de Córdoba, adscrito al ceiA3. Este trabajo de investigación ha sido dirigido y supervisado por el **Dr. José Luis Caballero Repullo** y el **Dr. Juan Muñoz Blanco**, ambos Catedráticos de Universidad y miembros del citado Departamento de Bioquímica y Biología Molecular de la Universidad de Córdoba.

Durante el periodo de investigación en el que se ha desarrollado esta Tesis Doctoral, el Ldo. FRANCISCO AMIL RUIZ, ha demostrado una gran capacidad de aprendizaje y trabajo de laboratorio que le han permitido abordar y desarrollar un amplio abanico de técnicas de biología molecular, biotecnología vegetal y bioinformática, que abarcan entre otras: tecnologías moleculares de extracción y amplificación de RNA y DNA, RT y tecnología de PCR, clonación mediante tecnología *Gateway*, expresión y obtención de proteínas recombinantes en sistemas heterólogos (*E. coli*, *Arabidopsis thaliana*), manipulación de plantas in vitro y transformación de explantos, obtención de plantas de fresa recombinantes, complementación y expresión de genes en *A. thaliana*, expresión génica transitoria en *Nicotiana benthaminana*, construcción y escrutinio de genotecas genómicas y sustractivas, tecnologías de cuantificación de la expresión génica a tiempo real (RTqPCR), hibridación Southern y Northern, tecnología Western, tecnologías ómicas de

microarrays y metabolómicas de análisis de compuestos mediante HPLC y LCMS, así como aplicaciones bioinformáticas mediante manejo de programas de búsqueda, análisis e interpretación de datos biológicos.

Con objeto de adquirir la mejor formación en las metodologías mencionadas, el Ldo. FRANCISCO AMIL RUIZ, ha realizado varias estancias en centros de investigación de prestigio, donde ha desarrollado parte del trabajo presentado en esta Tesis Doctoral bajo la supervisión de Doctores con demostrada experiencia en la aplicación de estas técnicas. Así, llevó a cabo el procesado de genotecas de ESTs, mediante el uso de un robot tipo Biomec2000, y comenzó la generación de una plataforma de microarrays de cDNA, bajo la supervisión del **Dr. Manuel Rey** (Newbiotechnic S.A., Sevilla) durante una estancia de 3 meses en la empresa (2004). Durante una estancia de 4 meses (2006) en el laboratorio de la **Dra. Xinnian Dong** (Biology Department, Duke University, NC, USA) adquirió formación en los trabajos con el sistema modelo Arabidopsis, desarrollando transformación y complementación de mutantes con genes heterólogos, así como realizando tests de resistencia frente a patógenos, expresión de proteínas y análisis mediante "*western blot*", y estudio de su interacción mediante "*yeast 2 hybrid system*". Diversas estancias (tres meses, 2008-2009) en el grupo de Protección Vegetal (IFAPA-Las Torres-Tomejil-CAPMA, Sevilla) le han permitido realizar estudios, bajo la supervisión de los **Doctores Fernando Romero y Berta de los Santos,** sobre los aspectos fitopatológicos de la interacción fresa-*Colletotrichum acutatum*, así como analizar la respuesta de esta planta a la activación de sus mecanismos de defensa mediante elicitación con compuestos químicos como SA y JA. En Julio de 2010, con la supervisión del **Dr. José Sánchez-Sevilla** (IFAPA-Churriana, Málaga), inició un primer estudio en la identificación y análisis de polimorfismo en marcadores SSRs y su posible relación con el diferente comportamiento, en términos de susceptibilidad, frente a la infección por *C. acutatum*, observado en distintos cultivares de fresa. Para su formación en el análisis de los datos obtenidos de microarrays, y el análisis funcional de los resultados, contó con la supervisión del **Dr. José Gadea-Vacas** (IBMCP-UPV/CSIC, Valencia), durante una estancia realizada en Abril 2011. Y para realizar los primeros estudios de metabolómica en tejidos vegetativos y reproductivos de fresa, realizó una estancia en Agosto-Septiembre de 2012 en el laboratorio de Biotecnología de Productos Naturales (TUM, Munich, Germany), supervisado por el **Dr. Wilfried Schwab**.

Adicionalmente, diversos cursos especializados, realizados durante este período, han favorecido el progreso en la formación del Ldo. FRANCISCO AMIL RUIZ. Por ejemplo:

"MDA course on Next Generation Sequencing Data Analysis for Transcriptomics", CIPF, Valencia (2013); "Fundamentals of IPA Training & Certification training", Plataforma Andaluza de Bioinformática – SCBI, Málaga (2013); Máster Técnico Superior en Prevención de Riesgos Laborales, Esp. Higiene Industrial, IMF, Madrid (2006); Curso de Formación Complementaria "Genómica Funcional I. El análisis de los microarrays. La bioinformática en la asignación de Funciones. Estudios de Proteómica y Metabolómica". V Máster Universitario en Biotecnología, UNIA (2006); Curso de "Formación Básica en Protección Radiológica", Universidad de Córdoba (2005); Curso de Formación Complementaria: "Genómica Funcional I. Genética directa y reversa". III Maestría en Biotecnología de Plantas. Universidad Internacional de Andalucía. (2003); Curso de Formación Complementaria: "Genómica Funcional II. Microarrays y Proteómica. Metabolómica". III Maestría en Biotecnología de Plantas. Universidad Internacional de Andalucía. (2003); Curso "Procesamiento de Datos de Expresión Génica", Universidad de Málaga (2003).

Durante todo el periodo de formación científica que ha realizado, el Ldo. FRANCISCO AMIL RUIZ ha demostrado una gran capacidad intelectual y de razonamiento científico, así como gran capacidad de aprendizaje, lo que le ha permitido adquirir una formación científica muy sólida y un alto nivel de experiencia en todas las tecnologías moleculares, le que le proporciona, sin lugar a duda, un sobrado bagaje científico para optar al título de Doctor con mención Internacional, así como un prometedor futuro como investigador.

• Los artículos de investigación derivados del trabajo desarrollado durante este período han sido:

Amil-Ruiz, F., Garrido-Gala, J., Blanco-Portales, R., Folta, K.M., Muñoz-Blanco, J. and Caballero, J.L. **(2013)** Identification and Evaluation of Reference Genes for Transcript Normalization in Strawberry Plant Defense Responses. *PLoS One*, *(in press).*

Amil-Ruiz, F., Garrido-Gala, J., Gadea, J., Blanco-Portales, R., Muñoz-Mérida, A., Trelles, O., Santos, B.d.l., Romero, F., Mercado, J.-Á., Pliego-Alfaro, F., Muñoz-Blanco, J. and Caballero, J.L. **(2013)** Incomplete activation of both SA- and JA-pathways by *Colletotrichum acutatum* causes ineffective defense response in strawberry. *(under review).*

Amil-Ruiz, F. and Caballero, J.L. **(2013)** Identification of Subsequent Molecular Mechanisms to Mount Defense Responses in Strawberry Against *C. acutatum*. *(in preparation).*

Moyano-Cañete, E., Bellido, M.L., García-Caparrós, N., Medina-Puche, L., **Amil-Ruiz, F.**, González-Reyes, J.A., Caballero, J.L., Muñoz-Blanco, J. and Blanco-Portales, R**. (2013)** FaGAST2, a Strawberry Ripening-Related Gene, Acts Together with FaGAST1 to Determine Cell Size of the Fruit Receptacle. *Plant and Cell Physiology*, **54**, 218-236.

Amil-Ruiz, F., Encinas-Villarejo, S., de los Santos, B., Muñoz-Mérida, A., Mercado, J.A., Trelles, O., Pliego-Alfaro, F., Romero, F., Muñoz-Blanco, J. and Caballero, J.L. **(2012)** Distinctive Transcriptome Response of Two Strawberry (*Fragaria* x *ananassa*) Cultivars to *Colletotrichum acutatum* Infection. *Acta Hort. (ISHS)*, **929**, 47-50.

Amil-Ruiz, F., Blanco-Portales, R., Muñoz-Blanco, J. and Caballero, J.L. **(2011)** The Strawberry Plant Defence Mechanism: A Molecular Review. *Plant and Cell Physiology*, **52**, 1873-1903.

Ruiz-Ramos, A., **Amil-Ruiz, F.**, Muñoz-Blanco, J., Caballero, J.L. and Maldonado-Alconada, A.M. **(2010)** Comparative proteomic analysis of Arabidopsis wild-type and Fawrky1 transgenic plants to characterize the function of the strawberry (Fragaria x ananassa) FaWRKY protein and its Arabidopsis homolog, AtWRKY75, two positive regulators of resistance. *Proteomica*, **5**, 160-161.

Encinas-Villarejo, S., Maldonado, A.M., **Amil-Ruiz, F.**, de los Santos, B., Romero, F., Pliego-Alfaro, F., Munoz-Blanco, J. and Caballero, J.L**. (2009)** Evidence for a positive regulatory role of strawberry (*Fragaria x ananassa*) Fa WRKY1 and Arabidopsis At WRKY75 proteins in resistance. *Journal of Experimental Botany*, **60**, 3043-3065.

Casado-Díaz, A., Encinas-Villarejo, S., Santos, B.d.l., Schilirò, E., Yubero-Serrano, E.-M., **Amil-Ruiz, F.**, Pocovi, M.I., Pliego-Alfaro, F., Dorado, G., Rey, M., Romero, F., Muñoz-Blanco, J. and Caballero, J.-L. **(2006)** Analysis of strawberry genes differentially expressed in response to *Colletotrichum* infection. *Physiologia Plantarum*, **128**, 633-650.

• Las comunicaciones presentadas en congresos y reuniones científicas han sido:

"*Jasmonate and salicylate signaling pathways seems to be manipulated in strawberry by Colletotrichum acutatum during infection"*. **Francisco Amil-Ruiz**, José Garrido-Gala, Antonio Muñoz-Mérida, José Gadea, Rosario Blanco-Portales, Oswaldo Trelles, José A. Mercado, Berta de los Santos, Fernando Romero, Juan Muñoz-Blanco, and José L. Caballero. **(2013).** II International Strawberry Congress. Antwerp, Belgium. *Accepted for Poster presentation.*

"*Analysis of Strawberry Genes Expression-stability and Evaluation as References for Transcript Normalization"*. **Francisco Amil-Ruiz**, José Garrido-Gala, Rosario Blanco-Portales, Kevin M. Folta, Juan Muñoz-Blanco, and José L. Caballero. **(2013).** II International Strawberry Congress. Antwerp, Belgium. *Accepted for Poster presentation.*

"*The strawberry defence to Colletotrichum acutatum: Searching for key genes"*. **Francisco Amil-Ruiz**, José Gadea, José Garrido-Gala, Antonio Muñoz Mérida, Berta de los Santos, Fernando Romero, Oswaldo Trelles, José Ángel Mercado, Fernando Pliego-Alfaro, Juan Muñoz-Blanco and José Luis Caballero. **(2012)**. VII International Strawberry Congress (ISS2012). Beijing, China. *Oral presentation.*

"*Deciphering the strawberry defense signalling pathways against pathogens"*. **Francisco Amil-Ruiz**, José Garrido-Gala, José Gadea, Antonio Muñoz Mérida, Berta de los Santos, Fernando Romero, Oswaldo Trelles, José Ángel Mercado, Juan Muñoz-Blanco and José Luis Caballero. **(2012)**. XI Reunión de Biología Molecular de Plantas. Segovia, Spain. *Poster presentation.*

¿Tiene efecto la auxina en la respuesta de defensa de la fresa?. José Garrido Gala, **Francisco Amil-Ruiz**, Juan Muñoz Blanco y José Luis Caballero. **(2012)**. XI Reunión de Biología Molecular de Plantas. Segovia, Spain. *Poster presentation.*

"*Identificación de dianas genéticas útiles en programas de mejora genética de la fresa mediante análisis de los cambios del transcriptoma de la planta tras la infección con Colletorichum acutatum"***. Francisco Amil-Ruiz**, Sonia Encinas-Villarejo, Berta de los Santos, Antonio Muñoz-Mérida, José A. Mercado, Oswaldo Trelles, Fernando Pliego-Alfaro, Fernando Romero, Juan Muñoz-Blanco y José L. Caballero. **(2011).** XIX Reunión de la Sociedad Española de Fisiología Vegetal (SEFV) – XII Congreso Hispano - Luso de Fisiología Vegetal. Castellón de la Plana, Spain. *Poster presentation.*

"*Estudio de resistencia inducida por productos a base de algas (gama ALGACAN) en líneas celulares vegetales y ensayos en cultivos de tomate"*. M. Polifrone, **F. Amil Ruiz**, R. Blanco Portales, J. Muñoz Blanco y J.L. Caballero**. (2011).** IX reunión de la SECIVTV. Tenerife, Spain. *Oral presentation.*

"*Can the Strawberry (Fragaria x ananassa) Fanpr1 Gene be used to Increase Resistance to Pathogens Using a "Cisgenic" Approach?"*. **Francisco Amil Ruiz** and José L Caballero. **(2010)**. IV Reunión de la Red Española Interacción Planta-Patógeno. Cercedilla, Spain. *Oral presentation.*

"*Comparative proteome analysis of Arabidopsis wild-type mutants and Fawrky1 transgenic plants to characterize the function of the strawberry (Fragaria x ananassa) FaWRKY protein and its Arabidopsis homolog, AtWRKY75, two positive regulators of resistance*". Alba Ruiz-Ramos, **Francisco Amil-Ruiz**, Juan Muñoz-Blanco, José Luis Caballero, Ana M. Maldonado Alconada. **(2010).** II Jornadas de Jóvenes Investigadores en Porteómica. Córdoba, Spain. *Poster presentation.*

"*Can the strawberry (Fragaria x ananassa) Fanpr1 gene be used to increase resistance to pathogens using a "cisgenic" approach?*". **Amil Ruiz, F.**, De los Santos, B., Mercado, J. A., Romero, F., Pliego Alfaro, F., Muñoz Blanco, J., Caballero, J. L. **(2010).** 28th International Horticultural Congress (ISSH). Lisbon, Portugal. *Oral presentation.*

"*Distinctive transcriptome response of two strawberry (Fragaria x ananassa) cultivars to Colletotrichum acutatum infection*". **Amil-Ruiz, F**., Encinas-Villarejo, S., de los Santos, B., Muñoz-Mérida, A., Mercado, J. A., Trelles, O., Pliego-Alfaro, F., Romero, F., Muñoz-Blanco, J., Caballero, J. L. **(2010).** 28th International Horticultural Congress (ISSH). Lisbon, Portugal. *Oral presentation.*

"*Characterization of a family of wrky transcription factors in strawberry (Fragaria x ananassa)*". Ruiz-Ramos, A., Amil-Ruiz, F., Maldonado, A., Garrido-Gala, J., De los Santos, B., Mercado, J. A, Romero, F., Pliego-Alfaro, F., Muñoz-Blanco, J., Caballero, J. L. **(2010).** 28th International Horticultural Congress (ISSH). Lisbon, Portugal. *Poster presentation.*

"*Cambios en el transcriptoma de dos cultivares de fresa (Fragaria x ananassa) tras la infección por Colletotrichum acutatum*". **Francisco Amil-Ruiz**, Sonia Encinas-Villarejo, Berta de los Santos, Antonio Muñoz-Mérida, José A. Mercado, Oswaldo Trelles, Fernando Pliego-Alfaro, Fernando Romero, Juan Muñoz-Blanco y José L. Caballero. **(2010).** XXXIII Congreso de la Sociedad de Bioquímica y Biología Molecular. Córdoba, Spain. *Poster presentation.*

"*Producción y evaluación de la capacidad bioelicitora de compuestos naturales en la planta de fresa*". Garrido-Gala J., José Manuel Hernández-Ros, de los Santos B., **Amil-Ruiz, F.**, Juan Soliveri, José Luis Copa-Patiño, Romero F., Muñoz Blanco J., Caballero J.L. **(2010).** XXXIII Congreso de la Sociedad de Bioquímica y Biología Molecular. Córdoba, Spain. *Poster presentation.*

"*Alternativas a la transgenia para la mejora biotecnológica de la planta de fresa: uso del gen Fanpr1 para potenciar la respuesta de defensa*". **Francisco Amil-Ruiz**, Berta de los Santos, Ana Maldonado, José A. Mercado, Fernando Romero, Fernando Pliego-Alfaro, Juan Muñoz-Blanco and José L. Caballero. **(2010).** XXXIII Congreso de la Sociedad de Bioquímica y Biología Molecular. Córdoba, Spain. *Poster presentation.*

"*Caracterización de genes de fresa tipo WRKY (FaWRKYs): evaluación de su función y uso para la mejora de la resistencia de la fresa a patógenos*". Alba Ruiz-Ramos, **Francisco Amil-Ruiz**, Sonia Encinas-Villarejo, Berta de los Santos, José A. Mercado, Fernando Pliego-Alfaro, Fernando Romero, Juan Muñoz-Blanco y José L. Caballero. **(2010).** XXXIII Congreso de la Sociedad de Bioquímica y Biología Molecular. Córdoba, Spain. *Poster presentation.*

"*Transcriptomic analysis of two strawberry (Fragaria x ananassa) cultivars determines key genes in response to Colletotrichum acutatum infection*". **Amil-Ruiz, F.**, Muñoz-Mérida, A., Trelles, O., Muñoz-Blanco, J., Caballero, J L. **(2010)**. Xth Spanish Symposium on Bioinformatics. Málaga, Spain. *Poster presentation.*

"*Molecular dissection of defense-related pathways against Colletotrichum acutatum in strawberry (Fragaria x ananassa)*". **Francisco Amil-Ruiz**; Sonia Encinas-Villarejo; Berta de los Santos; Antonio Muñoz-Mérida; José A. Mercado; Oswaldo Trelles; Fernando Pliego-Alfaro; Fernando Romero; Juan Muñoz-Blanco; José L. Caballero. **(2009).** 8º Plant Genomics European Meetings. Lisbon, Portugal. *Poster presentation.*

"*Identificación de genes de Fresa de cv. Camarosa y cv Andana, cuya expresión varía durante la interacción de las plantas con Colletotrichum acutatum: Selección de Genes Diana de Interés Agronómico*". **Francisco Amil Ruiz** and José L Caballero. **(2009).** II Jornada Cultivo de la Fresa: Investigación e innovación. Alcalá del Río, Spain. *Oral presentation.*

"Fanpr1 strawberry gene, an Arabidopsis thaliana npr1 homologue". **Francisco Amil-Ruiz**, Berta de los Santos, Fernando Pliego-Alfaro, Fernando Romero, Juan MuñozBlanco, and José L. Caballero. **(2008).** IX Reunión de Biología Molecular de Plantas. Santiago de Compostela, Spain. *Poster presentation.*

"Molecular and functional characterization of the Fanpr1 strawberry gene, an Arabidopsis thaliana npr1 homologue". **Francisco Amil-Ruiz**, Berta de los Santos, Fernando Pliego-Alfaro, Fernando Romero, Juan Muñoz- Blanco, and José L. Caballero. **(2008).** VI International Strawberry Symposium. Huelva, Spain. *Poster presentation.*

"Searching for the strawberry genes by functional genomic as valuable biotechnological tools". Muñoz Blanco, J., Caballero J.L, Blanco-Portales, R., Bellido-Cabello de Alba, M.L., Cumplido-Laso, G., Moreno-Suárez, B., García-Limones, C., **Amil-Ruiz, F.**, Encinas-Villarejo, S., Bombarelly, A., Sánchez-Sevilla, J.F., Valpuesta, V. **(2008).** VI International Strawberry Symposium. Huelva, Spain. *Oral presentation.*

"Increasing biomass and productivity of strawberry by transcriptional activation of genes associated with the plant defence response". **Francisco Amil-Ruiz**, Sonia Encinas-Villarejo, Ana María Maldonado, Berta de los Santos, Fernando Romero, Fernando Pliego-Alfaro, Juan Muñoz-Blanco and José L. Caballero. **(2008).** Plant Biomass for Food and Energy: Future and Reality. Baeza, Spain. *Poster presentation.*

"Utilización de la genómica funcional para la identificación de genes de fresa con aplicaciones biotecnológicas". Muñoz Blanco, J., Blanco-Portales, R., Bellido cabello de Alba, ML., López Ráez, JA., García Limones, C., Cumplido Laso, G., Moreno Suárez, B., Moyano Cañete, E., Encinas Villarejo, S., **Amil-Ruiz, F**. and Caballero J.L. **(2007).** XXX Congreso de la Sociedad Española de Bioquímica y Biología Molecular. Málaga, Spain. *Oral presentation.*

"Aproximación a la mejora de la resistencia a patógenos mediante el transcriptoma en la interacción Fresa-Colletotrichum acutatum: Caracterización molecular del gen de fresa Fanpr1 que presenta similitud con el gen npr1 de Arabidopsis thaliana, gen regulador en la respuesta de defensa". **Francisco Amil Ruiz** and José L Caballero. **(2006).** I Jornada Sobre el Cultivo de la Fresa. Alcalá del Río, Spain. *Poster presentation.*

"Análisis del transcriptoma en la interacción fresa-Colletotrichum acutatum: caracterización molecular del gen de fresa Fanpr1 que presenta similitud con el gen npr1 de Arabidopsis thaliana". **Francisco Amil-Ruiz**, Sonia Encinas Villarejo, Elena M. Yubero-Serrano, Elisabetta Schilirò, Berta de los Santos, Fernando Romero, Fernando Pliego-Alfaro, Juan Muñoz-Blanco y José Luis Caballero. **(2006).** VIII Reunión de Biología Molecular de Plantas. Pamplona, Spain. *Poster presentation.*

"El gen Fawrky de fresa, semejante a factores de transcripción tipo wrky: identificación funcional mediante transformación heteróloga en A. thaliana y patrón de expresión espacio-temporal en diferentes cultivares de fresa, en respuesta a la infección con Colletotrichum acutatum y a tratamientos con elicitores". Sonia Encinas Villarejo, Elisabetta Schilirò, Elena M. Yubero-Serrano, **Francisco Amil-Ruiz**, Ana Maldonado, Berta de los Santos, Fernando Romero, Fernando Pliego-Alfaro, Gabriel Dorado-Pérez, Juan Muñoz-Blanco y José Luis Caballero. **(2006).** VIII Reunión de Biología Molecular de Plantas. Pamplona, Spain. *Oral presentation.*

"Identification and characterisation in strawberry (Fragaria x ananassa duch.) of a pathogen-responsibe b-1,3-glucanase gene (Fabgln-1) that codifies for a gpi-anchored protein". Elisabetta Schilirò, Sonia Encinas Villarejo, **Francisco Amil-Ruiz**, Juan Muñoz-Blanco y José L Caballero. **(2006).** VIII Reunión de Biología Molecular de Plantas. Pamplona, Spain. *Poster presentation.*

"Strawberry genes in response to Colletotrichum acutatum infection: a genomic approach". Muñoz-Blanco, J., Casado-Diaz A, Encinas-Villarejo S, de los Santos B, Schiliro E., Yubero-Serrano EM, **Amil-Ruiz F**, Pliego-Alfaro F, Gabriel Dorado, Rey M, Romero F and Caballero J.L. **(2005).** Plant Genomics European Meetings. Amsterdam, Netherlands. *Poster presentation.*

"Caracterización molecular de un gen de fresa (FxaWRKY) semejante a factores de transcripción wrky, involucrado en respuesta de defensa de la planta a Colletotrichum acutatum". Sonia Encinas-Villarejo, Elisabetta Schiliró, Elena M. Yubero-Serrano, **Francisco Amil-Ruiz**, Berta de los Santos, Fernando Romero, Fernando Pliego-Alfaro, Gabriel Dorado-Pérez, Juan Muñoz-Blanco y José L Caballero. **(2004).** VII Reunión de Biología Molecular de Plantas. Benalmádena, Spain. *Poster presentation.*

"Caracterizacion molecular de un gen de fresa (FxaGLNb13) que codifica una beta, 1-3 glucanasa en respuesta a la infeccion por Colletotrichum acutatum". Elisabetta Schiliró, Sonia Encinas-Villarejo, Elena

M. Yubero-Serrano, **Francisco Amil-Ruiz,** Berta de los Santos, Fernando Romero, Fernando Pliego-Alfaro, Gabriel Dorado-Pérez, Juan Muñoz-Blanco y José L Caballero. **(2004).** VII Reunión de Biología Molecular de Plantas. Benalmádena, Spain. *Poster presentation.*

"*Identificación de genes de fresa (Fragaria x ananassa) inducidos por Colletotrichum acutatum durante el proceso de infección"*. **Francisco Amil-Ruiz**, Sonia Encinas-Villarejo, Antonio Casado-Díaz, Berta de los Santos, Elisabetta Schiliró, Elena M Yubero-Serrano, Mariana I Pocovi, Fernando Pliego-Alfaro, Manuel Rey, Gabriel Dorado-Pérez, Fernando Romero, Juan Muñoz-Blanco y José L Caballero. **(2004).** VII Reunión de Biología Molecular de Plantas. Benalmádena, Spain. *Oral presentation.*

"*Strawberry genes in response to infection by the fungus Colletotrichum acutatum"*. Encinas-Villarejo, S., Schilirò, E., Yubero-Serrano, E., **Amil-Ruiz, F.**, de los Santos, B, Pliego-Alfaro, F., Rey, M., Dorado, G., Romero, F., Muñoz Blanco, J and Caballero J.L. **(2004).** Workshop: Disease Resistance and Related Signalling Mechanisms in Plants. Centre for International Meetings on Biology. Madrid, Spain. Poster presentation.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 7 de Junio de 2013

Firma de los directores

Fdo.: Dr: José Luis Caballero Repullo

Fdo.: Dr. Juan Muñoz Blanco

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Summary

This thesis is focused on strawberry molecular studies aimed by the strong economic impact and social staple that represents this crop. With an annual production of 500000 tons and an economic weigh of 650 million €, Spain is the third producing country in world (FAOSTAT Agriculture Data [http://faostat.fao.org/]). Important losses in strawberry yields occur due to diseases and pests. Although resistant cultivars are a priority of most strawberry breeding programs, completely resistant cultivars have not yet been reported, relying pathogen control on the excessive use of chemical products (mostly environmental contaminants). Despite of the immediate necessity on developing new strategies to improve resistance in this crop plant, molecular knowledge is still scarce and most components and mechanisms of the strawberry defense network remain unknown and poorly understood.

The main aim of this work is to get molecular clues about how plant immunity is activated in strawberry to face pathogen attack. Understanding the molecular interplay between strawberry plant and microbes will successful contribute to identify candidate genes useful for developing biotechnological strategies and help breeding to increase resistance against specific pathogens. This work has been structured in eight chapters which contributed to mount and exploit new technical platforms to subsequently uncover the strawberry defense response at molecular level.

Chapter I is an intense and comprehensive compendium of all available information on strawberry immunity, and contribute to the field by discussing, updating and compiling research focused on the molecular aspects and events of the strawberry defense mechanisms against pathogens, concluding that major progress in the physiology, genetics and molecular biology of strawberry, is still needed to fully uncover the logic of its elaborate plant innate immune system. In this context, the use of high-throughput technologies will provide large amount of molecular information related with defense response in strawberry. However, the lack on commercially-available tools focused on crop plants such as strawberry, aimed us to produce our private platforms to be exploited in strawberry-based studies, as it is described in **Chapter II**. Thus, the generation of a ESTs collection enriched on defense-related genes, which allow the identification of new components of strawberry immunity, and provides information on those biological

processes activated by defense elicitors, constitutes valuable information for searching candidate genes involved in strawberry defense. In addition, a private microarray platform was fabricated from the strawberry ESTs collection previously obtained.

Very often, transcriptomic studies involve relative quantification of gene expression under a great variety of experimental conditions (RTqPCR). However, the accuracy and reliability of this methodology is strongly dependent upon the choice of an optimal endogenous reference gene. This essential information is an inescapable prerequisite for fine transcriptomic analysis in biological systems. However, there is no information whatsoever available on reliable endogenous reference genes to be used in studies involving strawberry-pathogen interactions, varieties, and many other experimental conditions of interest in strawberry. Information provided in **Chapter III** constitutes the first serious and systematic study in strawberry to identify and validate optimal reference genes for accurate normalization of gene expression in strawberry plant defense response studies. The resulting reference genes are strongly recommended as control genes for relative quantification of gene expression in strawberry plant-pathogen interaction and strawberry plant defense studies.

Chapter IV represents a first approach to increase knowledge in the molecular response of strawberry to pathogens. Thus, two *Fragaria* × *ananassa* cultivars, with different behavior to anthracnose in terms of susceptibility, were analyzed in the balance of four hormones (SA, JA, ABA and IAA) after infection. Plant hormones play crucial roles in defense to pathogens and pests. A controlled hormonal balance determines appropriate response to a particular plant-pathogen interaction, as singular events in the complex network of plant signaling are fine tune regulated by these compounds. Results show clear differences between the two cultivars analyzed. While the most susceptible cultivar (Camarosa) suffer an increase in both SA and JA hormones, the less susceptible one (Andana) decrease its SA contents and slightly increase JA concentration. The simultaneous increase in SA and JA, two hormones that have been clearly described as having negative crosstalk, may indicate a inefficient defense response in Camarosa, as it will be further described in **Chapter V**. However, the hormonal response detected in Andana is more complex, and results suggest that basal level of SA might be correlated

with differences in susceptibility between both strawberry cultivars. Transcriptomic regulation on Andana will be described further below in **Chapter VII**.

Those developed tools, together with knowledge obtained on strawberry immunity have been exploited in further analyses to explore strawberry transcriptome in response to biotic stimulus. Thus, **Chapter V** describes transcriptomic regulation on cv. Camarosa by *Colletotrichum acutatum* infection. Induction of key genes controlling important steps within SA and JA signaling pathways was mainly detected. Contrastingly, the induction of known SA and JA-responsive defense genes as *PR1, PR2, LOX2, JAR1,* and *PDF1* was strongly abolished. These data indicate that SA and JA pathways are partially promoted in strawberry against *C. acutatum* (coinciding which results of hormone regulation shown in **Chapter IV**), and evidence a putative strategy used by this pathogen to overcome the strawberry plant defense system and to spread within the host by manipulating the fine crosstalk between both hormonal pathways. In addition, our analysis has allowed the identification of subsequent molecular mechanisms to mount defense responses in strawberry against *C. acutatum*. **Chapter VI** describes the alteration in expression of an important number of *F* × *ananassa* genes representing most of the steps which are sequentially required for an efficient defense response. Plants have evolved a sophisticated innate immune system that is composed of multiple layers, and the integration of signals derived from these layers constitutes a crucial prerequisite for efficient defense. Thus, modification on the sensing apparatus, which propitiate correct recognition and identification of the invader by specific PRR and R receptors, occurs. Also, we have detect activation of a variety of signal transduction mechanisms, based on calcium, phosphate and ubiquitin binding proteins, and activation of specific hormone-dependent transcriptional factors, which generate a plant growth-to-defense transition and produces a strong impact on synthesis of new proteins and components of secretion to counteract the infection. All these components represent a model of subsequent steps exhibited in the strawberry cell during the defense response to *C. acutatum* attack.

Chapter VII has been focused in the dissection of the genetic basis of different phenotypes exhibited (in terms of susceptibility) by two strawberry cultivars, Camarosa (very highly susceptible) and Andana (less susceptible). Important and distinct transcriptomic changes leading to defense responses occur in both cultivars, but contrary to what was described previously to Camarosa cultivar (**Chapter V**), and coinciding with hormone determination obtained in **Chapter IV**, salicylic acid mediated defenses were

not activated in Andana against *C. acutatum*. In addition, an important number of genes more abundant specifically in Camarosa or in Andana cultivars have been identified, and may be responsible, in part, of differences in the efficiency demonstrated for defense responses mount by each cultivar. However, further analysis is needed to clearly associate those molecular components identified here with the differential susceptibility exhibited by these two strawberry cultivars.

As shown in **Chapter VIII,** we have identified five members of the NPR1-like family in strawberry. Members of the NPR1-like family are key players in salicylic acid (SA)-mediated resistance to pathogens in Arabidopsis and other plants. Very interestingly, overexpression of a control gene such as *NPR1* in Arabidopsis and other species (i.e. rice, tobacco, grapevine) has been described to increase the innate defense system in these plants. Therefore, modulation of the expression level of NPR1-like genes offers an attractive alternative to increase strawberry resistance. Thus, molecular and functional studies are being conducted to unravel the putative implication of *FaNPR1, FaNPR31, FaNPR32, FaNPR33* and *FaNPR5* in the strawberry plant physiology.

This manuscript represents a first compendium of results containing an important piece of knowledge in the molecular studies of defense response exhibited by the strawberry plant. I hope that this work will be of great interest for the scientific community, in special for those colleagues who focus their studies in this particular genus and for those others who could find on the technical approached here described an appropriate way to advance in their own studies.

Happy reading.

Resumen

Esta tesis está enfocada en estudios moleculares de la planta de fresa animada por el importante impacto económico y estabilizador social que representa este cultivo. Con una producción anual de 500000 toneladas y una relevancia económica de 650 millones de euros, España es el tercer país productor en el mundo (FAOSTAT Agriculture Data [http://faostat.fao.org/]). La producción de fresa sufre importantes pérdidas como consecuencia de las enfermedades y las plagas. Aunque la obtención de cultivares resistentes son una prioridad para la mayoría de los programas de mejora, aún no se han obtenido cultivares completamente resistentes, por lo que el control de los patógenos ha recaído en el uso excesivo de productos químicos (mayoritariamente contaminantes ambientales). Aún con la urgente necesidad de desarrollar nuevas estrategias para mejorar la resistencia en este cultivo, el conocimiento a nivel molecular de la mayoría de los componentes y mecanismos de la respuesta de defensa de la planta de fresa permanecen desconocidos y difícilmente entendibles.

El objetivo principal de este trabajo consiste en la obtención de pistas a nivel molecular a cerca de cómo la inmunidad de la planta de fresa es activada para enfrentarse al ataque de los patógenos. Conocer la interacción molecular entre la planta de fresa y los microbios contribuirá eficazmente a la identificación de genes candidatos útiles en el desarrollo de estrategias biotecnológicas y ayudará en los programas de mejora para incrementar la resistencia contra patógenos específicos. Este trabajo se ha estructurado en ocho capítulos que contribuyen en la tarea de montar y explotar nuevas plataformas técnicas para descubrir secuencialmente, a un nivel molecular, los distintos pasos en la respuesta de defensa desplegados por la planta de fresa.

El **Capítulo I** es un amplio e intenso compendio de toda la información disponible sobre la inmunidad de la fresa, y contribuye a este campo con la discusión, actualización y compilación de toda la investigación enfocada en los aspectos moleculares de los mecanismos de la defensa de esta planta. Este capítulo concluye que aún es necesario un importante esfuerzo en los estudios de la fisiología, la genética y la biología molecular en fresa, para llegar a discernir por completo la lógica de su muy elaborado sistema de inmunidad innata. En este contexto, el uso de tecnologías de gran escala proporcionará una gran cantidad de información molecular relacionada con la respuesta de defensa en

fresa. Sin embargo, la falta de herramientas disponibles comercialmente para su uso en cultivos como la fresa, nos promovió a producir nuestra propia plataforma para ser explotada en estudios sobre esta planta, tal como se describe en el **Capítulo II.** De esta manera, la generación de una colección de *ESTs* enriquecida en genes relacionados con la defensa, que permitió la identificación de nuevos componentes de la inmunidad de la fresa, y que proporcionó información sobre aquellos procesos biológicos activados tras la aplicación de elicitores químicos de la respuesta de defensa, constituye una información de gran valor en la búsqueda de genes candidatos que están involucrados en la defensa de la fresa. Adicionalmente, se generó una plataforma de *microarrays* a partir de la colección de *ESTs* obtenida previamente.

Muy a menudo, los estudios transcriptómicos incluyen cuantificación relativa de la expresión génica en una gran variedad de condiciones experimentales (RTqPCR). Sin embargo, la precisión y fiabilidad de esta metodología es muy dependiente de la elección de los genes de referencia más apropiados. Esta información esencial es un prerrequisito ineludible para los análisis transcriptómicos de los sistemas biológicos. Sin embargo, no existe ninguna información disponible sobre la fiabilidad de genes endógenos de referencia para ser utilizados en estudios de interacción planta-patógeno, en distintas variedades y en muchas otras condiciones experimentales de interés en la planta de fresa. La información proporcionada en el **Capítulo III** constituye el primer estudio serio y sistemático en fresa para identificar y validar genes de referencia óptimos para la normalización con precisión de la expresión génica en estudios de la planta de fresa y su respuesta de defensa. Los genes obtenidos de esta valoración son fuertemente recomendados como controles para la cuantificación relativa de la expresión génica en estudios de interacción planta-patógeno y respuesta de defensa en fresa.

El **Capítulo IV** representa la primera aproximación para incrementar el conocimiento en la respuesta molecular de la planta de fresa contra los patógenos. Así, dos cultivares de *Fragaria* × *ananassa*, con distinto comportamiento en términos de susceptibilidad frente a la enfermedad de la antracnosis, se analizaron en sus niveles basales, y en su balance tras la infección, de cuatro fitohormonas (SA, JA, ABA e IAA). Las hormonas vegetales juegan papeles cruciales en la defensa de la planta frente a los patógenos y las plagas. Un balance hormonal controlado determinará la respuesta más apropiada contra un patógeno

particular, ya que todos los eventos en el complejo entramado de señalización en la planta están delicadamente regulados por estos compuestos. Los resultados obtenidos mostraron claras diferencias entre los dos cultivares analizados en respuesta a la infección. Mientras que el cultivar más susceptible (Camarosa) incrementó los niveles de SA y JA, el menos susceptible (Andana) disminuyó su contenido en SA, y muy ligeramente incrementó la concentración de JA. El incremento simultáneo en SA y JA en Camarosa, dos hormonas que han sido claramente descritas por su mutua regulación negativa (*negative crosstalk*), podría indicar una respuesta de defensa ineficiente en este cultivar, tal como se detallará posteriormente en el **Capítulo V**. Sin embargo, la respuesta hormonal detectada en Andana es más compleja, y los resultados sugieren que los niveles basales de SA podrían correlacionarse con las diferencias en susceptibilidad entre los dos cultivares. La regulación transcripcional en Andana se describirá posteriormente en el **Capítulo VII**.

Estas herramientas que se han desarrollado, junto con el conocimiento generado a cerca de la inmunidad de esta planta, fueron explotadas en posteriores análisis para explorar el transcriptoma de la fresa en respuesta a estímulos bióticos. Así, el **Capítulo V** describe la regulación transcripcional en el cultivar Camarosa por la infección de *Colletotrichum acutatum.* Principalmente se detectó la inducción de genes clave en el control de importantes pasos en las rutas de señalización dependientes de SA y JA. En contraste, la inducción de los genes de defensa tradicionalmente descritos como marcadores de la respuesta a SA y JA (*PR1, PR2, LOX2, JAR1,* y *PDF1)* fue fuertemente suprimida. Estos datos indican que las rutas dependientes de SA y JA están promovidas parcialmente contra *C. acutatum*. Estos resultados son coincidentes con los obtenidos en los estudios de la regulación hormonal para este cultivar, que se muestran en el **Capítulo IV**, y evidencian una posible estrategia usada por este patógeno para sobrepasar el sistema de defensa de la planta de fresa, y propagarse por el huésped, con la manipulación de la mutua regulación existente entre las dos rutas hormonales (SA y JA dependientes). Adicionalmente, nuestro análisis ha permitido la identificación de los mecanismos moleculares que secuencialmente se promueven para montar la respuesta de defensa de la fresa contra *C. acutatum*. El **Capítulo VI** describe la regulación de un número importante de genes de *F* × *ananassa* que representan la mayoría de los pasos que son secuencialmente requeridos para una eficiente respuesta de defensa. Las plantas han evolucionado a un sistema de inmunidad innata muy sofisticado, compuesto por múltiples capas, y la integración de las señales derivadas de esas capas constituye un prerrequisito crucial para una defensa eficiente. De este modo, se ha detectado la modificación en el

sistema de sensores, que proporcionan el reconocimiento correcto y la identificación del invasor por los receptores específicos de tipo PRR y R. También hemos detectado la activación de una variedad de mecanismos de transducción de señal, basados en proteínas de unión a calcio, fósforo y ubiquitina, así como la activación de factores de transcripción dependientes de compuestos hormonales específicos, que generan la transición hacia la respuesta de defensa (*plant growth-to-defense transition*) y producen un impacto muy importante en la síntesis de nuevas proteínas y componentes del sistema secretor para contrarrestar la infección. Todos estos componentes representan un modelo de pasos consecutivos llevados a cabo por la célula de fresa en la respuesta de defensa contra *C. acutatum*.

El **Capítulo VII** se ha enfocado en la disección de la base genética de los diferentes fenotipos exhibidos (en términos de susceptibilidad) por los dos cultivares de fresa, Camarosa (muy susceptible) y Andana (menos susceptible). Se han detectado importantes y distintivos cambios transcriptómicos, relacionados con la respuesta de defensa, en ambos cultivares. Contrariamente a la regulación descrita para Camarosa (**Capítulo V**), y coincidiendo con los resultados obtenidos de las determinaciones hormonales (**Capítulo IV**), las defensas dependientes de SA no se activaron en Andana contra *C. acutatum*. Adicionalmente, un número importante de genes fueron detectados más abundantes específicamente en Camarosa o en Andana, y podrían ser responsables, al menos en parte, de las diferencias detectadas en la eficiencia para montar la respuesta de defensa demostrada por cada cultivar.

Tal como se muestra en el **Capítulo VIII**, hemos identificado cinco miembros de la familia *NPR1-like* en fresa. Los miembros de la familia *NPR1-like* son componentes clave en la resistencia a patógenos mediada por SA en Arabidopsis y en otras plantas. Se ha descrito que la sobreexpresión de un gen regulador como *NPR1* en Arabidopsis y otras especies (por ejemplo arroz, tabaco, uva) es capaz de incrementar la eficiencia del sistema de defensa innato en estas plantas. Por consiguiente, la modulación del nivel de expresión de los genes *NPR1-like* se ofrece como una atractiva alternativa para incrementar la resistencia en fresa. Por esto, se están llevando a cabo estudios moleculares y funcionales para descifrar la posible implicación de *FaNPR1, FaNPR31, FaNPR32, FaNPR33* y *FaNPR5* en la fisiología de la planta de fresa.

Este manuscrito representa un primer compendio de resultados, que contiene una pieza importante del conocimiento en los estudios moleculares de la respuesta de defensa activada por la planta de fresa. Yo espero que este trabajo sea de gran interés para la comunidad científica, especialmente para aquellos colegas que han enfocado sus trabajos en este género en particular, y también para aquellos otros que pudieran encontrar en las aproximaciones técnicas aquí descritas un método apropiado para avanzar en sus propios estudios.

Feliz lectura.

A mi familia

A mi esposa Ana Belén
A mi hijo Carlos

Agradecimientos / Acknowledgments

to the family

Cuando después de un esfuerzo enorme se recupera algún tipo de satisfacción... hay que dedicarlo a la familia. Porque ellos son los que te han perdido tantas veces a causa de tu dedicación. Porque siempre te han apoyado, incluso no entendiendo los sacrificios que has hecho. Y porque han sufrido igual, o quizá más, cuando las eventualidades parecían que te iban a superar. Simplemente porque son los que están ahí, siempre presentes, y sabes que siempre van a estar, haciendo de robusto soporte para sostenerte cuando este trabajo te dejaba agotado y exhausto, y no sólo físicamente. Y porque, en realidad, gran parte de este mérito, si lo hay, es sin duda de ellos.

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Objectives

The objectives of this Thesis are:

- **1.** To generate and analyze a strawberry EST collection enriched in transcripts related to plant defense response.
- **2.** To generate an in-home strawberry cDNA-based microarray platform.
- **3.** To identify and evaluate appropriate reference genes for transcript normalization in strawberry.
- **4.** To evaluate changes in plant hormones (SA, JA, and ABA) in strawberry challenged with *C. acutatum*.
- **5.** To identify genetic components and molecular mechanisms implicated in the strawberry defense response against *C. acutatum*.
- **6.** To isolate and characterize at the molecular level the function of the strawberry NPR1-like family of genes.

Objetivos

Los objetivos de esta tesis son:

- **1.** Generar y analizar una colección de ESTs de fresa enriquecida en transcritos relacionados con la respuesta de defensa.
- **2.** Generar una plataforma casera de *microarray* de cDNA para fresa.
- **3.** Identificar y evaluar genes de referencia apropiados para la normalización de transcritos en fresa.
- **4.** Evaluar los cambios en fitohormonas (SA, JA y ABA) en fresa inoculada con *C. acutatum*.
- **5.** Identificar los componentes genéticos y los mecanismos moleculares implicados en la respuesta de defensa de fresa frente a *C. acutatum*.
- **6.** Aislar y caracterizar a nivel molecular la función de la familia de genes *NPR1-like* en fresa.

Chapter I

Molecular Mechanisms of Strawberry Plant Defence

Part of this chapter has been published with modifications as:

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Editor-in-Chief's choice of the month.

doi:10.1093/pcp/pcr136, available FREE online at www.pcp.oxfordjournals.org

Abbreviations:

BR, brassinosteroid
BTH, benzothiadiazole
EST, expressed sequence tag
ET, ethylene
ETI, effector triggered immunity
FHT, flavanone 3-hydroxylase
Fra a, Fragariaxananassa alergen
HR, hypersensitive response
JA, jasmonate
LOX, lipoxygenase
LTP, lipid transfer protein
MAMP, microbe-associated molecular pattern
MeSA, methylsalicylate
NBS-LRR, nucleotide binding-leucine-rich repeat
OGA, oligogalacturonide
PA, proanthocyanidin
PAL, phenylalanine ammonia lyase
PAMP, pathogen-associated molecular pattern
PG, polygalacturonase
PGIP, polygalacturonase-inhibiting protein
POX, peroxidase
PPO, polyphenoloxidase
PR, pathogenesis-related
PRR, pattern recognition receptor
PTI, PAMP-triggered immunity
RGA, resistance gene analog
SA, salicylic acid
STK, serinethreonine kinase
TIR, Toll/interleukin-1 receptor-like
TLC, thin-layer chromatography.

Footnotes:

The nucleotide sequences reported in this chapter have been submitted to GenBank under accession numbers FaPR10-4 (JN415652) and FaCHI4-2 (JN415653).

ABSTRACT

Strawberry, a small fruit crop of great importance throughout the world, has been considered a model plant system for rosaceae, and is susceptible to a large variety of phytopathogenic organisms. Most components and mechanisms of the strawberry defence network remain poorly known. However, from current knowledge, it seems clear that the ability of a strawberry plant to efficiently respond to pathogens firstly rely on the physiological status of injured tissue (preformed mechanisms of defence), and in secondly, on the general ability of recognition and identification of the invaders by surface plant receptors, followed by a broad range of induced mechanisms, which include cell wall reinforcement, reactive oxygen species production, phytoalexin generation and pathogenesis related protein accumulation.

Dissection of these physiological responses to a molecular level will provide valuable information to improve future breeding strategies for new strawberry varieties and engineer strawberry plants for durable and broad-spectrum disease resistance. In turn, this will lead to a reduction in chemicals use and environmental risks.

Advances in the understanding of the molecular interplay between plant (mainly those considered model systems) and various classes of microbial pathogens have been achieved in the last two decades. However, major progress in the genetics and molecular biology of strawberry, is still needed to fully uncover the logic of its elaborate plant innate immune system. These fundamental insights will provide a conceptual framework for rational human intervention through new strawberry research approaches. This review, contribute to the field by discussing, updating and compiling research focused on the molecular aspects and events of the strawberry defense mechanisms against pathogens.

INTRODUCTION

Importance and Benefits of Strawberry as a Fruit Crop

Strawberry is a small fruit crop of great importance throughout the world. The strawberry belongs to the family *Rosaceae* in the genus *Fragaria,* containing 23 species (Folta and Davis 2006, Shulaev et al. 2008). In *Fragaria*, four basic fertility groups exist which are associated primarily with their ploidy level or chromosome number (Hancock 1999). The most common native species, *F. vesca* L., has 14 chromosomes and is considered to be a diploid (Oosumi et al. 2006). Other remarkable *Fragaria* species include the diploid *F. viridis* Duchesne (2n=2x=14 chromosomes) (Hodgson 2007), the hexaploid *F. moschata* Duchesne (musk strawberry, 2n=6x=42 chromosomes) (Hancock 1999), and the octoploid *Fragaria* x *ananassa* Duchesne (2n=8x=56) (Davis et al. 2007), the main cultivated species, that stems from the cross of the octoploids *F. virginiana* Duchesne from eastern North America, which was noted for its fine flavour, and *F. chiloensis* (L.) Mill. from Chile, noted for its large size (Hancock 1999). Numerous varieties of strawberries have been developed in the temperate zones of the world by different breeding programs.

In 2009, the strawberry world production reached approximately 4.2 million metric tons, with projected increases for subsequent years (FAOSTAT Agriculture Data [http://faostat.fao.org/, updated 17 may 2011]). Due to its broad horticultural importance this crop has been proposed as an interesting model for the development of basic genomics and recombinant DNA studies among rosaceous (Mezzetti 2009). Indeed, strawberry is unique within the Rosaceae, with a small basic (x=7) genome size (~240 Mb), and a short generation time for a perennial (Folta and Davis 2006), the availability of a robust and facile *in vitro* regeneration and transformation system (Alsheikh et al. 2002), and the recently reported genome sequence (Shulaev et al. 2011).

The hybrid octoploid (*F.* x *ananassa* Duch.), a perennial and herbaceous plant clonally propagated, covers almost the 60% of the world production, due to the organoleptic properties and health benefits of the fruit regarded as significant quality factors for both consumers and the food industry. Indeed, the benefits of the strawberry fruit consumption on cardiovascular, neurodegenerative, and other human diseases like aging, obesity, and cancer are documented (Maas et al. 1991, Zhang et al. 2008, da Silva Pinto et al. 2010).

The resistance to pathogens in strawberry

Strawberry cultivars exhibit great phenotypic diversity on their susceptibility to a large variety of phytopathogenic organisms, which limit both strawberry fruit quality and plant yield production (Simpson 1991, Maas 1998).

Natural sources of strawberry resistance to diseases have been reported among wild species (Harland and King 1957, Gooding et al. 1981, Maas 1998), and also in some varieties of cultivated *F.* x *ananassa* (Maas and Smith 1978, Melville et al. 1980, Wing et al. 1995, Nelson et al. 1996, Bell et al. 1997, Shaw and Gordon 2003, Mori et al. 2005, Particka and Hancock 2005, Zebrowska et al. 2006, Masny and Żurawicz 2009), but strawberry resistance to a variety of pathogens has been reported to be mostly polygenic quantitatively inherited (Maclachlan 1978, Barritt 1980, Denoyes-Rothan and Baudry 1995, Shaw et al. 1996, Lewers et al. 2003, Zebrowska et al. 2006), making it difficult to associate molecular markers with disease resistance genes. This is further complicated by the octoploid genome structure of the main cultivated strawberry species, *F.* x *ananassa*. However, high levels of conserved macrosynteny and colinearity have been observed between the octoploid and diploid *Fragaria* genomes (Rousseau-Gueutin et al. 2008), and molecular markers linked to a single dominant strawberry disease resistance gene that segregates in a disomic fashion have been reported (Denoyes-Rothan and Baudry 1995, van de Weg 1997a, van de Weg 1997b, Takahashi et al. 1997, Denoyes-Rothan et al. 2005).

Traditional breeding for resistance is time consuming and importantly, has not been shown durable in many plants (Quirino and Bent 2003). Moreover, due to the intensified focus on resistance, other substantial deficiencies for horticultural or productivity traits are usually co-selected (Shaw et al. 2005). Also, classical strawberry breeding is rather conservative due to difficulties in introgression of the resistance sources (Hancock and Bringhurst 1980, Hancock and Luby 1993). In addition, the development of "a narrow germplasm base" (i.e. cultivars introduced from North American breeding programs from 1960 to 1990 are descended from 53 founding clones with only 17 cytoplasm sources) (Dale and Sjulin 1990) has caused deleterious effects of inbreeding and genetic vulnerability to diseases, pests, and environmental stresses.

Ultimately the control of pathogens and pests of strawberry requires a combination of chemical and cultural methods. The effectiveness of chemicals for controlling diseases in fruiting fields is unclear. It may be that the incubation time between infection and disease is so long that most chemicals are ineffective in controlling diseases. Regular pesticide applications are also environmental contaminants and have harmful effects on human health. Thus, they are not yet considered an appropriate cultivation practice (González-León and Valenzuela-Quintanar 2007, Fernandes et al. 2011). In addition, plants make vitamins, polyphenolics and other antioxidants to protect themselves from dangers such as pests and drought. Many of these compounds are also healthy compounds for human consumption as they can act as antioxidants and may protect human cells against damage that can lead to heart disease, cancer and other diseases (Törrönen and Määttä 2002, Zhang et al. 2008, da Silva Pinto et al. 2010). Unlike wild plants and organically grown crops, it has been suggested that these healthy molecules are reduced in plants treated with pesticides, as they need to make less of these compounds (Asami et al. 2003).

Therefore, there is a growing need to develop alternative approaches for control of strawberry diseases. Advances in the last two decades in the understanding of the molecular interplay between plants (mainly those considered

model systems) and various classes of microbial pathogens have provided a conceptual framework for rational human intervention through new strawberry research approaches, including the use of natural plant elicitors (Terry and Joyce 2000, Babalar et al. 2007, Hukkanen et al. 2007, Shafiee et al. 2010), and biocontrol agents to enhance natural defence responses (Adikaram et al. 2002, Forster et al. 2004, Sesan 2006, Oliveira et al. 2007, Zhang et al. 2010, Huang et al. 2011, Tortora et al. 2011). Studies in strawberry providing molecular information to engineer strawberry plants for durable and broad-spectrum disease resistance are still scarce, and most components and mechanisms of the strawberry defence network remain completely unknown. Therefore, major progress in the genetics and molecular biology of strawberry, is still needed to fully uncover the logic of their elaborate plant innate immune system. This review, contribute to the field by updating and compiling research focused on the molecular aspects and events of the strawberry defence mechanisms against pathogens.

The Plant Innate Immunity in Strawberry: what is, and what is not known

Plant innate immunity is a term including all the molecular and cellular mechanisms that plants can display to prevent potential pathogen infection and pest attack, from preformed mechanical and chemical defences to the expression of induced resistance responses after detection of a great variety of microbial pathogen such as viruses, bacteria, fungi, oomycetes, nematodes, and insects. A schematic view of known strawberry defence mechanisms is shown in Figure 1.

Strawberry pathogens use a wide range of strategies to live (Maas 1998). Bacteria are able to enter through biological cell structures such a stomata and hydathodes (gas or water pores) or even gain access via wounds, and further proliferate in the intercellular spaces. Fungi can directly enter plant epidermal cells, or extend hyphae on top of, between, or through plant cells. Pathogenic and symbiotic fungi and oomycetes eventually invaginate feeding structures (haustaria) into the host cell plasma membrane. In a different complex way, nematodes and aphids feed by inserting a stylet directly into a plant cell. Viruses

need a direct transfer of sap through wounded plant tissues, and a biological vector such as an insect or nematode, to spread and infect on healthy plants.

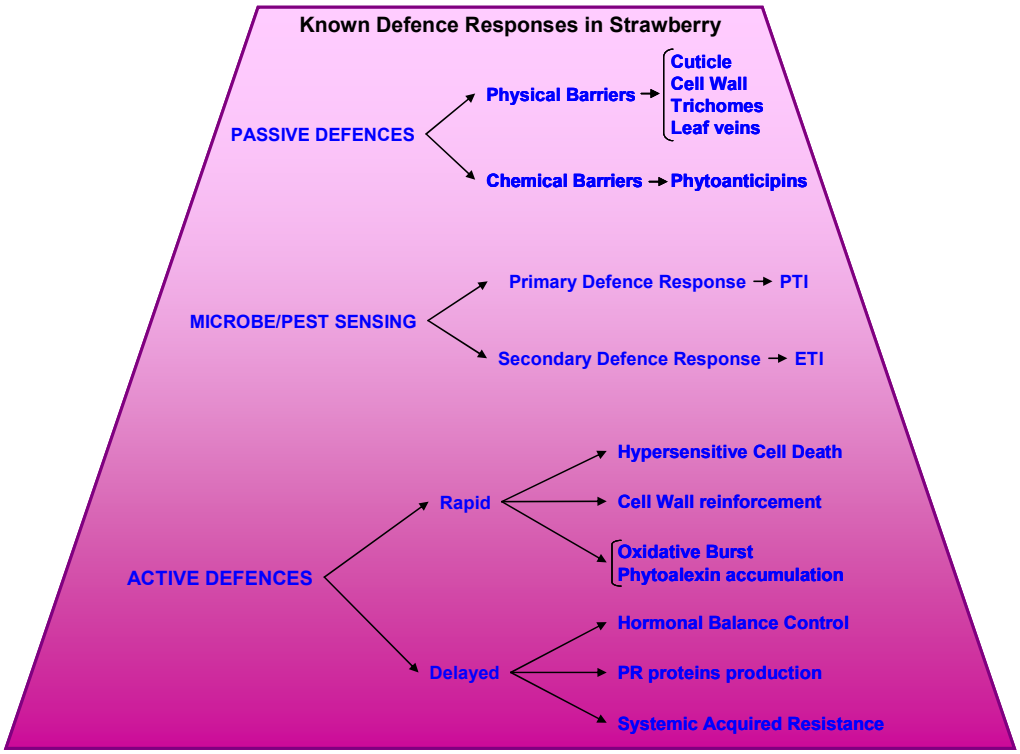

Figure 1. Schematic view of the known physiological responses exposed by the strawberry plant challenged by pathogens and pests.

Similar to animals, plants are able to recognize pathogens and swiftly activate defence. However, plant defence system differs notably from that in mammals (Nürnberger et al. 2004). Plants do not have mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell, and on systemic signals produced and dispersed from infection sites (Chisholm et al. 2006).

It may be assumed that strawberry plant must recognize pathogens and respond to diseases in a comparable manner to that known in other plants. Thus, the existence of structural and chemical barriers such as the cell wall and the cuticle

shield should prevent strawberry from most of invading organisms and it should conform a pre-existent passive defence mechanism that would include pre-synthesized toxins, toxic chemicals, antifungal proteins and enzymatic inhibitors (Dixon 2001, Nürnberger and Lipka 2005). Additionally, plants have developed induced defence systems to respond to microbes that manage to circumvent these preformed barriers. Generally, such challenged organisms are not able to invade a plant because of the activation of a primary defence response resulting in non-host resistance (Nürnberger and Lipka 2005). This primary active response (so called PAMP-triggered immunity, PTI) is initiated within the plant cell after pathogen interaction and perception of pathogen- or microbe associated molecular patterns (PAMPs or MAMPs) through pattern recognition receptors (PRRs) at the plant's cell surface. In turn, these events induce a molecular reprogramming of the cell and propitiate complex compound deposition in plant cell wall including callose, suberin, lignin and proteins, in addition to other metabolic changes leading to mount the plant immunity. Most of the microbes able to evade or suppress the primary defence response are recognized by the plant via the effector proteins that they secrete (avirulent factors or race-specific elicitors) to inhibit PTI. Additional plant receptors -called R proteins- can perceive such effectors to mount a second layer of defence called effector triggered immunity (ETI), which in most cases involves a hypersensitive response (HR), and a systemic activation of plant defences from the site of signal perception. All these events include the induction of specific signalling pathways and genes transcription, and the activation and production of proteins and chemicals with a clear defensive role, including pathogenesis related proteins, phytoalexins, and reactive oxygen species. Therefore, the primary and secondary defences responses in plants leading to resistance rely on a complex system of receptor-mediated pathogen perception and subsequent downstream signal transduction cascades, whereby cellular changes caused by the secondary defence response are generally most pronounced (Jones and Dangl 2006, Stulemeijer and Joosten 2008).

In cultivated strawberry (*F.* x *ananassa*, Duch.), breeders have tended to share their good quality fruit selections with others, including resistance, in the last two

centuries. Thus, it should have been expected the selection and maintenance within the strawberry cultivars of essential components for the primary and the secondary defence system. However, the knowledge of these molecular components and associated breeding markers in strawberry has been very limited, so far.

Strawberry Passive Defences

Preformed structural/mechanical barrier

Strawberry fruit is considered a "soft fruit" due to its delicate texture, coated by a very thin cuticle and presenting high susceptibility to physical damage.

Fruit firmness also relies on the composition and structure of cell wall, a rigid, cellulose-based support surrounding every cell. Heterogeneity of strawberry fruit in firmness and response to physical damage has been reported among cultivars (Gooding 1976, Ferreira et al. 2008), and a clear relation between skin strength or fruit firmness and susceptibility to pathogen infection has also been described (Gooding 1976, Barritt 1980). Indeed, pathogens use mechanical force or release cell wall degrading enzymes to break down these barriers, to access cellular nutrients. Table 1 shows a survey of known strawberry physical defence responses.

Changes in the cell wall composition and structure also occur naturally in strawberry during developmental stages. This armature is being continuously restructured by both enzymatic and non-enzymatic molecular processes (Rose et al. 2004). Thus, during strawberry fruit ripening, modification of the primary cell wall is required. Consequently, large variation in fruit firmness takes place during this physiological event.

In other plants, it is accepted that cell wall disassembly is a key component of susceptibility to pathogen (Cantu et al. 2008), and it is known that strawberry fruit (*Fragaria* x *ananassa* Duch.) varies in its inherent natural disease resistance (NDR) according to its physiological status (Gilles 1959). Indeed, the natural

Table 1. Strawberry Physical Defenses

Plant Structure	Strawberry Cultivar	Tissue	Pathogen/Pest	Defensive Activity	References
Cuticle and Cell Wall	*F.* x *ananassa*	Fruit	*Botrytis cinerea*	*clear relation between skin strength or fruit firmness and susceptibility to pathogen infection*	Gooding 1976 Barritt 1980
Cell Wall	Alba	Fruit	*Colletotrichum acutatum*	*natural modification in strawberry fruit cell wall during ripening process make the fruit more susceptible to the action of polygalacturonase enzymes from C. acutatum*	Guidarelli et al. 2011
	Apollo Sequoia Surecrop	Petiole	*Colletotrichum fragariae*	*thickening of the cell walls and a deposition of pectic material associated with fungal restriction in resistant cultivar*	Milholland 1982
	Pájaro	Leaf Petiole	*Colletotrichum fragariae*	*thickening of the cell wall of leaflets exposed to C. fragariae*	Salazar et al. 2007
	F. vesca	Fruit	*Botrytis cinerea*	*partial demethylation of strawberry cell wall oligogalacturonides is required for eliciting defence responses*	Osorio et al. 2008, 2011
Trichomes	Totem Zephyr Venta Tenira Induka Bogota Senga Sengana Kokinskaja Pozdnaja Korona *F. chiloensis*	Leaf	*Tetranychus urticae* Koch	*relationship between the oviposition and survival of the two spotted spider mite Tetranychus urticae Koch, and the number and density of glandular and non glandular trichomes*	Kishaba et al. 1972 Luczynski et al. 1990a,1990b Steinite and Levinsh 2003
Leaf Veins	64 *F.* x *ananassa* cultivars and clones	Leaf	*Xanthomonas fragariae*	*the spreading of Xanthomonas fragariae is effectively blocked by strawberry leaf veins*	Kennedy and King 1962a, 1962b

modification in strawberry fruit cell wall during ripening process has been reported to make the fruit cell wall more susceptible to the action of polygalacturonase enzymes from *Colletotrichum acutatum* (Guidarelli et al. 2011). Also, the timing of the ripening process may vary among strawberry genotypes, causing different softening rates (Rosli et al. 2004), and thus, different fruit susceptibility to pathogens has also been described among strawberry genotypes (Daugaard 1999, Casado-Díaz et al. 2006, Chandler 2006).

Changes in cell wall composition and structure are mainly due to the concerted action of a set of enzymes acting on the different cell wall polymers, and many of these enzymes have already been cloned in strawberry fruit (Table 2). It is also predicted that microorganism must secrete a similar set of counterpart hydrolytic enzymes to degrade the cuticles and disorganize the cell walls to allow the nutrient uptake and spread through the plant. Usually, plant cells respond to such entry attempts by using several defence responses including *de novo* cell wall biosynthesis, and deposition of the glucan polymer callose at the site of pathogen contact (Aist 1976, Kwon et al. 2008).

The dynamic changes in the structure and composition of the strawberry plant cell wall challenged with pathogens together with a functional analysis of strawberry cell wall modifying genes and enzymes have not been yet well studied to a molecular level, and is expected to be beneficial for the understanding of the complex process of defence response in this crop. Nonetheless, functional characterization of some of the strawberry cell wall genes mentioned in Table 2 has been performed either by ectopic expression or by antisense down-regulation technology. Thus, it has been reported biological roles for the endo-β-1,4-glucanase genes Cel1 and Cel2 (Woolley et al. 2001, Palomer et al. 2006, Mercado et al. 2010), the pectate lyase gene (FaPLC) (Jimenez-Bermudez et al. 2002, Sesmero et al. 2007, Santiago-Doménech et al. 2008, Youssef et al. 2009), and the polygalacturonase gene FaPG1 (García-Gago et al. 2009, Quesada et al. 2009).

Table 2. Strawberry Cell Wall Related Genes

Protein Family	Strawberry gene ID	Accession number	References	References for Functional Characterization or Direct Correlation with Fruit Firmness
expansin	FaExp-1	(AF163812)	Rose et al. 1997 Civello et al. 1999 Harrison et al. 2001	Dotto et al. 2006
	FaExp-2	(AF159563)		
	FaExp-3	(AF226700)		
	FaExp-4	(AF226701)		
	FaExp-5	(AF226702)		
	FaExp-6	(AF226703)		
	FaExp-7	(AF226704)		
polygalacturonase	FaPG1	(AF380299)	Redondo-Nevado et al. 2001 Figueroa et al. 2008 Quesada et al. 2009	Salentijn et al. 2003 Lefever et al. 2004 Villarreal et al. 2007, 2009 Figueroa et al. 2008 García-Gago et al. 2009 Quesada et al. 2009
	FaPG2	(AY280662)		
	FcPG1	(EF441274)		
pectate lyase	FaPLa	(AF339025)	Medina-Escobar et al. 1997 Benítez-Burraco et al. 2003 Figueroa et al. 2008	Jimenez-Bermudez et al. 2002 Benítez-Burraco et al. 2003 Sesmero et al. 2007 Figueroa et al. 2008 Santiago-Doménech et al. 2008 Youssef et al. 2009
	FaPLb	(AF339024)		
	FaPLc	(FXU63550)		
	FcPL1	(EF441273)		
pectin esterase	FaPE1	(AY324809)	Castillejo et al. 2004	Lefever et al. 2004 Osorio et al. 2008, 2011
	FaPE2	(AY357182)		
	FaPE3	(AY357183)		
	FaPE4	(AY357184)		
β-Galactosidase	Faβgal1	(AJ278703)	Trainotti et al. 2001	
	Faβgal2	(AJ278704)		
	Faβgal3	(AJ278705)		
β-xylosidase	FaXyl1	(AY486104)	Martínez et al. 2004	Martínez et al. 2004 Bustamante et al. 2006, 2009
aquaporin	FaPIP1	(GQ390798)	Mut et al. 2008 Alleva et al. 2010	
	FaPIP2	(GQ390799)		
arabinofuranosidase	FaAra1		Rosli et al. 2009	Rosli et al. 2009
	FaAra2			
	FaAra3			
endo-β-(1,4)-glucanase	FaCel1	(AF074923) (AF051346)	Harpster et al. 1998 Llop-Tous et al. 1999 Trainotti et al. 1999b	Trainotti et al. 1999a Woolley et al. 2001 Palomer et al. 2006 Mercado et al. 2010
	FaCel2	(AF054615)		

Also, a direct correlation between mRNA expression levels or enzyme activity and fruit firmness has been found in different cultivars for some of them, as to FaExp1-7 genes (Dotto et al. 2006), FaXyl1 (Martínez et al. 2004, Bustamante et al. 2006, Bustamante et al. 2009), FcPL1 (Figueroa et al. 2008), PME (Lefever et al. 2004), polygalacturonases (Salentijn et al. 2003, Lefever et al. 2004, Villarreal et al. 2007, Figueroa et al. 2008, Villarreal et al. 2009), arabinofuranosidases (FaAra1, FaAra2 and FaAra3) (Rosli et al. 2009), and the endo-β-1,4-glucanases (Trainotti et al. 1999a). So far, no further studies have been carried out with these strawberry genes, lines and cultivars, exploring their implication in the defence response to pathogens but the reported information that a partial demethylation of strawberry cell wall oligogalacturonides by the strawberry pectin methyl esterase

1 gene (FaPE1) is required for eliciting defence responses in wild *F. vesca* (Osorio et al. 2008, Osorio et al. 2011) (see further below).

Proteins with fundamental roles in plants also can play additional functions in defence. Thus, structural cell wall proteins such as extensins and proline-rich proteins (hydroxyproline-rich glycoproteins, HRGPs) play a role in cross-linking other components of the plant cell wall, and strengthen this protective layer against the attack of pathogens (Showalter 1993, Wei and Shirsat 2006, Deepak et al. 2010). It is known that these proteins are actively synthesized after wounding (Cheong et al. 2002) and pathogen infection (Maleck et al. 2000, Schenk et al. 2000) but the dynamical composition of the cell wall during different stages of plant development is thought to also lead to differences in susceptibility to pathogens. In strawberry fruit, synthesis of extensins seems to be independent of auxin control (Aharoni et al. 2002a), although Blanco-Portales et al. (2004) reported a strawberry FaHyPRP gene (hybrid proline-rich protein) whose expression was regulated by auxins. DNA microarray studies have revealed differences in the level of expression of strawberry HyPRP genes between soft and firm strawberry cultivars (cv. Gorella and cv. Holiday, respectively) (Salentijn et al. 2003). These results clearly support the role of these proteins in the strawberry cell wall reinforcement but a direct implication of these proteins in the mechanism of resistance to pathogens in strawberry needs to be further assessed.

Morphological features of strawberry plant leaves are also thought to affect herbivores as in other plants (Peters and Berry 1980). In many plants, the presence of trichomes, hairs or spines has been shown to be a very efficient mechanism of defence against herbivores and some pathogens and so, more pubescent leaves (containing a major number of non glandular trichomes) are more resistant to herbivores due to mechanical restrictions (Levin 1973, Dai et al. 2010). In strawberry, a negative relationship between the oviposition and survival of the two spotted spider mite *Tetranychus urticae* Koch, and the number and density of glandular and nonglandular trichomes in leaves has been reported (Luczynski et al. 1990). However, Kishaba and col. (1972) proposed that foliar pubescence might be related to spider mite susceptibility, and Steinite and Levinsh (2003)

have reported that the density of nonglandular trichomes is not the key factor for the resistance of strawberry cultivars but rather, the presence of preformed glandular trichomes containing oxidative enzymes.

In strawberry green tissues, leaf veins also seem to have a preventive function in defence, and effectively block the spreading of some pathogens. Thus, it has been reported that spreading of *Xanthomonas fragariae*, which causes angular leaf spots, is restricted by leaf veins in strawberry (Kennedy and King 1962a, Kennedy and King 1962b).

Preformed strawberry biochemical barrier

Preformed chemical barriers (phytoanticipins) appear to be decisive in plant passive defence mechanism. Plants produce a broad range of secondary metabolites, either as part of their normal program of growth and development or in response to stress, much of which have a proved toxic effect against pathogens and pests (Dixon 2001). Phenolics, sulphur compounds, saponins, cyanogenic glycosides, and glucosinolates conform this biological chemical barrier and act locally at the very early stages of pathogen attack. Most are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways (Kliebenstein 2004). The central phenylpropanoid pathway is leading to a major group of these valuable natural products, and flavonoids represent one of the largest classes within this group, which are also known to be involved in a multitude of other physiological functions (Winkel-Shirley 2001). Important products of the main phenylpropanoid branches in plants also include lignin, chlorogenic acid, salicylic acid and catecholamines, many of which have been proved to act as antimicrobials (Kliebenstein 2004).

In strawberry fruit, the phenylpropanoid pathway is switched on during the ripening process (see Singh et al. 2010 for a more comprehensive detail). Table 3 shows a set of known compounds putatively related with strawberry defence. Proanthocyanidins (PA) and many other compounds of the flavonoid pathway are actively synthesized and accumulate to high levels in strawberry fruit receptacle at early stages (green and white) of the strawberry fruit ripening, thereby giving

immature fruit an astringent flavour (Cheng and Breen 1991, Aharoni et al. 2002b, Almeida et al. 2007), contributing to plant defence (Terry et al. 2004, Halbwirth et al. 2006, Hukkanen et al. 2007)**.** PA in the strawberry consists of catechin units, which is a main flavonoid in strawberries (Ishimaru et al. 1995, Törrönen and Määttä 2002, Puhl and Treutter 2008, Wulf et al. 2008), and it is known to possess antimicrobial properties (Scalbert 1991, Yamamoto et al. 2000). Other compounds like euscaphic acid, tormentic acid and myrianthic acid, have also been identified through thin layer chromatography (TLC) bioassays and NMR spectral analysis in green stage strawberry fruit and flowers (Hirai et al. 2000, Terry et al. 2004). It is known that *Botrytis cinerea,* the causal agent of strawberry fruit rot, penetrates floral parts (petals, stigmas, styles, or stamens) of strawberries, raspberries, and grapes and remains quiescent until fruit ripens (Jarvis 1977, Elad and Evensen 1995). Terry et al. (2004) reported that extracts of strawberry flowers at post-anthesis showed greater antifungal activity than white bud and full bloom stages and proposed that antifungal compounds in strawberry flowers may play a role in initiating *B. cinerea* quiescence.

Several other authors have also found a positive correlation between resistance to *B. cinerea* and the concentration of PA in strawberry. Hébert et al. (2001, 2002) found that cultivars with higher concentrations of PA (mainly free and bound catechin and epicatechin) were more resistant to fungal infection. Jersch et al (1989) also found that aqueous extracts of immature strawberry cv. Chandler fruit did also have direct antifungal activity against *B. cinerea* conidial germination and mycelial growth, and suggested that a decline in PA concentration during fruit development governs *B. cinerea* quiescence through removing inhibition of a pathogen-derived polygalacturonase. They also observed that PA concentration was higher in the less susceptible strawberry cultivars. These results agree with the previously reported inactivation of a polygalacturonase enzyme from *B. cinerea* by strawberry phenolics (Harris and Dennis 1982). An inverse relationship between the PA content of immature strawberry fruits of various cultivars and the colonization of *B. cinerea,* was also observed by Di Venere (1998).

Table 3. Metabolites Related to Strawberry Defense.

Chemical Family	Compounds	Cultivar	Tissue	Pathogen/Pest	Defensive Activity	References
	not identified	Deutch Evern	Green Fruit	*Botrytis cinerea*	*preformed defense compound against Botrytis cinerea*	Gilles 1959
	not identified [1]	Surecrop Stelemaster Blakemore	Root	*Phytophthora fragariae* *Cladosporium cucumerinum*	*partially inhibitory to micelial growth in bioassay*	Mussell and Staples 1971
	not identified	Elsanta	Green Fruit	*Botrytis cinerea* *Cladosporium cladosporioides*	*antifungal activity against grey mould rot*	Adikaram et al. 2002
	not identified [2]	Chandler Sweet Charlie	Leaves	*Colletotrichum fragariae*	*these compounds conferred resistance to C. fragariae*	Vincent et al. 1999
	not identified	Chandler	Achenes	*Botrytis cinerea*	*these compounds inhibited radial growth of B. cinerea*	El Ghaouth et al. 1991
	fragarin	Chandler	Leaves	*Colletotrichum acutatum* *C.fragariae* *C.gloeosporioides*	*had a broad antibiotic spectrum, high activity against bacteria and fungal plant pathogens*	Filippone et al. 1999, 2001
	cyanogenic glycosides (source for HCN)	Elsanta	Achenes		*increase in transcript abundance of genes putatively involved in the metabolism of cyanogenic glycosides in the achenes*	Aharoni and O'Connell 2002
triterpenes	euscaphic acid tormentic acid myrianthic acid	Houkouwase	Unripe Fruit	*Colletotrichum musae* *C. fragariae*	*effective against infections with the fungus Colletotrichum*	Hirai et al. 2000
	not identified [1]	Elsanta	Achenes, Fruit and Flower	*Botrytis cinerea*	*preformed antifungal activity against the pathogen, Botrytis cinerea, and the bioassay organism, Cladosporium cladosporioides*	Terry et al. 2004
lipoxygenase pathway	(E)-hex-2-enal [3]			*C. acutatum* *Penicillium expansum* *Botrytis cinerea*	*inhibited spore germination and fungus growth, and altered the structures of the cell wall and plasma membrane, causing disorganization and lysis of organelles and, eventually, cell death of the pathogen*	Archbold et al. 1997 Fallik et al. 1998 Neri et al. 2006 Arroyo et al. 2007

Table 3. Continued.

Chemical Family	Compounds	Cultivar	Tissue	Pathogen/Pest	Defensive Activity	References
	ellagitannin ellagic acid quercetin kaempferol and others	Jonsok	Leaves		*production has been shown to be stimulated by foliar application of benzothiadiazole and glycine betaine*	Karjalainen et al. 2002 Hukkanen et al. 2007
	cathecol-based	Korona Senga Sengana Zephyr, and others	Leaves	*Tetranychus urticae Koch*	*higher resistance is associated with a trichome-localized inducible increase of catechol-based phenolics*	Steinite and Ievinsh 2002, 2003
		Totem	Leaves	*Tetranychus urticae Koch*	*development of the two spotted spider mite T. urticae Koch, was negatively correlated to foliar concentrations of phenolics*	Luczynski et al. 1990a, 1990b
	methyl salycilate	Sunrise Red Chief Scott	Leaves	*Tetranychus urticae Koch*	*increased approximately 10-fold after fruit harvest in plants more resistant to the two-spotted spider mite*	Hamilton-Kemp et al. 1988
phenolics		Korona	Flowers	*Anthonomus rubi*	*induced in higher amounts by weevil feeding*	Bichão et al. 2005
		Polka Honeoye	Leaves	*Phytonemus pallidus*	*emissions of methyl salicylate was greater from cyclamen mite-damaged strawberry plants than from intact plants*	Himanen et al. 2005
	methyl salycilate [(3)]			*Aphididae, Thripidae, Cicadellidae and others*	*enhanced natural enemy attraction but did not increase, nor decrease pest abundance*	Lee 2010
	quercetin	Howard and Surecrop type	Root	*Phythium irregulare* *Rhizoctonia solani* *Alternaria alternata*	*decisive to confer moderate resistance to root rot diseases*	Nemec 1973, 1976
	ellagitannins flavonoids	Herut	Achenes		*ellagitannins and flavonoids accumulate particularly in the achene during early and late development, respectively*	Fait et al. 2008

Table 3. Continued.

Chemical Family	Compounds	Cultivar	Tissue	Pathogen/Pest	Defensive Activity	References
phenolics	gallic acid	Chandler				
flavonoids	proanthocyanins catechin	Seascape Sweet Charlie	Fruit	*Botrytis cinerea*	*positive correlation between resistance to B. cinerea and the concentration of proanthocyanidins in strawberry fruit*	Hébert et al. 2001, 2002
	epicatechin	Annapolis				
	proanthocyanins catechins	Clea Pájaro	Fruit	*Botrytis cinerea*	*positive correlation between resistance to B. cinerea and the concentration of proanthocyanidins in strawberry fruit*	Di Venere et al. 1998
	proanthocyanidins	Senga Sengana	Fruit	*Botrytis cinerea*	*inhibition of Botrytis cinerea growth (quiescence) in green fruit*	Jersch et al. 1989
	flavonols	Jonsok	Leaves	*Botrytis cinerea*	*strawberry leaves with increased susceptibility to grey mould had decreased their contents in flavonols*	Hanhineva et al. 2009
	(+)-catechin	Morioka-16 Hokowase	Leaves	*Alternaria alternata*	*protective agent during induced resistance against Alternaria black spot*	Yamamoto et al. 2000
flavanols	proanthocyanidins catechins	Hybride	Leaves	*Mycosphaerella fragariae*	*precise localization of flavanols around fungal infections*	Feucht et al. 1992
	catechin derived procyanidins	Elsanta	Fruit	*Botrytis cinerea*	*inhibit growth of B. cinerea in immature strawberry fruits (quiescence)*	Puhl and Treutter 2008
	luteoliflavan					
	eriodictyol 7-glucoside					

(1) Some of these compounds probably correspond to the same phytoalexins found in strawberry cv. Houkouwase unripe fruit and reported by Hirai et al. (2000).

(2) These compounds might be similar to the preformed antifungal compounds found in strawberry green-stage-I fruit by Terry et al. (2004).

(3) Comercialized high purity chemical tested in bioasays.

More recently, Puhl and Treutter (2008) showed that the accumulation of catechin derived procyanidins was a fundamental factor to inhibit the growth of *B. cinerea* in immature strawberry fruits. In fact, grey mould symptoms occur only in ripe, red coloured fruits. They modified the concentration of flavanols in developing strawberry fruits by inhibiting the flavanone 3-hydroxylase (FHT), a prominent dioxygenase of the flavonoid pathway, which is involved in the biosynthesis of catechin precursors. The accumulation of novel flavonoids identified as luteoliflavan and eriodictyol 7-glucoside and enhanced levels of catechin were found when green fruits were treated with prohexadione-Ca, a bioregulator whose structure mimics that of 2-oxoglutarate which is able to inhibit dioxygenase enzymes which require 2-oxoglutarate as cosubstrate (Rademacher 2000, Roemmelt et al. 2003). Although the increase in catechin concentration seems to be contradictory to the occurring FHT bottleneck, similar observations on apple (Fischer et al. 2006) and grapevine (Puhl et al. 2008) have been explained by an additional strong inhibition of the flavonol synthase, which also is a 2-oxoglutarate dependent dioxygenase. Thus, an excess supply of substrates for the remaining FHT activity was assumed. The effect of the bioregulator was dependent of the fruit developmental stage, showing the higher increment of these compounds after flowering, within the stage of small green fruits, but having no effect thereafter. The increasing catechin and proanthocyanidin concentrations at small green stage restricted fungal growth, and became obvious that young fruits just at flowering do not accumulate flavanols to a sufficient level for preventing primary receptacle infection. Thus, the choice of the flowers as the favoured tissue for fungal invasion as well as the latency of the pathogen in green fruits can be regarded as the critical points in *B. cinerea* development. Indeed, the ability to develop latent infections on immature fruits, becoming quiescent until fruit ripens, has also been reported for other strawberry pathogens such as *Colletotrichum* spp. (Prusky 1996, Guidarelli et al. 2011).

Methyl salicylate it also has been suggested to be implicated in strawberry plant resistance. Thus, Hamilton-Kemp et al. (1988), detected a 10-fold increase on methyl salicylate relative amount when compared at flowering and after fruit

harvest strawberry, in plants that were more resistant to the two-spotted spider mite, *T. urticae* Koch. Surprisingly, this compound did not seem to have effect on spider mite behaviour, under bioassay at low concentrations.

It is believed that unripe fruit is highly protected by chemical barriers from herbivore and pathogens attack, to prevent widespread of not yet mature seeds. When fruit ripens, this protective layer usually decreases, and changes in the main branches of the phenylpropanoid pathway are produced allowing the synthesis of colour-, taste-, and aroma-related compounds used for the recruitment of seed dispersers. Contrary, the seed possess some chemical toxins and proteins although they are often well protected by physical structures, to ensure that the seed is not consumed along with the fruit (Terras et al. 1995). Thus, the strawberry achene, the true fruit, is heavily protected, not only by a sturdy and tough hedge, the pericarp, but it has a high concentration of toxic compounds that prevents it from being consumed by pathogens and pest (Aharoni and O'Connell 2002, Terry et al. 2004, Fait et al. 2008). Aharoni and O`connell (2002) reported an increase in transcript abundance of genes putatively involved in the metabolism of cyanogenic glycosides, a source for HCN (hydrocyanic acid) which can render a plant toxic, in achenes, pointing to their biosynthesis in the achene tissue. Also, Fait et al. (2008) detected defence related compounds of phenylpropanoids, ellagitannins and flavonoids, which accumulate particularly in the achene during early and late development, respectively. Terry et al. (2004), detected antifungal activity in all tissue types tested (viz. pith, cortex, epidermis) from strawberry green fruit, but specially and largely in the achenes.

Preformed antifungal compounds are also found in strawberry leaves. Vincent et al. (1999) found a positive correlation between the presence of these compounds (identity of these compounds was not determined) and strawberry resistance to *Colletotrichum fragariae*. They found that the amount of these preformed compounds varied between moderately resistant (Sweet Charlie) and susceptible (Chandler) cultivars to anthracnose, with approximately 15 times more antifungal activity present in the first one, suggesting that the resistance to *C.*

fragariae on different strawberry cultivars may be mediated by these preformed antimicrobials. Terry et al. (2004) suggested that these compounds might be similar to the preformed antifungal compounds they found in strawberry green-stage-I fruit. Yamamoto et al. (2000) reported that catechin preformed in strawberry leaves inhibited *Alternaria alternata*, and Hanhineva et al. (2009) observed that strawberry leaves with increased susceptibility to grey mould had decreased their contents in flavonols, thus, highlighting the role of flavonols in strawberry plant defence (Terry et al. 2004, Halbwirth et al. 2006, Hukkanen et al. 2007). Also, Luczynski et al. (1990) observed that the development of the two spotted spider mite *T. urticae* Koch, was negatively correlated to foliar concentrations of phenolics, especially cathecol-based, compounds.

Filippone et al. (1999) reported the isolation of a new type of antimicrobial compound constitutively present in strawberry leaves, called fragarin. This compound was isolated from a soluble fraction of this tissue and turned to be an amphipathic molecule of 316 Da that had a broad antibiotic spectrum, with a high activity against bacteria and fungal plant pathogens isolated from strawberry (*Colletotrichum gloeosporioides, C. fragariae* and *C. acutatum*) and other plants (*Clavibacter michiganensis* subsp. sepedonicus, strain C5, and *Pseudomonas corrugata,* isolated from tomatoes; *Pseudomonas syringae* isolated from onion, and *Erwinia* spp. isolated from rose leaves). These authors showed that fragarin was active against *C. michiganensis* by dissipating its membrane potential, and suggested that its action precedes or is simultaneous with cell death by altering the permeability and disrupting the membrane function (Filippone et al. 2001).

Quantitative differences on several phenolics are also present in strawberry root, and appears to be decisive to confer moderate resistance to root rot diseases caused by *Pythium irregulare*, *Rhizoctonia solani*, and *A. alternata*, (Nemec 1973, Nemec 1976).

Volatiles have also been related to defence in strawberry. Volatile aldehydes and alcohols are key compounds in the fresh and green sensorial notes of

vegetables and fruits (Rabetafika et al. 2008). They are produced by plants in response to various stresses and therefore may play a major role in plant defence mechanisms (Blée 2002).

Thus, it has been reported that (E)-hex-2-enal (trans-2-hexanal), a characterizing strawberry aroma volatile product, which is generated from the oxidative degradation of linolenic acid by a lipoxygenase (LOX) pathway, showed antifungal activity against *C. acutatum.* This volatile compound inhibited spore germination and fungus growth, and altered the structures of the cell wall and plasma membrane, causing disorganization and lysis of organelles and, eventually, cell death of the pathogen (Arroyo et al. 2007).

So far, the presence of a wide range of preformed defence compounds has been described in strawberry. Many of these preformed compounds are shared by different tissues like roots (Mussell and Staples 1971), leaves (Vincent et al. 1999), and green fruit (Hirai et al. 2000, Terry et al. 2004), so a similar preformed defence barrier seems to work against pathogens within the complete strawberry plant. In plants, over 100.000 low-molecular-mass compounds are produced as secondary metabolites (Dixon 2001). Such diversity makes it difficult to unravel specific products and pathways involved in defence (both, passive and active defences) within particular plant species. It is known that related plant families tend to use related chemical structures (for example isoflavonoids in the Leguminosae, sesquiterpenes in the Solanaceae), and some chemical classes are used across taxa (for example, phenylpropanoid derivatives) (Dixon 2001). A great deal of work is clearly still needed in this area, including effort to define products and genes, to determine branches of these pathways directly involved in the response to pathogens in strawberry.

Strawberry Plant Receptors: the PTI and ETI Responses

The strawberry non specific basal resistance (PTI)

In strawberry, the presence of extracellular surface plant pattern-recognition receptors (PRRs) that recognize microbe/pathogen-associated molecular patterns

(MAMPS or PAMPS), common to many classes of microbes, has been inferred from some indirect experiments but the characterization of these receptors and the transduction pathways they elicit is yet far to be well known. In this crop plant, the ability of chitosan to stimulate defence enzymes such as acidic chitinases have been reported on treated fruits (El Ghaouth et al. 1992), but close contact with tissue seems to be required for the elicitation. Strawberry receptors, which can presumably recognize chitin or chitin derivates compounds, were not able to detect the elicitor molecule through the nonporous strawberry cuticle, with act as a physical barrier preventing intimate interaction between the elicitor and the tissue, so direct application on freshly cut fruits is needed to develop the elicited plant response.

Adikaram et al. (2002) demonstrated enhanced disease resistance to grey mould rot (*B. cinerea*) in green strawberry fruit elicited both by *Aureobasidium pullulans* inoculation, and heat-killed cells of this yeast.

Some cell wall proteins with lectin domains have been described in strawberry (Trainotti et al. 2001, Martínez Zamora et al. 2008). Lectins are high affinity carbohydrate-binding proteins, which are able to recognize a great variability of ligands and interact directly with the cell wall. In many plants, lectins are described to be involved in plant defence and so, being implicated in facilitating PAMPs recognition (De Hoff et al. 2009). Curry et al. 2002 have provided evidences that these classes of proteins are involved in the strawberry defence response, and pathogens such as *C. fragariae* are recognized by this class of proteins.

Plant damage sensing is involved in basal defence response against pathogens and pests (Steinite and Ievinsh 2002). The ability of strawberry plants to be damage-elicited has been documented to confer resistance against pest (Kilkiewicz 1988, Greco and Sanchez 2003, Steinite and Ievinsh 2002), and pathogens (Terry et al. 2004, Myung et al. 2006). In other plants, during the process of plant–pathogen interaction, cell wall breakdown fragments of [1→4]-a-

linked oligogalacturonides (OGA) generated by either the plant or microbe, have been shown to elicit various plant defence responses (Côté and Hahn 1994, Aziz et al. 2004). How these responses are activated in strawberry need to be further studied. Recent evidences suggest partially demethylated cell wall pectin-derived oligogalacturonides as true elicitor molecules capable of activating strawberry plant basal defences (Osorio et al. 2008). The ectopic expression of the fruit-specific *F.* x *ananassa* pectin methyl esterase (FaPE1) in wild strawberry *F. vesca* induced a reduced degree of esterification of cell wall oligogalacturonides compared to those from wild-type fruits, and the transgenic *F. vesca* lines showed the salicylic acid signalling pathway constitutively activated and higher resistance to the necrotrophic fungus *B. cinerea*.

Oligomeric particles (10-15 monomers), are also induced by plant proteins with polygalacturonase inhibiting activity (PGIPs), which are included among the microbe-detecting molecules that are employed by the plant immune system to activate PTI (De Lorenzo and Ferrari 2002). PGIPs are thought to interfere with pathogen polygalacturonase activity, and to interrupt cell wall components degradation to monomers. A PGIP protein has also been isolated in strawberry and will be discussed further below, in section *Strawberry proteins with role in defence*.

The Strawberry Plant R-proteins: effector triggered immunity (ETI)

Recognizing of pathogen avirulent effectors (avr) has been reported in strawberry. A small cysteine-rich protein, PcF, identified in *Phytophthora cactorum*, was able to trigger necrosis in strawberry plants and also in tomato (Orsomando et al. 2001). This protein elicited the activity of the enzyme phenylalanine ammonia lyase (PAL) but its exact mode of action remains unclear (Orsomando et al. 2003). It has been reported that an avirulent isolate of *C. fragariae* has the ability to protect the strawberry *F.* x *ananassa* cv. Pájaro against the development of anthracnose (Salazar et al. 2007). Thus, culture supernatant derived from that strain was able to induce HR, oxidative burst, accumulation of salicylic acid, and callose deposition in strawberry cv. Pájaro. This elicitor was later identified as a

37 kDa protein, which belongs to the family of the subtilisin-like serin-proteases. It conferred resistance in different degrees to other strawberry cultivars, and it also induced the accumulation of hydrogen peroxide (H_2O_2), superoxide anion (O2.-) and callose deposition in *Arabidopsis thaliana* (Salazar et al. 2007, Chalfoun et al. 2009).

Martínez-Zamora et al. (2004) reported for the first time on resistance gene analogues (RGAs) in strawberry. Seven distinct families of RGAs of the NBS-LRR (nucleotide binding-leucine rich repeat domains) type, the most prevalent family of plant receptors (McHale et al. 2006), were identified from wild species *F. vesca* and *F. chiloensis*, and six different *F.* x *ananassa* cultivars, by genomic DNA amplification using degenerate primers. Fifty one clones presented significant homology to R gene sequences and RGAs from other species in the GenBank NR Database. All strawberry RGAs isolated were grouped into the TIR class of R genes, except one of them, which fell on the non-TIR branch. More recently Jung et al. (2010) have reported a cluster of four RGAs, contained in a strawberry (*F. vesca*) fosmid (34E24), with NBS and LRR domains, and conserved in all the rosids genomes they have compared with. They also have found that none of the genes have the TIR domain, so they may belong to the non-TIR class. Although no experimental evidences about correlation between the degree of resistance/susceptibility to a particular pathogen and the presence or absence of any particular class of RGAs has yet been shown, all the strawberry RGAs detected are closely related to R genes from other species, thus, some (if not all) of them may have pathogenesis response implications in strawberry resistance.

More recently, Martínez-Zamora et al. (2008) have also reported on the presence of serine-threonine kinase (STK) domain R gene receptors in strawberry. By using degenerate oligonucleotides to amplify conserved regions of the interspecific STK domain, they performed a broad screening on three related strawberry wild species (*F. vesca*, *F. chiloensis*, and *Potentilla tucumanensis*), and seven different *F.* x *ananassa* cultivars (Camarosa, Gaviota, Oso Grande, Sweet

Charlie, Pájaro, Milsei Tudla, and the breeding line US159 from Galleta et al. (1993)). They reported 31 putative strawberry STK clones (11 not redundant), and identified seven groups of STK genes out of the 11 not redundant ones. Five of them (containing seven unique sequences) were classified as Pto-like kinases. The two unique sequences corresponding to group 6, were classified as B-lectin receptor kinases, a novel class of plant R genes also involved in plant defence (De Hoff et al. 2009), and the other two sequences conforming the 7th group, were close related to the S-receptor-like protein kinases, involved in the mechanism by with hermaphrodite flowering plants avoid self-fertilization (Cui et al. 2000).

The first reported evidence of the synthesis of strawberry R proteins being regulated in response to pathogens can be found in Casado et al. (2006). They performed gene expression profiling and quantitative analysis of some strawberry genes coding for leucine-rich repeat (LRR) receptor-like proteins (Falrrp1, Falrrk1, and Falrrk2), after *C. acutatum* infection. The genes analysed showed a wide range of responses to the pathogen, which were tissue and cultivar dependents. Thus, the transcript level of *Falrrp1* and *Falrrk1* genes was higher in infected than in uninfected control fruit from cv Camarosa, indicating a clear upregulation of this gene after *C. acutatum* infection. In crown tissue, the expression of *Falrrk1* was modulated differently in the two cultivars analysed, cv. Andana and cv. Camarosa, and varied from up to down regulation along the time of pathogen interaction. These results highlight the importance of considering the spatial-temporal molecular studies in addition to the genotype, in order to fully understand the mechanism of strawberry defence.

In the last decades, advances on the understanding of molecular aspects leading to host genotype-specific resistance has been extensively produced in Arabidopsis and other model plants, and they have been mainly focused on the identification and functional characterization of plant resistance (R) proteins and their cognate pathogen effectors (Bent and Mackey 2007, Lukasik and Takken 2009). However, disease resistance based on single race-specific resistance (R) gene has not been shown durable in many crop species as members of the pathogen population

emerge that avoid recognition by the plant immune system, requiring the introduction of new resistance traits (Quirino and Bent 2003). Therefore, unravelling all the strawberry associated molecular components of the signalling pathways and genes they control related to the active defence is necessary to fully understand this process in this crop plant.

The Strawberry Active Defences

Cell wall fortification and HR

Milholland et al. (1982) first reported that strawberry cultivars with different susceptibility to anthracnose produced by *C. fragariae* (Apollo and Sequoia as resistant cultivars, and Surecrop as the susceptible one), presented clear histological differences after pathogen attack. While the most susceptible cultivars showed plant cellular collapse and necrosis, and successfully fungal invasion, the less susceptible ones, presented a thickening of the cell walls and a deposition of pectic material filling the intercellular spaces of the cortex. In addition, accumulation of tannins in the surrounding parenchyma cells was also found. All together, these changes were associated with fungal restriction to a few cells beneath the infection site. Although preformed structural and chemical components of the cell contribute to these mechanisms, actively synthesized de novo compounds are also implicated.

Salazar et al. (2007) also reported on morphological changes occurring on strawberry plant cv. Pájaro challenged with *C. fragariae*. The plant response started with an early oxidative burst within four hours after the inoculation with the fungus. They detected thickening of the cell wall of leaflets exposed to the microorganism, and mainly due to the enlargement of the parenchyma cells and the intercellular space rather than to an increase in the number of layers of the mesophyll. They also describe on the accumulation of pigments and of a new type of amorphous brown crystals in the intracellular mesophyll cells.

Cell wall fortification during infection, achieved by callose deposition (an amorphous, high-molecular-weight beta 1,3-glucan polymer) in cell wall

appositions (papillae), just below penetration sites, is a common defence response in plants (Luna et al. 2011).

Recently, a novel endo-β-1,3-glucanase gene (Faβgln1) from *F.* x *ananassa* cv. Chandler has been isolated upon infection with *C. acutatum* (Casado-Díaz et al. 2006). It encodes an unusual type of β-1,3-glucanase whose sequence structure contains a glycosylphosphatidylinositol (GPI) membrane anchor domain (J.L. Caballero, unpublished results). Nucleotide and protein sequence analyses identified this strawberry Faβgln1 as an acidic β-1,3-glucanase homologous to plant glycosyl hydrolases family 17. Although the (1→3)-β-D-glucanases are related to callose metabolism and plant defence, the exact biological role of these enzymes in relation to callose has not been yet clearly established (Minic and Jouanin 2006). Currently, the strawberry Faβgln1 gene is being fully characterized and curiously, its expression seems to be repressed in strawberry plant after challenged with *C. acutatum* (Casado-Díaz et al. 2006, J.L. Caballero, unpublished results).

Production of phytoalexins and other new antifungals

Evidences that strawberry has the capacity and ability to induce much of the genes encoding proteins with antifungal and antimicrobial activities, and enzymes that catalyse the new production of defence metabolites (phytoalexins), including chemical volatiles and those needed for the reinforcement of the cell wall, after detection of pathogen or cell damage by plant cell receptors have been reported.

Mussell and Staples (1971) detected production of phytoalexins in two strawberry cultivars, Surecrop and Stelemaster, with increased resistance to *Phytophthora fragariae,* challenged with the pathogen. Between 48-72h after inoculation, the only discernible symptom was a browning of root epidermal cells, which contained two undetectable compounds in healthy roots. On thin-layer chromatography (TLC) assays, these compounds showed a partially inhibitory effect on mycelial growth of *P. fragariae* but they were strong inhibitors of the growth of *Cladosporium cucumerinum*, a fungal pathogen that affects cucumbers.

When a strawberry susceptible cultivar was tested (Blakemore), only one of these two compounds was produced after a longer period (5-8 days) of *P. fragariae* inoculation. Apparently, the activity of phenylalanine ammonia lyase (PAL), which increases during the synthesis of many phytoalexins (Hadwiger et al. 1970), was not essential for the synthesis of these inhibitors in root tissue after infection of these two strawberry cultivars. Vincent et al. (1999) also reported detection of a phytoalexin compound after *C. fragariae* infection that was solely induced in the strawberry cv. Sweet Charlie, a cultivar with reported increased resistance to this pathogen.

Hirai et al. (2000) identified three triterpene antifungal compounds from unripe strawberry fruit wounded and inoculated with *Colletotrichum musae* as euscaphic acid, tormentic acid and myrianthic acid. These triterpene phytoalexins were effective against infections with the fungus *C. fragariae*. They pointed out that these compounds probably correspond to the same phytoalexins found in strawberry cv. Surecrop roots and reported by Mussell et al. (1971). This observation suggests that strawberry fruit may produce similar antifungal compounds to those in the roots.

Adikaram et al. (2002) showed that skin tissue from strawberry green fruit inoculated with *A. pullulans* had greater antifungal activity against grey mould rot than the control non inoculated tissue.

Yamamoto et al. (2000) proposed that induced catechin synthesis in response to strawberry leaf inoculation with a nonpathogenic strain of *A. alternata* was needed to inhibit penetration of the hyphae of this fungus into the leaf tissues. They concluded that the accumulation of (+)-catechin correlated with the time of spore inoculation of this non pathogenic fungus, causing most of the resistance response in the strawberry leaf.

Ellagitannins and ellagic acid conjugates are highly present in berries, including strawberry (Aaby et al. 2005, Aaby et al. 2007, Gasperotti et al. 2010,

Hager et al. 2010). Production of ellagitannin, ellagic acid and gallic acid derivates, quercetin and kaempferol conjugates has also been shown to be stimulated by foliar application of benzothiadiazole (BTH), a synthetic plant systemic acquired resistance (SAR) activator, and glycine betaine, an amino acid derivate from sugar beet (Karjalainen et al. 2002, Gorlach et al. 1996) and it suggests a contribution of these phenolic compounds in strawberry active defence.

Increased strawberry resistant to *T. urticae* Koch has been described to be dependent on the presence and higher activity of wound-induced enzymes such as polyphenol oxidase and peroxidase (Steinite and Ievinsh 2002, Steinite and Levinsh 2003). As mentioned before, these authors suggest that the higher resistance of some strawberry cultivars to this pest is associated with a trichome-localized inducible increase of catechol-based phenolics produced by the activity of these oxidative enzymes.

Also, induced volatiles are known to be important for strawberry plant to respond to the attack of herbivore predators, as in many other plants (Maffei 2010). More than 360 volatiles are produced by strawberry (Schwab et al. 2009). From them, only six has been identified so far as key flavour compounds in the typical strawberry-like odour, and also are species-specific significant volatiles: the (Z)-3-hexenal, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), methyl butanoate, ethyl butanoate, methyl 2-methylpropanoate, and 2,3-butanedione (Larsen et al. 1992). Also, linalool, nerolidol, α-pinene, and limonene are quantitatively predominant in strawberry, reaching up to 20% of the total fruit volatiles (Loughrin and Kasperbauer 2001). It has been suggested that many of the strawberry volatile compounds might serve a dual role as attractants for animals, insects and humans and as protectants against pests and pathogens (Aharoni et al. 2003, Kappers et al. 2005). Although these preformed molecules can be considered phytoanticipins, the synthesis of many of them and other new compounds can be increased “de novo” after pathogen attack and so, be part of the induced arsenal of the strawberry defence against pathogens.

Oxylipins are known to be synthesized "de novo" in response to various stresses, including wound injury (Andreou et al. 2009). Their substrates, polyunsaturated fatty acids, are liberated from membrane lipids and converted into various oxylipins via several enzymatic steps. In strawberry leaves and fruit, (E)-hex-2-enal is a major volatile oxylipin produced upon wounding and it is not detectable on intact strawberry tissue (Hamilton-Kemp et al. 2003). Thus, after bruising, strawberry fruit emitted (E)-hex-2-enal and its precursor cis-3-hexenal, and activities of the key enzymes, LOX and HPL were also increased (Myung et al. 2006). (E)-hex-2-enal is a good inhibitor of conidial germination of *Penicillium expansum*, one of the main fungus pathogen causing postharvest diseases in pears fruit (Neri et al. 2006), and it also has been reported to influence, either inhibiting or promoting, the development of the fungal pathogen *B. cinerea* Pers in strawberry fruit (Archbold et al. 1997, Fallik et al. 1998). Pérez *et al.* (1999) found that (E)-hex-2-enal is the endogenous more represented aldehyde on strawberry fruit during most developmental stages. Decreasing in its content during the process of fruit ripening was related with the appearance of anthracnose symptoms. In addition, Arroyo et al. (2007) evaluated the effect of eight of the volatile products characterizing the strawberry aroma, and generated by the oxidative degradation of linoleic and linolenic acids through the lipoxygenase (LOX) pathway, on the mycelial growth and conidia development (spore germination) of *C. acutatum* on strawberry fruit. A positive correlation between an increased content of (E)-hex-2-enal and an enhanced resistance of strawberry fruits to *C. acutatum* was found. They showed that this volatile compound altered the structures of the fungal cell wall and plasma membrane, causing disorganization and lysis of organelles and, eventually, cell death, and concluded that (E)-hex-2-enal was the most efficient of the volatile products in the control of *C. acutatum* infection. These results coincide with those reported by Fallik et al. (1998) and by Neri et al. (2006), in which (E)-hex-2-enal was related to enhance resistant to *B. cinerea* and to *P. expansum*, respectively, and open new perspectives in the biological control of pathogens by plant volatile compounds.

Also methyl salicylate (MeSA) is naturally produced by plants, including strawberry, in response to herbivores. Thus, increase of MeSA release and other volatiles has been detected in strawberry plants after injury (Hamilton-Kemp et al. 2003, see Table 3), infection with cyclamen mite (Himanen et al. 2005), and strawberry blossom weevil (Bichão et al. 2005). In other plants, it is well documented that MeSA and other volatiles are attractive to natural enemies, a plant defence strategy called "indirect defence", so being beneficial for pest control (Kessler and Baldwin 2002, James and Price 2004). For instance, MeSA reduced the aphid *Phorodon humili* Schrank in hop yard (Lösel et al. 1996), and it delayed the establishment of bird cherry-oat aphid, *Rhopalosiphum padi* (L.), in barley (Ninkovic et al. 2003). Alternatively, MeSA may also repel pests, and it seems to inhibit development of gray mold, *B. cinerea* Pers. ex Pers, on the fruit (Archbold et al. 1997). In strawberry, Jana C. Lee (2010) reported that MeSA enhanced natural enemy attraction but did not increase, nor decrease pest abundance. However, natural enemies of major strawberry pests responded to MeSA in the laboratory, including *Anaphes iole* Girault, an egg parasitoid of *Lygus hesperus* Knight (Williams et al. 2008), and *Phytoseiulus persimilis A*thias-Henriot, a predator mass released for control of twospotted spider mite, *T. urticae* Koch (de Boer and Dicke 2004).

Fadini et al. (2007, 2010) also demonstrated a positive communication through such as strawberry volatiles and *P. macropilis*, a predator of *T. urticae* Koch. This phenomenon remains to be further studied but there are evidences that strawberry have the capacity and ability to perform such defence strategy. Thus, Aharoni et al. (2003) demonstrated that ectopic overexpression of a strawberry dual linalool/nerolidol synthase gene (FaNES1) in chloroplasts of the *A. thaliana* significantly increased the amount of volatile terpenes such as linalool and its derivatives in leaves, and these transgenic plants were able to repel the attack of the aphid *Myzus persicae*. The recombinant FaNES1 enzyme generated (S)-linalool and trans-(S)-nerolidol from geranyl diphosphate (GDP) and farnesyl diphosphate (FDP), respectively. The authors demonstrated that unwound transgenic plants were able to easily attract the aphid predatory mite *P. persimilis*.

Kappers et al. (2005) targeted FaNES1, a strawberry linalool/nerolidol synthase, specifically to the mitochondria, and found that the majority of the predatory mites made their first visit to the transgenic plants, which demonstrates a clear preference for the undamaged transgenic plants.

These results suggest the possibility to protect strawberry plants from insect pests by stimulating the emission of VOCs produced upon feeding, which eventually attract 'bodyguard' predators as suggested by Kappers. Curiously, although similar genes have been found in wild and cultivated strawberry species, only FaNES1 is exclusively present and highly expressed during fruit ripening in cultivated octoploid varieties (Aharoni et al. 2003, Aharoni et al. 2004).

Strawberry proteins with role in defence.

An outstanding role in plant defence response to pathogen and pests is assigned to an important group of plant proteins regulated under biotic stress conditions. Components of this group, the so-called Pathogenesis Related Proteins (PRs), have been categorized into 18 families (van Loon et al. 2006). It is accepted that the term "Pathogenesis Related Proteins" includes all microbe-induced proteins and their homologues, even though some of them are generally constitutively present in the plant, and only increase during most infections. Among others, this is the case with enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POX), and polyphenoloxidase (PPO), which are often also referred to as PRs (van Loon et al. 2006).

Table 4 shows an update on recognized components of known families of pathogenesis-related proteins, in strawberry. As shown, members of almost all known plant PR families, have been reported in strawberry. However, the implication in strawberry defence have been mostly inferred from their induction pattern after pathogen attack, and the exact functional role remains to be yet set or experimentally proven for the majority of them.

Table 4. Strawberry Pathogenesis Related Proteins.

PR-protein Family [1]	Family Properties	Strawberry gene ID	Standarized gene name [2]	Accession number [3]	Characterization		References
					Protein activity	Gene expression regulation	
PR1	Unknown	FaPR1	-	(DV440399)	*no avaiable data (n.a.d.)*	*Upregulated by aplication of UV-C treatment. Related tc Botrytis resistance.*	Pombo et al. 2011b
PR2	β-1, 3-Glucanase	FaBG2-1	-	(AY170375)	*n.a.d.*	*Upregulated upon Colletotrichum fragariae or Colletotrichum acutatum infection, and by UV-C treatment.*	Khan et al. 2003 Pombo et al. 2011b
		FaBG2-3	-	(AY989819)			Shi et al. 2006 Pombo et al. 2011b
		FaBG2-2	-	(AY989818)		*n.a.d.*	Shi et al. 2006 Pombo et al. 2011b
		Fagln1	-	(AJ871767)		*Reduced level of transcripts after fruit infection by Colletotrichum acutatum.*	Casado-Díaz et al. 2006
PR3	Chitinase type I, II, IV, V, VI, VII	Fachit-1	FaCHI1-1	(AJ871765)	*n.a.d.*	*Downregulated on Colletotrichum acutatum infected fruits.*	Casado-Díaz et al. 2006
		FaChi2-1	FaCHI2-1	(AF147091)		*Induced upon Colletotrichum fragariae, Colletotrichum acutatum or Botrytis cinerea infection. FaChi2-2 increased in UV-C treated fruit immediately after the treatment.*	Khan and Shih 2004 Mehli et al. 2005 Zhang 2009 Pombo et al. 2011b
		FaChi2-2	FaCHI2-2	(AF320111)			
		class IV chitinase	FaCHIT4-1	(TA9333_57918)		*Upregulated in red and white strawberry fruits 24 h after Colletotrichum acutatum infection.*	Guidarelli et al. 2011
		M16D12	FaCHI4-2	(JN415653)		*Strong upregulation under biotic (Coletotrichum acutatum) and abiotic (JA) stress.*	umpublished results
PR5	Thaumatin-like	FaOLP1	FaOLP-1	(AAF13707)	*n.a.d.*	*Induction triggered by Colletotrichum fragariae and Colletotrichum acutatum.*	Wu et al. 2001 Zhang 2006
		FaOLP2	FaOLP-2	(DQ325524)		*Expressed at different levels in leaves, crowns, roots, green fruits and ripe red fruits. Upregulated by the signal molecules ABA, SA and by mechanical wounding, more prominently induced by salicylic acid. Non regulated by aplication of UV-C treatment.*	Zhang and Shih 2007 Pombo et al. 2011b
		PR5	FaPR5-3	(EU289405)		*Constitutive higher gene expression in strawberry trangenic (FaPE1) lines more resistant to Botrytis cinerea.*	Osorio et al. 2008
		Fapr5-1	FaPR5-1	(AJ871764)		*Upregulated in fruit and crown tisues by Colletotrichum acutatum infection.*	Casado-Díaz et al. 2006
		Fapr5-2	FaPR5-2	(AJ871763)		*Upregulated in fruits by Colletotrichum acutatum infection.*	Casado-Díaz et al. 2006
PR6	Proteinase-inhibitor	FaCPI-1	-	(AJ862660)	*Recombinant FaCPI-1 protein expressed in E. coli was a good inhibitor of papain and other cysteine proteinases and showed in vitro antifungal activity against Botrytis cinerea and Fusarium oxysporum*	*Expressed in fully expanded leaves, in roots and in achenes, but surprisingly not in the receptacle (pseudocarp) during fruit development.*	Martinez et al. 2005
PR8	Chitinase type III	FaChi3-1	FaCHI3-1	(AF134347)	*n.a.d.*	*Expressed constitutively at low levels in strawberry leaves, and increased expression in UV-C treated fruit immediately after the treatment.*	Khan et al. 1999 Khan 2002 Pombo et al. 2011b
PR9	Peroxidase	Faprox-1	FaPOX-1	(AJ871771)	*n.a.d.*	*Downregulated on Colletotrichum acutatum infected fruits.*	Casado-Díaz et al. 2006
		362ACC04	FaPOX-2	(AJ871760)		*Represented in mock portion of a sustractive strawberry (Fa cv. Andana) mock-Colletotrichum infected library.*	Casado-Díaz et al. 2006
		peroxidase	FaPOX-3	(DV439771)		*Increase expression in white fruit Fa cv. Alba upon Colletotrichum acutatum atack.*	Guidarelli et al. 2011

Table 4. Continued.

PR-protein Family [1]	Family Properties	Strawberry gene ID	Standarized gene name [2]	Accession number [3]	Characterization		References
					Protein activity	Gene expression regulation	
PR10	Ribonuclease-like	Fra a 1a	FaPR10-1.1	(DQ385511), (AM084674), (Q3T923)	*Demonstrated allergenic properties. Essential biological function in pigment formation in strawberry fruit (might be involved in processes leading to the formation and/or the accumulation of anthocyanins).*	*Fra a 1 protein is more than seven-fold more abundant in the red compared to white strawberry varieties. Fra a 1e mainly expressed in roots and the transcript levels decrease from the open flower stage to the ripe fruit. More transcipt level of an Fra a 1E gene in white non matured fruit when compared with red ripe fruit of Fxa cv. Camarosa and in cv. Alba where is also upregulated in red strawberry fruits 24 h after Colletotrichum acutatum infection.*	Karlsson et al. 2004 Hjernø et al. 2006 Musidlowska-Persson et al. 2007 Muñoz et al. 2010 Guidarelli et al. 2011
		Fra a 1b	FaPR10-1.2	(AM236313), (AM236314), (AM236315), (Q256S7)			
		Fra a 1c	FaPR10-1.3	(AM236317), (AM236318), (Q256S6)			
		Fra a 1d	FaPR10-1.4	(AM236316), (Q256S2)			
		Fra a 1e	FaPR10-1.5	(AM236319), (AM236320), (TA487_3747), (Q256S4)			
		DY673343	FaPR10-1.6	(DY673343)			
		Fra a 2	FaPR10-2	(GQ148818)	*An essential biological function in pigment formation in strawberry fruit (might be involved in processes leading to the formation and/or the accumulation of anthocyanins).*	*Highly transcribed in the late stages of fruit ripening, might be a hyperallergenic isoform.*	Muñoz et al. 2010
		Fra a 3	FaPR10-3	(GQ148819), (EU289406)		*Uniform expression pattern between strawberry tisues. Substantially higher (two- to five-fold) in ripe fruits of the white-fruited F. chiloensis cultivar than in the red variety. Similarly, in F. vesca mRNA level higher in white than in red fruits. Gene expression non afected in strawberry trangenic (FaPE1) lines more resistant to Botrytis cinerea.*	Osorio et al. 2008 Muñoz et al. 2010
		M23D11	FaPR10-4	(JN415652)	*n.a.d.*	*Strong upregulation under biotic (Coletotrichum acutatum) and abiotic (JA) stress.*	umpublished results
		EX672442	FaPR10-5	(EX672442)		*Increased of expression in infected red and white fruits of Fa cv. Alba by Colletotrichum acutatum.*	Guidarelli et al. 2011
		DY671909	FaPR10-6	(DY671909)			
		DY676200	FaPR10-7	(DY676200)			
		TA11697_57918	FaPR10-8	(TA11697_57918)			
PR13	Thionin	Faythio-1	FaTHIO-1	(AJ871768)	*n.a.d.*	*Downregulated on Colletotrichum acutatum infected fruits.*	Casado-Díaz et al. 2006
PR14	Lipid-transfer protein	LTP1	FaLTP-1.1	(DQ366727)	*Alergenic properties. Induced histamine release at a 100-fold higher concentration than peach LTP. Has less allergenic potency than peach and apple LTP, and therefore is an interesting tool for future immunotherapy.*	*Expression of LTP genes was observed in white and ripe fruit (including seeds) and leaves of strawberry cultivar Elsanta. Responds to abiotic treatments such as ABA and SA, but not to salt and heat stresses, also reported that the expression of the Fxaltp gene is stimulated by wounding and repressed by cold stress, and negatively regulated in strawberry crown tissue infected by Colletotrichum acutatum.*	Yubero-Serrano et al. 2003 Zuidmeer et al. 2006
		LTP2	FaLTP-1.2	(DQ366728)			
		LTP3	FaLTP-1.3	(DQ366729)			
		LTP4	FaLTP-1.4	(DQ366730)			
		LTP5	FaLTP-1.5	(DQ366731)			
		Fxaltp	FaLTP-1.6	(AJ315844)			
		LTP6	FaLTP-2	(DQ366732)			
		LTP	FaLTP-3	(TA11085_57918)	*n.a.d.*	*More highly expressed in white fruits than in red ones in cv. Alba.*	Guidarelli et al. 2011

[1] Only PR families with recognized members in strawberry are shown.

[2] A recomendation of standarized gene name is sugested for some of the strawberry genes (i.e. **FaCHI2-1.1**: **Fa** (species), **CHI** (gene type), **2** (class of gene type), **-1** (order it was discovered), **.1** (alele)).

[3] Institute for Genomic Reasearch (TIGR) and National Center for Biotechnology Information (NCBI) codes of transcript sequences.

Glucanases and chitinases conform the most abundant classes of strawberry PR genes with hydrolytic activity identified so far. Thus, three strawberry members (FaBG2-1, FaBG2-2, FaBG2-3) of class II of β-1,3-glucanase of plant PR2 family, have been cloned and partially characterized (Khan et al. 2003, Shi 2005, Shi et al. 2006). Genes FaBG2-1, and FaBG2-3 were shown to be induced after strawberry leaves infection with either *C. fragariae* or *C. acutatum*. A higher level of induction was detected when the former pathogen was tested (Shi et al. 2006). Previous studies have also shown that a gradual increase of total β-1, 3-glucanase activity occurred in strawberry from 2 h to 48 h post-infection in response to either of the two fungi (Shi 2005). Similarly, a gradual increase in total chitinase activity during the first 24 hours post-infection was also detected in strawberry challenged with either of these two pathogens. In addition, the overall chitinase activity was also induced to a significant level when strawberry plants were injured or treated with either salicylic acid or ethephon (Khan 2002). These results highlight the importance of chitinases in strawberry in response to both biotic and abiotic stresses. So far, three strawberry chitinase genes have been cloned, a class III chitinase from PR8 family (FaChit3-1) (Khan et al. 1999), and two class II chitinases from PR3 family (FaChi2-1, FaChi2-2) (Khan and Shih 2004). Similarly to the FaBG2-1 and FaBG2-3 genes, the FaChi2-1 and FaChi2-2 genes were induced upon *C. fragariae* or *C. acutatum* infection within 2–6 h or 24–48 h post-inoculation, respectively (Khan and Shih 2004). More recently, the cloning and sequencing of two FaChi2-1 alleles from Toyonaka and Akihime strawberry cultivars has also been reported, but no other information than sequence comparison with pea (L37876), kentucky bluegrass (AF000966), pepper (AY775335), parsley (AF141372), norway spruce (AY544781) and muskmelon (AF241538) orthologous genes is described (Zhang et al. 2009).

Two strawberry osmotin-like coding genes, FaOLP1 and FaOLP2, belonging to plant PR5 family have been cloned (Wu et al. 2001, Zhang and Shih 2007). The expression of FaOLP1 has been examined upon fungal infection (Zhang 2006). Thus, both *C. fragariae* and *C. acutatum*, triggered a substantial induction of FaOLP1 in strawberry leaves at 24-48 h post-inoculation, suggesting the

involvement of FaOLP1 in strawberry defence against these fungi. The spatial expression pattern of FaOLP2 has also been studied in strawberry plant (Zhang and Shih 2007). Thus, high level of FaOLP2 transcripts was detected in crown and leaf while relatively low level was detected in root and ripe red fruit, and very low level in green fruit. Interestingly, FaOLP2 was up-regulated by ABA, SA, and mechanical wounding within 2–6 h post-treatment, and was more prominently induced by SA than by the other abiotic stimuli, indicating that this strawberry gene responds to abiotic stresses (Zhang and Shih 2007). Surprisingly, no expression studies aimed to support the implication of this strawberry FaOLP2 gene in response to biotic stresses have been published yet.

A strawberry member of the PR6 family has been cloned and characterized (Martinez et al. 2005). This strawberry Cyf1 gene (FaCPI-1 gene) encodes a phytocystatin, a protein with proteinase inhibitor activity. Plant phytocystatins have been implicated in the endogenous regulation of protein turnover (Arai et al. 2002, Corre-Menguy et al. 2002), programmed cell death (Solomon et al. 1999, Belenghi et al. 2003), and also, in defence mechanisms against insects and pathogens (Vain et al. 1998, Gutierrez-Campos et al. 1999). It has been speculated that alterations in the fungal membrane permeability could be the origin of the antifungal properties on this family of plant defence proteins (Giudici et al. 2000, van der Vyver et al. 2003). Curiously, the strawberry Cyf1 gene was originally obtained from a developing fruit of *F.* x *ananassa* cv. Elsanta (Martinez et al. 2005). Northern blot and *in situ* hybridization analyses indicated that the Cyf1 gene is expressed in fully expanded leaves, in roots and in achenes, but surprisingly not in the receptacle (pseudocarp) during fruit development. However, the recombinant FaCPI-1 protein expressed in *E. coli* was a good inhibitor of papain and other cysteine proteinases and showed *in vitro* antifungal activity against *B. cinerea* and *Fusarium oxysporum*. Previous studies have shown that the ectopic expression of a peptidase inhibitor from cowpea (CpTi, cowpea trypsin inhibitor) in strawberry was effective against insects (Graham et al. 1997, Graham et al. 2002). Therefore, the inhibitory properties shown by the strawberry

FaCPI-1 protein highlight the importance of this endogenous FaCyf1 gene as a valuable tool for fungal strawberry diseases control.

Members of PR10 family have also been described in strawberry. Thus, seven strawberry genes homologous to proteins from PR10 group, called Fra a 1 (five isoforms: a to e), Fra a 2, and Fra a 3, have been reported (Hjernø et al. 2006, Musidlowska-Persson et al. 2007, Muñoz et al. 2010). Apart from their known allergenic properties (Karlsson et al. 2004, Musidlowska-Persson et al. 2007), an essential biological function in pigment formation in strawberry fruit has been recently proposed for some member of this strawberry family (Muñoz et al. 2010). By transient expression analysis in strawberry fruit, Muñoz et al. (2010) directly linked genes Fra a 1e, Fra a 2, and Fra a 3 to flavonoid biosynthesis. It was also suggested that these genes could function either as carriers of flavonoid pathway intermediates or as (co-) transporters of anthocyanins into the plant vacuole. However, more recently some Fra a alleles have also been shown to be induced in strawberry plant upon pathogen attack. Thus, Fra a 1 (gene DY673343) and Fra a 1E (gene TA487_3747) were up-regulated in red ripe fruit of *F.* x *ananassa* cv. Alba 24 h after *C. acutatum* infection (Guidarelli et al. 2011) (see also below). Also a new member of PR10 family (FaPR10-4) strongly upregulated under biotic (*C. acutatum*) and abiotic (jasmonic acid) stress have been cloned from strawberry crown tissue and is currently being characterized (J.L. Caballero, personal communication).

Yubero-Serrano et al. (2003) described the cloning and characterization of a strawberry Fxaltp gene (PR14 family), which responds to abiotic treatments such as ABA and SA, but not to salt and heat stresses. It was also reported that the expression of the Fxaltp gene is stimulated by wounding and repressed by cold stress. The Fxaltp gene showed a tissue dependent regulatory mechanism, and responded differently to these abiotic treatments in fruit and leaves, highlighting the importance of the spatial expression studies to fully understand the role of this and other strawberry genes in defence. The Fxaltp gene, now renamed FaLTP1.6 gene (J.L. Caballero, personal communication), belongs to type 1 of extracellular

plant nsLTPs. Curiously, allergenic properties have been also proved to this class of strawberry genes (Zuidmeer et al. 2006). Thus, FaLTP1 (alleles LTP1 to 5 and Fxaltp1) and FaLTP2 (allele LTP6) proteins induced histamine release at a 100-fold higher concentration than peach LTP, and have less allergenic potency than peach and apple LTP, therefore are proposed as an interesting tool for future immunotherapy. A wide range of extracellular roles has been suggested for members of this family of plant proteins, including a specific defensive function against bacterial and fungal pathogens (García-Olmedo et al. 1995, Molina et al. 1996, Kirubakaran et al. 2008, Sarowar et al. 2009), as well as a putative role in the early recognition of plant intruders and in systemic resistance signalling (Buhot et al. 2001, Blein et al. 2002, Maldonado et al. 2002, Sarowar et al. 2009). However, the exact *in vivo* role remains unclear for most of them. Interestingly, the Faltp1 gene is negatively regulated in strawberry crown tissue infected by *C. acutatum* (J.L. Caballero personal communication).

In a recent study, Pombo et al. (2011) directly related the enhancement of gene expression and enzymatic activity of a set of strawberry genes with the increase of strawberry resistance against *B. cinerea*. They studied the effect of UV-C treatment on the growth of *B. cinerea* during strawberry fruit postharvest decay and analysed the activity of enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POX) and β-1,3-glucanase, and as well as the level of gene expression of FaPAL6 (Pombo et al. 2011a) and PR genes such as FaChi2-2, FaChi3, FaBG2-1, FaBG2-3, and FaPR1 (Tables 4 and 5). An improvement in fruit resistance against this pathogen was observed in collected fruit after this physical treatment. In addition, except for genes FaChi2-1 and FaOLP2, both the expression level and the enzymatic activity increased for all these genes and enzymes, supporting a defensive role of all of them against this fungal pathogen.

A cell wall-related strawberry (*F.* x *ananassa*) fruit gene coding for a polygalacturonase inhibiting protein (FaPGIP) has been cloned and described to play a role in strawberry defence (Mehli et al. 2004). It is known that PGIPs are

bound by ionic interactions to the extracellular matrix of plant cells (Shanmugam 2005). These plant proteins display leucine rich repeat (LRR) domains and have a high affinity for fungal endopolygalacturonases (PGs), which are important pathogenicity factors (O'Connell et al. 1990). In fact, PGs are among the first enzymes secreted by *B. cinerea* upon infection (van der Cruyssen et al. 1994, Rha et al. 2001). Seven different variants of FaPGIP from five strawberry cultivars (Elsanta, Korona, Polka, Senga sengana, Tenira) were identified, and divided into three major groups (FaPGIP1a, FaPGIP1b, FaPGIP1c, FaPGIP2a, FaPGIP2b, FaPGIP2c, and FaPGIP3) (Mehli et al. 2004, Schaart et al. 2005, Table 5). After fruit inoculation with *B. cinerea*, all five strawberry cultivars studied displayed a significant induction of the overall FaPGIP gene expression.

Specific analysis showed that all the FaPGIP variants studies were upregulated when white-stage fruits were inoculated with the pathogen. In addition, by using either of the two FaPGIP allelic sequences FaPGIP1a or FaPGIP2a, these authors produced genetically modified strawberry lines with expression of this FaPGIP gene regulated by the strong and constitutive *CaMV 35S* promoter (Schaart 2004). The strawberry transgenic lines expressed a less susceptible phenotype against *B. cinerea* than the control one not transformed. These results strongly support a defensive role of this strawberry FaPGIP gene. According to other plant PGIPs, the strawberry FaPGIP gene also showed spatial and fruit developmental regulation. Curiously, in crops as pear (Abu-Goukh et al. 1983), raspberry (Johnston et al. 1993), apple (Yao et al. 1999), and cantaloupe (Fish and Davis 2004), the PGIP gene expression is higher in immature than in mature fruit but the opposite is true for strawberry where FaPGIP gene presents the highest level of expression in healthy mature fruit (Mehli et al. 2004). This fact may reflect a strawberry plant specific strategy focused to enhance fruit protection during the most helpless and soft stages.

Table 5. Other Strawberry Defense-Related Proteins.

Protein Family	Strawberry gene ID	Standarized gene name [1]	Accession number [2]	Characterization		References
				Protein activity	Gene expression regulation	
Phenylalanine ammonia-lyase	FaPAL1	FaPAL-1.1	(AB360390)	*no avaiable data (n.a.d.)*	*n.a.d.*	umpublished (GenBank sequences)
	FaPAL3	FaPAL-1.2	(AB360392)			
	FaPAL4	FaPAL-1.3	(AB360393)			
	FaPAL5	FaPAL-1.4	(AB360394)			
	FaPAL2	FaPAL-2.1	(AB360391)			
	FaPAL6	FaPAL-2.2	(AJ871757) (HM641823)	*Correlation between gene expression and accumulation of higher anthocyanin amount in Camarosa cultivar.*	*Represented in infected portion of a sustractive strawberry (Fa cv. Andana) mock-Colletotrichum infected library. Upregulated by aplication of UV-C treatment.*	Casado-Díaz et al. 2006 Pombo et al. 2011a, 2011b
Polygalacturonase-inhibiting protein	FaPGIP1.a	FaPGIP-1.1		*genetically modified strawberry overexpressing FaPGIP which is less susceptible to grey mould.*	*Mature fruit showed the highest constitutive gene expression levels (the gene is developmentally regulated). After inoculation with B. cinerea, all five cultivars studied ('Elsanta', 'Korona', 'Poika', 'Senga sengana', 'Tenira') displayed a significant induction of gene expression, this upregulation was accompanied by a significant change in FaPGIP allele frequencies when compared with non-treated fruits. Gene expression non afected in strawberry trangenic (FaPE1) lines more resistant to Botrytis cinerea.*	Mehli et al. 2004, 2005 Schaart 2004 Schaart et al. 2005 Osorio et al. 2008
	FaPGIP1.b	FaPGIP-1.2				
	FaPGIP1.c	FaPGIP-1.3				
	FaPGIP2.a	FaPGIP-1.4				
	FaPGIP2.b	FaPGIP-1.5				
	FaPGIP2.c	FaPGIP-1.6				
	FaPGIP3	FaPGIP-1.7				
	FaPGIP	FaPGIP-1.8	(EU117213)	*n.a.d.*	*n.a.d.*	Zhang et al. 2010
	FrveA7 PGIP	FaPGIP-1.9	(AF196892)	*n.a.d.*	*n.a.d.*	Osorio et al. 2008
	FrveA6 PGIP		(AF196891)	*n.a.d.*	*n.a.d.*	Mehli et al. 2004
	PGIP	FaPGIP-1.10	(AY534684)	*n.a.d.*	*upregulated in red and white strawberry fruits 24 h after Colletotrichum acutatum infection*	Guidarelli et al. 2011
	ABV04088	FaPGIP-1.11	(ABV04088)	n.a.d.	n.a.d.	Zhang et al. 2010b
	gene20317	FvPGIP-2				Strawberry genome release Shulaev et al. 2011
WRKY transcription factor	FaWRKY1	-	(AJ871772)	*FaWRKY1 can play a role as important element mediating defence response to C. acutatum in strawberry.*	*Induced by Colletotrichum acutatum in fruit and crown tissues from very susceptible (cv. Camarosa) and moderately susceptible (cv. Andana) cultivars, treatments with elicitors, and wounding.*	Casado-Díaz et al. 2006
Hypersensitive-induced response protein	Fahir-1	-	(AJ871769)	*n.a.d.*	*Induced by Colletotrichum acutatum in fruit and crown tissues from very susceptible (cv. Camarosa) and moderately susceptible (cv. Andana) cultivars.*	
LRR receptor-like proteins	Falrrk-1	-	(AJ871784)	*n.a.d.*	*Down regulated in fruits and crown tisues infected by C. acutatum.*	
	Falrrk-2	-	(AJ871783)		*Down regulated in infected fruits by C. acutatum.*	
xyloglucanase-inhibiting protein	TA10709_57918	-	(TA10709_57918)	*n.a.d.*	*Significantly upreguiated after inoculation with C. acutatum in both white and red fruit stages.*	Guidarelli et al. 2011
cytochrome p450 monoxygenases	TA9078_57918	-	(TA9078_57918)			
aldehyde dehydrogenase	TA12321_57918	-	(TA12321_57918)			
flavonol synthase	TA9432_57918	-	(TA9432_57918)			
tropine reductase	DY673561	-	(DY673561)			
alpha/beta amyrin synthase	TA11548_57918	-	(TA11548_57918)			
3-hydroxy-3-methylglutaryl(HMG) coenzyme-A synthase	CO381295	-	(CO381295)			
lectin family	TA10594_57918	-	(TA10594_57918)		*Enhanced expression in white stage fruit upon C. acutatum infection.*	
glutatione S-transferase	CO79212	-	(CO79212)		*Up-reguiated in red challenged fruits with C. acutatum.*	
snaking-1	CO378568	-	(CO378568)			

[1] A recomendation of standarized gene name is sugested for some of the strawberry genes (i.e. **FaCHI2-1.1**: **Fa** (species), **CHI** (gene type), **2** (class of gene type), **-1** (order it was discovered), **.1** (alele)).

[2] Institute for Genomic Reasearch (TIGR) and National Center for Biotechnology Information (NCBI) codes of transcript sequences.

Hormonal and signalling pathways involved in the strawberry defence response

Molecules such as salicylic acid (SA), jasmonate (JA) and ethylene (ET) are well known as plant response regulators of biotic stresses. SA-dependent signalling pathway is critical in establishing the hypersensitive response HR and the systemic pathogen resistance, and prevent progression of pathogens mainly with biotrophic and hemibiotrophic lifestyles, while JA- and ET-dependent signalling pathways are mainly induced in response to necrotrophic pathogens, mechanical wounding and herbivore predation (Glazebrook 2005). Abscisic acid, auxin, gibberellic acid (GA), cytokinin, brassinosteroids (BR), and peptide hormones are also part of the hormonal arsenal used by plants in defence signalling pathways (Bari and Jones 2009). Extensive crosstalk between these hormone-dependent signalling pathways fine tune regulates the plant defence response.

Similar to other plants, SA seems to work as a defence inducer in strawberry. Treatments of strawberry plants with benzothiadiazole S-methyl ester (BTH), a SA analogue, highly increased the concentration of SA in leaves (Hukkanen et al. 2007). Strawberry plants treated with this hormonal compound improved the postharvest quality of fruit (Babalar et al. 2007, Cao et al. 2010b, Shafiee et al. 2010), and exhibited changes in chemical composition, mainly phenolic compounds such as ellagitannins (Cao et al. 2010a, Cao et al. 2011), enhancing total antioxidant capacity of the fruit (Asghari and Babalar 2009) and the level of expression of specific genes related with defence, which lead to a reduction in microbial population (Zhang and Shih 2007, Hukkanen et al. 2007, Encinas-Villarejo et al. 2009, Cao et al. 2010b). Exogenous application of SA at non-toxic concentration to strawberry fruits also enhanced resistance to pathogens as *B. cinerea*, and effectively reduced fungal decay (Asghari and Aghdam 2010, Babalar et al. 2007).

Also methyl jasmonate increases the level of phenolic compounds such as chlorogenic acid and rutin, and induces strawberry resistance to two-spotted spider mite (*T. urticae* Koch) (Warabieda et al. 2005).

It has been described that repression of auxin responsive genes is part of the SA-mediated disease-resistance mechanism (Wang et al. 2007). In strawberry, auxins have mainly been implicated in developmental processes, acting as key regulators for growth and fruit ripening (Aharoni et al. 2002a, Mezzetti et al. 2004). However, recent evidences by Osorio et al. (2011) also associate auxins to plant defence response in strawberry. Thus, resistance of *F. vesca* transgenic FaPE1 lines to *B. cinerea* was correlated to a significant decrease in the auxins content as well as an enhanced expression of some auxin-repressed genes in transgenic fruit.

Ethylene has been considered a ripening hormone in other plants but the role of ethylene in strawberry fruit ripening has been considered as negligible, and strawberry is considered a non-climacteric fruit. However, it has been reported that the achenes of red strawberry fruit produce ethylene at low concentrations (Iannetta et al. 2006). Interestingly, SA treated strawberries effectively reduced fruit ethylene production (Babalar et al. 2007), a physiological mechanisms resembling that of auxin genes.

Positive or negative cross talk between SA and JA/ET signalling pathways is dependent on the specific pathogen, and protein factors such as NPR1 (non expressor of PR1) or WRKY play important roles in this antagonistic interaction (Spoel et al. 2007). Thus, WRKY70 proteins have been shown to act as a positive regulator of SA-dependent defences and a negative regulator of JA-dependent defences (Li et al. 2004). Recently, two *F.* x *ananassa* WRKY70 gene analogues has been cloned (J.L. Caballero, unpublished). Preliminary expression analyses indicate that both strawberry genes are induced in plants cv. Andana infected with *C. acutatum*, and also respond to SA treatments, and suggest that these FaWRKY70 genes may take part of the SA signalling network of strawberry defence. Also, another strawberry FaWRKY707 gene is strongly induced on *C. acutatum* infected fruits (J.L. Caballero, unpublished). FaWRKY707 presents high similarity to AtWRKY33, which is rapidly and strongly induced by fungal and bacterial PAMPs in Arabidopsis (Lippok et al. 2007), and acts as a positive

regulator of JA- and ET-mediated defence signalling but as a negative regulator of SA-mediated responses (Zheng et al. 2006). The identification of these WRKY orthologous factors in strawberry indicates that key regulatory members of defence mechanisms are also presents in strawberry, and suggest that antagonistic relationship between the known plant defence-related signalling pathways might also be working in strawberry in response to pathogens, but this needs to be further analysed.

Emerging evidence suggests that gibberellin (GA) signalling components play major roles in control plant immune responses (i.e. by modulating SA and JA dependent defence responses (Navarro et al. 2008, Tanaka et al. 2006)). In addition, brassinosteroids (BRs), which are plant hormones structurally related to the animal steroid hormones (Bajguz 2007), enhances resistance to pathogens in tobacco, rice (Nakashita et al. 2003), tomato and potato (Krishna 2003), and may be in cross-talk with other hormone signalling in mediating defence responses in plants as ABA and ET (Krishna 2003). Although some of the genes involved in hormonal regulated processes of gibberellin, auxin, ethylene and brassinosteroid signalling have been reported in strawberry (Bombarely et al. 2010, Csukasi et al. 2011), no detailed information is available to date about their putative implication in the strawberry plant defence response.

Transcriptomic approaches for defence-related gene discovery in strawberry

So far, few studies in strawberry have been published focused to pursue high throughput gene discovery related with the mechanism of defence. Casado et al. (2006) reported the first study aimed to identify strawberry genes with altered expression in response to *C. acutatum* infection. Using a subtractive hybridisation approach, a large number of strawberry genes involved in signalling, transcriptional control, defence, and many genes with unknown function were isolated. Spatial and temporal gene expression profiles after *C. acutatum* infection yielded a first insight on some of the genes responding to this pathogen, and showed that the strawberry response was dependant on the tissue and cultivar analysed. Thus, strawberry genes belonging to PR5 (*Falpr5*-1 and *Falpr5*-2, two

thaumatin-like proteins) and PR10 (Falpr10-1, a ribonuclease-like gene) families as well as genes *Fahir*-1 (encoding a hypersensitive-induced response protein) and *Fawrky1* (encoding a protein with similarity to WRKY transcription factors) were found to be induced in fruit and crown tissues from very susceptible (cv. Camarosa) and moderately susceptible (cv. Andana) cultivars, but their expression pattern was found to be different between both cultivars, being either stronger and/or quicker in the less susceptible one. Interestingly, strawberry members of PR2 (Fagln-1, a β-1,3-glucanase), PR3 (Fachit-1, a class 1 chitinase), PR9 (Faprox-1, a peroxidase), and PR13 (Faγthio-1, a γ-thionin) families, as well as genes Falrrk-1 and Falrrk-2, encoding two LRR receptor-like proteins, were clearly down regulated in infected fruits. Genes Fachit-1 and Falrrk-1 were also significantly inhibited in cv. Camarosa infected crown tissues. Chitinases and related β-glucanases are known to be rapidly induced in plant upon pathogen infection or treatment with elicitors (Leubner-Metzger and Meins 1999, Khan et al. 2003, Shi et al. 2006, Khan and Shih 2004, Mehli et al. 2005, Zhang et al. 2009, Pombo et al. 2011b), and downregulation of β-1,3-glucanase genes has only been reported for tobacco (class I) genes by treatment with abscisic acid (Leubner-Metzger et al. 1995, Rezzonico et al. 1998) and by combination of auxin and cytokinin (Vögeli-Lange et al. 1994) (a wider dynamic range of gene expression information can be obtained in Casado et al. (2006)). Thus, the results described by Casado et al. suggest that *C. acutatum* progression can be dependent upon a reduction of the active defences of strawberry, and highlight the importance of further studies on these strawberry genes to fully understand the process of infection and strawberry plant defence against this pathogen.

Recently, the strawberry Fawrky1 gene has been further characterized (Encinas-Villarejo et al. 2009). The Fawrky1 gene is up-regulated in strawberry following *C. acutatum* infection, treatments with elicitors, and wounding. A Fawrky1 full-length cDNA was cloned which encodes a IIc WRKY transcription factor (FaWRKY1). The ectopic expression of FaWRKY1 in Arabidopsis mutants in its orthologous gene Atwrky75 has provided some positive clues of its function in plant defence. Thus, the overexpression of this strawberry gene in Atwrky75

mutants and wild type reverted the enhanced susceptibility, and even increased resistance to avirulent strains of *Pseudomonas syringae*, demonstrating an active role of this FaWRKY1 protein in the activation of basal and R-mediated resistance in Arabidopsis. Further experimental results provided by these authors strongly suggest that FaWRKY1 can play a role as important element mediating defence response to *C. acutatum* in strawberry (Encinas-Villarejo et al. 2009). Currently, new experiments to unravel the exact function of this FaWRKY1 gene are in progress (J.L. Caballero, unpublished).

Very recently, Guidarelli et al. (2011) have performed microarrays analysis of white and red fruit strawberries after 24 h of their interaction with *C. acutatum*. These authors have provided new data of strawberry genes regulated upon *C. acutatum* infection. Thus, a DNA microarray of more than 93300 oligo-probes was produced using ESTs from TIGR Plant Transcript Asemblies database (http://plantta.jvci.org/) (4197 of *F.* x *ananassa*, release 2; 13366 of *Fragaria vesca*, release 3; 124 of *Malus domestica*, release 2). Many genes encoding for PR proteins were found to be upregulated in both white and red infected fruit upon infection. Thus, genes coding for a xyloglucanase-inhibiting protein (gene TA10709_57918), for several isoforms of the PR-10 proteins family (genes TA11697_57918, EX672442, DY671909, and DY676200), as well as for cytochrome p450 monoxygenases (gene TA9078_57918), which are known to play important roles in plant detoxification pathways, were induced. In addition, several metabolism genes coding for toxic aldehydes scavengers, such as an aldehyde dehydrogenase (ALDH) (gene TA12321_57918), for enzymes involved in the synthesis of stress-related flavonol and alkaloid compounds, such as the flavonol synthase (gene TA9432_57918) and the tropine reductase (gene DY673561), respectively, and for enzymes involved in the biosynthesis of terpenoids defence compounds, such as the alpha/beta amyrin synthase (gene TA11548_57918) and the 3-hydroxy-3-methylglutaryl(HMG)coenzyme-A synthase (gene CO381295), were also found to be significantly upregulated after inoculation with the pathogen in both white and red fruit stages. The expression of many other strawberry genes related with biotic stress defence was increased only

in one of the two fruit stages, and so the transcript level of genes coding for a peroxidase (PR-9 family, gene DV439771), and a member of the lectin family (gene TA10594_57918), enhanced in white stage fruit whereas genes coding for Fra a protein isoforms (PR10 family, genes DY673343 and TA487_3747), a glutathione *S*-transferase (gene CO79212), a snaking-1 a polygalacturonase-inhibiting protein (gene AY534684), and a class IV chitinase (PR-3 family, gene TA9333_57918) were up-regulated in red challenged fruits (see Guidarelli et al. (2011) for a more extensive list of differentially regulated strawberry genes).

Regardless of the availability of transcriptomic information from the interaction strawberry plant-*C. acutatum*, to date, no direct evidence the strawberry plant defence response nor functional gene characterization has been reported for the majority of the identified genes.

Conclusions and Perspectives

Despite the worldwide importance of strawberry and the lack of fully resistant cultivars to any disease in this crop, the molecular mechanism and components of the defence signalling pathways exhibited by this plant to face a diverse array of pathogen attack strategies is yet scarce and very poorly understood. In response to both biotic and some forms of abiotic stress, it is clear that strawberry can exhibit similar molecular mechanisms reported in other higher plants. Thus, strawberry is able to activate primary (PTI) and secondary (ETI) defence systems as members of both layers of plant defence have been identified. However, little is yet known about the exact function of these individual components, and many genes and factors still remains undiscovered. In this sense, several authors have directed their efforts in proving the positive effect that the ectopic expression of known plant defence-related genes can have on increasing resistance in strawberry. It can be predicted that a similar counterpart gene either with the same or a similar role in defence could be present in the strawberry genome.

Many examples of strawberry transgene-mediated resistance against pathogens have been reported using the heterologous strategy. Thus, the expression of a

variety of plant chitinases from tomato, rice or bean, the thaumatin II gene from *Thaumatococcus daniellii* Bennett, and a PGIP gene from pear fruit has been shown to reduce the damage caused by some fungal pathogens in strawberry. Also the introduction of a cowpea protease inhibitor gene in strawberry improved protection against herbivores (see Table 6 for details).

New breeding strategies using the ectopic expression of heterologous genes in strawberry can indeed also help to obtain important varieties of this crop with increased resistance but acceptance of a transgenic modification in a fresh fruit for human consumption is far to be achieved. Therefore, the finding of the strawberry orthologous genes, not only will help to unravel the molecular mechanisms underlying the activation of defence responses in this plant but, in addition, a cisgenic approach (Schaart et al. 2004) using these endogenous genes can be a useful tool to obtain strawberry varieties with increased resistance, which can fit the consumer acceptance of a healthy fruit for human consumption.

Furthermore, the identification and characterization of specific and partial resistance traits, as race specific R genes responsible for the monogenic resistance found to *P. fragariae*, *C. acutatum* and *A. alternata* (Denoyes-Rothan and Baudry 1995, van de Weg 1997a, van de Weg 1997b, Takahashi et al. 1997, Denoyes-Rothan et al. 2005), together with studies on identification of genome regions containing sets of genes that control resistance or quantitative trait loci (QTL), which have been undertaken to polygenic quantitative inheritance of resistance (Maclachlan 1978, Barritt 1980, Denoyes-Rothan and Baudry 1995, Shaw et al. 1996, Lewers et al. 2003, Zebrowska et al. 2006), offer promising assistance in conventional breeding programmes searching for disease resistance in this crop, and it has been very recently reviewed by Korbin (2011).

Table 6. Examples of strawberry transgene-mediated resistance.

Gene Family	Transgene ID	Host Plant	Accession Number	Transgene References	Modified Strawberry Cultivar	Defensive Activity in Strawberry	References
chitinase	pcht28	*Lycopersicon chilense*	L19342	Chen et al. 1994	Joliette	*ectopic expression produce higher resistance to Verticillium dahliae as compared to nontransgenic controls*	Chalavi et al. 2003
	RCC2	*Oryza sativa*	X56787	Nishizawa and Hibi 1991 Nishizawa et al. 2003	Toyonoka	*transgenic strawberry showed enhanced resistance against Sphaerotheca humuli causal of powdery mildew*	Asao et al. 1997
	ch5B	*Phaseolus vulgaris*	AAA33756	Broglie et al. 1986, 1989	Pájaro	*high level of resistance to gray mold disease (B. cinerea) displayed by strawberry transgenic lines*	Vellicce et al. 2006
thaumatin II	thau II	*Thaumatococcus daniellii Bennett*	J01209	Edens et al. 1982	Firework	*strawberry plant transformation produced a significantly higher level of resistance against gray mold (B. cinerea)*	Schestibratov and Dolgov 2005
polygalacturonase-inhibiting protein	pPGIP	*Pyrus communis*	L09264	Stotz et al. 1993	Pegasus	*although transgenic tomatoes expressing this PGIP gene were previously proved to control B. cinerea infection, in strawberry a small number of the transgenic lines showed reduced susceptibility to infection by B. cinerea on detached flowers*	Simpson et al. 1999 Powell et al. 2000
protease trypsin inhibitor	CpTi	*Vigna unguiculata*	AJ271752	Lawrence et al. 2001	Melody and Symphony	*overexpressing lines improved protection against feeding by vine weevil larvae (Otiorhynchus sulcatus)*	James et al. 1992 Graham et al. 1995, 1997, 2001, 2002

The strawberry ESTs and microarray data collection already available (Casado-Díaz et al. 2006, Bombarely et al. 2010, Guidarelli et al. 2011) constitutes a valuable information for searching candidate genes involved in strawberry defence. The recent publication of the complete sequence of *F. vesca* genome represents an enormous scientific contribution to this aim (Shulaev et al. 2011). However, progress in the field of basic genomic in the diploid species, *F. vesca*, is still necessary and it is of great interest. Currently, a second generation of "in-house" microarray has been developed using a set of selected strawberry unigenes from the ESTs information provided by Casado et al. (2006), and new transcriptomic analysis are being performed using infected and uninfected crown tissue from *F.* x *ananassa* cultivars with different susceptibility to *C. acutatum* (J.L. Caballero, unpublished). Certainly, the strawberry transcriptomic approaches will be benefited from the *F. vesca* genome information as improved DNA chips, containing high-density arrays of short synthetic oligonucleotides, can be developed and used as powerful tool to identify novel defence genes.

Proteomic and metabolomic approaches offer complementary methodologies that need to be addressed in strawberry in helping to understand the molecular mechanisms underlying the defence response of this plant. In this sense, non-targeted analysis of metabolite composition in strawberry has been recently improved (Hanhineva et al. 2008), but the application of metabolomic technologies to obtain a description in chemical defences deployed by this plant against pathogens needs to be further implemented. Indeed, only analysis of particular groups of secondary metabolites has been reported for each individual case of study (Hanhineva et al. 2010).

Combined results produced by the application in strawberry of these high-throughput technologies will also yield new insights on the role played by genes and compounds in strawberry plant defence, and this approach should be further explored. Indeed, very recently, analyses of metabolic and transcriptional changes in the receptacle of FaPE1 transgenic *F. vesca* fruits have provided new relevant information of the molecular changes associated with the resistance to this

pathogen (Osorio et al. 2011). *F. vesca* transgenic lines overexpressing the FaPE1 gene, a *F.* x *ananassa* gene encoding a pectin methyl esterase related with the making of the architecture of the strawberry plant cell wall, were previously shown to have increased resistance to *B. cinerea* (Osorio et al. 2008). The transcriptomic and metabolomic analyses of the ripe receptacle of these transgenic lines have showed an increased expression of genes related to plant defence such as genes encoding PR10 proteins, WRKY transcription factor, and metallothioneins, which was in parallel with the channelling of metabolites to aspartate and aromatic amino acids as well as phenolics, flavanones, and sequiterpenoids (see Osorio et al. (2011) for a more detailed description of genes and compounds). Taken together these results, a wider overview of changes in metabolites and transcripts is obtained, helping to assign important candidate genes to putative metabolic pathways.

In recent years, description of high efficiency transformation protocols for strawberry (Oosumi et al. 2006) has also allowed to use new research strategies such as reverse genetics for functional genomic analyses in this crop (Oosumi et al. 2010). The authors report the development of efficient T-DNA tagging in *F. vesca* as a model for insertional mutagenesis in Rosaceae, and efficiently use the TAIL-PCR method (Liu et al. 1995, Liu and Chen 2007), to amplify *F. vesca* genomic sequence flanking T-DNA insertion. About 60% of T-DNAs were integrated into genetic regions, with 154 of 213 (72%) of the T-DNA tagged genomic sequences showing homology to plant genes, proteins and ESTs. These authors have shown that T-DNA integration process in strawberry is not random but directed by sequence microsimilarities in the host genome. By using this T-DNA tagging technology, a wide range of strawberry mutagenic lines and phenotypes is expected. This certainly will help molecular studies in all the strawberry fields of interest. Other new emerging technologies such as RNA sequencing (Ozsolak et al. 2009, Ozsolak and Milos 2011), which eliminates several challenges posed by microarray technologies and accurately offers a global view of the whole transcriptome changes, would certainly be beneficial for unravelling the complexity of defence response in strawberry.

In summary, the use of high-throughput technologies will provide large amount of molecular information related with defence response in strawberry in the very near future. This important piece of information needs to be further processed, and efficient and accurately analysed to successfully identify important strawberry candidate genes. In particular, a thorough characterization of strawberry control genes encoding important transcription factors and key enzymes which translate recognition of pathogens into appropriate transcriptional outputs is encouraged. To accomplish this needs, the efficient use of the transient expression technology in strawberry (Hoffmann et al. 2006, Muñoz et al. 2010, Hoffmann et al. 2011) is expected to reduce the time to unravel the complex network of defence signalling pathways in this important crop. Simultaneously, as strawberry traits such as resistance are controlled by multiple genes (Faedi et al. 2002, Folta and Davis 2006), key regulatory genes offer the possibility of being used as important genetic markers for genetic diversity analysis and selective breeding, which might allow to engineer new strawberry varieties with improved resistance and healthy quality in a shorter period, leading to reduce chemicals use and environmental risks.

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Chapter II

Generation and Analysis of Defense-Related ESTs from Octoploid Strawberry (*Fragaria* × *ananassa*) and Generation of a Strawberry cDNA Microarray Platform

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Part of this chapter has been published with modifications as:

Amil-Ruiz, F., Encinas-Villarejo, S., de los Santos, B., Muñoz-Mérida, A., Mercado, J.A., Trelles, O., et al. (2012) *Distinctive Transcriptome Response of Two Strawberry (Fragaria x ananassa) Cultivars to Colletotrichum acutatum Infection*. Acta Hort. (ISHS) 929: 47-50.

Casado-Díaz, A., Encinas-Villarejo, S., Santos, B.d.l., Schilirò, E., Yubero-Serrano, E.-M., **Amil-Ruiz, F.**, et al. (2006) *Analysis of strawberry genes differentially expressed in response to Colletotrichum infection. Physiologia Plantarum* 128: 633-650.

Abbreviations:

SA: Salicilic acid

MeJA: Methyl jasmonate

EST: Expressed sequence tag

KEGG: Kyoto encyclopedia of genes and genomes

ABSTRACT

Strawberry (*Fragaria* × *ananassa*) production is strongly affected by fungal diseases and pests (e.g., *Colletotrichum* spp.), forcing the excessive use of chemical products (mostly environmental contaminants) to control them. Resistant cultivars are a priority of most strawberry breeding programs. However, little is known about the genetic basis of strawberry resistance to pathogens, and completely resistant cultivars to most of them have not been yet reported. Although molecular markers of disease resistance in strawberry has been reported, the octoploid genetic structure of commercial strawberry makes it difficult to associate molecular markers with disease resistance genes.

The generation of a strawberry ESTs collection enriched in defense-related genes has supposed a first insight at the molecular level of the mechanisms underlying defense response in this plant under biotic stress. Thus, 4677 high quality sequences representing 3249 strawberry unigenes were obtained from partial screening of six expression libraries generated from experimental conditions related with defense. The identification of an important number of molecular components associated to defense in strawberry was obtained. In addition, functional analysis of such sequences suggest activation of mechanical defenses through cell wall reinforcement in strawberry cellular suspensions chemically elicited (SA and MeJA), underlining the relevance of cell wall structure in strawberry plant defenses.

To gain insights into the genetic mechanisms of strawberry defense, an in-house cDNA microarray based on a 3K strawberry probe set has been constructed from the strawberry ESTs collection previously obtained. Subsequent analyses using this microarray platform have shown good repeatable and reproducible data. Therefore, this platform has been exploited in further analyses of transcriptomic approaches to explore strawberry response to biotic stimulus. Valuable information has been generated for effective control strategies to increase resistance in strawberry.

INTRODUCTION

Despite its importance as both a cash crop and important staple, little is known about the genetic basis that control strawberry resistance to pathogens. As seen in Chapter I, it seems clear that the ability of a strawberry plant to efficiently respond to pathogens firstly rely on the physiological status of injured tissue (preformed mechanisms of defense), and secondly, on the general ability of recognition and identification of the invaders by surface plant receptors. Them, strawberry receptors are able to activate a broad range of induced mechanisms including cell wall reinforcement, reactive oxygen species production, phytoalexin generation and pathogenesis related protein accumulation.

Knowledge about how the strawberry metabolism is fine controlled by transcriptomic changes in response to pathogenic organisms is a valuable piece of information for the understanding of this complex network of defense signals. Over the last decade, microarrays have proved to be a valuable tool to analyse the expression of thousands of genes simultaneously, helping to elucidate the underlying networks of gene regulation that lead to a wide variety of defense responses (Wang et al. 2006, Sarowar et al. 2011). Although a diverse array of commercially available platforms have been developed for many plants of interest, and based on different technological strategies, none of them is strawberry compatible.

So far, few studies in strawberry using high throughput technology have been published aimed to discover genes related with defense. Very recently, Guidarelli et al. (2011) have performed microarray analysis of white and red fruit strawberries after 24 h of their interaction with *C. acutatum*, providing new data of strawberry genes regulated upon *C. acutatum* infection. To date, however, no direct evidence neither functional gene characterization has been reported for the majority of the identified genes in the interaction strawberry plant-*C. acutatum*.

Here, we report on the analysis of a strawberry ESTs collection which has been enriched in defense-associated genes, and on the construction of an octoploid strawberry-based microarray platform using a set of selected unigenes from the ESTs information already obtained.

MATERIALS AND METHODS

Generation of strawberry ESTs collection and bioinformatics

Generation of subtracted libraries, as well as description on plant materials and pathogen treatments was done following the specific instructions that we reported in Casado et al. (2006), and DNA sequencing and computer analysis as described in Conesa et al. (2005) and Forment et al. (2008) (Conesa et al. 2005, Casado-Díaz et al. 2006, Forment et al. 2008).

Strawberry probeset and microarray platform

More than 3200 clones were used for plasmid DNA purification and specific sequences (ESTs) were amplified by PCR. Obtained amplicons were purified and concentrated to 200-300ng/ul by "PCR 96 Cleanup Kit" (Millipore). Concentration and specificity of the amplification were tested by Nanodrop ND-100 Spectrophotometer and ReadyAgarose 96 Plus Gel 3% (BioRad), respectively. A set of external controls was added to the probeset, Lucidea Universal ScoreCard (Amersham Biosciences), in order to calibrate the microarray images. All probeset components were mixed with DMSO (1:1 volume) and rearayed into 384-wells plates by Biomek® 2000 (Beckman Coulter). The strawberry probeset was printed by MicroGridII Pro Arrayer (BioRobotics) in microarray compatible slides (UltraGAPS Coated Slides (Corning)). Quality control, labeling, hybridization, and scanning were carried out by the SCAI, University of Córdoba (http://www.uco.es/servicios/scai/index.html), following the Genomic Unit guidelines. Hybridization procedure was preformed using Lucidea APS (Automated Slide Processor, Amersham). Microarray images were acquired by

GenePix Microarray Scanner 4000B (Axon Instruments) and analyzed using GenePix 6.0 software (Molecular Devices).

SYBRGreen I staining and sample labeling

Printed slides used to determine the appropriate concentration and purification method were stained by 3-minutes immersion on SYBRGreen I (Molecular Probes) diluted 10^{-5} in MiliQ water, followed by an abundant wash on MiliQ water to remove those fluorophore molecules not intercalated into DNA. Sample labeling was done using SuperScript Plus Indirect cDNA Labeling System (Invitrogen) and fluorophores Alexa Fluor 555 and Alexa Fluor 647 (Invitrogen), following manufacture guidelines as described previously (Amil-Ruiz et al. 2012).

SECTION ONE:

STRAWBERRY ESTs COLLECTION. Generation and analysis of ESTs from octoploid strawberry (*Fragaria* × ananassa)

RESULTS

Six ESTs libraries were constructed from strawberry (*F* × *ananassa*) in response to *C. acutatum* infection (Casado-Díaz et al. 2006), and in response to chemical elicitors of plant defences (unpublished). Four of these libraries contain genes associated to defense from cultivars Camarosa and Andana challenged with *C. acutatum*. The last two, contain genes from cellular suspensions of cv. Chandler treated with well-known elicitors of plant defenses, such as salicylic acid and methyl jasmonate (Table 1).

Selection of clones, sequencing and pre-processing

The six strawberry libraries were partially screened for positive clones carrying cDNA fragments. Positive clones represented 75-80% of the screened colonies. Individual clones were randomly selected, tagged and stored separately. Up to 18000 clones (~3000 approximately from each library) were isolated.

Table 1. Description of cDNA strawberry libraries.

Library	Cultivar	Tissue	Treatment	Reference
AC	Andana	Crown	Mock	Casado-Díaz et al. 2006
AI	Andana	Crown	*C. acutatum*	Casado-Díaz et al. 2006
CC	Camarosa	Crown	Mock	Casado-Díaz et al. 2006
CI	Camarosa	Crown	*C. acutatum*	Casado-Díaz et al. 2006
UC	Chandler	Cellular suspensions	Mock	unpublished
UT	Chandler	Cellular suspensions	Chemical elicitors	unpublished

A first insight of genes implicated in strawberry defense response was obtained by partially sequencing 6000 of these isolated clones. Pre-processing of the sequences, including base calling and removal of low-quality and contaminant sequences such as adaptors, cloning vectors and sequences from bacterial host (Forment et al. 2008), rendered a total of 4677 high quality and clean ESTs for further analysis (Figure 1 and Table 2). Sequences were an average of 397bp in length (range from 51 to 910bp), and the majority of them were over 300 bp in length (more that 75%) (Figure 1).

Accuracy was evaluated by the frequency of appearance of an undetermined nucleotide (N/bp). In the six libraries analyzed, the accuracy ranged from one every 320 to 14769 bp, with an average of 521 bp (Table 2). Markedly differences were found between libraries generated from strawberry crown tissue compared with those generated from cellular suspensions. In the four crown-based libraries, one undetermination every 388 bp in average was detected, with a maximum of N/320bp in AC and a minimum of N/507bp in CI. In the two cellular suspensions-based libraries, the accuracy was excellent, with values of N/14.7kb and N/11.6kb for UT and UC, respectively.

Table 2. Analysis of strawberry ESTs. [a]AI and AC, libraries of strawberry cv. Andana infected and mock, respectively; CI and CC, libraries of strawberry cv. Camarosa infected and mock, respectively; UT and UC, libraries of strawberry cv. Chandler´s cellular suspensions elicitors and mock treated, respectively. [b]The final number of unique sequences decreases considering the six libraries together. Accordingly, 138 new contigs arise, and redundancy is also increased in 390 sequences. Values are mean ± SD.

	Strawberry Libraries[a]												**TOTAL[b]**	
	AI		**AC**		**CI**		**CC**		**UT**		**UC**			
High quality sequences	860		768		828		908		811		502		4677	
EST length (bp ± SD)	400	± 135	396	± 128	391	± 140	394	± 144	419	± 127	371	± 128	397	± 135
bp/N average	355		320		507		370		14769		11653		521	
Singletons	655	(76%)	621	(81%)	558	(67%)	702	(77%)	204	(25%)	352	(70%)	2564	(55%)
Contigs	85		65		116		90		144		47		685	
Unigenes	740	(86%)	686	(89%)	674	(81%)	792	(87%)	348	(43%)	399	(79%)	3249	(69%)
Redundancy (%)	14%		11%		19%		13%		57%		21%		31%	
Library-specific unigenes	545		516		452		581		181		289			
Novelty (%)	63%		67%		55%		64%		22%		58%			

EST length range (bp)	Absolute Frecuency	Relative Frecuency
51 to 100	31	0,66%
100 to 200	325	6,95%
200 to 300	803	17,17%
300 to 400	1226	26,21%
400 to 500	1203	25,72%
500 to 600	777	16,61%
600 to 700	267	5,71%
700 to 800	37	0,79%
800 to 900	6	0,13%
900 to 1000	2	0,04%
Total	4677	100,00%

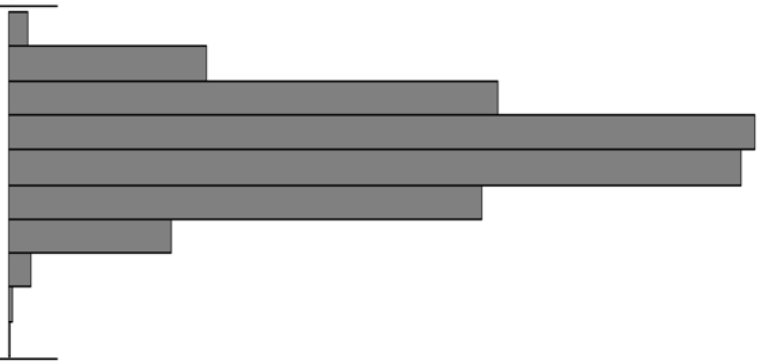


Figure 1. Strawberry ESTs Length Distribution.

Assembly

ESTs usually correspond to only partial cDNA sequences but even when normalized during library construction, they can be typically redundant due to the random selection of the sequenced clones. Therefore, a clustering step was needed to obtain a non-redundant set of unique consensus sequences, or unigenes, through assembling the overlapping ESTs. To determine the level of redundancy in our libraries, ESTs obtained from every individual library were initially analyzed and individually assembled into clusters consisting of overlapping and contiguous DNA sequences. Each cluster was counted as a different individual species or unigene, and each individual species that did not exhibit similarity to a database sequence with similarity to a cluster (singletons) was also counted as unigene (Huang and Madan 1999, Tang et al. 2009). As expected and based on the normalization process during the libraries construct (Diatchenko et al. 1996), most of these genes, were not assembled into contigs, thus representing unique sequences. Values of different species of ESTs or unigenes for inoculated and mock-treated libraries were, respectively, 740 and 686, for Andana, 674 and 792, for Camarosa, and 348 and 399, for cellular suspensions of Chandler (Table 2). While redundancy was still very low (from 11 to 19%) for the four crown-derived libraries (AI, AC, CI and CC) and for the UC library derived from mock treated cellular suspensions (21%), significantly higher level of redundancy was observed for UT library (57%). Figure 2 shows the number of ESTs per unigene distribution. Thus, only around 11% of the contigs (2% of the total of unigenes) are composed by 5 ESTs or more.

Seventy six out of 685 contigs were composed by 5 or more ESTs and their associated functions are discussed below (Table 3). As expected, and due to the UT library saturation mentioned above (Table 2), most of the overrepresented contigs carry DNA sequences belonging to this single library (Table 3). A total of 679 ESTs conform the subset of 76 different contigs, but the single contribution of each library to this subset of ESTs was not homogeneous. Thus the single contribution of UT library supposed over 53% (362) of the total ESTs, while the single contribution of the other five libraries ranged from 6.5% (44, AC) to 12%

(83, UC). More over, around 42% of the contigs are composed in at least a 75% by ESTs from UT library.

Range (num ESTs/contig)	Num of Contigs	Relative Frecuency
2 to 3	560	81,75%
3 to 4	49	7,15%
4 to 6	39	5,69%
6 to 8	12	1,75%
8 to 10	9	1,31%
10 to 12	4	0,58%
12 to 17	7	1,02%
17 to 22	2	0,29%
22 to 39	3	0,44%
Total	685	

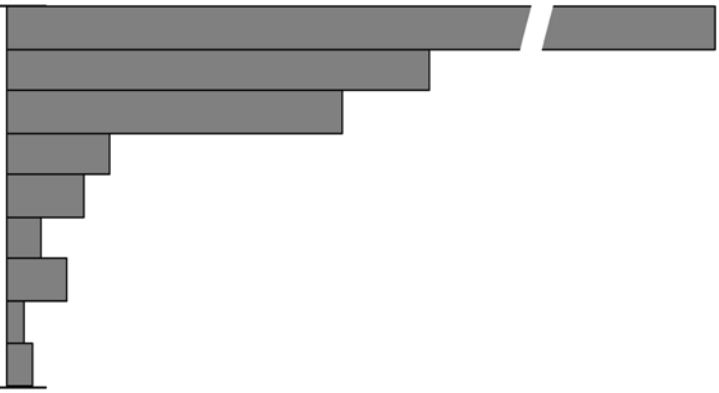


Figure 2. Strawberry ESTs distribution per contig.

Functional annotation

Annotation of the 3249 strawberry unigenes was performed by blasting against the UniProt Knowledgebase database (UniProtKB, UniProt release 2012_05 - May 16, 2012), which is a comprehensive resource for protein sequence and annotation data, and represent the central hub for the collection of functional information on proteins, with accurate, consistent and rich annotation. This database consists of two sections: a section containing manually-annotated records with information extracted from literature and curator-evaluated computational analysis, and a section with computationally analyzed records that await full manual annotation.

Table 3. (next page) Overrepresented unigenes (contigs made up of 5 or more ESTs). Shadow in grey, yellow, green and red: contigs composed in at least 75% of ESTs from UT single library, UC single library, CC+AC (crown mock) libraries and CI+AI (crown infected) libraries, respectively.

Functional Groups	Contig ID	Fv orthologe	Anotation /Associated Functions	Conting Length	Number of ESTs	Number of libraries	Abundancy per Library					
							CC	CI	AC	AI	UC	UT
Defense Responses												
Mechanical Defenses (Cell wall modification and Phenylpropanoid pathway)	Contig572	gene20700	AtCAD7, Elicitor activated 3, Plant defence, RPM1 dependent	901	39	1	0	0	0	0	0	39
	Contig582	gene24025	AtCAD5, Lignification, Response to insects	894	32	1	0	0	0	0	0	32
	Contig599	gene00496	AtCAD1, Lignification, Response to wounding	1385	9	1	0	0	0	0	0	9
	Contig520	gene20700	AtCAD 8, Response to Bacteria	470	5	2	0	1	0	0	0	4
	Contig541	gene20550	Cellulose synthase like protein E1, Related to plant disease, Response to wounding	650	10	1	0	0	0	0	0	10
	Contig516	gene04118	ATP-citrate lyase A-3, Acetyl-CoA biosynthetic process, Wax biosynthetic process	719	5	1	0	0	0	0	0	5
	Contig646	gene05164	AtBXL1, Beta D Xylosidase, Secondary cell wall metabolism	774	21	2	1	0	0	0	20	0
	Contig632	gene05164	Beta-xylosidase 1, Secondary wall thickening	357	7	1	0	0	0	0	7	0
	Contig67	gene22465	Endochitinase, Deposition of lignin	1147	6	4	0	2	2	1	0	1
Pathogenesis Related Proteins	Contig525	gene07064	Patogenesis related protein 10, Fra a 3	734	32	2	0	1	0	0	0	31
	Contig551	gene07085	FaPR10-4, MeJA responsive	750	16	2	0	2	0	0	0	14
	Contig499	gene07065	Patogenesis related protein 10, Fra a 2	438	15	3	1	2	0	0	0	12
	Contig554	gene07066	Patogenesis related protein 10, Fra a like protein	647	10	2	0	0	0	1	0	9
	Contig358	gene07082	Patogenesis related protein 10, Fra a like protein	355	9	2	0	0	0	2	0	7
	Contig509	gene30434	Polyphenol oxidase, Defence response, Lignin biosynthetic process	438	6	1	0	0	0	0	0	6
	Contig276	gene07080	Patogenesis related protein 10, Fra a 1-B	842	10	3	1	0	0	1	8	0
Response to Oxidative Stress	Contig57	gene10383	Glutathione S-transferase, Induced by drought stress, oxidative stress, and high doses of auxin and cytokinin	1320	8	3	0	1	0	1	0	6
	Contig566	gene28763	Glutathione S-transferase PHI 9, Defense response to bacterium	671	5	1	0	0	0	0	0	5
	Contig603	gene08384	Glutathione S-transferase TAU 8, Response to cadmium	539	8	1	0	0	0	0	0	8
	Contig622	gene32646	Carbonic anhydrase 2, Innate immunity signaling, Defense response to bacterium and fungus	1023	7	1	0	0	0	0	0	7
	Contig623	gene10776	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	533	6	1	0	0	0	0	0	6
	Contig620	gene10141	Zinc-binding dehydrogenase, Response to oxidative stress	504	5	1	0	0	0	0	0	5
	Contig657	gene14095	Metallothionein 2B, May provide protection against metal toxicity and oxidative stress	452	9	1	0	0	0	0	9	0
	Contig278	gene19619	AtPRXR1, Peroxidase	615	15	4	6	2	5	2	0	0
	Contig677	gene30155	Fructose-bisphosphate aldolase	628	7	3	0	2	0	0	1	4
Response to Stress	Contig18	gene08617	Dehydrin 2, Induced early on in response to dehydration stress and ABA	746	6	4	1	2	1	2	0	0
	Contig197	gene06814	Methionine sulfoxide reductase (MSS4-like), Translationally controlled tumor protein, Auxin homeostasis, Defense response to bacterium	506	6	3	1	0	0	0	1	4
	Contig389	gene15165	Isoflavone reductase, Proanthocyanidin biosynthesis	974	14	6	4	3	2	1	2	2
	Contig152	gene26994	ADP/ATP carrier protein 1, Mitochondrial	574	11	4	1	3	0	4	0	3
	Contig453	gene23293	Aquaporin PIP2-1, Response to drought	661	11	4	3	5	1	2	0	0
Signalling												
Calcium Related	Contig149	gene02575	Calmodulin binding protein	365	21	1	0	0	0	21	0	0
	Contig533	gene05089	Calmodulin-like, Response to salt and drought	359	10	1	0	0	0	0	0	10
Glucosyltransferases	Contig602	gene24225	UDP-glucose glucosyltransferase	765	14	1	0	0	0	0	0	14
	Contig507	gene34574	UDP-glucosyl transferase 85A, Required for resistance to *Hyaloperonospora parasitica*	588	7	1	0	0	0	0	0	7
	Contig508	gene00708	UDP-Glycosyltransferase, Response to ABA	710	5	1	0	0	0	0	0	5
Ubiquitin Related	Contig225	gene19551	Polyubiquitin 10, Induced by SA independently of NPR1	689	8	4	2	0	2	0	2	2
	Contig537	gene12767	Ubiquitin supergroup;Ribosomal protein L40e	574	5	3	0	0	1	2	0	2
Protein synthesis												
	Contig583	gene24875	Zinc-binding 60S ribosomal protein L44	266	11	1	0	0	0	0	0	11
	Contig309	gene28055	Ribosomal protein L16p/L10e, Response to UV-B	582	8	3	0	2	1	5	0	0
	Contig223	gene30590	Ribosomal protein S11	721	5	3	2	0	2	1	0	0
	Contig346	gene01350	Ribosomal protein L7Ae/L30e/S12e/Gadd45	711	5	2	4	1	0	0	0	0
	Contig555	gene23217	Elongation factor-1A, SMV resistance-related protein, Calmodulin binding, Related to TIR receptors, ABA-ET signalling	936	14	5	1	6	2	2	0	3
	Contig113	gene03801	Translation initiation factor SUI1	738	6	3	1	2	0	0	3	0
	Contig84	gene28639	Elongation factor 1-alpha, GTP binding, Calmodulin binding	755	6	3	0	1	2	3	0	0
	Contig172	gene10075	Eukaryotic translation initiation factor 5A-2, Involved in pathogen-induced cell death and development of disease symptoms	546	6	3	3	2	1	0	0	0
	Contig440	gene18780	Ribosomal protein L35Ae	370	6	3	0	0	2	0	2	2
	Contig258	gene30096	Ribosomal protein L24, Auxin mediated signaling pathway	447	6	3	2	1	0	0	3	0
	Contig460	gene04747	Ribosomal protein L34e	531	6	2	0	0	2	0	0	4
	Contig142	gene03525	Ribosomal protein 40S-S8	539	5	3	0	1	0	1	0	3
Transport / Delivery Machinery												
	Contig390	gene01798	Alpha-tubulin 4 chain, Response to Cadmium	509	9	4	1	1	0	4	3	0
	Contig667	gene26908	Tubulin alpha-2 chain	392	5	2	0	1	0	0	4	0
	Contig28	gene18570	Actin 7, Induced by auxin	780	6	3	3	0	2	1	0	0
Regulation, Hormone Related and Others												
	Contig123	gene07254	Pyruvate dehydrogenase, Transketolase family protein, Defense response to bacterium	394	5	2	0	1	0	0	0	4
	Contig132	gene09418	S-adenosyl-L-methionine-dependent methyltransferases, Methylsalicylate biosynthesis, Role in defense	751	5	2	0	1	0	0	0	4
	Contig407	gene30512	Glutamine synthetase, Response to cadmium ion and to salt stress	615	6	2	0	0	1	0	0	5
	Contig544	gene18966	ATP-binding cassette transporter	437	6	1	0	0	0	0	0	6
	Contig638	gene18038	Carboxylesterase, Giberelin receptor	705	11	1	0	0	0	0	11	0
	Contig55	gene20785	14-3-3KAPPA, Brassinosteroid signaling	628	5	3	1	1	0	3	0	0
	Contig261	gene34297	Chlorophyll A/B binding protein 3	478	8	3	4	2	2	0	0	0
	Contig604	gene17371	3-hydroxy-3-methylglutaryl coenzyme A reductase, Cytokinin biosynthesis	932	5	4	0	0	1	1	1	2
	Contig494	gene31580	Pyrophosphate-energized vacuolar membrane proton pump 1, Auxin signalling	997	5	3	0	0	1	2	2	0
	Contig31	gene19595	FaLTP4	633	8	4	2	2	3	1	0	0
	Contig641	gene07312	Aldolase-type TIM barrel family protein, Response to cadmium ion	730	5	2	0	0	3	0	2	0
	Contig344	gene13949	Histone H2b	250	5	3	1	3	0	0	1	0
	Contig412	gene14152	Histone H4	566	5	2	0	2	3	0	0	0
Unknown												
	Contig588	gene11307	Hypothetical protein	433	8	1	0	0	0	0	0	8
	Contig600	gene31859	Hypothetical protein	536	5	1	0	0	0	0	0	5
	Contig584	No hit found	No hit found	697	14	5	2	1	2	2	0	7
	Contig569	gene20833	No hit found	691	10	1	0	0	0	0	0	10
	Contig607	gene30397	No hit found	565	8	1	0	0	0	0	0	8
	Contig598	gene14900	No hit found	487	5	1	0	0	0	0	0	5
	Contig66	No hit found	No hit found	386	5	3	0	2	0	2	1	0
	Contig476	gene33864	No hit found	411	5	1	5	0	0	0	0	0
	Contig271	gene18240	No hit found	423	6	3	2	2	0	0	0	2
	Contig179	gene10077	No hit found	380	5	2	0	3	0	0	0	2
	Contig53	gene26409	No hit found	720	5	3	0	1	0	2	0	2

1

For the sake of continuity and name recognition, the two sections are referred to as "UniProtKB/Swiss-Prot" (reviewed, manually annotated) and "UniProtKB/TrEMBL" (unreviewed, automatically annotated), respectively (Figure 3).

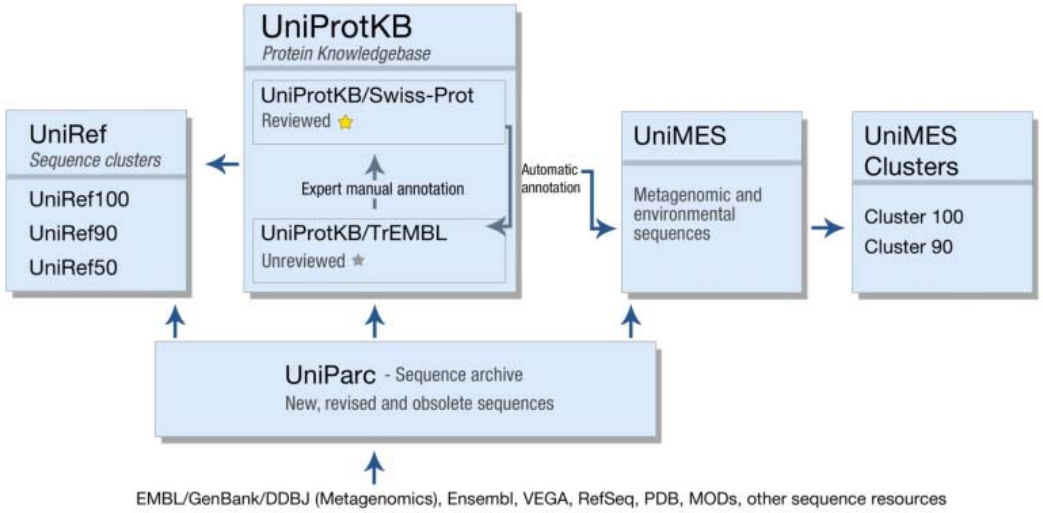


Figure 3. The Universal Protein Resource (UniProt), a comprehensive resource for protein sequence and annotation data, databases scheme (image from http://www.uniprot.org).

Following this strategy, 2437 unigenes (representing 75% of our strawberry unigene collection) were successfully annotated after applying e-value drop cut-off < E^{-5} (see Supplemental Table 1 for details). Most of the sequences found with similarity, belong to the unreviewed and automatically annotated subgroup into UniProtKB database (Figure 4).

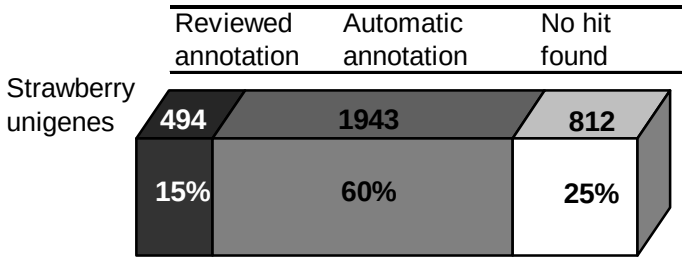


Figure 4. Functional classification of strawberry unigenes by UniProtKB.

Close to 56% of unigenes similar to sequences found in public databases have their most similar partner in sequences from two species, either *Ricinus communis* or *Populus trichocarpa* (representing 29% and 27% of the matched genes, respectively, see Figure 5). The sequences belonging to other four species (including *Fragaria* × *ananassa*, *Vitis vinifera, Glycine max* and *Arabidopsis thaliana*) represent a set of 23% of our strawberry unigenes (Figure 5). The last 21% unigenes matched sequences from a variety of 138 different species whose single contribution is below a 1.7% of the total of sequences with similarity (all of them were included into the group of "others" in Figure 5).

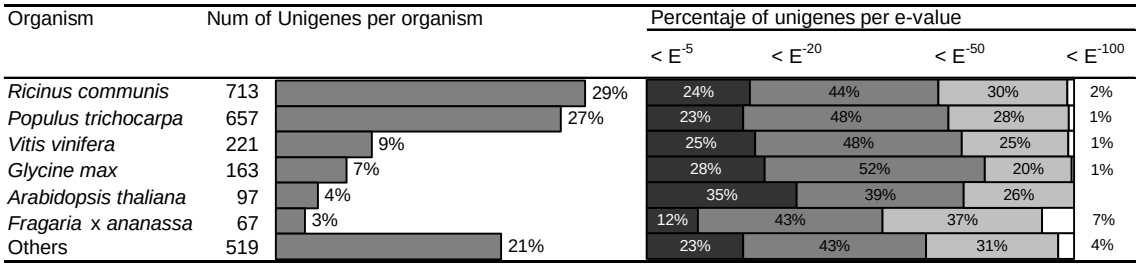


Figure 5. Number of strawberry unigenes with found similar sequences in the screened databases at different e-value (E) cutt-off. More representative organisms into the Blast results are showed. Those organisms which single contribution is under 1.7% were grouped into "Others" (See supplemental Table 1 for details).

The distribution of E-value was quite homogeneous between different species, with values of $< E^{-20}$ for almost 65-77% of the homologous genes. As it was expected, higher similarity between our unigene set and UniProtKB sequences from *Fragaria* species was found, so the distribution of E-value break the tendency showed in Figure 5 for *Fragaria* × ananassa species, where 88% of sequences have an e-value lower than $< E^{-20}$. However, the low number of strawberry sequences deposited on the database, in comparison with other plant species such as *R. communis, P. trichocarpa* and *V. vinifera*, has produced enrichment on genes obtained from other species. Table 4 shows the number of entries for strawberry species in comparison with the other contributing species.

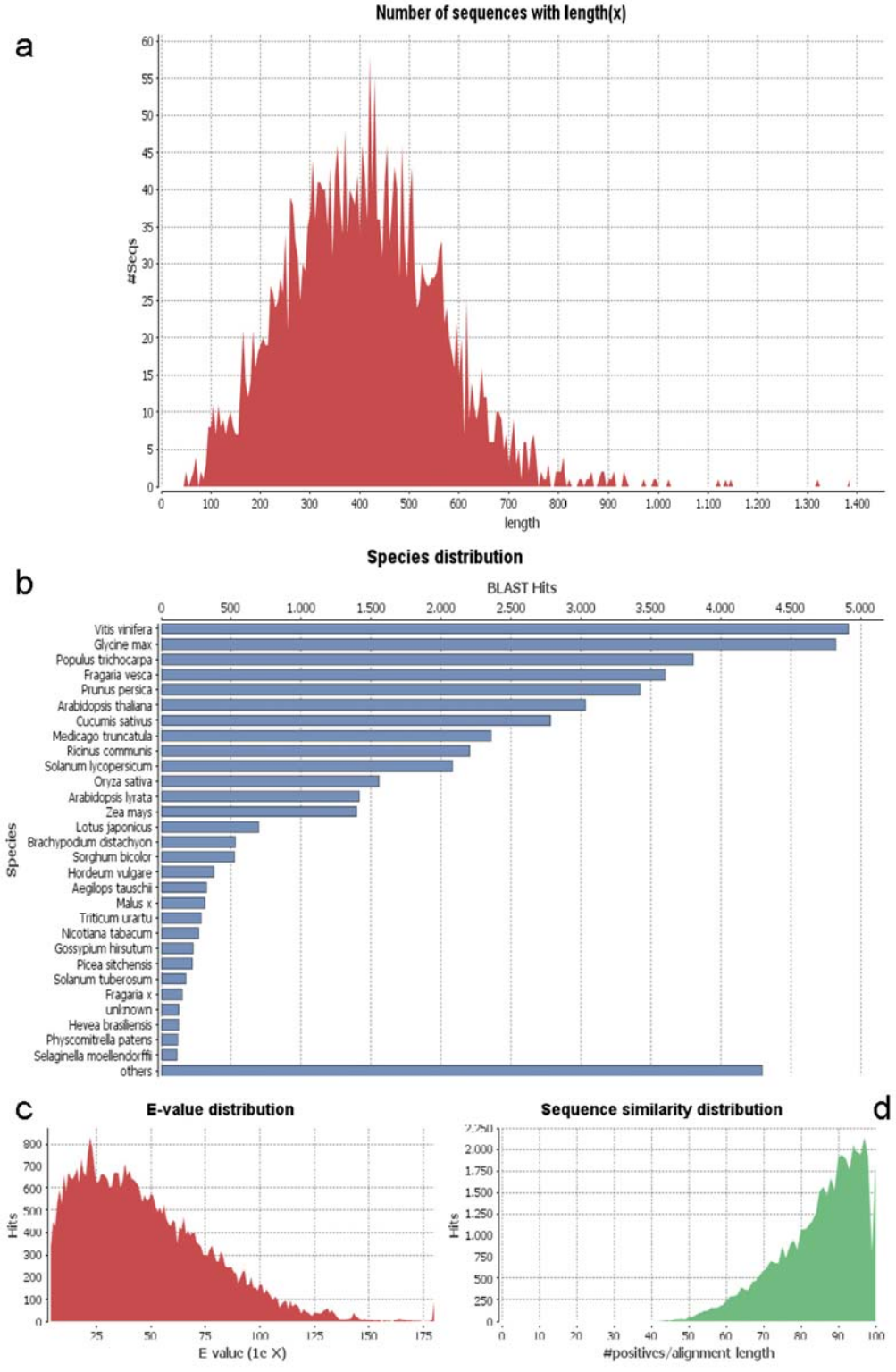
Table 4. Entries in UniprotKB of some plant species and comparison with strawberry deposited sequences.

	reviewed (UniProtKB/Swiss-Prot)	unreviewed (UniProtKB/TrEMBL)	Total entries
Arabidopsis thaliana	11206	43425	54631
Vitis vinifera	164	54052	54216
Populus trichocarpa	127	44070	44197
Ricinus communis	73	31381	31454
Glycine max	375	12767	13142
Fragaria* x *ananassa	31	378	409
Fragaria vesca	11	124	135

Additional functional annotation

Additional functional information was associated to the strawberry ESTs collection by Blast2GO (Conesa et al. 2005). Thus, valuable information was associated to all 3249 sequences following subsequent Blast2GO steps, such as Blasting (blastx to nr database), Mapping and Annotating results to our ESTs collection. Figures 6 and 7 summarize the statistics and annotation results using Blast2GO, respectively. Additionally, InterProScan and Enzyme Code and KEGG information was added. Figure 8 shows five examples of KEGG assignment. Complete annotation is available in Supplemental File 1 (Blast2GO compatible).

Figure 6. (next page) Blast2GO statistics in blastx results against nr database. a) Distribution of analyzed sequences by length, b) distribution of species contributing to blast hits, c) distribution of obtained e-values, d) distribution of sequence similarities.


a
Number of sequences with length(x)
#Seqs
length
b
Species distribution
BLAST Hits
Species
Vitis vinifera
Glycine max
Populus trichocarpa
Fragaria vesca
Prunus persica
Arabidopsis thaliana
Cucumis sativus
Medicago truncatula
Ricinus communis
Solanum lycopersicum
Oryza sativa
Arabidopsis lyrata
Zea mays
Lotus japonicus
Brachypodium distachyon
Sorghum bicolor
Hordeum vulgare
Aegilops tauschii
Malus x
Triticum urartu
Nicotiana tabacum
Gossypium hirsutum
Picea sitchensis
Solanum tuberosum
Fragaria x
unknown
Hevea brasiliensis
Physcomitrella patens
Selaginella moellendorffii
others
c
E-value distribution
Hits
E value (1e X)
d
Sequence similarity distribution
Hits
#positives/alignment length

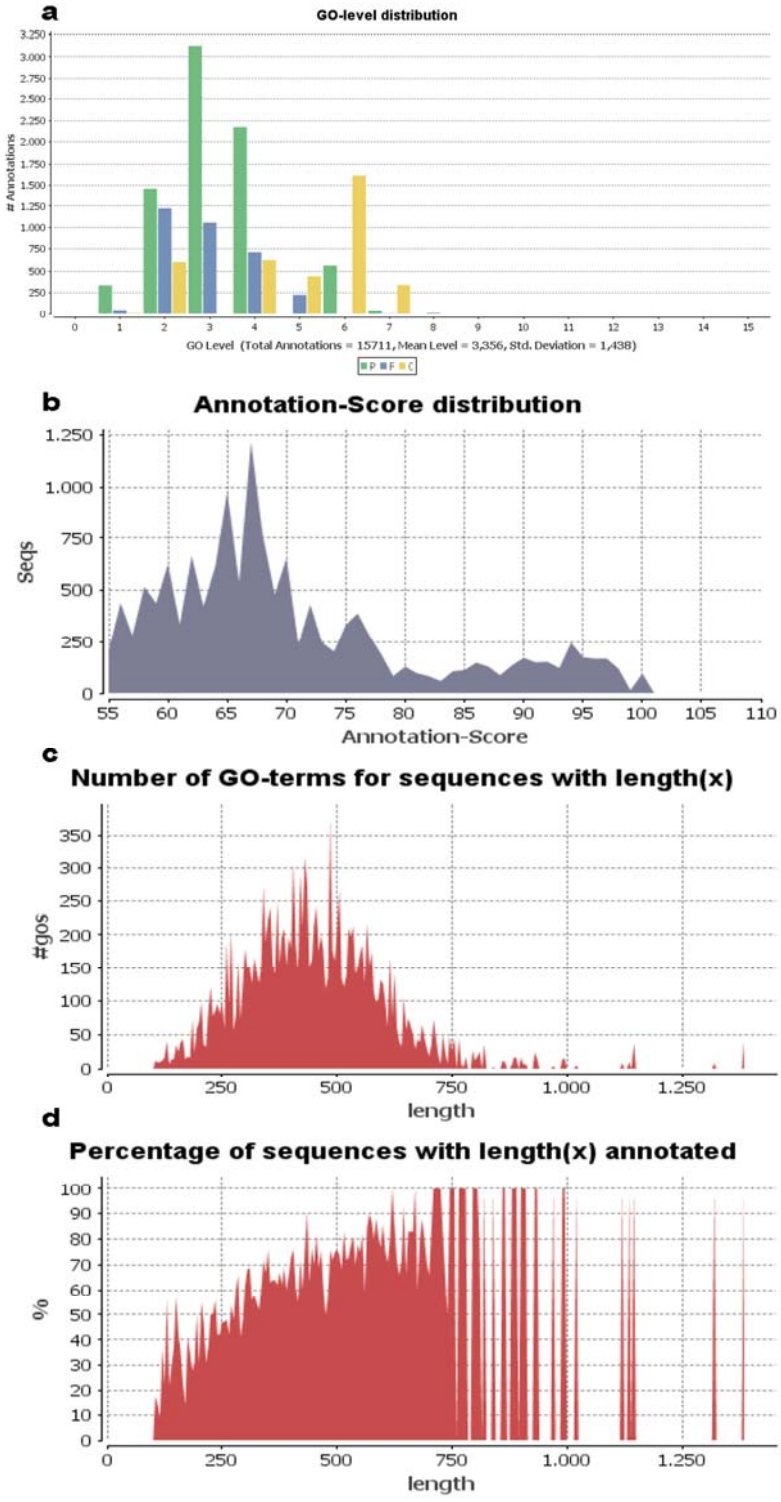

a
GO-level distribution
Annotations
GO Level (Total Annotations = 15711, Mean Level = 3,356, Std. Deviation = 1,438)
P F C
b
Annotation-Score distribution
Seqs
Annotation-Score
c
Number of GO-terms for sequences with length(x)
#gos
length
d
Percentage of sequences with length(x) annotated
%
length

Figure 7. (previous page) Summary of Blast2GO Annotation. a) Assigned GO-level distribution, b) distribution of annotation-Score, c) number of GO-terms associated to each sequence in relation to their length, d) percentage of sequences annotated in relation with their length.

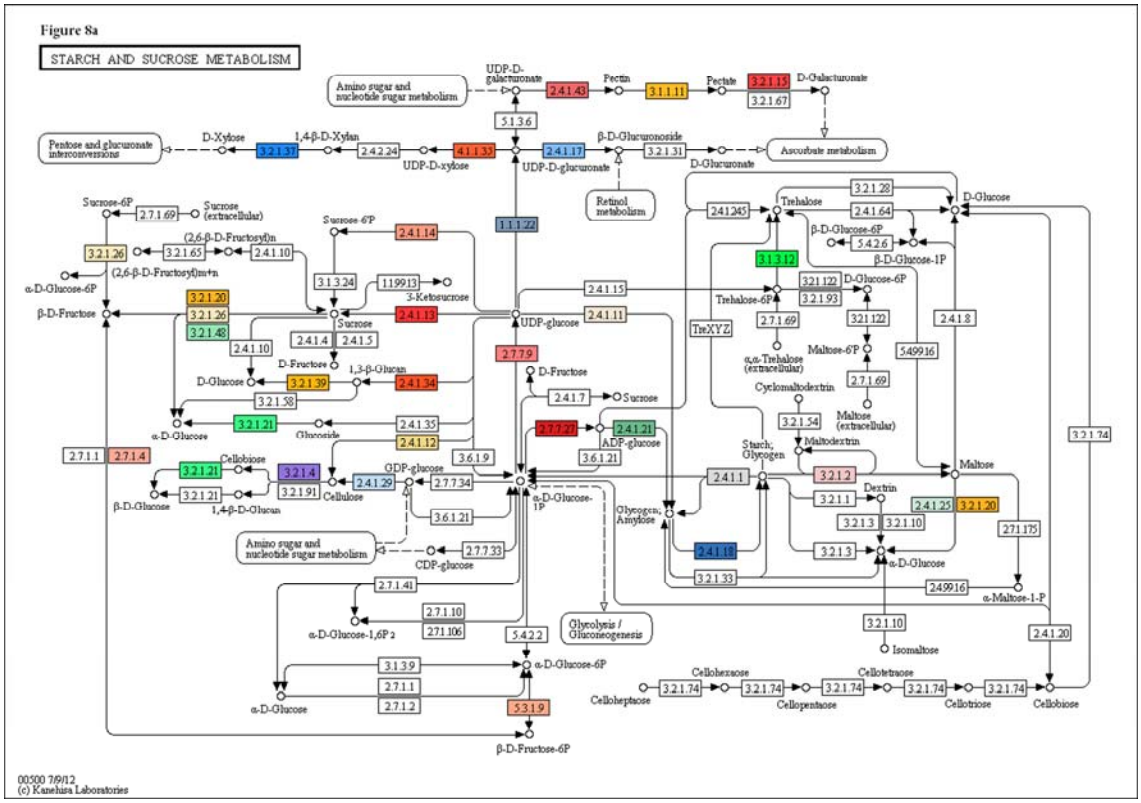


Figure 8. (and subsequent pages) Examples of KEGG pathway assignment to our strawberry ESTs collection for an additional functional annotation. (a) Starch and sucrose metabolism, (b) Flavonoids biosynthesis, (c) Inositol phosphate metabolism, (d) Phosphatidylinositol signaling system, (e) Oxidative phosphorilation.

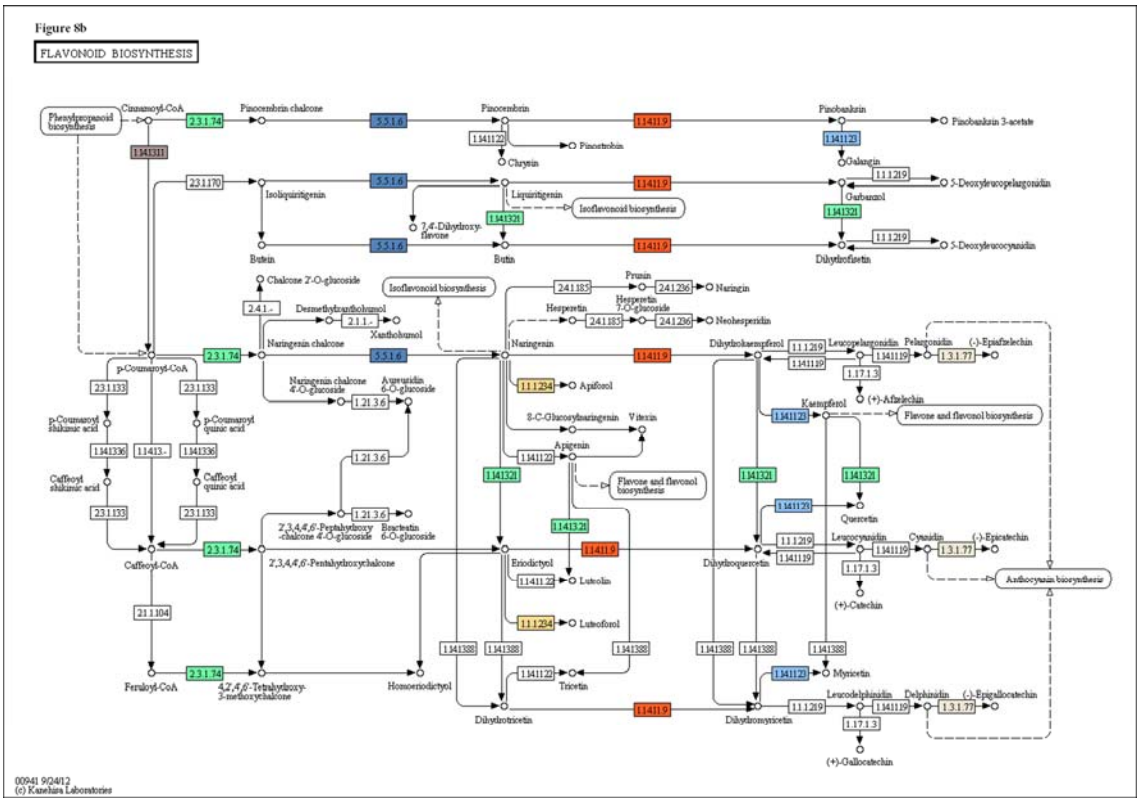


Figure 8b
FLAVONOID BIOSYNTHESIS
00941 9/24/12
(c) Kanehisa Laboratories

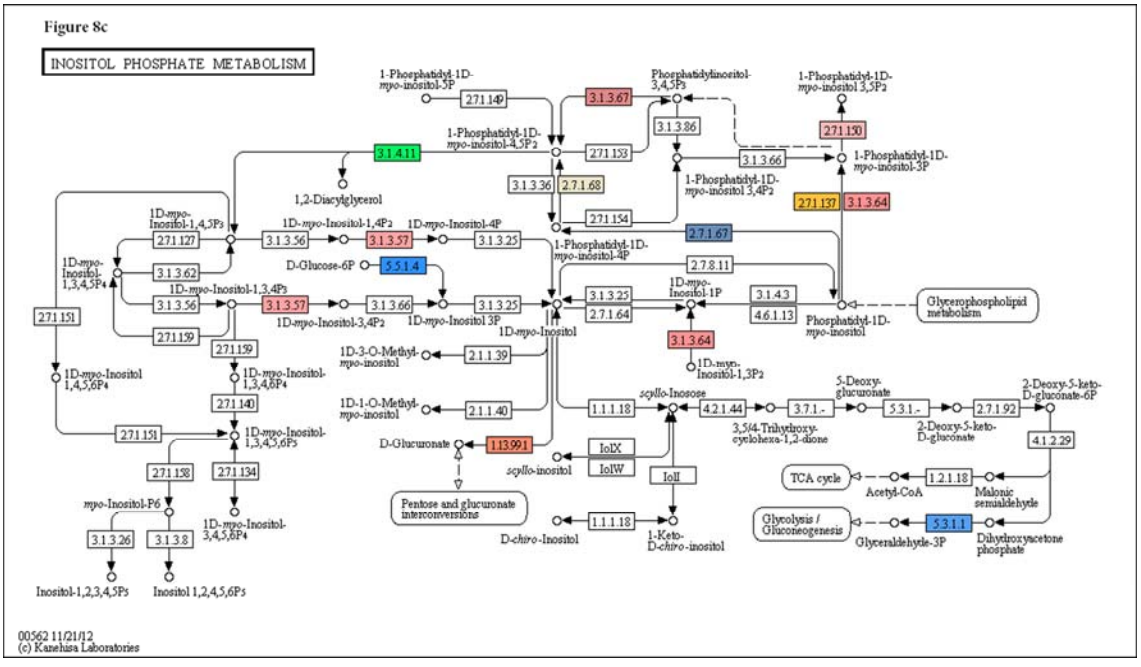


Figure 8c
INOSITOL PHOSPHATE METABOLISM
00562 11/21/12
(c) Kanehisa Laboratories

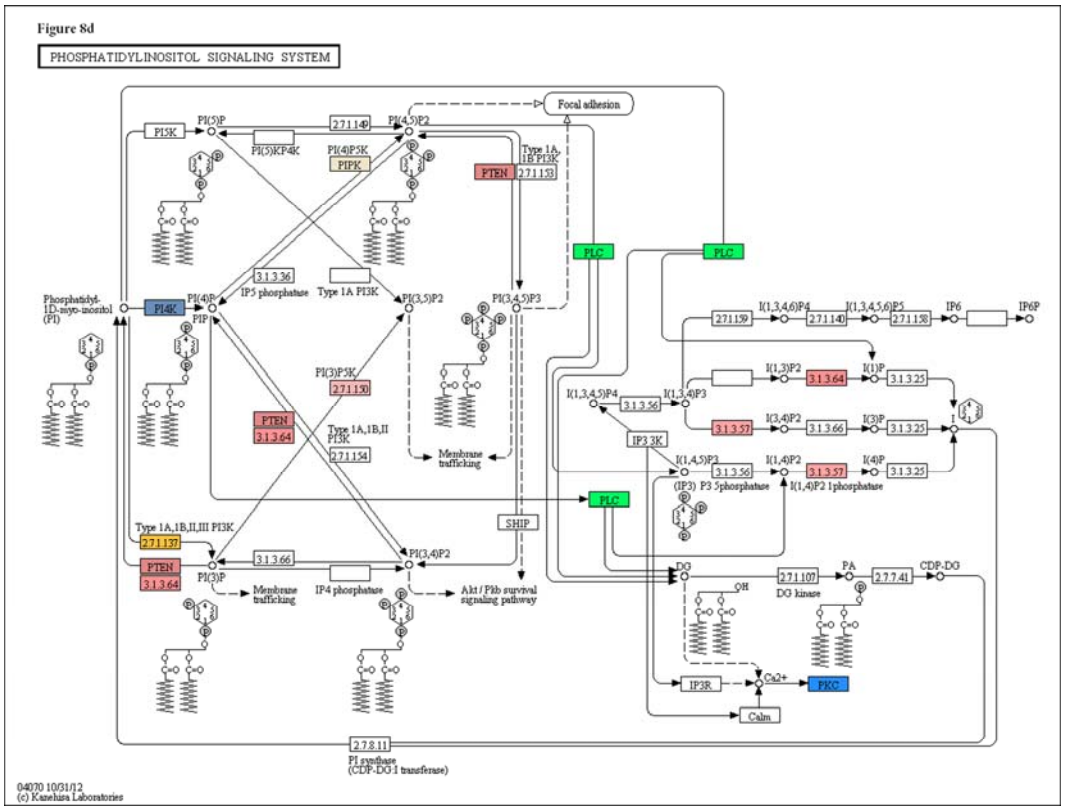


Figure 8d
PHOSPHATIDYLINOSITOL SIGNALING SYSTEM
Focal adhesion
PI3K
PI(5)P
PI(4,5)P2
PI(5)KPI4K
PI(4)P3K
PIPK
PTEN
Type 1A, 1B PI3K
3.1.3.36
IP5 phosphatase
Type 1A PI3K
PLC
Phosphatidyl-1D-myo-inositol (PI)
PI4K
PI(4)P
PIP
PI(3,5)P2
PI(3,4,5)P3
PI(3)P5K
PTEN
Type 1A,1B,II PI3K
Membrane trafficking
I(1,3,4,6)P4
I(1,3,4,5,6)P5
IP6
IP6P
I(1,3)P2
I(1)P
I(1,3,4,5)P4
I(1,3,4)P3
IP3 3K
I(3,4)P2
I(3)P
I(1,4,5)P3
I(1,4)P2
I(4)P
(IP3) P3 5phosphatase
I(1,4)P2 1phosphatase
SHIP
Type 1A,1B,II,III PI3K
PI(3)P
IP4 phosphatase
Akt / Pkb survival signaling pathway
DG
DG kinase
PA
CDP-DG
IP3R
Ca2+
PKC
Calm
PI synthase (CDP-DG 1 transferase)
04070 10/31/12
(c) Kanehisa Laboratories

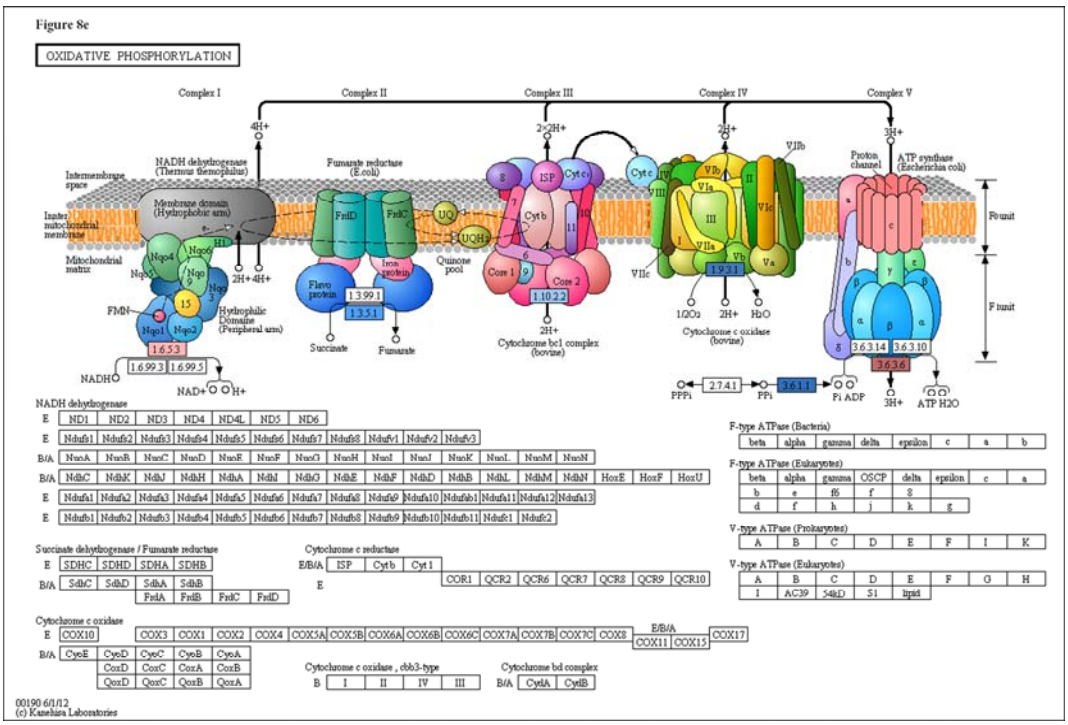


Figure 8e
OXIDATIVE PHOSPHORYLATION
Complex I
Complex II
Complex III
Complex IV
Complex V
NADH dehydrogenase (Thermus thermophilus)
Fumarate reductase (E.coli)
Proton channel
ATP synthase (Escherichia coli)
Intermembrane space
Inner mitochondrial membrane
Membrane domain (Hydrophobic arm)
Mitochondrial matrix
Hydrophilic Domain (Peripheral arm)
Quinone pool
Cytochrome bc1 complex (bovine)
Cytochrome c oxidase (bovine)
Succinate
Fumarate
NADH
NAD+
ATP H2O
NADH dehydrogenase
Succinate dehydrogenase / Fumarate reductase
Cytochrome c reductase
Cytochrome c oxidase
Cytochrome c oxidase , cbb3-type
Cytochrome bd complex
F-type ATPase (Bacteria)
F-type ATPase (Eukaryotes)
V-type ATPase (Prokaryotes)
V-type ATPase (Eukaryotes)
00190 6/1/12
(c) Kanehisa Laboratories

DISCUSSION

Analysis of defense-related ESTs from octoploid strawberry

Sequencing information has produced very important data for plant biologists in both basic and applied studies of plant physiology. Despite of the importance of the cultivated strawberry throughout the world, most components and mechanisms of the strawberry defense network remain poorly understood due to its growing characteristics and the inherent difficulty of working with an octoploid (Amil-Ruiz et al. 2011). The generation of a ESTs collection is a relatively quick and powerful method to identify key genes of interest, estimate relative expression levels of transcripts (the so called "digital northern" (Audic and Claverie 1997), and also, to develop a cDNA microarray platform (Alba et al. 2004). However this method has certain limitations, such as the obtaining of redundant sequences from the most common transcripts and the need of a pre-processing and cleaning process of the raw sequences. In this study, we have analyzed more than 4600 high quality sequences from *F* × *ananassa*, assembled in more than 3200 unigenes. All these sequences proceeded from our own sequencing project aimed to identify genetic components of defense response in the cultivated strawberry. Although pests and pathogens cause important losses in strawberry production, genetic information in a defense context is still scarce. Thus, the molecular information provided in this study is highly valuable for further studies to elucidate the molecular aspects of defense response in strawberry, and to improve its resistance through either biotechnological or traditional breeding approaches.

Two strawberry cultivars, exhibiting different behavior to the fungal pathogen *C. acutatum* (cvs. Andana and Camarosa), were used as a genetic resource searching for genes implicated in specific defense response against this pathogen. In addition, elicitation with SA and MeJA (well-known inductors of defense responses in plants) was carried out in cellular suspensions from cv. Chandler, with the aim of identify genetic components necessary for an efficient defense response that could be strategically repressed by this hemibiotrophic pathogen during its infection process.

The pre-processing step applied to our strawberry ESTs collection indicates that values of EST length (average of 397bp) are highly similar to those found in other ESTs sequencing projects in strawberry, apple, *Populus* and kiwifruit, were the average length of the edited sequences was 343 to 612, 468, 470 and 503 bases, respectively (Sterky et al. 2004, Newcomb et al. 2006, Crowhurst et al. 2008, Bombarely et al. 2010). In addition, the accuracy of such sequences, with appearance of an undetermined nucleotide every 521 bp in average, is equivalent to that found in other strawberry ESTs collections (N/388bp) (Bombarely et al. 2010). Even more, the sequences corresponding to clones obtained from the cellular suspensions libraries are extremely high accurate, and undeterminations were found one every several kilobases. Thus, all data obtained from the pre-processing step indicate that 4677 sequences are highly accurate and good quality sequences, with length size ranging within the media of other EST collections.

After the assembly step, a collection of non-redundant strawberry unigenes was generated. Analysis of redundancy indicated that, in general, our strawberry libraries could be further exploited. The redundancy observed in UT (57%) suggests a very close to saturation status of this particular library, maybe due to an experimental design focused in a restricted set of genes that are biologically regulated during the process under study, or well due to a very strong subtraction process on the library construction. When the six libraries were analyzed altogether, the number of unigenes decreased as some sequences from a particular library were found to be present in the other libraries. However, the percentage of total unigenes was still high and there was a quite low level of redundancy (Table 2). In addition, the low average number of ESTs found per contig (3) reinforces that our libraries are far from saturation and so, they could be further exploited by sequencing in order to identify new genes of interest (Figure 2 shows the number of ESTs per contig distribution).

In summary, based on the number of ESTs isolated in our study and the low level of redundancy obtained, we can conclude that we have identified a high

proportion of genes in strawberry related with the mechanism of defense response to *C. acutatum*, including new members of sensing machinery, signal transduction mechanisms, transcriptional control, and direct defenses. Part of these results have already been published (Casado-Díaz et al. 2006).

The annotation of our sequence collection has incorporated additional functional information for almost 75% of the strawberry unigenes. The low blast e-value found for most of the strawberry unigenes (e-value < E^{-20} for 1111 of the unigenes) suggest high conservation in the sequences of these unigenes between taxa (Figure 5). However the reduced contribution of strawberry sequences to the total of entries in UniProtKB (Table 4), especially to the reviewed sequences group (only 42 sequences from *F* × *ananassa* and *F. vesca*), strongly limited our information in strawberry. However, the public availability of the *F. vesca* complete genome sequence in 2011 (Shulaev et al. 2011) has provided a valuable resource to generate this functional information.

ESTs collections can be useful to obtain gene expression information (the so-called "digital-northern"). Thus a proportional correlation between the number of ESTs of a particular unigene and its mRNA abundance in the sample used for library construction, is assumed (Oblessuc et al. 2012). This effect can be exploited to calculate quantitative changes in transcriptome. Indeed, ESTs collections generated from non-normalized libraries can be used in this sense. In our study, however, during the construct of the subtractive libraries, a normalization step was applied in order to equilibrate the presence of genes more infrequently expressed (low copy genes), and to reduce the redundancy (Diatchenko et al. 1996, Mahalingam et al. 2003). It is of special interest to mention that unigenes found overrepresented in our data set represent genes that escape to the proper normalization process of the library, and although we cannot use them to calculate proper relative values, they are, indeed, highly responsive to the treatments.

The information generated by sequencing clones from the six libraries was a previous valuable step for the cDNA microarray platform construction. This microarray has been very useful in massive analysis of transcriptome changes produced in strawberry under biotic stress conditions, as it will be described in further chapters of this study.

Identification of defense-associated functions overrepresented in the strawberry ESTs collection

Functional annotation of the unigenes allowed us to know for the first time which physiological processes were overrepresented in the unigene set. In this sense, we focused in contigs containing 5 or more ESTs (Table 3). Molecular components responsible of reinforcing mechanical defenses, such as cell wall modification and phenylpropanoid metabolism enzymes, were regulated by defense-elicitor treatment. Thus, contigs 572, 582, 599, 520 and 541, which code for enzymes implicated in ligning and cellulose biosynthesis (cinnamyl alcohol dehydrogenases family (CAD), and cellulose synthase) were overrepresented in UT library (defense-elicitor treated), while contigs 646 and 632 (beta-xylosidases), coding for enzymes responsible for hydrolysis of cell wall components, were found overrepresented in UC library (mock treated). In strawberry, this activity has been clearly associated with softening of the ripen fruit, as beta-xylosidase transcripts and activity were absent in immature stages, and strongly increased from white to red stage (Martínez et al. 2004). In addition, beta-xylosidase transcript level and activity was clearly correlated with the softest cultivar Toyonaka, when compared with Camarosa, two strawberry cultivars with contrasting fruit firmness (Bustamante et al. 2006). In addition, ATP-citrate lyase A-3 (contig 516), implicated in wax biosynthetic process, was also detected in UT. As firmness of the cell wall is supposed to benefit plant defense by avoiding pathogen or pest invasion, increase of CAD and cellulose synthase activities will probably reinforce the shield structure, and it seems to be accompanied by a decrease in cell wall degrading enzymes such as beta-xylosidase. These results indicate that activation of strawberry defense responses by elicitor treatment implicate reinforcement of cell wall through both, new synthesis and reduction in

degradation of components of such mechanical barrier to prevent pathogen entrance.

In addition, overrepresentation of genes belonging to PR10 family (contigs 525, 551, 499, 554 and 358), and polyphenol oxidase (contig 509) was detected in UT. Curiously, one PR10 family member (contig 276) was mainly detected in mock treated library (UC) showing opposite expression pattern that the rest of the family members here detected. These genes are considered classical markers of plant activation of defense responses (van Loon et al. 2006), suggesting that the elicitor treatment has efficiently produced strawberry defense activation.

Response to oxidative stress was also activated during the treatments. Thus, overrepresentation of members of glutathione S-transferase family (contigs 57, 566 and 603), and other genes associated to REDOX protection such as oxygenase (contig 623), zinc-binding dehydrogenase (contig 620) and carbonic anhydrase 2 (contig 622) was found in response to treatments. Opposite behaviour was detected for metallothionein 2b (contig 657) and peroxidase-R1 (contig 278), which were detected in mock-derived libraries (UC and non infected (CC and AC), respectively). Interestingly, carbonic anhydrase 2 functions in innate immunity signaling and defense response to bacterium and fungus through the action of calmodulin proteins (Fett and Coleman 1994, Ma et al. 2008). Two members of this family of calcium binding proteins were also detected in response to elicitor treatment (UT, contig 533) and to *C. acutatum* infection (AI, contig 149), and might indicate that immunity activation occurs in strawberry by such signaling components. Additionally, contigs 602, 507 and 508, coding for glucosyltransferases, which have been clearly related to plant defense responses in plants (Zhang et al. 2007, von Saint Paul et al. 2011), were overrepresented in UT, and might be implicated in signal transduction mechanisms during the defense response in strawberry.

The above results demonstrate that the experimental design for UT library construction and the chemical treatments used for elicitation have produced a

strong defense response in the strawberry cellular suspensions treated, and have enriched our EST collection in defense related genes.

All in all, this strawberry ESTs collection provides an important source of genetic information related to plant defense responses in this crop. As previously stated, cultivated strawberry is an octoploid species with at least two genomes involved in its origin, one is thought to be an ancestor of *F. vesca* or *F. manchurica*, and the other an ancestor of *F. iinumae*, or potentially other species (Rousseau-Gueutin et al. 2008). Thus, identification in *F* × *ananassa* of alleles from genetic components of defenses represents a great potential that might be useful as traits for breading (Adams et al. 2003).

SECTION TWO:

STRAWBERRY MICROARRAY PLATFORM. Generation of a cDNA microarray platform based on the *F* × ananassa ESTs collection

RESULTS AND DISCUSSION

Probe set preparation, slide printing and spot quality validation

The analysis of genetic information obtained in the above Section One was used to generate a cDNA-based microarray platform. Thus, ESTs with biological function related to plant defenses were selected to build up the probe set for the microarray platform as described in Methods.

A first set of microarray printings was done to determine the best conditions of both cDNA concentration and purification method. Every slice, once printed, was stained with SYBRGreen I solution, washed with MiliQ water and scanned by GenePix 4000B (Axon). Figure 9, shows a brief view of the evaluation carried out during this process. Once amplified by PCR, we determined the most appropriate method for cDNA purification. Figure 9 (a and b) shows the effect of residual

salts in not properly purified samples. Thus, contaminants generated amorphous salt crystals, which disrupt spot morphology and blocked slide surface preventing appropriate DNA binding to slide. As a second step, once purified, appropriate concentration for cDNA samples was determined by printing serial dilutions of about 200 probes (Figure 9 c). Results suggested that a concentration between 200-300ng/ul was recommended to obtain uniform spot morphology and appropriate intensity, and to prevent the generation of any artifact (Figure 9 d).

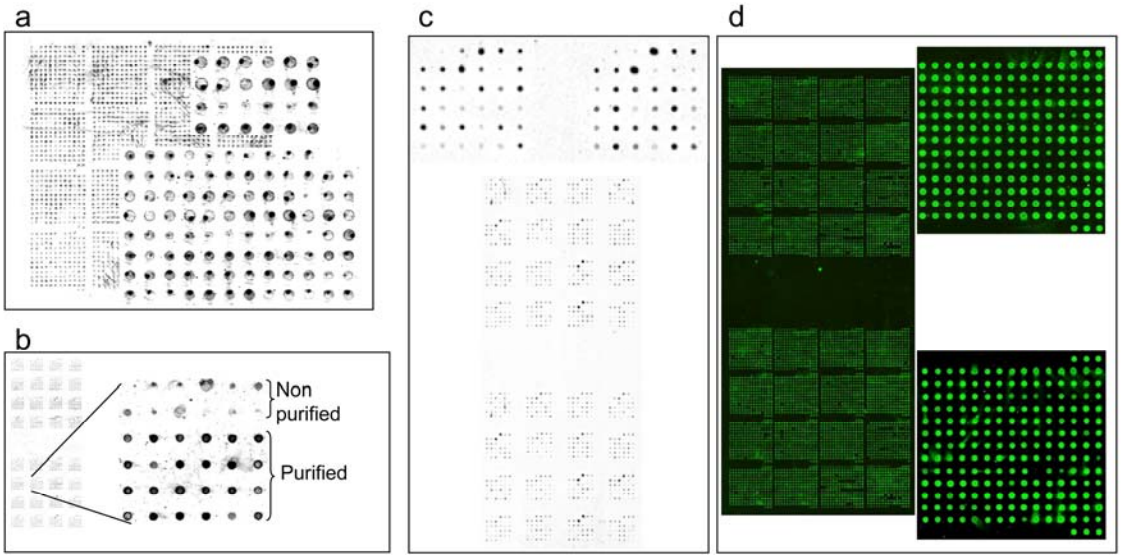


Figure 9. Determination of appropriate conditions of strawberry probeset for microarray printing. a) Deposition of salt crystals altering the spot morphology in non purified samples, b) blocking effect on slide surface by sample contaminants in non properly purified cDNAs, c) determination of appropriate cDNA concentration for microarray printing, d) slide printed with purified samples of appropriate cDNA concentration and stained with SYBRGreen I.

Quality evaluation of the strawberry microarray

Prior transcriptomic analyses, the quality of the microarray platform was evaluated using two comparisons: two biological replicates of the same cDNA-type labeled with the same dye, and the same cDNA from a third biological replicate labeled with either Alexa Fluor 555 or 647 dyes (the so called dyeswap). Figure 10 shows an example of a microarray hybridization image in which cDNA

from mock treated and infected plants (5 days post inoculation) of cultivar Camarosa was labeled with Alexa Fluor 555 and 647 dyes, respectively. The microarray quality was estimated using the Pearson correlation coefficient along the three replicates, including dye-swap, for the two strawberry cultivars analyzed, Camarosa and Andana. The Pearson correlation coefficient (R2) of log transformed normalized ratios in direct replicates was set around 0.9, and in dye-swap replicates around 0.8, which represents a good score of microarray quality (Figure 11). These values indicated that the cDNA microarray platform developed for cultivated strawberry had good quality and the results obtained here were repeatable and reproducible.

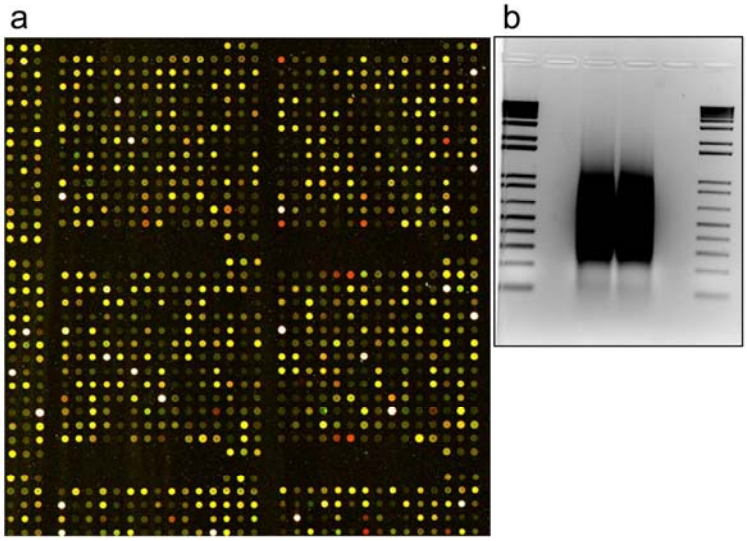


Figure 10. Strawberry microarray hybridization and cDNA image. (a) Microarray scanned after hybridization with cDNA from mock treated and infected plants (5 days post inoculation) of cultivar Camarosa labeled with Alexa Fluor 555 and 647 dyes, respectively. (b) Electrophoresis of such cDNAs showing molecules size range. DNA marker is 1Kb Plus (Invitrogen).

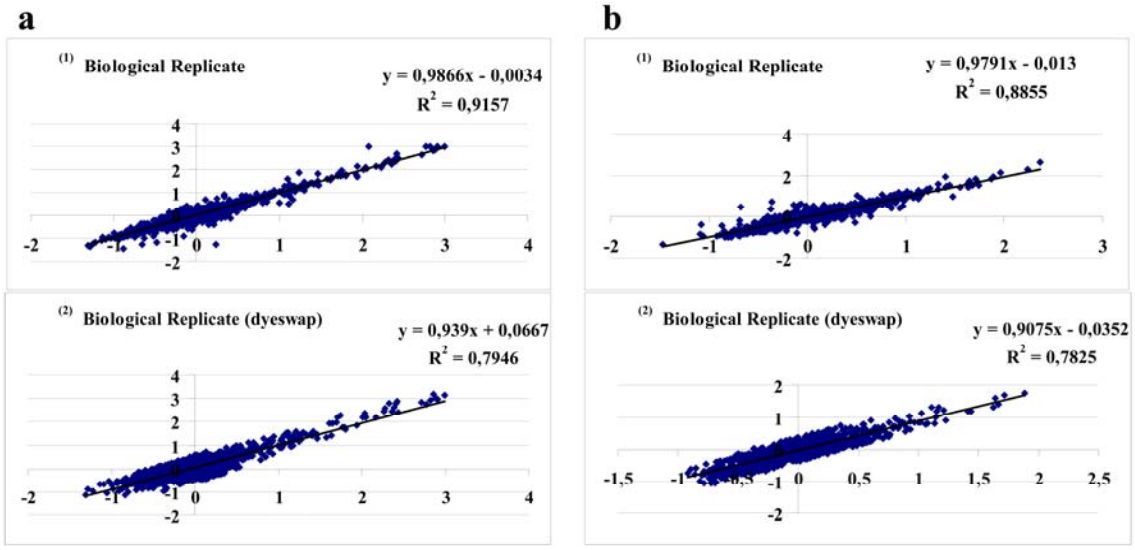


Figure 11. Strawberry microarray quality evaluation. The quality of the microarray platform was evaluated by the two comparisons: (1) two biological replicates of the same cDNA-type labeled with the same dye, and (2) the same cDNA from a third biological replicate labeled with either Alexa Fluor 555 or 647 dyes (dyeswap). The correlation coefficients of log transformed normalized ratios between the replicates and different dyes (dyeswap) were calculated. Microarray hybridizations were done for the two strawberry cultivars analyzed, Camarosa (a) and Andana (b).

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Chapter III

Identification and Evaluation of Superior Reference Genes for Transcript Normalization in Strawberry Plant Defense Responses

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ABSTRACT

In the last few years, an increasing number of important molecular studies in strawberry are being reported, as this worldwide horticultural important crop species has been proposed as an interesting model for the development of basic genomics and recombinant DNA studies among Rosaceae. Very often, these studies involve relative quantification of gene expression as this methodology is extensively used to estimate the expression of genes under experimental conditions of interest. However, its accuracy and reliability is dependent upon the choice of an optimal endogenous control gene. So far, there is no information available on suitable endogenous reference genes to be used for studies involving strawberry-pathogen interactions. The present data constitutes the first systematic study in strawberry to identify and validate optimal reference genes for accurate normalization of gene expression in strawberry plant defense response studies. Thirteen potential pre-selected strawberry reference genes, and different tissues and strawberry cultivars under biotic stress, ripening and senescence, and SA and JA treatments were considered. Evaluation of their goodness was deeply analyzed by five different methodologies available to date, and individual information was merged with appropriate algorithm to take advantage of the goodness offered by these five methods. The resulting superior reference genes is strongly recommended to be used as control genes for relative quantification of gene expression in strawberry plant-pathogen interaction and plant defense studies under all the experimental conditions here described, and also as a starting pool for assessing suitable reference genes under new conditions.

INTRODUCTION

Transcriptomic analyses are nowadays essentials to understand complex biological processes occurring in plants. Although massive techniques such as microarrays have provided a global view of the entire transcriptome regulation, the relative quantification of gene expression by quantitative reverse transcription (RTqPCR) is a fundamental step to validate microarrays data, and this technique is used as a primary source of in-depth molecular expression information for smaller set of genes due to its wide range of quantification, reproducibility, and higher precision and accuracy (Czechowski et al. 2004, Gachon et al. 2004, Bustin et al. 2005). However, this approach requires a set of very stably expressed reference genes for data normalization of the target gene under specific experimental conditions. Failure to use an appropriate reference or internal control gene may result in biased gene expression profiles, as well as low precision. Consequently, either only gross changes in gene expression level are declared statistically significant, or the pattern of gene expression is inaccurately characterized (Vandesompele et al. 2002, Bustin et al. 2009).

To date, some of the best known and most frequently used reference gene transcripts for RTqPCR in plants and animals include those coding for 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase, elongation factor-1α, actin, and α- and β-tubulin (Goidin et al. 2001, Bustin 2002, Kim et al. 2003, Andersen et al. 2004, Brunner et al. 2004, Dheda et al. 2004, Radonić et al. 2004, Guénin et al. 2009). So far, these genes have been considered stably expressed housekeeping genes, and they have been widely used as reference genes in plants. However, many reports have also indicated that their expression is unstable under some experimental conditions and plant systems, affecting the results and introducing a significant level of error when the expression pattern of a target gene has to be determined (Czechowski et al. 2005, Gutierrez et al. 2008). So, the finding and characterization of the most appropriate and good internal reference genes for normalization in every particular experimental plant system under study is a prerequisite, and a compulsory step for obtaining reliable and reproducible results,

and perform accurate RTqPCR analyses following the golden rules which have been detailed recently in Udvardi et al. (2008).

During the last few years, efforts have been made to identify suitable reference genes for quantification of gene expression in model plant species such as Arabidopsis (Hong et al. 2010), but also in crop plants, as pea (Die et al. 2010), banana (Podevin et al. 2012, Chen et al. 2011), sulla (Cordoba et al. 2011), zucchini (Obrero et al. 2011), and citrus (Mafra et al. 2012). However, no good reference genes have yet been identified and tested in many other species of high agricultural interest such as strawberry, a small fruit crop of great importance throughout the world (FAOSTAT Agriculture Data [http://faostat.fao.org/, updated 7 aug 2012]).

Due to its broad horticultural importance, strawberry has been proposed as an interesting model for the development of basic genomics and recombinant DNA studies among Rosaceae (Mezzetti 2009, Amil-Ruiz et al. 2011). Consequently, in the last few years, an increasing number of important molecular studies in this species are being reported. Many of these studies have performed RTqPCR analysis using traditional reference genes described in other plant species, to understand a wide variety of molecular events occurring in strawberry plant development, such as fruit ripening and fruit aroma production, and also in response to many biotic and abiotic stresses (Khan and Shih 2004, Guidarelli et al. 2011, Lin-Wang et al. 2010, Casado-Díaz et al. 2006, Encinas-Villarejo et al. 2009). However, little information is yet available on strawberry endogenous reference genes but none whatsoever is reported about their optimal suitability for comparative analyses and proper evaluation of target genes in this crop.

An appropriate reference gene should be expressed with minimal change regardless of the experimental conditions. Because there is no reference gene that is universally stable in expression, it is necessary to identify candidate genes specifically chosen for transcript normalization for the conditions under study (Wong and Medrano 2005, Hruz et al. 2011). Also, when using only one reference

gene, its stability cannot be properly evaluated. The use of multiple reference genes does not only produce more reliable data but permits an evaluation of the stability of these genes, as well.

In the present study, a subset of strawberry putatively good reference genes for RTqPCR normalization in plant defense studies were identified and tested in a range of forty-eight situations distributed along seven experimental conditions including fruit ripening stages, biotic stress after *Colletotrichum acutatum* infection, and treatments with plant hormones such as SA and MeJA. Also, different cultivars of strawberry (*Fragaria* × *ananassa*), and growth conditions were tested. The use of some of these suitable genes to ensure an accurate normalization of transcript level under a given condition in strawberry gene expression studies by RTqPCR technology is strongly recommended. Also, we provide primer sequences for these strawberry good reference genes.

RESULTS

Selection of candidate reference genes in strawberry for gene expression analysis.

Due to the lack of previous studies on reference genes for RTqPCR analysis in this crop, some strawberry candidate genes were pre-selected on the basis of information previously generated in our group, and obtained from a range of microarrays experiments (Amil-Ruiz et al. 2012, Amil-Ruiz et al., unpublished). Thus, strawberry genes were previously harvested due to their high degree of stability on gene expression among biological replicates and experimental conditions. Moreover, due to the fact that low abundant genes generally show high variation in their basal expression (Fan et al. 2009), only genes showing medium-high basal expression level were considered. From them, only genes whose primer designed fit the conditions described further below, were considered suitable. In addition, a pursuit of functional diversity among the chosen candidate genes was performed, as this is strongly recommended to avoid a putative co-

regulation effect among genes under evaluation in the particular experimental assay, and it is, in fact, a prerequisite to use one of the statistical procedures (the geNORM algorithm) reported to identify stably expressed genes (Vandesompele et al. 2002).

Under all these restrictive conditions, thirteen preselected candidate genes were finally chosen (Table 1). These genes encode molecular components associated to a wide variety of biological functions in plant cell physiology such as 18S rRNA (gene FaRIB413), a ribosome complex component; glyceraldehyde-3-phosphate dehydrogenase (genes FaGAPDH1 and FaGAPDH2), an essential enzyme for carbohydrate metabolism in cytoplasm; elongation factor-1α (gene FaEF1α), a component of the protein synthesis machinery; actin (gene FaACTIN), α-tubulin (gene FaTUBα) and β-tubulin (gene FaTUβ), major components of microfilament and microtubule of the cytoskeleton, respectively; the ubiquitin conjugating enzyme E2 (gene FaUBQ1), a basic component of the ubiquitin-mediated protein labeling system; chromatin remodeling protein CHC1 (gene FaCHC1), an essential part of the chromodomain remodeling complex; S-adenosyl-L-methionine-dependent methyltransferase (gene FaMT1), an enzyme implicated in secondary metabolism; a strawberry ortholog of the Arabidopsis AtBZIP61 regulatory transcription factor (gene FaBZIP1); a mitochondrial import inner membrane translocase (gene FaTIM1); a protein with a forkhead-associated domain and unknown molecular function (gene FaFHA1). In addition, the FaWRKY1 gene, a previously reported strawberry gene known to respond to all the different biological conditions used in this study (Encinas-Villarejo et al. 2009), was chosen as a target gene to test the validity of these strawberry candidate genes as good reference genes in RTqPCR analyses.

Primers designed of candidate reference genes

The RTqPCR primer pairs for each putative reference gene, as well as for FaWRKY1, were designed following common criteria, and were tested to generate clear and unique PCR products in RTqPCR reactions (Table 1 and Figure 1).

Table 1. Information of selected genes after evaluation, and characteristics of PCR products and primers used in this analysis.

Fragaria x *ananassa* gene ID	*Fragaria vesca* orthologe (a)	Gene description	Oligo orientation	Sequence (5´ - 3´)	Primer melting temp (ºC) (b)	Product size (bp)	Optimal anealing (ºC) (c)	PCR product melting temp (ºC) (d)	PCR efficiency ± SD (e)	Ref (f)
FaGAPDH2	gene07104	Glyceraldehyde-3-phosphate dehydrogenase	sense chain	CCCAAGTAAGGATGCCCCCATGTTCC	82,1	117	65	85	1,769 ± 0,028	Khan and Shih (2004)
			anti-sense chain	TTGGCAAGGGGAGCAAGACAGTTGGTAC	81,2					
FaUBQ1	gene08438	Ubiquitin E2	sense chain	CCCATCTCCGACAACCGCCACATCTAAA	83,1	130	66	89,5	1,727 ± 0,028	
			anti-sense chain	CCGCCGCCCCAATCTTCTGTACTCC	82,7					
FaGAPDH1	gene18492	Glyceraldehyde-3-phosphate dehydrogenase	sense chain	GGCTTCTATCTCAACCGGCTCGTCT	77,7	121	65	85	1,925 ± 0,025	Grellet-Bournonville et al. (2012)
			anti-sense chain	CTTCCCACTGCTCCCTGATCTCTGATAC	77,3					
FaEF1α	gene28639, gene28622, gene23217	Elongation factor 1-alpha	sense chain	TGGATTTGAGGGTGACAACATGA	73,1	145	65	87	1,798 ± 0,028	Guidarelli et al. (2011)
			anti-sense chain	GTATACATCCTGAAGTGGTAGACGGAGC	73,3					
FaTUBα	gene01798, gene05604, gene03851, gene26908	Tubulin alpha	sense chain	CATGGCTTGCTGTTTGATGTACCGTG	78,5	156	65	87	1,765 ± 0,027	
			anti-sense chain	GGGACAACAGTGGGTGGCTGGTAGT	79,1					
FaTUBβ	gene07781, gene13266, gene20192, gene08531, gene18775	Tubulin beta	sense chain	ACACTGTTGTGGAGCCTTACAATGCTAC	74,7	172	65	85,5	1,773 ± 0,024	
			anti-sense chain	GACATTGTTGCGGAGATCAAGTGAT	74,8					
FaACTIN	gene26612, gene18390, gene22626, gene18570, gene14112, gene01836	Actin	sense chain	GGGCCAGAAAGATGCTTATGTCGG	77	152	65	85	1,801 ± 0,029	Lin-Wang et al. (2010)
			anti-sense chain	GGGCAACACGAAGCTCATTGTAGAAC	76,2					
FaCHC1	gene25887	SWI/SNF complex component	sense chain	CATCTGTTTCCGCCACAACCTATACA	75,1	156	65	83,5	1,762 ± 0,03	
			anti-sense chain	TTTGTTTTTCTCTGAGTTGGCCATTAG	74,1					
FaMT1	gene10517	S-adenosyl-L-methionine-dependent	sense chain	AGGAGATAAGATAGCATTCGAAGTACCC	71,5	153	65	83,5	1,782 ± 0,028	
			anti-sense chain	CTGTACTTAGGATCACAAGGCTTGAAC	70,9					
FaBZIP1	gene17796	Basic leucine zipper transcription factor	sense chain	AGGGTCAACAAAACCAGAATGGGGATAA	77,7	151	65	85,5	1,712 ± 0,027	
			anti-sense chain	CTGCGTTCCAGCTCTGAAATGTATTGC	77,8					
FaTIM1	gene17570	Mitochondrial import inner membrane translocase	sense chain	GCTCCGCCACTTACGCCGCTAATT	80,2	100	65	86,5	1,811 ± 0,028	
			anti-sense chain	AGATCATCAGGCCCCGTCTTTCTCGTT	80					
FaFHA1	gene17571	SMAD/FHA domain-containing protein	sense chain	ATTGCATGCTAAGTTGGTGGAACAGTA	73,9	179	65	85,5	1,744 ± 0,025	
			anti-sense chain	GACCCTTAGACCTTGTGTTGATGACAA	74,6					
FaRIB413	gene33863	RNA interspacer (16S–23S) region	sense chain	ACCGTTGATTCGCACAATTGGTCATCC	83,4	149	65	91	1,784 ± 0,032	Casado-Díaz et al. (2006)
			anti-sense chain	TACTGCGGGTCGGCAATCGGACC	81,9					
FaWRKY1	gene07210	WRKY75 like transcription factor	sense chain	ACAGCAGTAAGATTAGGGATGAAGAAGGGAC	76,2	196	65	85,5	1,824 ± 0,025	Encinas-Villarejo et al. (2009)
			anti-sense chain	GCTTCTTCACATTGCAACCCTGATGCGTC	83,8					

(a) Genes found in *Fragaria vesca* genome that can be amplified by the designed primers pair are shown. (b) Teorical information of melting temperatures calculated by Oligo Primer Analisys software version 6.65 for each primer. (c) Recomened optimal anealing temperature was calcutalted by gradient PCR run on RTqPCR termocycler and subsequent PCR efficiency optimization. (d) PCR-product melting temperature as showed by melting curves at RTqPCR runs. (e) PCR efficiencies were calculated by LigRegPCR. (f) Known references that have use for the first time the same analyzed gene under evaluation in a RTqPCR assay.

All primers were conceived within the CDS of the selected genes, always avoiding regions expanding over conserved sequence domains or presenting high sequence similarity to other genes. Thus, for genes belonging to gene families or with identified multiple copies present in the genome of the wild strawberry (*F. vesca*), recently released (Shulaev et al. 2011), the least conserved region was used to assure amplification of a single gene by PCR. In four cases (FaEF1α, FaTUBα, FaTUBβ and FaACTIN), it was not possible to differentiate between either multicopy or nearly identical genes although unique amplicons were obtained (Table 1, Figure 1). In six cases including the control gene (FaGAPDH1, FaTUBβ, FaBZIP1, FaTIM1, FaFHA1, FaWRKY1) primers were designed to span an exon-exon junction.

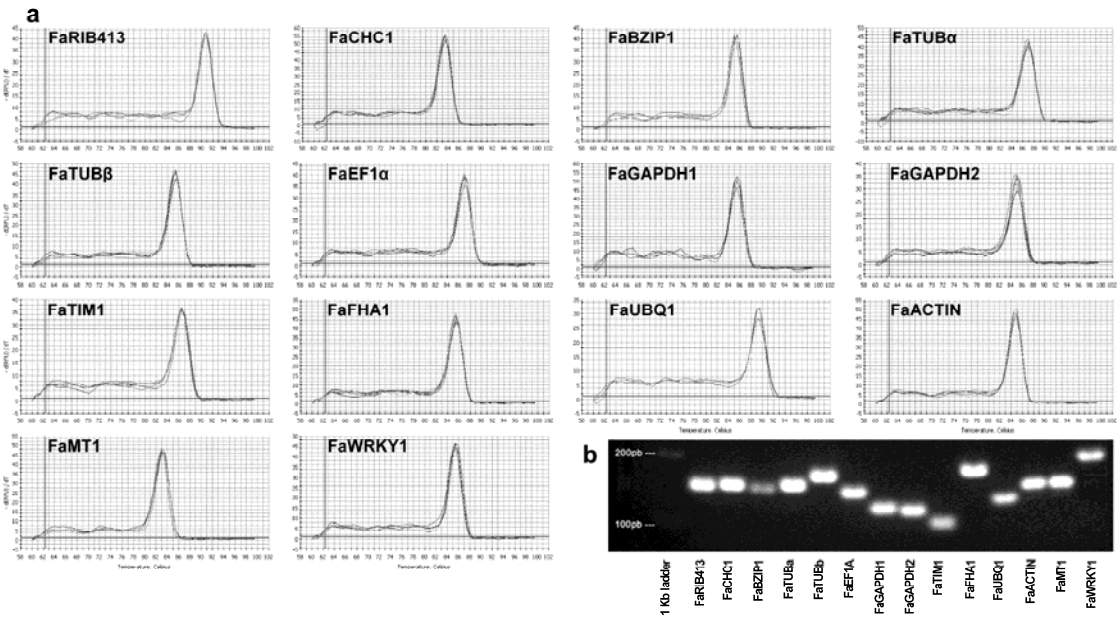


Figure 1. **Dissociation curves and agarose gel analysis of the amplicons tested in this study.** (a) Melting curve analysis of 13 potential reference genes along with control gene for validation (FaWRKY1) was carried out to confirm the absence of multiple amplicon species after RTqPCR. Each line represents a melting curve of amplicons from two technical replicates of two biological replicates in the given experiments. (b) Agarose gel electrophoresis of RTqPCR products after 40 cycles of PCR.

To ensure maximum specificity and efficiency during PCR amplification, primers were designed to have melting temperatures over 70 ºC, and were required to generate short amplicons, usually between 100 and 200bp (Table 1). The most appropriate annealing temperature for every primer pair was calculated by RTq-gradientPCR, and only primer pairs with optimal efficiency at annealing temperatures of above 65ºC were considered for subsequent RTqPCR analyses. Primer pair for gene FaRIB413 was previously designed in our group (Casado-Díaz et al. 2006), and tested to meet all of the above criteria. The specificity of the primers was tested by PCR using first-strand cDNAs synthesized from total RNA isolated from the biological samples. All the thirteen selected strawberry candidate reference genes, plus gene FaWRKY1, produced a single peak in the melting curve analysis (Figure 1). An additional electrophoresis analysis confirmed the presence of a single amplicon of the expected size.

The PCR efficiency of each primer pair was calculated using LinRegPCR, a method that utilizes absolute fluorescence data captured during the exponential phase of amplification of each real-time PCR reaction (Ramakers et al. 2003). Table 1 shows the calculated PCR efficiencies for the primer pairs we have studied. Each given efficiency value represents an average ± SD calculated from 192 amplification plots (i.e. two technical replicates of two biological replicates of a total of 48 different experimental conditions). For all primer pairs, values ranged from 1.712 to 1.925, with very low standard deviation. These values indicated comparable amplification efficiencies among the 96 diverse cDNA samples tested (Table 1), and suggested that the designed primer pairs efficiently amplified their target genes. Therefore, the mean primer pair efficiency value was considered for all subsequent studies, including estimations of the relative expression level of the reference genes under evaluation.

Experimental conditions and RNA preparation for RTqPCR

The suitability of the selected strawberry candidate reference genes for RTqPCR normalization was verified in several strawberry tissues as fruit, crown, petiole, in-vitro entire young plant and cellular suspensions, from different strawberry varieties and experimental conditions. Thus, a variety of physiological stages, as ripening, natural and controlled fungal infection, and hormonal treatments were also contemplated (Table 2). Two independent biological replicates were performed for each experimental condition. Them a significant number between 10 to 18 independent samples per experiment was analyzed (Table 2).

Total RNA was isolated from all strawberry samples, and the quality and quantity of the extracted RNA was determined spectrophotometrically by NanoDrop 1000 Spectrophotometer (Thermo scientific). All the extracted RNA samples showed high degree of purity, without residual contamination by organic compounds, accordingly to Accerbi et al. (2010). To assure equal concentration of RNA in all samples prior to the RT reactions, all RNA samples were diluted to 200ng/ul and reassessed three times in a serial dilution of 1:0, 1:5 and 1:25, to ensure fidelity of the measure. The integrity of each RNA sample was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Deutschland), all the samples showed RIN values over 8 (data not shown) and so, suitable for RTqPCR analysis.

All the RNA samples were tested to be free of genomic DNA contamination after DNase I treatment by performing a qPCR analysis using the primer pairs of FaGAPDH2 and FaRIB413 genes as control. Thus, amplicons corresponding to these two genes were undetectable in all the RNA samples after 40 cycles of these PCR reactions, either checked by qPCR or by agarose gel electrophoresis (data not shown). These results assured that amplicons generated by PCR amplification after the RT reaction of any RNA isolated from strawberry samples used for RTqPCR analysis was produced only from cDNA synthesis.

Table 2. Summary of strawberry varieties, tissues and experimental conditions used in this study.

Biological process	Cultivar	Culture type / Tissue	Biological stages / Time points after treatments	Experimental conditions	
Ripening and senescence	Camarosa	Fruit	G, W, R, OR and SE	Fruit ripening in field	RCF[a]
Defense against fungal infection	Camarosa	Fruit	Red stage fruits: Mock / Infected grades 1, 2, 3 and 4	Red fruit naturaly infected with *C. acutatum* in field	FCF[a, e]
Defense against fungal infection	Camarosa	Crown	Mock: 1, 3, 5 and 7dpi / Infected: 1, 3, 5 and 7dpi	Growth chamber *C. acutatum* infection under controled conditions	FCC[b, e]
Defense against fungal infection	Camarosa	Petiole	Mock: 1, 3, 5 and 7dpi / Infected: 1, 3, 5 and 7dpi	Growth chamber *C. acutatum* infection under controled conditions	FCP[b, d, e]
Defense against fungal infection	Andana	Petiole	Mock: 3, 5 and 7dpi / Infected: 1, 3, 5 and 7dpi	Growth chamber *C. acutatum* infection under controled conditions	FAP[d]
Hormone response	Camarosa	Young in-vitro plant	Mock: 12, 24, 48hpt / SA (5mM): 12, 24, 48hpt / MeJA (2mM): 12, 24, 48hpt	Mock, SA and MeJA treatment	HCY[c, e]
Hormone response	Chandler	Cellular suspensions	Mock: 4 and 6hpt / SA (0,75mM): 4 and 6hpt / MeJA (0,1mM): 4 and 6hpt	Mock, SA and MeJA treatment	HCC[c]

RCF, Ripening-Camarosa-Fruit; FCF, Fungal-Camarosa-Fruit; FCC, Fungal-Camarosa-Crown; FCP, Fungal-Camarosa-Petiole; FAP, Fungal-Andana Petiole; HCY, Hormone-Camarosa-Young-in-vitro; HCC, Hormone-Chandler-Cellular-suspensions. G1: smal green, W: white, R: red, OR: over-ripened, SE: senescent. (a) Comparison in gene expression between overripening-derived senescense and infection-derived necrosis, (b) Comparison in gene expression between cultivars under biotic stress, (c) Comparison in gene expression between cultivars under hormonal treatment, (d) Comparison in gene expression between different plant tissues, (e) Comparison in gene expression between infected and hormone treated plants.

Expression stability of the candidate reference genes under different experimental conditions

All preselected candidate reference genes were evaluated by RTqPCR analyses in all the experimental conditions summarized in Table 2. Four replicates per sample, this is, two technical replicates of each of two biological replicates, were used in this study. The generated results were subjected to the following previously reported analytical methods: analysis of ‘‘Stability index’’ (Brunner et al. 2004), geNORM (Vandesompele et al. 2002) implemented in qBASEplus software (Hellemans et al. 2007), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and the comparative Δ-Ct (Silver et al. 2006).

Statistical analysis of gene expression by “stability index” calculation

Figure 2 shows the expression level of candidate reference genes in the seven experimental conditions named in Table 2. Mean Cq values for each gene in every experimental condition, together with coefficient of variation (CV), slope, and stability index (SI), according to Brunner, (2004) are given in Table 3.

The analysis of variation, as reflected in the coefficient of variation (CV), showed highly predictability of all candidate reference genes in every of the seven experimental conditions, and also considering them all together, with almost all CV values below 6%. Exceptions were genes FaGAPDH1 and FaGAPDH2, within the ripening experimental conditions, and genes FaTUBα, FaGAPDH1, FaBZIP1 and FaTIM1, within the “all together” conditions (Table 3).

Table 3. (next page) Summary of statistics evaluating stability of gene expression. Genes are ordered into each experiment analyzed, top to bottom, from those tending to show the highest stability to those showing the lowest, based on the stability index. a) "n" represents the number of total data analyzed from each experiment, including all idependent samples that compose the experimental design, two biological replicates, each one run twice (four data per sample, two biological and two technical replicates of each). b) Obtained data based on analysis of Cq values. SD, standard deviation. CV, Coefficient of variation. c) Slope of regression of gene means. Intercepts are also given for the estimated regression lines. d) Stability index is the product of CV

Table 3. (cont.) and slope (multiplication of columns 3 and 4). Genes whose expression depends least in a predictable way on sample (slope), are preferred as controls. Asterisk marks those best candidate genes with stability index below 0,0x.

		Mean [b]	SD	CV (%)	Slope [c]	Intercept	Stability index [d]
	Ripening-Camarosa-Fruit (n=20) [a]						
*	FaRIB413	8,341	0,239	2,860	0,004	8,329	0,011
*	FaCHC1	23,085	0,201	0,869	0,021	23,024	0,018
*	FaTUBβ	22,334	0,359	1,609	0,015	22,289	0,024
	FaACTIN	23,894	0,309	1,294	0,144	24,326	0,186
	FaTIM1	22,602	0,359	1,587	0,151	23,054	0,239
	FaMT1	25,622	0,449	1,753	0,143	25,193	0,251
	FaEF1α	17,406	0,413	2,371	0,161	17,889	0,382
	FaFHA1	23,258	0,643	2,765	0,204	23,870	0,564
	FaTUBα	22,899	1,174	5,128	0,556	24,567	2,851
	FaBZIP1	30,089	1,485	4,936	0,607	28,270	2,994
	FaUBQ1	26,677	1,249	4,680	0,812	29,113	3,800
	FaGAPDH2	17,073	1,071	6,274	0,622	18,939	3,903
	FaGAPDH1	24,080	1,715	7,120	1,115	27,425	7,939
	Fungal infection-Camarosa-Fruit (n=20)						
*	FaGAPDH1	23,530	0,316	1,345	0,005	23,545	0,007
*	FaTUBα	21,462	0,322	1,499	0,019	21,518	0,028
*	FaUBQ1	25,599	0,405	1,583	0,047	25,458	0,074
	FaGAPDH2	16,274	0,331	2,031	0,062	16,090	0,125
	FaACTIN	23,539	0,314	1,335	0,136	23,133	0,181
	FaEF1α	16,556	0,250	1,510	0,130	16,166	0,196
	FaTIM1	24,031	0,372	1,549	0,131	23,638	0,203
	FaCHC1	23,929	0,467	1,953	0,121	23,568	0,235
	FaTUBβ	21,668	0,387	1,784	0,133	21,271	0,236
	FaBZIP1	27,780	0,478	1,719	0,164	27,288	0,282
	FaFHA1	23,606	0,545	2,308	0,213	22,969	0,490
	FaRIB413	8,635	0,323	3,736	0,158	8,161	0,590
	FaMT1	25,910	0,745	2,876	0,425	24,635	1,222
	Fungal infection-Camarosa-Crown (n=32)						
*	FaUBQ1	27,734	0,486	1,752	0,037	27,567	0,065
*	FaRIB413	7,873	0,241	3,057	0,027	7,752	0,083
	FaGAPDH1	25,569	0,453	1,771	0,064	25,282	0,113
	FaCHC1	24,988	0,492	1,968	0,067	24,687	0,131
	FaEF1α	17,786	0,386	2,173	0,062	17,509	0,134
	FaGAPDH2	19,286	0,352	1,825	0,090	18,880	0,165
	FaMT1	22,968	0,571	2,486	0,068	22,664	0,168
	FaFHA1	23,875	0,651	2,728	0,062	24,156	0,170
	FaTIM1	25,885	0,813	3,139	0,116	25,363	0,364
	FaTUBβ	22,086	0,622	2,818	0,136	21,472	0,384
	FaTUBα	20,298	0,585	2,883	0,136	19,687	0,392
	FaACTIN	24,440	0,563	2,303	0,211	23,493	0,485
	FaBZIP1	25,229	0,929	3,682	0,279	23,975	1,026
	Fungal infection-Camarosa-Petiole (n=32)						
*	FaTUBα	20,767	0,423	2,036	0,007	20,737	0,014
*	FaACTIN	23,676	0,364	1,536	0,013	23,528	0,020
*	FaRIB413	8,816	0,237	2,685	0,027	8,695	0,072
*	FaBZIP1	24,874	0,620	2,493	0,034	25,025	0,084
*	FaEF1α	17,574	0,437	2,485	0,034	17,727	0,084
	FaGAPDH2	18,321	0,357	1,946	0,064	18,611	0,125
	FaMT1	22,583	0,517	2,288	0,065	22,293	0,148
	FaTUBβ	22,009	0,574	2,609	0,100	21,561	0,260
	FaCHC1	24,874	0,735	2,954	0,139	25,499	0,411
	FaUBQ1	27,470	0,766	2,788	0,169	28,246	0,472
	FaTIM1	25,223	0,755	2,993	0,193	26,091	0,577
	FaFHA1	24,264	0,774	3,191	0,224	25,270	0,713
	FaGAPDH1	25,419	1,120	4,405	0,263	26,600	1,156

		Mean [b]	SD	CV (%)	Slope [c]	Intercept	Stability index [d]
	Fungal infection-Andana-Petiole (n=28)						
*	FaGAPDH1	26,129	0,632	2,418	0,004	26,143	0,009
*	FaGAPDH2	18,484	0,374	2,024	0,025	18,585	0,051
*	FaACTIN	23,640	0,428	1,812	0,030	23,760	0,054
*	FaEF1α	18,780	0,417	2,219	0,026	18,886	0,059
*	FaMT1	23,992	0,506	2,108	0,028	23,879	0,060
*	FaFHA1	24,251	0,570	2,349	0,036	24,107	0,085
	FaTUBβ	21,575	0,374	1,734	0,090	21,216	0,155
	FaCHC1	26,339	0,404	1,536	0,120	26,816	0,184
	FaBZIP1	26,410	0,446	1,688	0,110	25,971	0,185
	FaTIM1	26,864	0,481	1,790	0,116	26,401	0,207
	FaUBQ1	27,650	0,642	2,322	0,115	27,085	0,266
	FaRIB413	8,790	0,444	5,053	0,061	9,036	0,310
	FaTUBα	20,281	0,595	2,931	0,211	19,436	0,620
	Hormonal treatment-Camarosa-Young in-vitro plant (n=36)						
*	FaGAPDH1	25,817	0,479	1,856	0,024	25,938	0,045
*	FaUBQ1	28,954	0,518	1,789	0,026	29,085	0,047
*	FaGAPDH2	19,183	0,278	1,451	0,038	18,993	0,055
*	FaRIB413	8,838	0,523	5,912	0,016	8,760	0,093
	FaCHC1	26,297	0,482	1,832	0,080	25,895	0,147
	FaTUBα	23,058	0,586	2,542	0,093	22,591	0,237
	FaFHA1	25,649	0,615	2,396	0,101	25,145	0,242
	FaEF1α	18,593	0,442	2,375	0,119	17,996	0,284
	FaMT1	25,669	0,726	2,829	0,165	24,846	0,466
	FaTIM1	27,336	0,800	2,928	0,176	26,457	0,514
	FaTUBβ	23,573	0,775	3,286	0,218	22,484	0,716
	FaBZIP1	27,459	0,845	3,079	0,262	26,150	0,806
	FaACTIN	25,122	0,979	3,899	0,325	23,499	1,265
	Hormonal treatment-Chandler-Cellular suspensions (n=24)						
*	FaTIM1	25,850	0,974	3,766	0,003	25,862	0,013
*	FaGAPDH2	17,889	0,300	1,679	0,013	17,843	0,022
*	FaRIB413	8,426	0,299	3,551	0,021	8,498	0,073
*	FaUBQ1	27,222	0,566	2,079	0,039	27,322	0,081
	FaCHC1	24,163	0,427	1,767	0,129	23,712	0,228
	FaBZIP1	25,344	0,523	2,063	0,163	25,914	0,336
	FaEF1α	16,478	0,413	2,505	0,151	15,950	0,377
	FaTUBα	20,364	0,539	2,649	0,236	19,538	0,625
	FaMT1	24,533	0,550	2,240	0,282	23,545	0,632
	FaFHA1	23,116	0,612	2,646	0,246	22,256	0,650
	FaACTIN	23,145	0,776	3,352	0,343	21,943	1,151
	FaGAPDH1	20,908	0,836	3,999	0,401	22,313	1,605
	FaTUBβ	20,592	0,964	4,681	0,436	19,067	2,040
	All seven experiments (n=192)						
*	FaACTIN	24,011	0,883	3,676	0,004	23,905	0,015
	FaRIB413	8,542	0,490	5,736	0,056	8,306	0,323
	FaTUBβ	22,073	1,067	4,835	0,069	22,252	0,333
	FaEF1α	17,716	0,904	5,100	0,082	17,270	0,416
	FaMT1	24,338	1,399	5,747	0,097	24,857	0,560
	FaFHA1	24,140	1,037	4,298	0,144	23,426	0,619
	FaTUBα	21,292	1,305	6,131	0,158	21,937	0,970
	FaGAPDH1	24,722	1,856	7,509	0,157	25,119	1,175
	FaUBQ1	27,492	1,134	4,124	0,295	26,149	1,217
	FaGAPDH2	18,270	1,063	5,818	0,267	17,007	1,551
	FaCHC1	25,000	1,189	4,756	0,333	23,479	1,583
	FaBZIP1	26,547	1,789	6,741	0,489	28,697	3,297
	FaTIM1	25,650	1,591	6,201	0,619	22,923	3,839

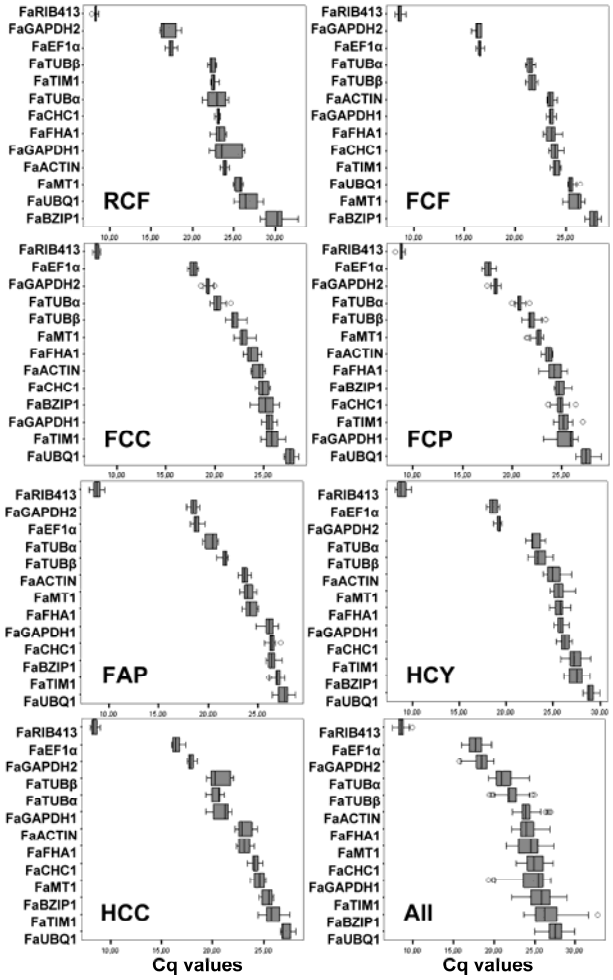


Figure 2. Expression levels of candidate reference genes in different experimental sets. Box plot graphs of Cq values for each reference gene tested in all strawberry samples and subsets. Cq values are inversely proportional to the amount of template and are shown as the first and third quartile. Vertical lines indicate the range of values, and median values are indicated by the black lines. Circles indicate outliers. RCF, Ripening-Camarosa-Fruit; FCF, Fungal-Camarosa-Fruit; FCC, Fungal-Camarosa-Crown; FCP, Fungal-Camarosa-Petiole; FAP, Fungal-Andana-Petiole; HCY, Hormone-Camarosa-Young-in-vitro; HCC, Hormone-Chandler-Cellular-suspensions; All, samples from all seven experiments analyzed together.

The mean expression level for each gene in each tested sample was regressed against the overall means for the different samples (Figure 3). The slope of the predicted regression lines provided an estimate of the degree to which the gene is

sensitive to general expression-promoting conditions. Assuming that both constancy over samples (low slope) and high predictability (low CV) are desired, we have used the "stability index" (SI) (product of slope and CV) to evaluate gene stability, according to Brunner, (2004). Genes with the lowest stability index will usually provide the best reference genes or controls.

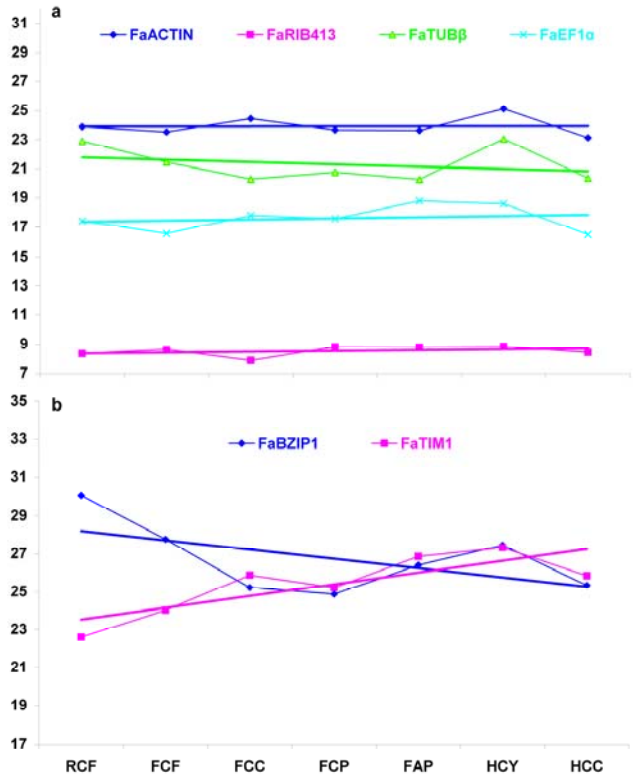


Figure 3. **Regression analysis for several genes showing predicted regression lines and actual means over all experiments.** The most stable and consistent control genes would have the lowest slope and closest fit to the regression line. (a) FaACTIN (first in top) had the highest stability and FaRIB413, as well as FaEF1α and FaTUBβ, have also very good values of stability (from first in bottom to second in top). (b) Genes FaBZIP1 and FaTIM1 had the lowest stability index. See Table 2 for descriptions of tissue samples that here are labelled only by such initials.

Results show that every experimental condition has several predicted candidate genes with a very good stability index (Table 3, marked by asterisks). Thus, during fruit ripening process, candidates FaRIB413, FaCHC1 and FaTUBβ

showed very good SI values (0.011, 0.018, and 0.024, respectively). Genes FaGAPDH1, FaTUBα and FaUBQ1 also shown to be excellent reference genes for fungal infection studies in red fruit (SI of 0.007, 0.028, and 0.074, respectively). In vegetative tissues challenged with the fungal, variations in number and diversity of convenient reference genes was also found. Thus, genes FaUBQ1 (SI, 0.065) and FaRIB413 (SI, 0.083), were found to be the best candidates for normalization on crown tissue of cultivar Camarosa but genes FaTUBα (SI, 0.014), FaACTIN (SI, 0.020), FaRIB413 (SI, 0.072), FaBZIP1 (SI, 0.084), FaEF1α (SI, 0.084) were also very good candidates on petiole tissue of this cultivar. However, on petiole tissue from cultivar Andana, the set of predicted good candidate reference genes turned to be mostly different. Thus, very good candidates were genes FaGAPDH1 (SI, 0.009), FaGAPDH2 (SI, 0.051), FaACTIN (SI, 0.054), FaEF1α (SI, 0.059), FaMT1 (SI, 0.060), and FaFHA1 (SI, 0.085). Only genes FaACTIN, FaEF1α were found to be the best reference genes for normalization in petiole tissue of both strawberry cultivars. In addition, genes FaUBQ1, FaGAPDH2, and FaRIB413 were found to be the best reference genes for SA and JA studies either in in-vitro plant (SI, 0.047, 0.055, and 0.093, respectively) or in cell suspension treatments (SI, 0.081, 0.022, and 0.073, respectively), and different cultivars. Genes FaGAPDH1 (SI, 0.045) and FaTIM1 (SI, 0.013) wear also found good candidates for the in-vitro plants and cellular suspensions experiments, respectively.

Also, we have considered an “all together” analysis where all seven experimental variants have been contemplated. In this analysis, gene FaACTIN showed the lowest stability index (SI, 0.015), and seems to be the best overall reference gene.

Variations among the best reference genes over the different tissues, developmental stages and environmental conditions studied have been previously found in other plant systems (Brunner et al. 2004). Also, differences in the expression pattern of genes related with plant defense response have been previously reported to operate over different strawberry tissues and cultivars

challenged with *C. acutatum* (Casado-Díaz et al. 2006). Thus, one should be aware of all these variations when different strawberry tissues or cultivars are studied either with or without challenged with pathogens, and appropriate reference genes for a given set of experimental conditions should be selected in order to obtain biologically significant changes in gene expression by real-time RTqPCR analyses.

Although, genes with the best values of "stability index" represent the best option for normalization, many of the other strawberry candidate genes can also be considered acceptable as controls but accordingly to the SI value obtained in this study (Table 3). In addition, the level of expression of the reference genes compared to that of the genes being analyzed is an important factor to be considered in certain cases (Brunner et al. 2004). In our study, the two most stably expressed strawberry genes in all seven experiments together represented opposite ends of the spectrum. FaRIB413 is highly expressed (mean Cq = 8.542), whereas FaACTIN is expressed at a much lower level (mean Cq = 24.011) (Table 3, Figure 3). Thus, they may be selected as appropriate reference genes to test high and low expressed target genes, respectively. Indeed, we had previously reported FaRIB413 as an internal control for expression studies in strawberry using several tissues and experimental conditions either in northern and RTqPCR analyses (Benítez-Burraco et al. 2003, Casado-Díaz et al. 2006, Encinas-Villarejo et al. 2009). Accordingly, we had already recommended that for studies of strawberry genes expressed at relatively low levels a dilution factor of up to 4000 times of the cDNA template samples should be carried out prior FaRIB413 amplification in order to use this gene as reference for good comparative Cq analyses (Casado-Díaz et al. 2006). This now can be improved using gene FaACTIN as control instead of FaRIB413.

Expression stability and calculation of hypothetical normalization factor by geNorm^{PLUS}

We calculate the stability coefficient (M values) and the coefficient of variation (CV values) of each gene, which are inversely related to their expression stability,

using the qBase software (Hellemans et al. 2007) but taking into account the previously calculated specific PCR efficiency of each gene. The average stability coefficient (M_A), defined as the average value of the M values (average pairwise variation of a gene with all other tested reference genes of all combinations of a gene and high-ranking reference genes), of the relative quantities of the thirteen genes under evaluation were analyzed with geNormPlus (qBase software, Vandesompele et al. 2002, Hellemans et al. 2007).

Figure 4 represents the average stability coefficients (M_A) of the thirteen candidate reference genes tested under every particular analyzed condition. All thirteen genes showed acceptable expression stabilities ($M_A \leq 1$), as described in Hellemans and coworkers for heterogeneous samples (Hellemans et al. 2007), with the exception of genes FaBZIP1 and FaGAPDH1 when all seven experimental conditions were analyzed together. Table 4 shows genes ranked by their M_A and CV values. The M_A results revealed that optimal candidate reference genes differed among the analyzed experimental conditions. Thus, FaACTIN (0.182) seems to be the most stable gene in fruit ripening analyses, meanwhile FaTIM1 (0.143) is in fruit natural infection, FaGAPDH2 (0.234) and FaRIB413 (0.300) in Camarosa crown and petiole infected tissues, respectively, FaMT1 (0.247) in Andana infected petiole, FaEF1α (0.242) in hormonal treatments of in-vitro plants, FaEF1α (0.242) and FaTUBα (0.242) in elicited cellular suspensions of cultivar Chandler, and finally, FaGAPDH2 (0.594) in the "all together" conditions.

However, two common well-established sets of candidates with good and poor stable values were detected in all the experimental conditions (Table 4). A similar result was detected when CV values were considered (Table 4). Thus, FaEF1α always appears well positioned in all the experimental conditions within the lowest M_A values, and also FaACTIN is very stably expressed in ripening and mostly all infection conditions (except in crown tissue of cultivar Camarosa) (Table 4). Oppositely, genes FaGAPDH1 and FaBZIP1 mostly showed high M_A values (a lower stability) in all analyzed conditions. Curiously, FaFHA1 is stably

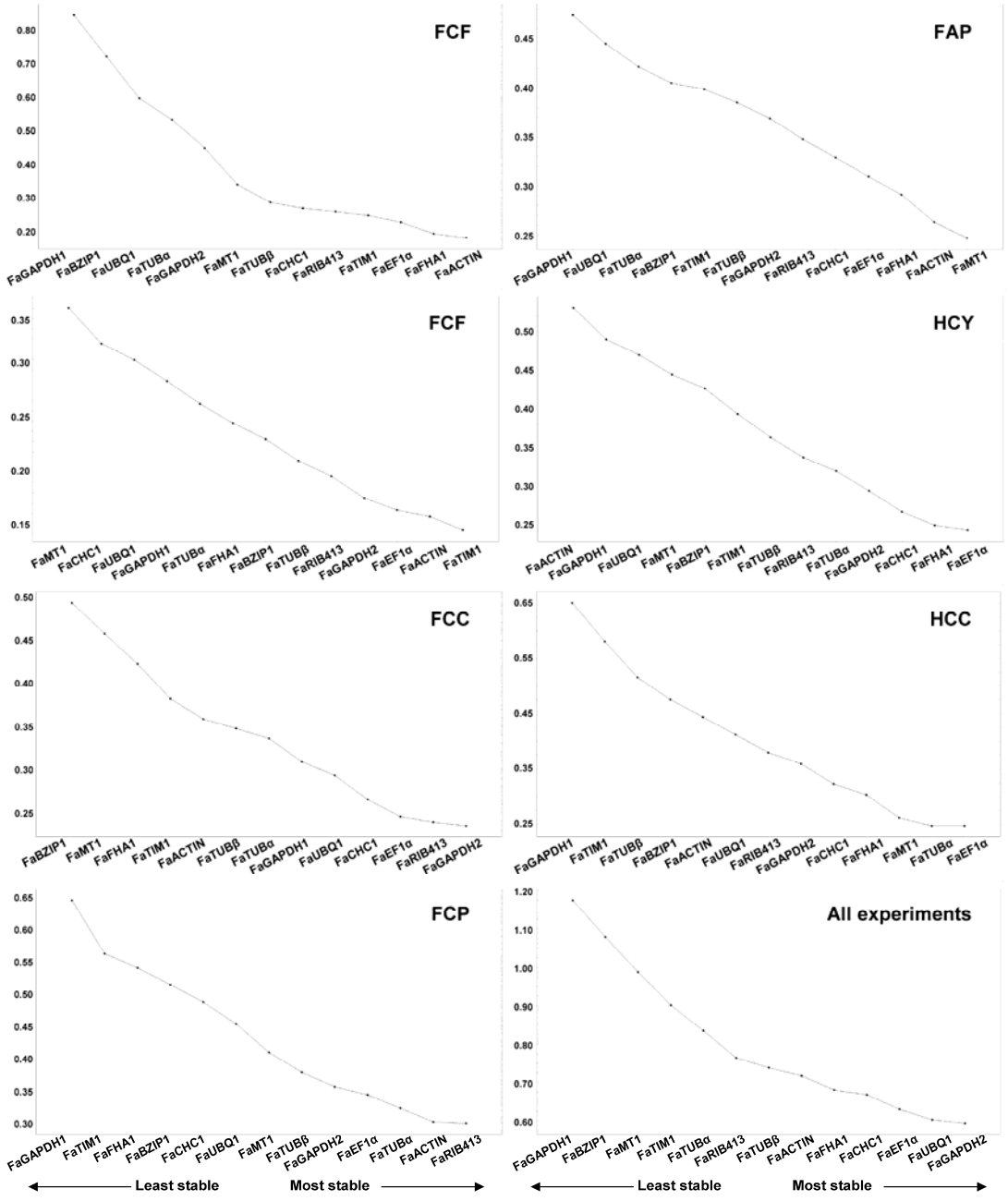


Figure 4. **Average expression stability value (M_A) of each gene.** Specific M_A values were calculated under seven single experimental conditions tested, and also considering all samples together. The average expression stability values (M_A) of genes tested under the given experimental conditions are shown as given by geNormPLUS analysis. The lowest M_A value indicates the most stable expression.

Table 4. Reference genes ranked in order to their average expression stability (MA) and coefficient of variation (CV) respectively. Increasing stability from left to right. See Table 2 for experiments description.

Ranking by M_A values from geNorm PLUS													
RCF	FaGAPDH1	FaBZIP1	FaUBQ1	FaTUBα	FaGAPDH2	FaMT1	FaTUBβ	FaCHC1	FaRIB413	FaTIM1	FaEF1α	FaFHA1	FaACTIN
	(0.845)	(0.712)	(0.597)	(0.533)	(0.449)	(0.34)	(0.287)	(0.269)	(0.259)	(0.248)	(0.228)	(0.195)	(0.182)
FCF	FaMT1	FaCHC1	FaUBQ1	FaGAPDH1	FaTUBα	FaFHA1	FaBZIP1	FaTUBβ	FaRIB413	FaGAPDH2	FaEF1α	FaACTIN	FaTIM1
	(0.361)	(0.325)	(0.31)	(0.289)	(0.267)	(0.248)	(0.232)	(0.211)	(0.196)	(0.175)	(0.163)	(0.156)	(0.143)
FCC	FaBZIP1	FaMT1	FaFHA1	FaTIM1	FaACTIN	FaTUBβ	FaTUBα	FaGAPDH1	FaUBQ1	FaCHC1	FaEF1α	FaRIB413	FaGAPDH2
	(0.493)	(0.458)	(0.422)	(0.382)	(0.359)	(0.348)	(0.336)	(0.309)	(0.293)	(0.266)	(0.245)	(0.239)	(0.234)
FCP	FaGAPDH1	FaTIM1	FaFHA1	FaBZIP1	FaCHC1	FaUBQ1	FaMT1	FaTUBβ	FaGAPDH2	FaEF1α	FaTUBα	FaACTIN	FaRIB413
	(0.645)	(0.563)	(0.541)	(0.515)	(0.488)	(0.454)	(0.41)	(0.379)	(0.357)	(0.345)	(0.324)	(0.302)	(0.3)
FAP	FaGAPDH1	FaUBQ1	FaTUBα	FaBZIP1	FaTIM1	FaTUBβ	FaGAPDH2	FaRIB413	FaCHC1	FaEF1α	FaFHA1	FaACTIN	FaMT1
	(0.474)	(0.445)	(0.421)	(0.405)	(0.399)	(0.385)	(0.369)	(0.348)	(0.329)	(0.31)	(0.291)	(0.263)	(0.247)
HCY	FaACTIN	FaGAPDH1	FaUBQ1	FaMT1	FaBZIP1	FaTIM1	FaTUBβ	FaRIB413	FaTUBα	FaGAPDH2	FaCHC1	FaFHA1	FaEF1α
	(0.531)	(0.49)	(0.469)	(0.444)	(0.426)	(0.393)	(0.362)	(0.337)	(0.319)	(0.293)	(0.266)	(0.248)	(0.242)
HCC	FaGAPDH1	FaTIM1	FaTUBβ	FaBZIP1	FaACTIN	FaUBQ1	FaRIB413	FaGAPDH2	FaCHC1	FaFHA1	FaMT1	FaTUBα	FaEF1α
	(0.651)	(0.581)	(0.516)	(0.476)	(0.445)	(0.413)	(0.38)	(0.359)	(0.323)	(0.304)	(0.262)	(0.247)	(0.247)
All samples	FaGAPDH1	FaBZIP1	FaMT1	FaTIM1	FaTUBα	FaRIB413	FaTUBβ	FaACTIN	FaFHA1	FaCHC1	FaEF1α	FaUBQ1	FaGAPDH2
	(1.174)	(1.079)	(0.987)	(0.901)	(0.835)	(0.764)	(0.738)	(0.717)	(0.681)	(0.669)	(0.631)	(0.603)	(0.594)
Ranking by CV values from geNorm PLUS													
RCF	FaGAPDH1	FaBZIP1	FaUBQ1	FaMT1	FaTUBα	FaGAPDH2	FaTUBβ	FaCHC1	FaRIB413	FaTIM1	FaFHA1	FaACTIN	FaEF1α
	(0.844)	(0.703)	(0.493)	(0.487)	(0.441)	(0.337)	(0.299)	(0.292)	(0.234)	(0.17)	(0.17)	(0.155)	(0.093)
FCF	FaMT1	FaGAPDH1	FaTUBα	FaCHC1	FaUBQ1	FaFHA1	FaTIM1	FaACTIN	FaBZIP1	FaTUBβ	FaRIB413	FaGAPDH2	FaEF1α
	(0.352)	(0.236)	(0.219)	(0.206)	(0.188)	(0.171)	(0.144)	(0.139)	(0.118)	(0.116)	(0.116)	(0.1)	(0.073)
FCC	FaBZIP1	FaMT1	FaFHA1	FaTIM1	FaUBQ1	FaCHC1	FaACTIN	FaGAPDH1	FaTUBβ	FaGAPDH2	FaTUBα	FaEF1α	FaRIB413
	(0.374)	(0.369)	(0.311)	(0.278)	(0.244)	(0.204)	(0.204)	(0.201)	(0.164)	(0.147)	(0.145)	(0.136)	(0.111)
FCP	FaGAPDH1	FaMT1	FaFHA1	FaCHC1	FaTIM1	FaTUBβ	FaBZIP1	FaACTIN	FaUBQ1	FaTUBα	FaEF1α	FaRIB413	FaGAPDH2
	(0.758)	(0.372)	(0.325)	(0.308)	(0.304)	(0.297)	(0.257)	(0.246)	(0.237)	(0.205)	(0.191)	(0.166)	(0.136)
FAP	FaGAPDH1	FaUBQ1	FaTUBα	FaRIB413	FaACTIN	FaCHC1	FaGAPDH2	FaMT1	FaBZIP1	FaTIM1	FaTUBβ	FaFHA1	FaEF1α
	(0.393)	(0.319)	(0.266)	(0.24)	(0.239)	(0.215)	(0.206)	(0.205)	(0.201)	(0.183)	(0.175)	(0.154)	(0.12)
HCY	FaACTIN	FaGAPDH1	FaTIM1	FaUBQ1	FaMT1	FaBZIP1	FaRIB413	FaTUBβ	FaTUBα	FaGAPDH2	FaFHA1	FaCHC1	FaEF1α
	(0.375)	(0.373)	(0.34)	(0.295)	(0.282)	(0.277)	(0.224)	(0.203)	(0.181)	(0.18)	(0.173)	(0.15)	(0.122)
HCC	FaGAPDH1	FaTIM1	FaTUBβ	FaACTIN	FaBZIP1	FaMT1	FaGAPDH2	FaUBQ1	FaFHA1	FaRIB413	FaTUBα	FaEF1α	FaCHC1
	(0.685)	(0.47)	(0.406)	(0.314)	(0.282)	(0.254)	(0.229)	(0.223)	(0.209)	(0.2)	(0.176)	(0.159)	(0.155)
All samples	FaGAPDH1	FaTIM1	FaMT1	FaBZIP1	FaCHC1	FaTUBα	FaGAPDH2	FaUBQ1	FaRIB413	FaTUBβ	FaACTIN	FaFHA1	FaEF1α
	(1.703)	(1.072)	(0.721)	(0.595)	(0.581)	(0.533)	(0.521)	(0.474)	(0.419)	(0.376)	(0.332)	(0.279)	(0.259)

expressed in all conditions except in all infected tissues from cultivar Camarosa, and FaRIB413 is also very stable but only in infected crown and petiole tissues from the same cultivar. On the other hand, gene FaTIM1 presented high M_A values in all conditions but the two fruit experiments, in which showed to be very stable, and gene FaMT1 presented low stability in all Camarosa experimental conditions, but low M_A values when cultivar Andana and Chandler is considered.

We have also determined both the optimal and the minimal number of reference genes needed to calculate a hypothetical optimal normalization factor suitable in each analyzed condition, as described by Vandesompele (Vandesompele et al. 2002). Figure 5, shows that the optimal number of these needed reference genes differed in each experimental conditions but a combination of them is assumed to be an ideal reference gene. Thus, in fruit ripening analyses, $V_{5/6}$ was the lowest pairwise variation value (0.041). Therefore, the hypothetical normalization factor in these experimental conditions would be the geometric mean of the five or six more stable genes (see Figure 4 and Table 4, for the ranking of more stable genes for this and other experimental condition). Other lowest pairwise variation values were, $V_{11/12}$ (0.03) for the infected fruit experiment, $V_{8/9}$ (0.036) and $V_{11/12}$ (0.047) for Camarosa crown and petiole infected tissues, respectively, $V_{9/10}$ (0.035) for Andana infected petioles, $V_{9/10}$ (0.043) for hormonal treatment of in-vitro plants experiment, $V_{6/7}$ (0.053) for elicited cellular suspensions, and finally, $V_{7/8}$ (0.086) when all experiments were considered together.

In practical, however, the number of genes required should be low enough to make experimental procedures affordable, and high enough to merit confidence in the conclusions. This means that if the pairwise variation value for n genes is below the recommended cut-off of 0.15, additional genes are considered not to considerably improve normalization (Vandesompele et al. 2002). Thus, the minimal number of reference candidates in each single experiment was determined as two in all the experimental conditions (marked with an arrowhead in Figure 5) but four in the all-together conditions. In each experimental

condition, these genes were FaACTIN and FaFHA1 ($V_{2/3}$ value of 0.098) for fruit ripening, FaTIM1 and FaACTIN ($V_{2/3}$ value of 0.055) for fruit infection, FaGAPDH2 and FaRIB413 ($V_{2/3}$ value of 0.078) for Camarosa crown infection, FaRIB413 and FaACTIN ($V_{2/3}$ value of 0.116) for Camarosa petiole infection, FaMT1 and FaACTIN ($V_{2/3}$ value of 0.112) for Andana petiole infection, FaEF1α and FaFHA1 ($V_{2/3}$ value of 0.095) for in-vitro plants treated with hormones, and FaEF1α and FaTUBα ($V_{2/3}$ value of 0.091) for elicited cellular suspensions. For the all-together conditions the minimal reference genes were FaGAPDH2, FaUBQ1, FaEF1α, and FaCHC1 ($V_{4/5}$ value of 0.113).

Evaluation of expression stability by ΔCt method, Normfinder and BestKeeper approaches

In order to accurately assess the usefulness of the thirteen preselected reference genes, other three analytical methods were applied to our data set. The comparative ΔCt method (Silver et al. 2006), which ranks the reference genes by their mean standard deviation in the pairwise comparisons, the NormFinder (Andersen et al. 2004), which ranks the set of candidate normalization genes according to their expression stability in a given sample set and a given experimental design, and the Bestkeeper algorithm (Pfaffl et al. 2004), which performs pairwise comparison using the geometric mean of the Cp (Cq), values.

Table 5 shows the results obtained from all three methods. Both ΔCt and NormFinder analyses coincided by selecting the best set of reference genes for each experimental condition. Essentially, the best were FaTIM1 for ripening, FaEF1α for infected fruits, FaEF1α and FaGAPDH2 for Camarosa crown and petiole infected tissues, respectively, FaACTIN for Andana infected petioles, FaRIB413 for in-vitro hormone-treated plants, FaRIB413 for cellular suspension treatments, and finally, FaEF1α when all the experiments were analyzed together. Similar results were also obtained when BestKeeper algorithm was used. Taken together the results from these three methodologies, gene FaEF1α seemed to be the most stably expressed reference gene meanwhile genes FaGAPDH1 and FaBZIP1 were the least stable ones.

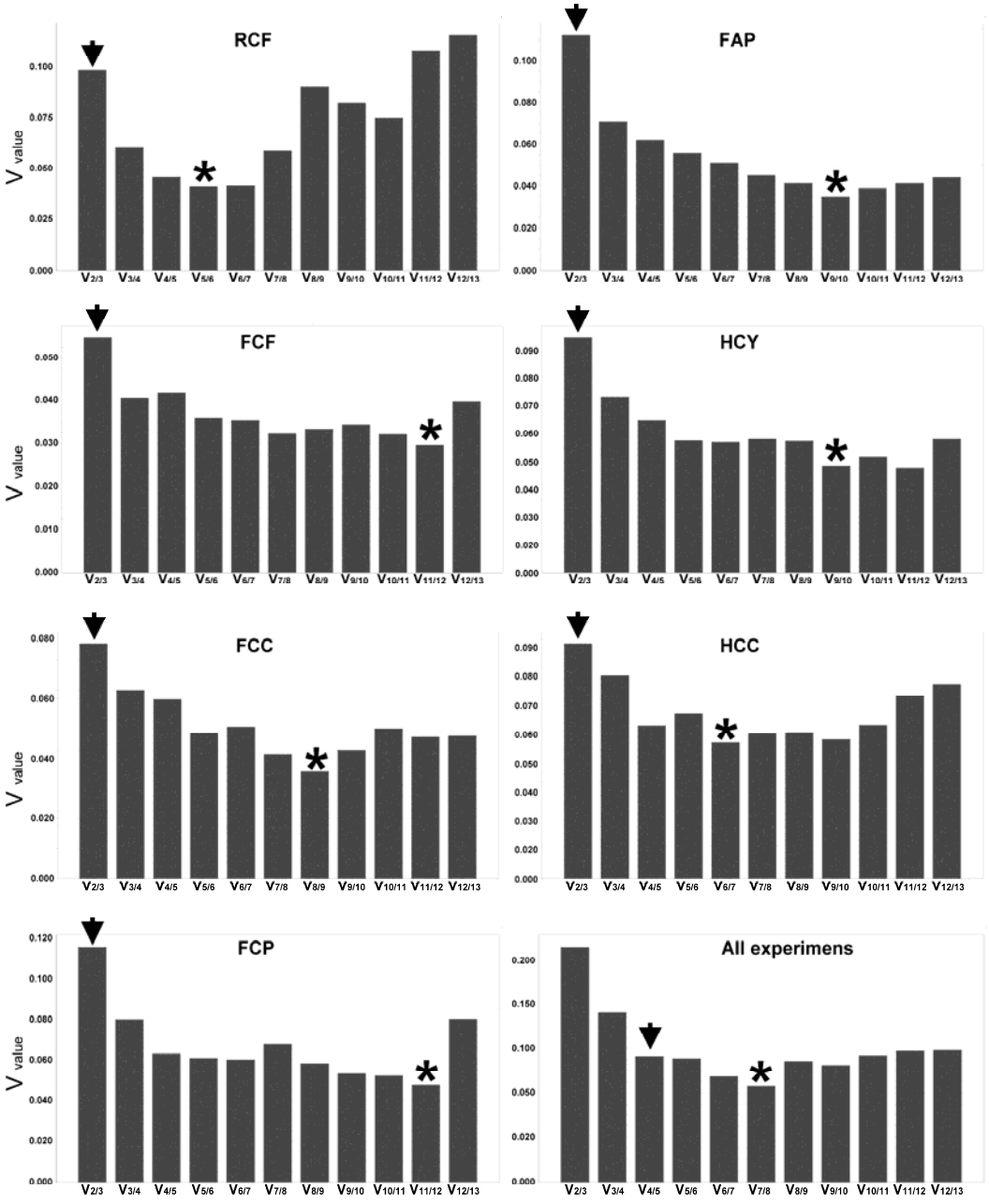


Figure 5. **Determination of the number of genes to calculate a hypothetical normalization factor.** Pairwise variation (Vn/n+1) analysis was carried out to determine de number of reference genes required for accurate normalization. An asterisk indicates the lowest V value in each experiment. An arrowhead indicates the minimum number of genes required to pass the suggested cut-off value (0.15) [4]. See Table 2 for experiments description.

Table 5. Ranking of reference genes resulting from evaluation of their expression stability in given experiments by ΔCt, Normfinder and BestKeeper methods. Increasing stability from left to right.

Ranking by STDEV values from ΔCt													
RCF	FaBZIP1	FaGAPDH1	FaUBQ1	FaTUBα	FaCHC1	FaMT1	FaFHA1	FaGAPDH2	FaTUBβ	FaEF1α	FaRIB413	FaACTIN	FaTIM1
	(1.64)	(1.60)	(1.29)	(1.15)	(1.03)	(0.99)	(0.99)	(0.96)	(0.93)	(0.85)	(0.84)	(0.82)	(0.82)
FCF	FaBZIP1	FaMT1	FaFHA1	FaTIM1	FaCHC1	FaUBQ1	FaGAPDH2	FaTUBβ	FaTUBα	FaRIB413	FaGAPDH1	FaACTIN	FaEF1α
	(0.72)	(0.69)	(0.65)	(0.62)	(0.62)	(0.61)	(0.59)	(0.57)	(0.57)	(0.54)	(0.54)	(0.50)	(0.47)
FCC	FaBZIP1	FaFHA1	FaGAPDH1	FaTIM1	FaTUBβ	FaCHC1	FaACTIN	FaRIB413	FaMT1	FaGAPDH2	FaTUBα	FaUBQ1	FaEF1α
	(1.12)	(1.08)	(1.07)	(1.04)	(0.99)	(0.96)	(0.96)	(0.95)	(0.94)	(0.88)	(0.88)	(0.88)	(0.82)
FCP	FaGAPDH1	FaFHA1	FaTIM1	FaBZIP1	FaTUBβ	FaCHC1	FaMT1	FaACTIN	FaUBQ1	FaEF1α	FaTUBα	FaRIB413	FaGAPDH2
	(1.28)	(1.11)	(1.07)	(1.04)	(1.02)	(1.00)	(0.96)	(0.90)	(0.89)	(0.89)	(0.87)	(0.83)	(0.76)
FAP	FaGAPDH1	FaFHA1	FaUBQ1	FaBZIP1	FaTIM1	FaRIB413	FaGAPDH2	FaCHC1	FaMT1	FaTUBα	FaEF1α	FaTUBβ	FaACTIN
	(0.94)	(0.82)	(0.80)	(0.75)	(0.74)	(0.74)	(0.72)	(0.70)	(0.68)	(0.68)	(0.60)	(0.59)	(0.59)
HCY	FaACTIN	FaTIM1	FaBZIP1	FaGAPDH1	FaTUBβ	FaMT1	FaUBQ1	FaFHA1	FaCHC1	FaTUBα	FaEF1α	FaGAPDH2	FaRIB413
	(0.89)	(0.85)	(0.83)	(0.83)	(0.82)	(0.82)	(0.80)	(0.80)	(0.73)	(0.71)	(0.71)	(0.70)	(0.68)
HCC	FaGAPDH1	FaTIM1	FaTUBβ	FaACTIN	FaUBQ1	FaMT1	FaGAPDH2	FaBZIP1	FaFHA1	FaTUBα	FaCHC1	FaEF1α	FaRIB413
	(1.14)	(1.13)	(1.06)	(0.92)	(0.89)	(0.84)	(0.81)	(0.79)	(0.78)	(0.75)	(0.72)	(0.70)	(0.70)
All samples	FaBZIP1	FaGAPDH1	FaMT1	FaTIM1	FaTUBα	FaCHC1	FaGAPDH2	FaUBQ1	FaTUBβ	FaRIB413	FaFHA1	FaACTIN	FaEF1α
	(2.02)	(1.90)	(1.79)	(1.70)	(1.50)	(1.39)	(1.37)	(1.34)	(1.34)	(1.32)	(1.28)	(1.24)	(1.21)
Ranking by stability values from NormFinder													
RCF	FaBZIP1	FaGAPDH1	FaUBQ1	FaTUBα	FaCHC1	FaMT1	FaFHA1	FaTUBβ	FaGAPDH2	FaRIB413	FaEF1α	FaACTIN	FaTIM1
	(1.533)	(1.498)	(1.103)	(0.845)	(0.738)	(0.638)	(0.638)	(0.571)	(0.535)	(0.396)	(0.379)	(0.267)	(0.243)
FCF	FaBZIP1	FaMT1	FaFHA1	FaTIM1	FaCHC1	FaUBQ1	FaGAPDH2	FaTUBα	FaTUBβ	FaGAPDH1	FaRIB413	FaACTIN	FaEF1α
	(0.610)	(0.565)	(0.523)	(0.466)	(0.466)	(0.444)	(0.430)	(0.397)	(0.387)	(0.343)	(0.341)	(0.277)	(0.177)
FCC	FaBZIP1	FaGAPDH1	FaFHA1	FaTIM1	FaTUBβ	FaACTIN	FaCHC1	FaRIB413	FaMT1	FaUBQ1	FaGAPDH2	FaTUBα	FaEF1α
	(0.907)	(0.856)	(0.840)	(0.784)	(0.745)	(0.673)	(0.670)	(0.662)	(0.630)	(0.573)	(0.571)	(0.554)	(0.429)
FCP	FaGAPDH1	FaFHA1	FaTIM1	FaTUBβ	FaBZIP1	FaCHC1	FaMT1	FaACTIN	FaEF1α	FaUBQ1	FaTUBα	FaRIB413	FaGAPDH2
	(1.119)	(0.890)	(0.821)	(0.807)	(0.800)	(0.723)	(0.673)	(0.605)	(0.559)	(0.552)	(0.543)	(0.429)	(0.272)
FAP	FaGAPDH1	FaFHA1	FaUBQ1	FaBZIP1	FaTIM1	FaRIB413	FaGAPDH2	FaCHC1	FaTUBα	FaMT1	FaEF1α	FaTUBβ	FaACTIN
	(0.809)	(0.661)	(0.637)	(0.564)	(0.550)	(0.548)	(0.507)	(0.478)	(0.463)	(0.439)	(0.300)	(0.283)	(0.277)
HCY	FaACTIN	FaTIM1	FaGAPDH1	FaBZIP1	FaMT1	FaUBQ1	FaTUBβ	FaFHA1	FaCHC1	FaTUBα	FaEF1α	FaGAPDH2	FaRIB413
	(0.726)	(0.643)	(0.639)	(0.633)	(0.616)	(0.614)	(0.600)	(0.581)	(0.461)	(0.460)	(0.432)	(0.425)	(0.413)
HCC	FaGAPDH1	FaTIM1	FaTUBβ	FaACTIN	FaUBQ1	FaMT1	FaGAPDH2	FaBZIP1	FaFHA1	FaTUBα	FaCHC1	FaEF1α	FaRIB413
	(1.013)	(0.972)	(0.932)	(0.746)	(0.647)	(0.604)	(0.525)	(0.490)	(0.473)	(0.401)	(0.356)	(0.324)	(0.297)
All samples	FaBZIP1	FaGAPDH1	FaMT1	FaTIM1	FaTUBα	FaCHC1	FaGAPDH2	FaUBQ1	FaTUBβ	FaRIB413	FaFHA1	FaACTIN	FaEF1α
	(1.795)	(1.626)	(1.493)	(1.397)	(1.075)	(0.932)	(0.918)	(0.840)	(0.787)	(0.734)	(0.686)	(0.578)	(0.538)
Ranking by SD of Cp from BestKeeper													
RCF	FaGAPDH1	FaBZIP1	FaTIM1	FaMT1	FaTUBα	FaCHC1	FaTUBβ	FaUBQ1	FaFHA1	FaGAPDH2	FaEF1α	FaACTIN	FaRIB413
	(1.52)	(1.36)	(1.34)	(1.26)	(1.09)	(1.06)	(0.95)	(0.89)	(0.88)	(0.85)	(0.82)	(0.76)	(0.35)
FCF	FaMT1	FaBZIP1	FaCHC1	FaTIM1	FaTUBβ	FaGAPDH2	FaUBQ1	FaRIB413	FaFHA1	FaGAPDH1	FaACTIN	FaTUBα	FaEF1α
	(0.60)	(0.56)	(0.48)	(0.48)	(0.42)	(0.32)	(0.32)	(0.32)	(0.20)	(0.18)	(0.18)	(0.18)	(0.00)
FCC	FaBZIP1	FaTUBβ	FaTIM1	FaACTIN	FaFHA1	FaGAPDH1	FaGAPDH2	FaCHC1	FaMT1	FaRIB413	FaUBQ1	FaEF1α	FaTUBα
	(0.84)	(0.73)	(0.72)	(0.69)	(0.64)	(0.61)	(0.59)	(0.59)	(0.53)	(0.53)	(0.46)	(0.46)	(0.40)
FCP	FaGAPDH1	FaFHA1	FaCHC1	FaTIM1	FaTUBβ	FaBZIP1	FaUBQ1	FaEF1α	FaACTIN	FaMT1	FaTUBα	FaRIB413	FaGAPDH2
	(0.88)	(0.73)	(0.69)	(0.66)	(0.63)	(0.63)	(0.47)	(0.47)	(0.41)	(0.38)	(0.33)	(0.30)	(0.22)
FAP	FaGAPDH1	FaFHA1	FaMT1	FaTIM1	FaTUBα	FaRIB413	FaUBQ1	FaEF1α	FaACTIN	FaGAPDH2	FaCHC1	FaBZIP1	FaTUBβ
	(0.65)	(0.56)	(0.50)	(0.49)	(0.46)	(0.46)	(0.43)	(0.41)	(0.34)	(0.27)	(0.27)	(0.27)	(0.24)
HCY	FaACTIN	FaBZIP1	FaTUBβ	FaTIM1	FaMT1	FaTUBα	FaUBQ1	FaRIB413	FaEF1α	FaFHA1	FaCHC1	FaGAPDH1	FaGAPDH2
	(0.78)	(0.73)	(0.72)	(0.60)	(0.57)	(0.56)	(0.49)	(0.48)	(0.46)	(0.44)	(0.43)	(0.40)	(0.35)
HCC	FaTUBβ	FaTIM1	FaACTIN	FaUBQ1	FaGAPDH1	FaMT1	FaFHA1	FaGAPDH2	FaBZIP1	FaTUBα	FaCHC1	FaEF1α	FaRIB413
	(0.83)	(0.78)	(0.75)	(0.68)	(0.67)	(0.56)	(0.56)	(0.49)	(0.44)	(0.42)	(0.38)	(0.15)	(0.15)
All samples	FaGAPDH1	FaBZIP1	FaTIM1	FaMT1	FaTUBα	FaCHC1	FaTUBβ	FaUBQ1	FaFHA1	FaGAPDH2	FaEF1α	FaACTIN	FaRIB413
	(1.52)	(1.36)	(1.34)	(1.26)	(1.09)	(1.06)	(0.95)	(0.89)	(0.88)	(0.85)	(0.82)	(0.76)	(0.35)

STDEV and SD, represent standard deviation; Cp and Ct, represent Cq for different methods.

Combination of all five methods used for selective classification of reference genes by RankAggreg

Taking into account the strengths and weaknesses of every algorithm when applied individually, we finally have used the combined stability measurements generated by all five approaches ("stability index", geNorm^{PLUS}, ΔCt method, Normfinder, and BestKeeper) to establish a consensus rank of reference genes by applying RankAggreg (Pihur et al. 2009). The input to this statistical package was a matrix of rank-ordered genes according to the different stability measurements previously computed by each of the five methods described above.

RankAggreg calculated Spearman footrule distances and the software reformatted this distance matrix into an ordered list that matched each initial order as closely as possible. This consensus rank list was obtained by means of the Cross-Entropy Monte Carlo algorithm present in the software.

As shown in Figure 6, results of the merged data revealed that the most appropriate reference genes from all the preselected candidates tested for normalization are FaRIB413 and FaACTIN for analysis of strawberry fruit ripening, FaEF1α and FaACTIN for defense response studies in fruit, FaEF1α and FaGAPDH2, and FaGAPDH2 and FaRIB413, for defense response studies in crown and petiole, respectively, of cultivar Camarosa, FaACTIN and FaTUBβ, for defense response studies in petiole of cultivar Andana, FaGAPDH2 and FaRIB413 for SA and JA treatment of in-vitro plants, and FaEF1α and FaRIB413 for SA and JA treatment of cellular suspensions. Finally, FaEF1α and FaACTIN are the most stably expressed genes when all 48 experimental conditions are evaluated together.

Contrary, the lowest recommended reference genes are FaGAPDH1 and FaBZIP1 for analysis of strawberry fruit ripening, FaMT1 and FaBZIP1 for defense response studies in fruit, FaBZIP1 and FaGAPDH1, and FaGAPDH1 and FaFHA1 for defense response studies in crown and in petiole, respectively of

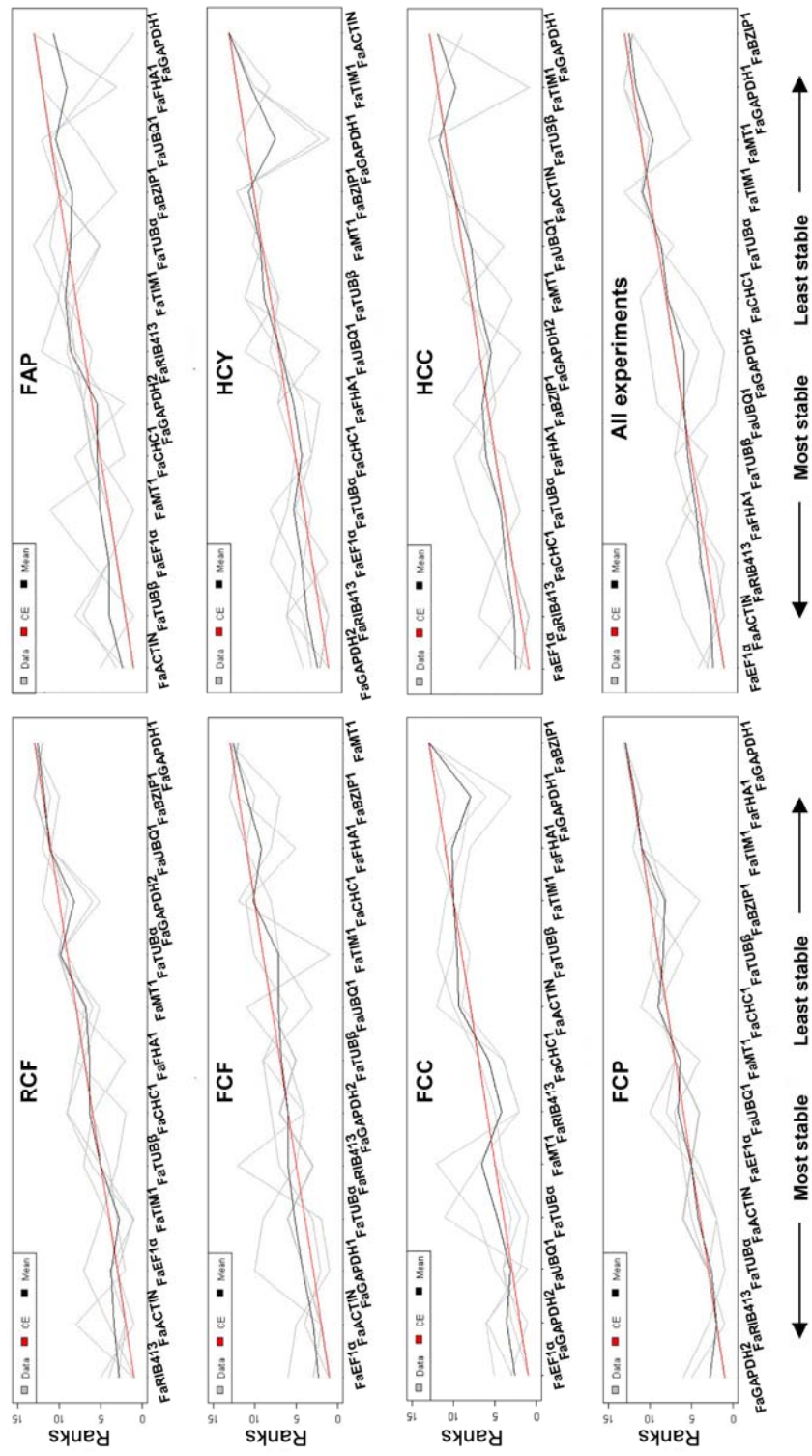
cultivar Camarosa, FaGAPDH1 and FaFHA1 for defense response studies in petioles of cultivar Andana, FaACTIN and FaTIM1 for SA and JA treatment of in-vitro plants, and FaGAPDH1 and FaTIM1 for SA and JA treatment of cellular suspensions. Finally, FaBZIP1 and FaGAPDH1 was the least recommended when all the experiment are considered together.

Taken together these results, we propose genes FaRIB413, FaACTIN, FaEF1α and FaGAPDH2 as superior reference genes for accurate transcript normalization in strawberry (*Fragaria* × *ananassa*) under the present experimental conditions.

Validation of the selected superior reference genes

In order to validate the selected superior reference genes, the relative expression level of the strawberry gene encoding the transcription factor FaWRKY1 (AtWRKY75 ortholog, Encinas-Villarejo et al. 2009) was determined in all the experimental sets of evaluated conditions. The strawberry gene FaWRKY1 acts as positive regulator of defense response during compatible and incompatible interactions in Arabidopsis and, very likely, FaWRKY1 is an important element mediating defense responses to *C. acutatum* in strawberry. We also know that FaWRKY1 gene is significantly upregulated in strawberry tissues under *C. acutatum* attack, and after SA and MeJA treatments (Encinas-Villarejo et al. 2009, Amil-Ruiz et al., unpublished data).

FaWRKY1 was normalized to either a combination of the two best candidates ranked by RankAgreg algorithm as recommended by geNorm (Figures 5 and 6), or the least recommended one, to analyze the bias effect on target expression analysis by inappropriate reference gene. FaWRKY1 primer sequences and other characteristics are listed in Table 1. As predicted, the expression profile of FaWRKY1 is strongly affected by the choice of the reference gene.


RCF
FCF
FCC
FCP
FAP
HCY
HCC
All experiments
Data
CE
Mean
Ranks
Most stable
Least stable

Thus, in the strawberry fruit ripening conditions (RCF) as well as for infected petioles of cultivar Camarosa (FCP) and elicited cellular suspensions (HCC), the expression level values were very similar to those previously reported (Encinas-Villarejo et al. 2009), when the reference genes were the two superior recommended ones (FaRIB413 and FaACTIN, FaGAPDH2 and FaRIB413, FaEF1α and FaRIB413, respectively), either individually or combined as geometric mean (Figures 7a, 7d and 7g). By contrary, a strong bias in the FaWKRY1 expression pattern was obtained when the least recommended gene (FaGAPDH1 in all three cases) was used for normalization. Thus, the use of FaGAPDH1 as reference gene somehow neutralizes the detectable induction of FaWRKY1 during fruit ripening and senescence, in the response to infection and after elicitation with SA and MeJA compounds.

Interestingly, in other three experimental conditions (FCF, FCC and HCY) the use of the least stable reference gene (FaMT1, FaBZIP1 and FaACTIN respectively) seem to have opposite influence in the perception of the expression values of the FaWRKY1 target gene, and anomalously increases the level of induction of this target gene (Figures 7b, 7c and 7f). This is probably due to slightly but opposite variations in their corresponding mRNA levels during the analyzed process, but which, nonetheless, has significant impact in the final relative quantification of the expression of the particular target gene under analysis. Only in the Andana petioles under fungal infection (FAP experiment), differences in the expression values of FaWRKY1 were not significant when both the best (FaACTIN) and the worst (FaGAPDH1) reference candidates were considered.

Figure 6. (previous page) Rank aggregation of gene lists using the Monte Carlo algorithm. Visual representation of rank aggregation using Monte Carlo algorithm with the Spearman footrule distances. The solution of the rank aggregation is shown in a plot in which genes are ordered based on their rank position according to each stability measurement (grey lines). Mean rank position of each gene is shown in black, as well the model computed by the Monte Carlo algorithm (red line). See Table 2 for experiments description.

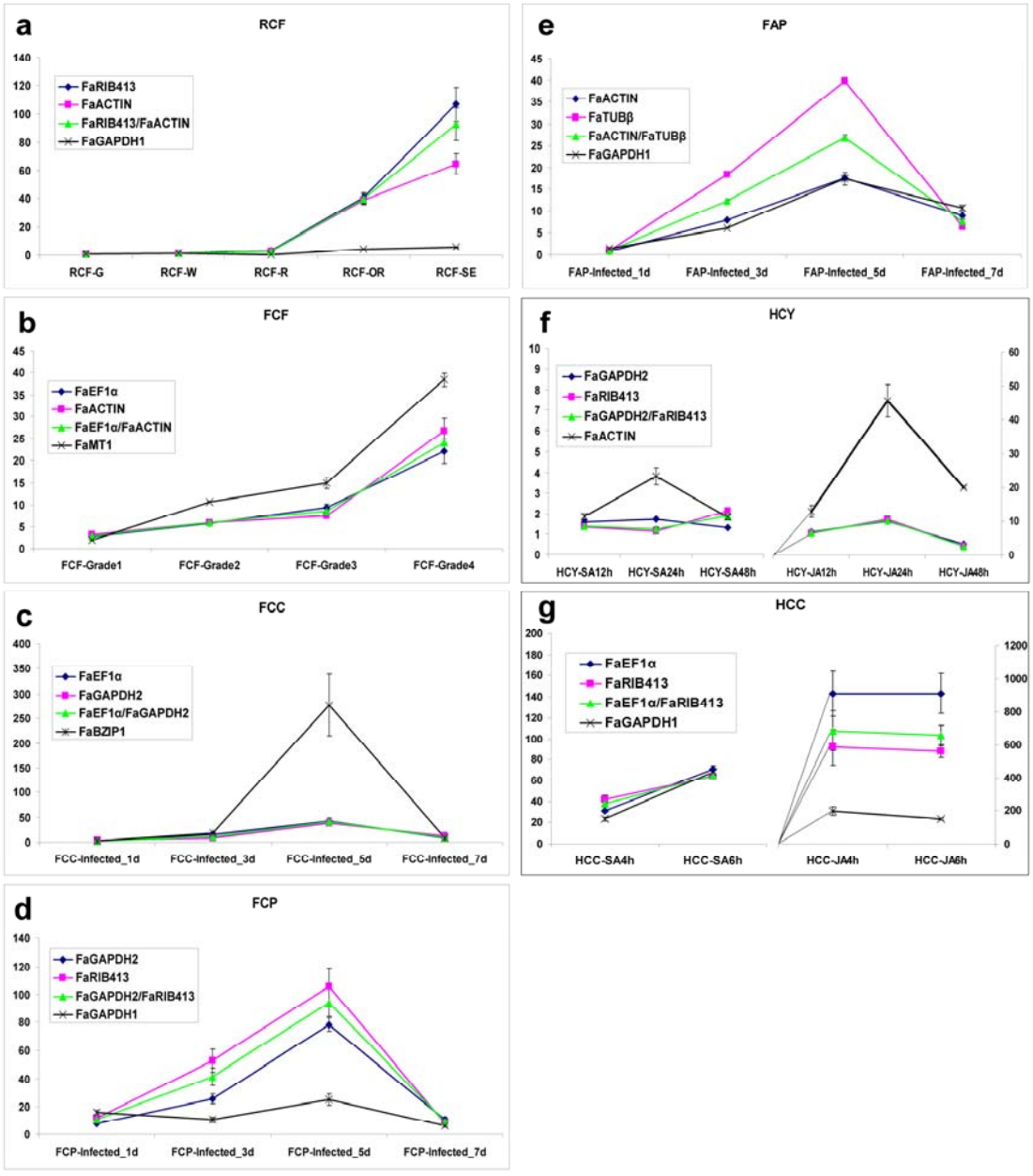


Figure 7. **Transcript level relative quantification of the FaWRKY1 transcription factor**. FaWRKY1 gene expression was finely analyzed in strawberry under the seven independent experimental conditions used in this study. Error bars show standard deviation calculated from two biological replicates. Normalization factors were calculated as the geometric mean of the expression levels of the two most stable reference genes as recommended in Figure 6 for each single experiment. Normalization to each gene individually is also shown. Additionally, the least

In summary, the analyses of these reference genes under all these stringent criteria implies that these strawberry genes were stably expressed under each considered experimental condition, and thus we suggest they can be used as superior reference genes for normalization in strawberry (*Fragaria* × *ananassa*), according to the criteria here described. Also, they can be used as starting pool of ideal genes to test for more accurate normalization in strawberry under other different experimental conditions.

DISCUSSION

Recommended reference genes in a strawberry-defense response context

This work has mainly been focused to the evaluation of a set of strawberry predicted good reference genes to be used as successful control genes in strawberry plant-defense response studies. Therefore, a variety of biological samples representing experimental conditions usually carried out to evaluate plant defense responses has been used. Thus, the effect of natural pathogen infection and also the senescence or fruit decay process are represented by experiments of fruit ripening and fruit natural infection by *C. acutatum* in growing fields. Other tissues from Camarosa and Andana strawberry cultivars under fungal infection conditions were also included in this study, allowing comparisons between vegetative tissues within a cultivar, and between same tissues in different cultivars.

Also, strawberry cultivars grown under different systems (in-vitro plants and cellular suspensions) were compared after treatment with either SA or JA, two phytohormones implicated in the activation of two well-known plant defense signaling pathways.

stable reference gene was used for normalization of each experiment to demonstrate the effect of unstable reference genes in the quantification of the relative amount of mRNA for the target gene. Every sample was calibrated with their corresponding mock sample (see Table 2 for experimental details). Black lines linked to the X axis have been added to f and g to illustrate range of gene induction and facilitate visual understanding.

To determine which reference gene is best suited for transcript normalization in a given subset of biological samples, different methods, statistical procedures and software packages have been reported. Every algorithm has its own strengths and flaws when applied individually. Thus, geNORM uses pair-wise comparisons and geometric averaging across a matrix of reference genes and biological samples to determine the best reference gene for a given set of samples by calculating an expression stability value (M_A), and propitiates accurate normalization of RTqPCR data (Vandesompele et al. 2002, Hellemans et al. 2007). This, however, make it sensitive to co-regulation, which tends to select those genes with the highest degree of similarity in their expression profiles (Andersen et al. 2004). On the other hand, it has the advantages that it is minimally affected by expression intensity of the candidate genes (Mehta et al. 2010), and it can determine the optimal number of genes required to more accurately normalization of RTqPCR data, based in pairwise variation (V), being only two genes needed when $V<0.15$ (Vandesompele et al. 2002). Unlike geNORM, NormFinder is not affected by correlated expression of the candidate genes. However, this last one gains in robustness as the sample number is increased, while geNorm don't need large sample size since it uses pair-wise comparison. Bestkeeper algorithm also performs pairwise comparison using the geometric mean of the Cp (Cq) values, but different expression levels can generate heterogeneous variance between groups, and this can invalidate the use of Pearson correlation coefficient (Lefever et al. 2009, Bustin et al. 2009). The other two methods, ΔCt and "stability index", perform studies about the variation of ΔCt in pairwise genes or simple Ct respectively. The comparative ΔCt method rank the reference genes by their mean standard deviation in the pairwise comparisons, while the "stability index" approach introduces statistics and linear regression analysis to rank the candidates by the product of the coefficient of variation and slope of regression of gene means against overall means for the different samples.

We have applied RankAggreg (Pihur et al. 2009) to establish a consensus rank of reference genes by combination of all five above methods. This approach strengthens the value of the recommended candidates to normalize target gene

expression in any of the conditions here described. Thus, results in Figure 6 show genes recommended in each particular experiment, suggesting they can be used as superior reference genes for this kind of studies.

The comparative analysis between using the most and the least appropriate reference gene in a given experiment (Figure 7) evidences the magnitude of the bias produced by normalization with an unstable gene, and also highlight how the incorrect use of reference genes without any previous validation can lead to misinterpretation of data. For this reason we strongly recommend to perform a validation of the putative reference genes prior any quantitative expression studies, as it is also recommended by other authors (Dekkers et al. 2012, Mafra et al. 2012, Matta et al. 2011, de Oliveira et al. 2012, Podevin et al. 2012). The use of merged lists in an unsupervised way and giving identical weight to the out-put of the different five methods used to evaluate the stability of the proposed references strengthens their recommendation to be a starting list of candidates to normalize the given experiment in similar conditions to those we describe here.

Some of the genes here studied have never been reported as reference genes for RTqPCR in strawberry, but particular other ones have been used in previously reported strawberry studies (see Table 1), although no experimental work was performed to validate their usefulness as control genes in the analyzed strawberry process. In particular, the FaRIB413 gene has been extensively used for northern and RTqPCR normalization in strawberry (Benítez-Burraco et al. 2003, Casado-Díaz et al. 2006, Osorio et al. 2008, Encinas-Villarejo et al. 2009, Csukasi et al. 2011, Moyano-Cañete et al. 2013). FaRBI413 encodes a highly abundant ribosomal RNA (Cq around 8 in our study, Table 3), which does not contain a poly(A) tail, making it unsuitable for RTqPCR analysis aimed at differentiating the expression levels of rare genes, and also for the synthesis of cDNA using oligo(dT) primers. Therefore, although FaRIB413 presents very good values of expression stability in almost all of the experiments analyzed in our study (Table 6), and with the exception of analyzing very abundant target genes, from now on we strongly recommend the use of an alternative strawberry reference with Cq

values as close as possible to the Cq values showed by the target gene under study.

Very recently, an actin gene was used by Lin-Wang et al. (2010), for normalization of RTqPCR studies in different strawberry plant tissues. Authors selected this gene as a reference gene "*because of its consistent transcript level throughout fruits and leaves*". From our results, FaACTIN presents high stability in all fruit experimental conditions, such as ripening and infection, in Andana petiole tissues, and also considering all the experiments together, which could represent the analysis reported by Lin-Wang et al. (2010). However, this FaACTIN gene was not appropriate when vegetative tissues of cultivar Camarosa (crown and petioles) were exposed to fungal infection, or by phytohormone elicitation either of strawberry plants or cellular suspensions.

Also, a strawberry elongation factor 1α gene (EF1a) was used by Guidarelli et al. (2011), to normalize raw expression data in an RTqPCR experiment with fruits of the very susceptible strawberry cultivar Alba inoculated with *C. acutatum*. Although authors did not assess the stability of expression of this gene by none of the available methods, they detected that this gene had "*the most constant expression levels (absolute ΔCt < 1 among treatments)*", and assumed this candidate gene for data normalization. From our results, FaEF1α is indeed recommended as the best candidate for normalization of experiments based on strawberry fruits under biotic interaction. Therefore, our analysis validates the study carried out by Guidarelli et al. (2011).

In addition, FaGAPDH1 and FaGAPDH2 genes have been previously used as reference genes in a plant-pathogen interaction context (Khan and Shih 2004, Grellet-Bournonville et al. 2012, Mamaní et al. 2012, Zamora et al. 2012). In the case of FaGAPDH2 gene reported by Khan et al. (2004), our results support the use of this gene as control in the experimental conditions reported by these authors, (i.e. strawberry vegetative tissues inoculated with *Colletotrichum*) (see Figure 6). However, the use of FaGAPDH1 as a single reference gene in

strawberry experimental treatments with phytohormones or after fungal inoculation, as reported by Grellet-Bournonville et al. (2012), Mamaní et al. (2012) and Zamora et al. (2012), should had been avoided as this gene has shown the lowest values of stability in almost all the experimental conditions we have analyzed, and some inaccurate results could have been brought about.

In conclusion, stably expressed genes were selected from two independent strawberry biological replicates of a total of forty eight samples, representing seven different experimental conditions. Our results make a relevant contribution to the scientific plant community as the best candidates of superior reference genes in strawberry, ranked accordingly to their respective expression stability, in a variety of samples representing major conditions typically used in a plant-defence context, have been characterized and validated. The identification of other stable reference pools under different experimental conditions would build a useful community resource for gene expression analysis in this crop.

MATERIALS AND METHODS

Plant materials and growth conditions

Plant material, *Fragaria* × *ananassa* cultivars Chandler, Camarosa and Andana were used. *Colletotrichum acutatum*, a major strawberry pathogen was used for natural infection and controlled inocculation. All the plant culture and growth conditions, *C. acutatum* experimental conditions, and treatments with chemicals have been previously described (Casado-Díaz et al. 2006, Encinas-Villarejo et al. 2009), and are summarized in Table 2. Briefly, strawberry cellular suspensions (cv. Chandler) were prepared from *in vitro* growing calli. Five days old cell suspensions were treated with MeJa (0.1 mM), SA (0.75 mM) or water (as control). Alicuots were taken at 2 hour intervals and cells were frozen in liquid nitrogen. Samples at 4 and 6 hours were used in this work because they match with a strong relative expression of the FaWRKY1 target gene, and many other strawberry genes currently under study in our lab. Axenic *in-vitro* plants from cv.

Camarosa were aseptically sprayed with water, MeJa (2 mM) and SA (5 mM) solutions and collected at 12, 24 and 48 hours post-treatment. Strawberry fruits were collected from a growing field in several ripening stages and pooled by stage. Red stage strawberry fruits naturally-infected by *Colletotrichum acutatum* and exhibiting different increasing degrees of fungal necrotic lesions were collected and fruits having similar symptoms were pooled. No specific permissions were required for these activities. None human manipulation was applied to strawberry field prior to sample collection. Field studies did not involve endangered or protected species. Eight-week-old strawberry plantlets were placed in 20 cm diameter plastic pots containing sterilized peat and grown for a minimum of six additional weeks prior to mock or pathogen inoculation by spraying a spore suspension of 10^6 CFU ml^{-1}. Crowns and petioles were collected 1, 3, 5 and 7 days after treatment. All samples were flash frozen in liquid nitrogen and stored at -80 ºC until needed.

Total RNA extraction and cDNA synthesis

Total RNA from strawberry fruits and vegetative tissues, as well as cell suspension cultures, was isolated according to Manning Manning 1991, treated with DnaseI (Invitrogen) to remove the residual contaminating DNA, and further purified with the RNeasy MinElute Cleanup Kit (QIAGEN). Purified RNA was quantified by the NanoDrop 1000 Spectrophotometer (Thermo scientific) and the integrity checked by agarose gel electrophoresis and the Agilent 2100 Bioanalyzer (Agilent Technologies, Deutschland). First-strand cDNA synthesis were carried out by the iScript cDNA Synthesis kit (Bio-Rad) using as template 1µg of purified total RNA per 20 µL of reaction volume. RT reactions were diluted 5-fold with nuclease-free water prior to be used in the qPCR.

Real-time qPCR

Specific primer pairs set for the genes tested were designed using Oligo Primer Analysis software version 6.65, tested by dissociation curve analysis, and verified

for the absence of non-specific amplification. More details are provided in results. RTqPCR runs were performed in MyIQ and iCycler real-time PCR systems (Bio-Rad) using 96-well plates and 20 µL final reaction volume per well. Two µL template cDNA was added to the PCR reaction mixture containing 0.4 µM of each primer and 10 µL of 2X SsoAdvanced™ SYBR® Green supermix (Bio-Rad). The protocol was: an inicial step of enzyme activation/DNA denaturation of 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, 65°C for 15 sec and 72°C for 15 sec, and a final standard dissociation protocol to obtain the melting profiles. Data were acquired by means of the MyIQ v1.004 and iCycler v3.1 software's (Bio-Rad).

Computational data analysis

Data analysis strategy is described in detail in the results section. Reaction efficiency calculus was done using LinRegPCR version 2012.3 (Ramakers et al. 2003, Ruijter et al. 2009). Resulting mean PCR efficiencies per amplicon were taken. Reference genes validation was performed using previously described software applications, included the MS Excel VBA applets NormFinder v0.953 (Andersen et al. 2004) and BestKeeper v1 (Pfaffl et al. 2004), and the geNorm (Vandesompele et al. 2002) algorithm provided in qBasePlus v2.4 package (Hellemans et al. 2007). Other statistical procedures were performed with the free software R v2.15.2 (http://www.R-project.org), with the packages RankAggreg 0.4-3, clValid 0.6-4 and gtools 2.7.0; and SPSS software ver 15.0 for Windows.

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Chapter IV

Multiple Hormone Analysis Indicates Involvement of Jasmonate and Salicylate, but not Absisic Acid Signalling in the Defence Response of Strawberry to *Colletotrichum acutatum* in a Cultivar Dependent Manner

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ABSTRACT

Plant hormones play crucial roles in defense to pathogens and pests. A controlled hormonal balance determines appropriate response to a particular plant-pathogen interaction, as singular events in the complex network of plant signaling are fine tune regulated by these compounds. In many plants, significant progress has been made in understanding the biological significance of changes in the level of some of these compounds, such as salicylic acid (SA) and jasmonates (JA), closely related to biotrophic and necrotrophic defenses-related pathways, respectively. However, the role that these compounds can play in relation to the mechanism of defense in strawberry is poorly understood. More over, understanding their biological significance in a specific interaction against a hemibiotrophic organism, such as *C. acutatum*, is far to be clear.

To increase knowledge in the molecular response of strawberry to the hemibiotrophic fungus *C. acutatum*, two *Fragaria* × *ananassa* cultivars with different behavior to anthracnose in terms of susceptibility, cvs. Camarosa and Andana, were analyzed after infection in the balance of four hormones, SA, JA, ABA and IAA. Thus, basal amount and changes in the level of these phytohormones in both cultivars challenged with *C. acutatum* have been analyzed. Results suggest that the basal level of SA might be correlated with differences in susceptibility between both strawberry cultivars. The relevance of the three hormones, SA, JA and ABA, together with the possible crosstalk between their respective pathways is discussed.

INTRODUCTION

Plant hormones are a group of naturally occurring organic substances that influence physiological processes at low concentrations. Phytohormones play important roles in regulating complex signaling networks involving developmental processes and plant responses to a wide range of environmental stresses including biotic and abiotic stresses. Since the identification of gibberellin (GA) as the responsible compound for the phenotype of excessive growth exhibited by rice seeds infected with the fungus *Gibberella fujikuroi* detected in the 19th century, other plant compounds have been isolated and identified as hormones acting in plant response to disease. Significant progress has been made in identifying the key components and understanding signaling activity in salicylic acid (SA), jasmonates (JA) and ethylene (ET), whose are well known to play crucial roles in plant disease and pest resistance (Glazebrook 2005; Lorenzo and Solano 2005; Broekaert et al. 2006; Loake and Grant 2007; Balbi and Devoto 2008). However little is known about the roles of other hormones such as abscisic acid (ABA), auxin, gibberellin (GA), cytokinin (CK) and brassinosteroid (BL) in plant defense (Robert-Seilaniantz et al. 2007, Bari and Jones 2009), about interactions between different plant hormone-mediated signaling pathways (Robert-Seilaniantz et al. 2011), and about plant responses to various pathogens infecting at the same time (Spoel et al. 2007).

Once the plant detects attempted invasion of an infectious agent (such as a bacterium, fungus or virus), it responds to the presence of the invader by turning on a complex set of defensive reactions to prevent widespread of the pathogen (Durrant and Dong 2004), including localized production of reactive oxygen species, generation of pathogenesis related proteins, releasing of toxic chemicals (phytoalexins), and even promoting programmed cell death nearby the point of infection. Appropriate regulation of defense response is greatly important for plant fitness, as its activation has deleterious effects on plant growth (Heil et al. 2000, Tian et al. 2003). To fine control these specific responses after infection, a

balanced production in certain phytohormones is required. These hormones are also responsive for the "alarm signal" in the activation of the systemic response in the complete plant system. In this sense, SA plays major role in plant defense and is generally involved in the activation of defense responses against biotrophic pathogens, as well as the establishment of systemic acquired resistance (SAR, Grant and Lamb 2006). By contrast, JA is usually associated with defense against necrotrophic pathogens (Glazebrook 2005). Depending on the type of plant-pathogen interaction, and the pathogen lifestyle, each hormone plays its role as positive or negative actor in a crosstalk that strongly influences the outcome of defense response. However, the underlying molecular mechanisms are far to be completely understood. How the plant regulates the level of phytohormones in response to various pathogens attacking at the same time, or how it is done in response to a pathogen which behavior can not be clearly categorized as a biothrophic or necrotrophic one, are important questions that still need to be answered.

Many authors have focused their investigations to find out the biological function of some of these phytohormones in the strawberry plant. Thus, SA application increase strawberry fruit total antioxidant activity (Asghari and Aghdam 2010), offer low-temperature and salt stress protection (Karlidag et al. 2009a, Karlidag et al. 2009b) and improved postharvest fruit quality (Shafiee et al. 2010). ABA has been related with response to root growth restrictions (Giannina et al. 1998), determination of plant juvenility (Mohamed et al. 1991), fruit development and ripening (Li et al. 2011), and also with drought stress (Terry et al. 2007). Many studies have associated fruit ripening with methyl derivatives of JA (Gansser et al. 1997, Pérez et al. 1997, Yilmaz et al. 2003, Mukkun and Singh 2009, de la Peña Moreno et al. 2010). In addition, JA has an inhibitory effect on pollen germination (Yildiz and Yilmaz 2002), functions in strawberry damage-self sensing response (Heil et al. 2012), and alleviates water stress effects (Wang 1999).

However, the role of these compounds in relation to the mechanism of defense in strawberry is poorly understood. Nonetheless, some studies have tried to unravel defense related activities to these compounds. Thereby, SA application reduced postharvest *Botrytis* infection (Babalar et al. 2007), but its endogenous level had apparently none relation with induced strawberry resistance to powdery mildew (Hukkanen et al. 2007). Biotic interaction with *B. cinerea* increased ABA contents of ripe strawberry fruit (Terry et al. 2007). Also, this hormone regulates strawberry stomata closure (Kubik and Plonka 1984, Yadava 1987), and activates defense response to bacterial and fungal pathogens (Gudesblat et al. 2009). Very few studies have been reported about jasmonates in a strawberry plant-defense context. Thus, application of MJ controlled *B. cinerea* (Moline et al. 1997, Stanley 1998, Zhang et al. 2006), and induced strawberry resistance to the two-spotted spider mite (*T. urticae* Koch) (Warabieda et al. 2005, Warabieda and Olszak 2010). Although all this information is of great interest a major piece of work is still needed to clearly unravel the biological significance of the hormonal balance in each specific strawberry-pathogen interaction.

Even though some pathogens can be clearly classified as biotrophs of necrotrophs, many others first develop a biotrophic interaction with the host plant and later switch to a destructive necrotrophic lifestyle depending on the conditions in which they find themselves or the stages of their life cycles (Münch et al. 2008, Lee and Rose 2010), they are the so called hemibiotrophic pathogens. *Colletotrichum acutatum* (microbial agent causing anthracnose) is a clear example of this kind of pathogen exhibiting a hemibiotrophic lifestyle (Curry et al. 2002). *C. acutatum* is major pathogen of fruit crops and has a very wide host range, causing economically important losses of temperate, subtropical and tropical fruits worldwide (Dyko and Mordue 1979, Bailey and Jeger 1992, Wharton 2004). This fungus is between the top 10 fungal pathogens in molecular plant pathology (Dean et al. 2012).

With the aim of increase knowledge in the strawberry hormonal response to the hemibiotrophic fungus *C. acutatum*, two *F* × *ananassa* cultivars with different

behavior to anthracnose in terms of susceptibility (the very susceptible cultivar Camarosa, and in the less susceptible one Andana) were analyzed in the balance of four hormones, SA, JA, ABA and IAA (auxin), in response to infection. In order to ascribe a putative biological defense function to these hormones, their basal amount and their changes in the plant after infection have been compared with differences in the susceptibility between the cultivars under study.

RESULTS

We have studied the involvement of endogenous plant hormones during the first steps of anthracnose disease establishment. In infected plants of cultivar Camarosa, the symptoms on leaves were observed 2 days after inoculation, with 20-30% of symptomatic leaves after 3 days, and extensive disease in the 5th day after inoculation. Infected plants of cultivar Andana showed the first symptoms of disease 3 days after inoculation, with 40-50% of symptomatic leaves in the 5th day (data not shown).

We have measured absicic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) within the same sample, 3 and 5 days after inoculation with *C. acutatum* and water (mock). Regrettably, indole-3-acetic acid (IAA) levels were undetectable in all samples of our system. Although these plant hormones have already been shown to be implicated in some plant-fungus interactions (reviewed in Bari and Jones 2009, Robert-Seilaniantz et al. 2011), this is the first study of their involvement in the response of strawberry cultivars with different sensitivity to *C. acutatum* infection (see Figure 1 for the structures of the four acidic plan hormones analyzed).

As detailed in "*Materials and Methods*" section, after plant vegetative tissue was homogenized with ultrapure water (proved as an effective solvent for the initial extraction, Gómez-Cadenas et al. 2002, Durgbanshi et al. 2005), a partition against diethyl ether was performed, and acidic phytohormones were recovered in the organic phase, while the large amounts of sugars and amino acids present in

plant samples will remain in the aqueous phase. However, the limitation of this step was that most sugar hormone conjugates will likely also be excluded (tossed away with the water phase), so our hormone quantifications represent only free SA, JA and ABA portions, in the given sample. The accuracy and precision of the method was determined previously in order to validate it in the analysis of plant samples (Durgbanshi et al. 2005).

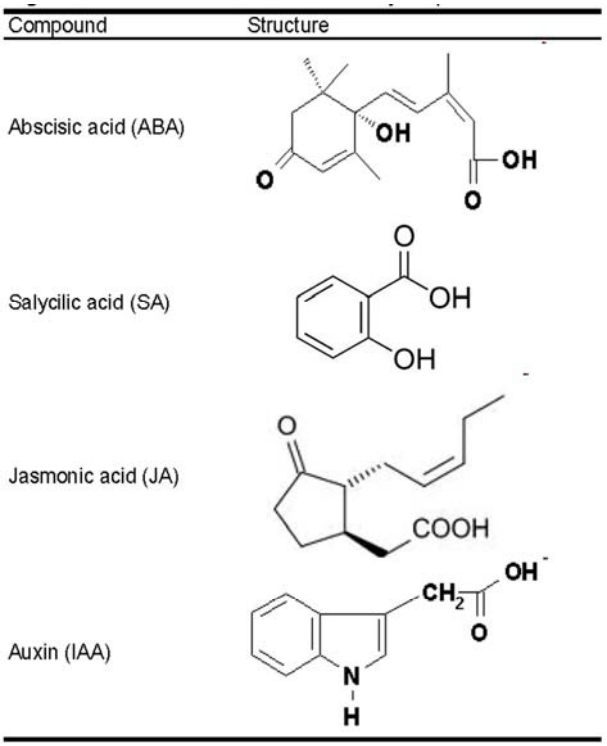

Figure 1. Schematic structures of the analysed plant hormones.

The linearity of this procedure was assayed by analyzing the calibration curves. These curves were obtained by using solutions containing increasing amounts of each plant hormone and a fixed amount of the corresponding deuterium-labeled internal standard. The calibration curves (see Figure 2) indicated a linear behavior in the concentration ranges chosen (R^2 values always higher than 0.983).

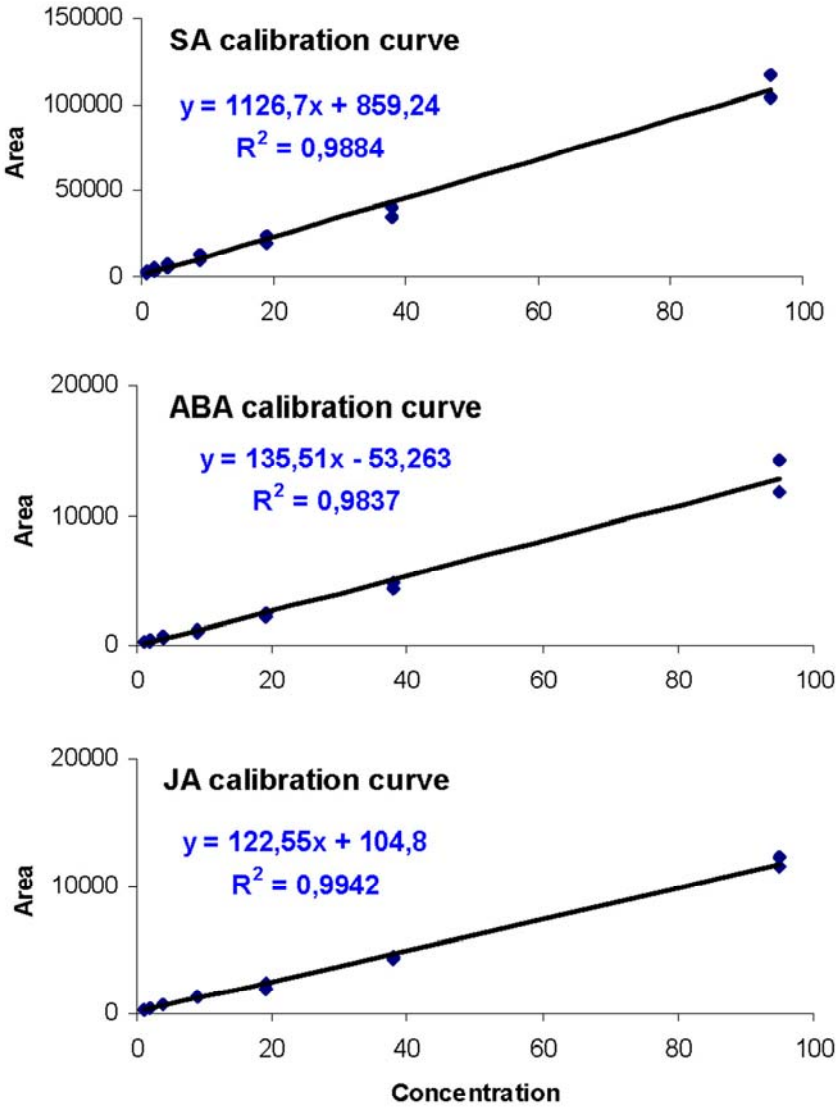

Figure 2. Hormonal compounds calibration curves.

Salicylic Acid in strawberry cvs. Camarosa and Andana

Basal level of Salicylic acid

Basal level of free SA found in full plants of Camarosa and Andana cultivars reaches 60 and 110 ng g^{-1} dry weigh respectively. Thus, the less susceptible cultivar (Andana) has almost double level of free SA in basal conditions than the very susceptible one (Table 1, Figure 3).

Table 1. Contents of SA, ABA and JA [ng g^{-1} (dry weigh)] 3 and 5 days post inoculation (dpi) in vegetative tissues of strawberry very susceptible cultivar Camarosa and less susceptible cultivar Andana. Mean ± SD.

	cv. Camarosa			
	Mock		Inoculated	
	3dpi	5dpi	3dpi	5dpi
SA	74,42 ± 2,97	52,33 ± 2,42	202,21 ± 2,98	354,77 ± 14,84
JA	320,42 ± 27,46	401,93 ± 7,97	771,39 ± 135,05	1707,03 ± 433,40
ABA	343,46 ± 14,97	433,19 ± 17,46	417,06 ± 25,93	448,80 ± 50,45

	cv. Andana			
	Mock		Inoculated	
	3dpi	5dpi	3dpi	5dpi
SA	107,69 ± 9,36	119,82 ± 8,87	76,61 ± 6,48	70,07 ± 7,96
JA	205,79 ± 17,73	65,17 ± 8,40	205,29 ± 6,54	292,78 ± 9,40
ABA	521,23 ± 55,42	328,90 ± 16,46	495,72 ± 40,86	345,77 ± 12,18

Salicylic acid level in response to C. acutatum

In relation to the SA synthesis, we have detected differences in behavior between the two strawberry cultivars examined after infection. While the very susceptible cultivar Camarosa strongly induced SA production early in response to *C. acutatum* infection, with 3 to 7 times more phytohormone in infected plants than in control ones, the less susceptible one Andana, which has higher basal level of this phytohormone, showed a reduction in its free SA content which decreased to a level similar to that detected in Camarosa (Table 1, Figure 4).

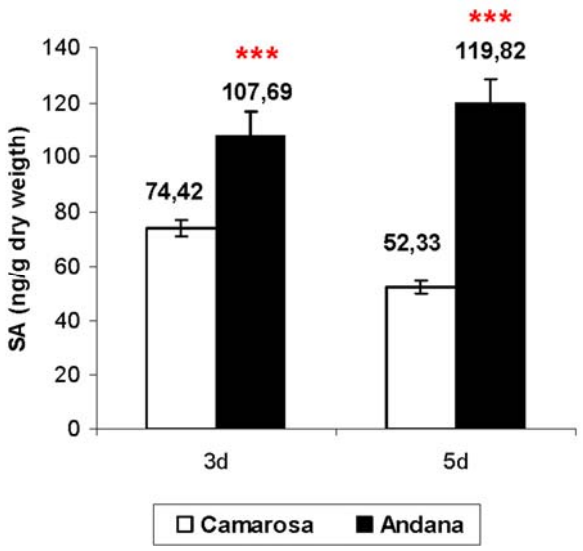

Figure 3. Salicylic acid basal levels in Camarosa and Andana strawberry cultivars. One-way Analysis of Variance (ANOVA) (Tukey-Kramer Multiple Comparisons Test): $p < 0.001$, ***.

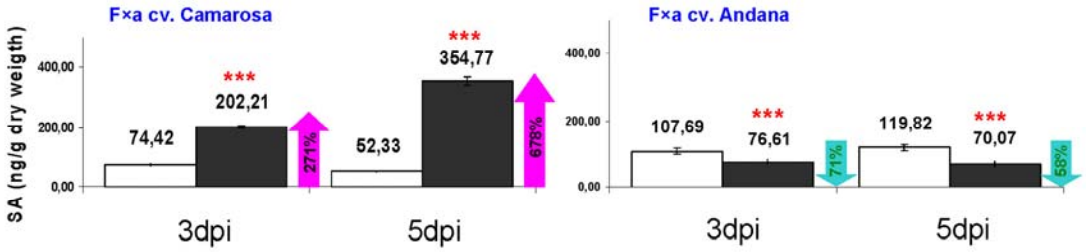

Figure 4. Regulation of SA synthesis in the two strawberry cultivars. White bars represent mock samples, and dark bars represent inoculated samples. Dpi, days post inoculation. Arrows show relative levels of hormone after inoculation to respective mock treatments. One-way Analysis of Variance (ANOVA) (Tukey-Kramer Multiple Comparisons Test): $p < 0.001$, ***.

In Camarosa, this important induction in the synthesis of SA seems to occur during the early stages of infection and a 271% increase in free SA concentration was significantly detected in plants at 3dpi (202.21 ng/g dw, in infected plants *vs*. 74.42 ng/g dw, in mock treated plants). Also, in infected plants the level of SA increased up to 678% at 5dpi compared with that of mock treatment (354.77 ng/g

dw *vs*. 52.33 ng/g dw, respectively). To this stage, the disease had already reached and extended through all tissues of the plant.

Jasmonic Acid in strawberry cvs. Camarosa and Andana

Basal level of Jasmonic acid

Basal level of free JA was found significantly higher in Camarosa than in Andana cultivar both at 3dpi (320.42 and 205.79 ng/g dw, respectively), and at 5dpi (401.93 and 65.17 ng/g dw, respectively) (Table 1, Figure 5). These values are in agreement with those previously found in strawberry leaves by other authors (Heil et al. 2012).

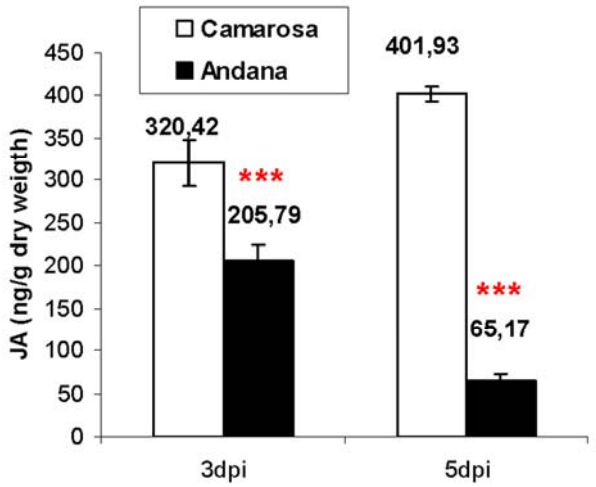

Figure 5. Jasmonic acid basal levels in Camarosa and Andana strawberry cultivars. One-way Analysis of Variance (ANOVA) (Tukey-Kramer Multiple Comparisons Test): $p < 0.001$, ***.

Jasmonic acid level in response to C. acutatum

In relation to the JA synthesis, we also have detected a distinctive behavior between the two strawberry cultivars examined after infection. While the very susceptible cultivar Camarosa strongly induced JA production early in response to *C. acutatum* infection (2.5 and 4 times more phytohormone at 3 and 5dpi, respectively), the less susceptible one Andana maintained the level detected in

mock treatment at 3dpi, and only showed a tiny but significant increase in JA at 5dpi (Table 1, Figure 6).

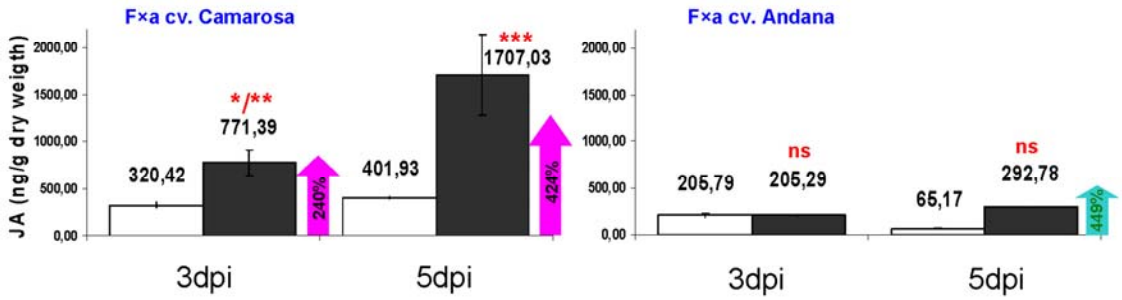

Figure 6. Regulation of JA synthesis in the two strawberry cultivars. White bars represent mock samples, and dark bars represent inoculated samples. Dpi, days post inoculation. Arrows show relative levels of hormone after inoculation to respective mock treatments. One-way Analysis of Variance (ANOVA) (Tukey-Kramer Multiple Comparisons Test): $p < 0.001$, ***.

In Camarosa, and similarly to SA synthesis, this important induction in the synthesis of JA seems to occur during the early stages of infection. Thus, a 240% increase in free JA concentration was significantly detected in plants at 3dpi (771.39 ng/g dw, in infected plants *vs*. 320.42 ng/g dw, in mock treated plants). In addition, in infected plants the level of JA increased up to 424% compared with that of mock treatment (1707.03 ng/g dw *vs*. 401.93 ng/g dw, respectively), in the stage (5dpi) where the disease had already reached and extended through all tissues of the plant.

By contrary, Andana cultivar does not dramatically increase free JA content during the time period under study. Instead, Andana appears to maintain its JA levels at 3 dpi (205.79 and 205.29 ng/g dw in mock treated and infected plants, respectively), and slightly accumulated JA content at 5dpi (from 65.17 to 292.78 ng/g dw). Although this increase is statistically significant, the JA value in mock treated plants at 5dpi was found especially low. So far, we cannot explain this issue. However, the level of JA at 5dpi in inoculated plants remains significantly

below the level of JA found in Camarosa uninfected plants (mock values), indicating differences in control of JA synthesis in response to this pathogen.

Abscisic Acid in strawberry cvs. Camarosa and Andana

Basal level of Abscisic acid

Basal level of ABA found in plants of Camarosa and Andana cultivars was similar, and values ranged from 343.46 ng/g dw to 433.19 ng/g dw, and from 328.90 ng/g dw to 521.23 ng/g dw, respectively (Table 1, Figure 7). These ABA values are in agreement with those reported by other authors in strawberry vegetative tissues (Mohamed et al. 1991) and fruits (Jia et al. 2011). So, the ABA basal level detected in these strawberry cultivars does not correlate with differences observed in their susceptibility to *C. acutatum*.

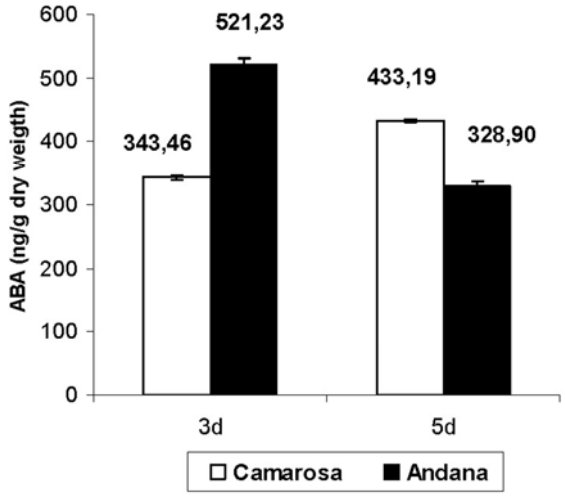

Figure 7. Abscisic acid basal levels in Camarosa and Andana strawberry cultivars.

Abscisic acid level in response to C. acutatum

Data obtained of ABA level in strawberry samples were robust, with values of 343.46 and 433.19 ng/g dw, and 417.06 and 448.80 ng/g dw, at 3 and 5dpi in mock and infected plants of Camarosa cultivar, respectively; and 521.23 and 380.90 ng/g dw, and 495.72 and 345.77 ng/g dw, at 3 and 5dpi in mock and

infected plants of Andana cultivar, respectively (Table 1, Figure 8). However, no significant changes in ABA level were detected even after 5 dpi, suggesting that in these strawberry varieties ABA signaling does not seem to be activated after *C. acutatum* infection.

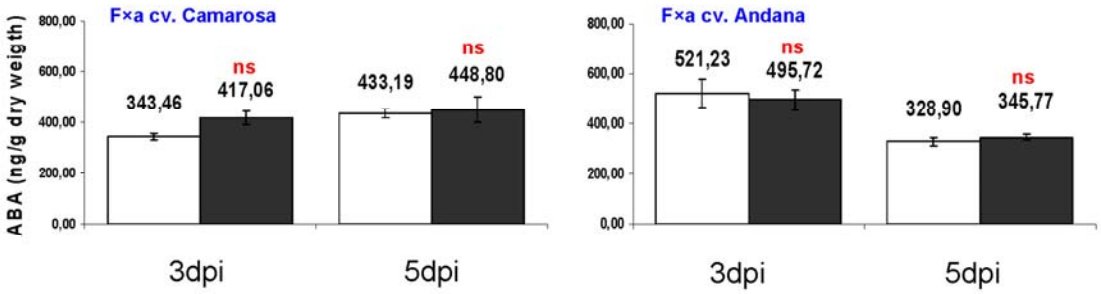

Figure 8. Regulation of ABA synthesis in the two strawberry cultivars. White bars represent mock samples, and dark bars represent inoculated samples. Dpi, days post inoculation. One-way Analysis of Variance (ANOVA) (Tukey-Kramer Multiple Comparisons Test): $p < 0.001$, ***.

DISCUSSION

Measurement of changes in free hormone content in plant tissues offers a clear view of the control of their "*de novo*" production, and often provides an idea of the putative biological roles that these metabolites can play in the process under study. In this study, we have measured changes in the content of three important hormones (SA, JA and ABA) in two strawberry cultivars, Camarosa and Andana, challenged with *C. acutatum*.

SA basal level correlates with differences in susceptibility to *C. acutatum*

We have found that the differences in susceptibility to *C. acutatum* described for the two strawberry cultivars used in this study seem to correlate with differences in their basal level of SA. Thus, higher basal level of SA (nearly two fold) was detected in Andana (less susceptible) than in Camarosa (very susceptible). These values are significantly lowers than those found previously in strawberry leaves of

cv. Jonsok by Hukkanen et al. (2007), (Hukkanen et al. 2007) with values of 1-3 ug g^{-1} fw (from ten times more), and it maybe due to differences in the methodology used, tissue or cultivar analysed (we have used the entire plant vs. leaves, and Camarosa and Andana vs. Jonsok). Differences in the growth conditions might also explain differences in basal level of free SA in the analyzed strawberry samples.

Increase in SA level by exogenous application before fungal inoculation has been shown to produce some degree of protection against *Colletotrichum* and fungal decay in strawberry (Babalar et al. 2007, Asghari and Babalar 2010, Mamaní et al. 2012).

Resistance to disease as consequence of higher basal level of SA has also been reported in other plants as potato, Arabidopsis and rice. Thus, high concentration of basal SA (1–3 ug/g fresh weight) in potato has been suggested that make it more resistant to *Phytophthora infestans* (Coquoz et al. 1995, Vleeshouwers et al. 2000, Navarre and Mayo 2004). Additionally, some Arabidopsis mutants with high basal levels of SA constitutively express SAR, conferring resistance to a broad range of pathogens (Bowling et al. 1994). In rice, the high basal level of SA seems to correlates with resistance to blast fungus, and is not regulated by infection (Silverman et al. 1995).

Hormonal changes during *C. acutatum* infection

Although, ABA may have implications in the strawberry defensive status to bacterial and fungal pathogens by regulating stomata closure (Kubik and Plonka 1984, Yadava 1987, Terry et al. 2007), we have not detected significant alteration in ABA contents after *C. acutatum* inoculation in none of the cultivars studied.

We have detected induction in free SA in cv. Camarosa, challenged with *C. acutatum*. Meanwhile endogenous SA production is required for local defense

activation against biotrophic pathogens and in effective SAR establishment (reviewed in Durrant and Dong 2004, An and Mou 2011), JA production is mainly required for defense against necrotrophic pathogens. Therefore, it seems reasonable to think that induction of SA mediated defense in Camarosa during the first steps of infection, in which the hemibiotrophic pathogen (*C. acutatum*) displayed a biotrophic lifestyle, should slow down the spread of this pathogen.

However, SA production needs to be timing defined and not unnecessarily prolonged to avoid toxic effects in plant cells, high costs in terms of plant fitness (Heil et al. 2000, Heidel and Dong 2006). Thus, negative effects of permanent and uncontrolled SA pathway activation in plant fitness have been published (Heidel and Dong 2006). Also, mechanisms of negative feedback regulation in SA pathway to control excessive endogenous SA production have been reported (Delaney et al. 1995, Wang et al. 2006). None of these mechanisms seem to work properly in Camarosa under *C. acutatum* infection.

On the contrary, Andana cultivar reduced its SA contents in response to infection up to similar values to Camarosa basal levels. However, an early pick of induction of free SA after *C. acutatum*, is not dismissed. Further experiments need to be done to ensure that a reduction of SA basal level in Andana after infection is due to a negative regulation of this pathway by the pathogen and plant resetting of plant metabolism to mitigate SA cytotoxic effect as featured in other plants (Delaney et al. 1995, Wang et al. 2006).

Noteworthy the maintenance of high level of SA induction at later times of infection should become counterproductive in strawberry when pathogen switched to a necrotrophic lifestyle. Indeed, necrotrophic pathogens benefit from these conditions due to the nature of negative crosstalk between SA/JA defense mechanisms (Spoel et al. 2003, Glazebrook 2005, Mur et al. 2006 , Spoel et al. 2007, Koornneef and Pieterse 2008, Koornneef et al. 2008). Surprisingly, JA level increased significantly in Camarosa during its interaction with *C. acutatum*.

Hormone crosstalk in the context of strawberry-*C. acutatum* interaction

It is important to note that cultivar Camarosa challenged by *C. acutatum* suffers an important induction in both SA and JA contents. Increments in both hormones at the same time apparently contrast with their in vivo effectiveness as indeed this cultivar present a very susceptible phenotype and the fungus is able to extend through plant tissues. Curiously, the interaction between these two different types of resistance is expected to be mainly antagonistic as the induction of one must attenuate the other (Feys and Parker 2000, Kunkel and Brooks 2002, Robert-Seilaniantz et al. 2011). However, this do not happens in strawberry cv. Camarosa where both synthetic pathways seem to be active. Consequently, activation of both SA and JA-mediated defense signaling pathways and higher resistance is to be expected. By contrary, a high susceptibility of this cultivar to anthracnose is detected. All in all, it is reasonable to think that *C. acutatum* must strategically manipulate part of these pathways in order to spread within strawberry.

Spoel et al. (2007) (Spoel et al. 2007) demonstrated that simultaneous inoculation of Arabidopsis thaliana with a biotrophic and a necrotrophic pathogen resulted in impaired resistance to the necrotrophic pathogen. This showed that the SA pathway that was activated by the biotrophus suppressed the level of JA-dependent resistance against the necrotrophus. Applying this model to our system, a simultaneous activation of SA and JA defense pathways should be very unsuccessful for the plant, because JA pathway suppression by SA accumulation will be counterproductive in a moment of necrotrophic behavior of the fungus and advanced disease symptoms.

Very similar to our findings during *C. acutatum*–strawberry interaction, El Oirdi et al. (2011) have shown that during *Botrytis*-tomato compatible interaction, inappropriate induction in SA contents as result of pathogenic manipulation of plant defense mechanisms, facilitates establishment of this necrotrophus and cause disease (El Oirdi et al. 2011). The authors showed that after an exopolysaccharide production by the pathogen, which acts as an elicitor of the SA pathway, SA is

strongly increased up to 2.5 times over basal concentration. This SA pathway activation impaired JA based defenses by interruption of JA pathway downstream of the JA production. Moreover, despite of an increase in JA contents of nine fold compared to that of the mock treated plants, JA defenses were impaired, and the fungus could gradually extends through plant tissues.

Curiously, Hukkanen et al. 2007 reported that free SA concentration was not affected in the susceptible strawberry cultivar Jonsok after powdery mildew inoculation (Hukkanen et al. 2007). This result contrast with ours and highlight the importance of further studies in regulation of hormonal equilibrium in strawberry in the context of plant defense.

Very different and difficult to understand is the response exhibited by Andana cultivar to *C. acutatum* infection. A reduction in SA contents, while no changes in JA or ABA concentrations, was stimulated by *C. acutatum*. However, important components of JA synthesis and defense signaling pathways have not been detected in our study. Thus, MJ derivatives (due to its volatility) and all the JA-conjugates have been discarded during the extraction procedure, as stated before. Interestingly, the JA-conjugates have been strongly correlated to plant defense response (Staswick and Tiryaki 2004). So further research is still needed to determine JA conjugates during this strawberry-*C. acutatum* interaction.

CONCLUDING REMARKS

To fully understand the molecular basis underlying the different response of both strawberry cultivars to *C. acutatum* is a complex task. SA and JA defense signaling pathways are activated by biotrophic and necrotrophic pathogens, respectively. However, *C. acutatum* is considered a hemibiotrophic pathogen. Therefore, during its first biotrophic stage of infection, the plant SA defense-signaling pathway is expected to be switched on. The success of the pathogen infection depends on the balance of how quick it is able to elude the plant defenses and how quick the plant is able to mount the defense barrier. With low

basal level of SA in plant, the progress of the infection should be faster than with high basal level, as a higher basal level of SA may provide the plant a valuable state of ready-to-respond to invader and the capacity to delay the biotrophic stage of infection to establish the complete plant defense barrier. Thus, at a first stage, the basal level of SA in the plant might modulate the needs for new synthesis of this signal molecule to fully activate defenses against pathogen. Accordingly, we have observed that infection proceeded faster in Camarosa than in Andana, and a higher and significant increase in SA was also detected in Camarosa, in response to this pathogen.

On the other hand, to quick spread over the plant tissues, the pathogen should activate its own molecular mechanisms to counteract the preformed plant defense barrier and to control the plant defense pathway. At low basal levels of SA the pathogen might spread over the plant tissue so fast that it will be able to trespass the incipient biotrophic plant defense barrier and enter in a different and necrotrophic style of life. Therefore, to avoid further infection, plant also should fully activate the JA signaling pathway. However, increases in SA level during this plant-pathogen interaction could benefit the pathogen spreading if they occur at the right moment. Thus, it is known that some components of the SA pathway negatively interact with important components of the JA defense pathway. So, although the synthesis of both SA and JA was activated after infection in the very susceptible cultivar Camarosa, the observed increase of SA might negatively regulate important components of the JA defense pathway, providing the pathogen with the right conditions to spread all over the plant tissues.

How *C. acutatum* copes with plant defense in the strawberry cultivar Andana seems to be more complex and need further research. The first stage of *C. acutatum* infection is indeed delayed in this cultivar compared with that in Camarosa and correlate with its higher basal level of SA.

MATERIAL AND METHODS

Plant Materials and Growth Conditions

Strawberry plants (*Fragaria* × *ananassa* very susceptible, cv. Camarosa, and less susceptible, cv. Andana) were obtained from meristem tissue culture and maintained in vitro free of pathogens. The plants were growth in basal medium contained the macro-elements of the N30K mineral formulation (Margara 1984) with MS microelements and vitamins (Murashige and Skoog 1962) and stored into individual and hermetic ECO2box white filter (Cat. E1650.0001, Duchefa Biochemie BV, The Netherlands). Culture conditions were as described in (Barceló et al. 1998). Four moth old plants of 4-6 cm in size were sub-cultured into fresh media and acclimated for 3 more weeks before their use.

Fungal Materials and *Colletotrichum* Controlled Inoculation of Strawberry

The *C. acutatum* isolate CECT 20240 was obtained from strawberry crown and grown as described in Casado-Díaz et al. (2006) (Casado-Díaz et al. 2006). Control plants were touch with a cotton ball, while inoculation was done by direct *C. acutatum* mycelial contact. Plant hormones were analyzed in the full plant (vegetative tissue) in both cultivars under study. Samples were collected 3 and 5 days post inoculation (dpi) and immediately frozen in liquid nitrogen and stored at -80°C for analysis.

Hormone Determination in Strawberry Tissues

The extraction and purification procedures, as the chromatographic methods were done as described in Durgbanshi et al. (2005) (Durgbanshi et al. 2005). In brief: 2,5-3 grams of frozen tissue were directly lyophilized. The tissue was immediately homogenized in 5 mL of ultrapure water. Centrifugation (5000g, 10 min) followed to pellet debris. The pH of the supernatant was adjusted to 2.8 with 15% CH3COOH and the supernatant partitioned twice against an equal volume of diethyl ether. After the aqueous phase was discarded, the organic fraction was evaporated in a vacuum at room temperature and the solid residue resuspended in 1 mL of a water/methanol (90:10, v/v) solution which was filtered through a 0.22

μm cellulose acetate filter. A 20 μL aliquot of this solution was then directly injected into the HPLC system. High performance liquid chromatography was performed using a Waters (Milford, MA) Alliance 2690 system, which consists of an autosampler and a quaternary pump. Aliquots (20 μL) were injected on a Nucleosil ODS reversed-phase column. Phytohormones were eluted with a gradient of methanol and 0.01% CH3COOH in water that started from 10:90 (v/v) and linearly reached 60:40 (v/v) in 10 min. In the following 4 min, the gradient increased to 80:20 (v/v). Isocratic conditions of 80:20 were then retained during the last 2 min of the run. The initial conditions were restored and allowed to equilibrate for 5 min, giving a total time of 21 min per sample. The solvent flow rate was 0.3 mL/min with working pressures around 70-100 bar.

The endogenous contents of plant hormones quoted are mean values from 2 measurements of 2 extracts of one experiment. The One-way Analysis of Variance (ANOVA) with a Student-Newman-Keuls Multiple Comparisons Test, Bonferroni Multiple Comparisons Test and Tukey-Kramer Multiple Comparisons Test were performed using GraphPad InStat3 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) to calculate the significant differences between control and inoculated plants.

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Chapter V

Incomplete Activation of Both SA- and JA-Pathways by *Colletotrichum acutatum* Causes Ineffective Defense Response in Strawberry

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ABSTRACT

Plant resistance to pathogenic agents usually operates through a complex network of defense mechanisms mediated by a diverse array of signaling molecules. Whereas the salicylic acid (SA) signaling pathway is mainly activated against biotrophic pathogens, the jasmonic acid (JA) signaling pathway is activated against necrotrophic pathogens.

To gain insights into the physiological and molecular processes which strawberry is able to activate in response to the hemi-biotrophic pathogen *C. acutatum*, a global transcriptional analysis and measurements of the acidic hormones SA, and JA were accomplished in *Fragaria* × *ananassa* after challenge with this pathogen. Induction of key genes controlling important steps within SA and JA signaling pathways was mainly detected. Contrastingly, the induction of known SA and JA-responsive defense genes as PR1, PR2, LOX2, JAR1, and PDF1 was strongly abolished. Both, salicylic acid and jasmonic acid accumulated in strawberry after infection. These data indicate that SA and JA pathways are partially promoted in strawberry against *C. acutatum*, and evidence a putative strategy used by this pathogen to overcome the strawberry plant defense system and to spread within the host by manipulating the fine crosstalk between both hormonal pathways.

INTRODUCTION

Strawberry exhibits great phenotypic diversity on its susceptibility to a large variety of phytopathogenic organisms including *Colletotrichum* spp., which are major pathogens of this crop (Simpson, 1991; Maas, 1998). This fact limits strawberry fruit quality and plant yield production, forcing the excessive use of chemical agents to control diseases.

Regular pesticide applications are not yet considered an appropriate cultivation practice as mostly are environmental contaminants, and adversely affect human health (González-León & Valenzuela-Quintanar, 2007; Fernandes *et al.*, 2011). In addition, many compounds like vitamins, polyphenolics and other antioxidants that plants make to protect themselves from dangers, are also healthy compounds for human consumption as they can act as antioxidants and may protect human cells against damage that can lead to heart disease, cancer and other diseases (Törrönen & Määttä, 2002; Zhang *et al.*, 2008; da Silva Pinto *et al.*, 2010). It has been suggested that these healthy molecules are reduced in plants treated with pesticides, as they need to make less of these compounds (Asami *et al.*, 2003).

As in many other crops, natural resistant resources and breading for this trait constitute the best environmentally friendly alternative to face diseases in cultivated strawberries but totally resistant cultivars to *C. acutatum* spp. have not been yet reported in the hybrid octoploid *Fragaria* × *ananassa* Duch., the main strawberry species worldwide propagated (FAOSTAT [http://faostat.fao.org/]) (Freeman *et al.*, 2001). In addition, strawberry resistance to a variety of pathogens has been reported to be mostly polygenic quantitatively inherited (Amil-Ruiz *et al.*, 2011), making it difficult to associate molecular markers with disease resistance genes.

Resistance development is the result of specific and dynamic molecular interactions between the plant and the pathogen. Understanding the molecular interplay between plant and microbes has successful contributed to identify candidate genes useful for developing biotechnological strategies and help breeding to increase resistance against specific pathogens in many plants (mainly those considered model systems). Plant resistance to pathogenic agents usually operates through a complex network of defense mechanisms. Compounds such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are well known to regulate pathways that allow a fine-tuning of plant defence to mount appropriate responses to different pathogens (Robert-Seilaniantz *et al.*, 2011). Appropriate regulation of defence response is greatly important for plant fitness, as its activation has deleterious effects on plant growth (Heil *et al.*, 2000; Tian *et al.*, 2003). To fine control these specific responses after infection, a balanced production in certain phytohormones is required. Whereas the SA signaling pathway is mainly activated against biotrophic pathogens, the JA/ET signaling pathway is activated against necrotrophic pathogens. Antagonism between these signaling pathways also occurs. In strawberry, the isolation of individual genes related with plant defense has been previously reported (recently reviewed by Amil-Ruiz *et al.*, 2011). Also, Casado-Díaz et al. (2006) first reported on the isolation of a large set of genes with altered expression during the interaction of strawberry and *C. acutatum*. However, most components, and mechanisms of the strawberry defence network remain unknown and poorly understood. Thus, major progress in the physiology, genetics and molecular biology of strawberry, is still needed to fully uncover the logic of its elaborate plant innate immune system.

Over the last decade, microarrays have proved to be a valuable tool to analyze the expression of thousands of genes simultaneously, helping to elucidate the underlying networks of gene regulation that lead to a wide variety of defense responses. The usefulness of this approach has been demonstrated by numerous discoveries of key regulatory genes for defense signaling as well as valuable end-point genes whose products display direct action against pest and diseases (Wang *et al.*, 2006; Sarowar *et al.*, 2011). Very recently, this technique has been used in

strawberry to analyze differences in gene expression between white and red fruit after 24h of their interaction with *C. acutatum* and provided some data of factors likely responsible of pathogen quiescence during fruit immature stages (Guidarelli *et al.*, 2011).

In this report a *F* × *ananassa* microarray has been used to specifically analyze the expression profiles of pathogen-responsive genes in strawberry crown tissue, the main tissue of natural infection, challenged with *C. acutatum*. The synthesis of known signaling molecules such as SA and JA was also examined, and the expression of specific sets of important genes related with defense was monitored by reverse transcription real-time quantitative-polymerase chain reaction (RTqPCR) in crown and petiole, and in plants elicited with SA and JA hormone compounds, at different time points after pathogen infection and treatments. Our studies reveal important physiological changes occurring in strawberry challenged with *C. acutatum*, and evidence aspects, which support for the first time the putative strategy used by this hemi-biotrophic pathogen to overcome the strawberry plant defense system and to spread within the host.

RESULTS

Expression Profiling of *C. acutatum*-Infected Strawberry Crowns

A functional genomic approach employing a proprietary microarray was applied to investigate the early defense responses in strawberry crown tissue after *C. acutatum* infection. For this purpose, plants from the susceptible *Fragaria* × *ananassa* cultivar Camarosa (Casado-Díaz *et al.*, 2006), were used. Two different experimental conditions, infected vs mock, were analyzed after five days of treatments. To assure that pathogen infection was established, a set of infected plants were maintained for longer times, and in all of them pathogen was always re-isolated from crown tissue.

Table 1 shows a summary of identified genes with the highest induction or repression levels after crown infection. A total of 147 genes were differentially expressed following criteria described in Material and Methods. Of these, 118 genes were induced, and 29 genes were repressed. Thus, the activation of gene expression is the predominant mechanism of transcriptional gene regulation detected in strawberry crown (cv. Camarosa) under this biotic stress condition. This pattern of gene expression has been described for other plant-biotic interactions (Koroleva *et al.*, 2005).

To assign a putative biological function to every detected differentially expressed gene, their corresponding orthologous genes from the wild species *F. vesca*, which genome has been recently released (Shulaev *et al.*, 2011), were identified by blasting the EST sequence associated to each singular spot within the array to the overall collection of *F. vesca* predicted genes (Altschul *et al.*, 1990; Shulaev *et al.*, 2011; http://www.rosaceae.org/).

In order to enrich this process, the putative orthologs from *A. thaliana* were also identified for every *F* × *ananassa* gene as a vast functional information is available for the former species (TAIR10: http://www.arabidopsis.org/) (Table 1). Mostly, all ESTs matched with *F. vesca* predicted genes with a range of sequence identity between 95-99%. From all, thirteen *F* × *ananassa* ESTs did not match any sequence within the coding region of the complete database of predicted genes, either because these ESTs represented sequences from the UTR regions of such genes or because they represented not predicted genes (Supplemental Table S1). In addition, six *F. vesca* gene predictions were found to putatively contain more than one unique CDS (gene19270, gene13677, gene05017, gene06367, gene12874, gene25662). Thus, their translated product, were represented by different genes in the transcriptome of Arabidopsis and other species (Supplemental Table S1). Altogether, these new predicted *F. vesca* genes should be appropriately annotated within future reports on *F. vesca* genome studies.

Table 1a. Early up-regulated genes by *Colletotrichum acutatum* in crown tissue of strawberry cultivar Camarosa. Genes were considered as differentially expressed if they fulfilled a FDR < 0.05 after a SAM test analysis and the fold-change was higher that 1.75-fold between the compared conditions. Values represent the ratio of cv. Camarosa mock vs. infected, CC Vs. CI. Regulated genes were grouped acordingly to their role in different steps of the defence response against *C. acutatum (see Table S4 for associated references)* . Asterisk marks those genes which take part in more than one unique functional group. Color code of each group of genes is associate with group shaded in Figure 2.

Strawberry gene ID	*F. vesca* Orthologue	*A. thaliana* Orthologue	Gene Description	Relation with Defense/ Biological Function	CC vs. CI	
					Fold Change	FDR qvalue
Invasion sensing						
M13C5*	gene07245	AT5G13160	Serine/threonine-protein kinase PBS1	Receptor kinase, R protein-guard model	**4,17**	0
M19F7*	gene15497	AT4G33210	SLOMO (SLOw MOtion) F-box/LRR-repeat protein	Fbox/LRR protein, plant receptor, Proteasome complex	**2,65**	7,93E-03
M2F10*	gene19270[a]	AT4G00340	Receptor-like protein kinase 4	Receptor kinase, Signal transduction regulation	**2,43**	7,93E-03
M14D5	gene13911	AT1G30240	Proline-, glutamic acid- and leucine-rich protein 1	LRR protein, plant receptor	**2,35**	7,93E-03
M6C2	gene01890	AT5G42090	Lung seven transmembrane receptor family protein	Plant receptor	**2,35**	7,93E-03
ELRR-39	gene25524	AT5G21090	CPR30 Leucine-rich repeat (LRR) family protein	LRR protein, plant receptor	**2,14**	7,93E-03
M29F3	gene16731	AT3G14460	LRR and NB-ARC domains-containing disease resistance protein	CC-NBS-LRR class of R proteins, plant receptor	**2,00**	7,93E-03
M18E3	gene20858	AT3G14460	Proline-, glutamic acid- and leucine-rich protein 1	CC-NBS-LRR class of R proteins, plant receptor	**1,84**	7,93E-03
Signal Transduction						
M23A9	gene14522	AT4G35790	Phospholipase D delta	Phospholipase D, Transduction of stress responses	**8,26**	0
M27D3	gene18784	AT5G01160	RING/U-box E3 ubiquitin-protein ligase	E3 ligase, Proteasome complex	**7,44**	0
M16B7	gene00744	AT1G69960	Serine/threonine-protein phosphatase PP2A catalytic subunit	Ser/Thr protein phosphatase 2A catalytic subunit	**5,20**	0
M19D11	gene10418[a]	AT3G03940	Casein Serine/threonine-protein kinase	Calcium binding kinase	**4,59**	0
M13C5*	gene07245	AT5G13160	Serine/threonine-protein kinase PBS1	Receptor kinase, R protein-guard model	**4,17**	0
EDS1-936*	gene09503	AT3G48090	EDS1-specific diacylglycerol lipase alpha	Lipase, SA pathway regulator	**3,82**	0
M23A6	gene32391	AT4G11740	Ubiquitin-like superfamily protein	Ubiquitin, Proteasome complex	**3,49**	7,60E-03
M27C10	gene30942$_{3utr}$	AT5G25510	Serine/threonine protein phosphatase 2A regulatory B subunit	Ser/Thr protein phosphatase 2A catalytic subunit	**3,44**	0
M8G2	gene10067	AT4G30960	CIPK-Serine/threonine-protein kinase 6	Calcium binding kinase SOS2	**3,18**	0
M4F10	gene21532	AT1G65430	E3 ubiquitin-protein ligase ARI8	E3 ligase, Proteasome complex	**3,15**	0
M8G7	gene24036	AT4G36990	TBF1 Heat shock factor protein	Major molecular switch for plant growth-to-defense transition	**3,06**	7,93E-03
M24D7*	gene28350	AT5G40150	Peroxidase superfamily protein	Class III peroxidase	**2,76**	7,93E-03
M10E2	gene02575	AT1G27460	NPGR1-No pollen germination related 1	Calmoduling binding protein	**2,73**	7,93E-03
M3D5	gene23778	AT1G05180	NEDD8-activating enzyme E1 regulatory subunit / AXR1 (Auxin resistant 1) NAD(P)-binding Rossmann-fold superfamily protein	Fbox, JA signaling, Proteasome complex	**2,73**	7,93E-03
M19F7*	gene15497	AT4G33210	SLOMO (SLOw MOtion) F-box/LRR-repeat protein	Fbox/LRR protein, plant receptor, Proteasome complex	**2,65**	7,93E-03
M25E7	gene01516	AT1G15780	Bromodomain-containing protein	Interact with calcium binding protein kinase	**2,47**	7,93E-03
M8D11*	gene06214	AT1G60490	Phosphatidylinositol 3-kinase	Protein kinase, Protein trafficking, Secretory Pathway	**2,46**	7,93E-03
M13H9	gene12681	AT5G57020	Myristoyl-CoA:protein N-myristoyltransferase	Co-traslational addition of myristic acid	**2,45**	7,93E-03
M2F10*	gene19270[a]	AT4G00340	Receptor-like protein kinase 4	Receptor kinase, Signal transduction regulation	**2,43**	7,93E-03
M7G11	gene04753	AT1G69640	Sphingoid base hydroxylase 1 (SBH1)	Synthesis of membrane components	**2,42**	7,93E-03
M4E10*	gene16110	AT3G52430	Phytoalexin deficient 4, Lipase	Lipase, Chemical defenses, SA pathway regulator	**2,33**	7,93E-03
M4C3	gene15015	AT5G10930	CIPK-Serine/threonine-protein kinase 5	Calcium binding kinase	**2,25**	7,93E-03
M14H1	gene07894	AT3G51860	Vacuolar cation/proton exchanger 3	Proton/Calcium antiporter	**2,20**	7,93E-03
M7B6	gene05859	AT1G80210	BRCC36A - homologous recombination	Homologous recombination, Deubiquitinating activity, Proteasome complex	**2,20**	7,93E-03
M21H5	gene01441	AT5G56180	Actin-related protein 8	Fbox/Actin/helicase domain, Proteasome complex, XXXRNAmetabolism	**2,05**	7,93E-03
M4E6	gene12959	AT4G33240	1-phosphatidylinositol-4-phosphate 5-kinase	Protein kinase, Protein trafficking, Endomembrane homeostasis	**2,04**	7,93E-03
M28C8*	gene12445	AT1G05260	Peroxidase superfamily protein	Class III peroxidase	**1,98**	7,93E-03
M17E3	gene06367[a]	AT4G24830	Argininosuccinate synthase	NO synthesis, Signal transduction	**1,98**	7,93E-03
M3E6*	gene27591	AT1G71695	Peroxidase superfamily protein (Prx12)	Class III peroxidase	**1,92**	7,93E-03
M10B6	gene01594	AT3G13460	YTH domain family protein 2	Calcium transport to nucleus, regulate gene expression	**1,86**	7,93E-03
M13F3	gene28416	AT3G27925	Protease DegP1	Protease	**1,79**	7,93E-03
M1H8	gene12874[a]	AT5G53360	E3 Ubiquitin protein ligase SINAT3	E3 ligase, Proteasome complex	**1,75**	9,42E-03
New Protein Synthesis and Secretion						
M21B3	gene01340	AT5G13080	WRKY DNA-binding protein 75	Transcription factor	**5,79**	0
M8H8	gene10702	AT4G17960	ATP-dependent RNA helicase DBP10	RNA metabolism	**5,61**	0
M26G7	gene31909	AT2G25970	RNA binding KH domain-containing protein	RNA metabolism	**5,35**	0
M22D9	gene22758	AT3G51980	Armadillo repeat superfamily protein-Hsp70 nucleotide exchange factor fes1	Protein folding	**4,80**	0
J_4-9	gene07210	AT5G13080	WRKY DNA-binding protein 75	Transcription factor	**3,89**	0
M11C6	gene03828	AT1G69620	60S Ribosomal protein L34	Protein synthesis	**3,79**	0
M6G7	gene32154	AT3G48030	Hypoxia-responsive Zinc finger (C3HC4-type RING finger) family protein	Transcription factor	**3,75**	0
M10C12	gene08531	AT1G75780	Tubulin beta-1 chain	Citosqueleton	**3,62**	0
M9F6	gene29752	AT1G28420	Homeobox protein orthopedia	Transcription factor	**3,44**	0
M1A2	gene24354	AT1G62020	Coatomer subunit alpha	Protein transport	**3,31**	0
M23C4	gene02623	AT4G37750	AINTEGUMENTA gene - AP2 like transcription factor	Transcription factor	**3,20**	7,60E-03
M18A9	gene30367	AT5G46190	RNA-binding KH domain-containing protein	RNA metabolism	**2,85**	7,93E-03
M7G4	gene23202	AT3G52250	Duplicated homeodomain-like superfamily protein	RNA metabolism	**2,75**	7,93E-03
M23C7	gene25539$_{3utr}$	AT4G33865	40S ribosomal protein S29	Protein synthesis	**2,57**	7,93E-03
M17H1*	gene13547	AT3G56400	WRKY DNA-binding protein 70	Transcription factor, SA-JA crosstalk	**2,53**	7,93E-03
M18F1	gene09051	AT1G47490	RNA-binding protein 47C	RNA metabolism	**2,49**	7,93E-03
M8D11*	gene06214	AT1G60490	Phosphatidylinositol 3-kinase	Protein kinase, Protein trafficking, Secretory Pathway	**2,46**	7,93E-03
M11H4	gene22626	AT3G12110	Actin 11	Citosqueleton	**2,42**	7,93E-03
M8H3*	gene13803	AT2G38470	WRKY DNA-binding protein 33	Transcription factor, JA pathway	**2,41**	7,93E-03
M14B5	gene29081	AT1G59740	Peptide transporter PTR	Protein secretion	**2,39**	7,93E-03
M5B8	gene24582	AT5G22950	Vacuolar protein sorting-associated protein 24	Protein secretion	**2,22**	7,93E-03
M12E12*	gene21365	AT3G56400	WRKY DNA-binding protein 70	Transcription factor, SA-JA crosstalk	**2,19**	7,93E-03
M3A1	gene30880	AT3G16060	Kinesin-related protein	Citosqueleton	**2,12**	7,93E-03
M19E4	gene05323	AT2G44710	RNA-binding (RRM/RBD/RNP motifs) family protein	RNA metabolism	**2,12**	7,93E-03
M18C5	gene04135	AT1G66140	Zinc finger protein 4	Transcription factor	**2,08**	7,93E-03
M3E11	gene25805	AT1G18650	Plasmodesmata callose-binding endo-1,3-beta-glucosidase protein 3 (PdCB3)	Cell-to-cell trafficking	**2,02**	7,93E-03
M12B6	no hit found[a]	AT3G25940	DNA-directed RNA polymerase TFIIB zinc-binding protein	RNA metabolism	**2,01**	7,93E-03
M7D1	gene10625	AT3G05590	60S ribosomal protein L18-2	Protein synthesis	**2,00**	7,93E-03
M20A3	gene21473	AT5G16715	Valyl-tRNA synthetase	Protein synthesis	**1,98**	7,93E-03
M8A6	gene00998	AT1G77030	DEAD-box ATP-dependent RNA helicase 29	RNA metabolism	**1,93**	7,93E-03
M9E2	gene15731	AT1G80070	Pre-mRNA-processing-splicing factor SUS2	RNA metabolism	**1,92**	7,93E-03
M28B7	gene16235$_{5utr}$	AT2G22430	Homeobox-leucine zipper protein ATHB-6	Transcription factor	**1,89**	7,93E-03
M1C12*	gene28174	AT2G38470	WRKY DNA-binding protein 33	Transcription factor, JA pathway	**1,86**	7,93E-03
M6A9	gene00185	AT5G67300	Transcription factor MYB44	Transcription factor	**1,83**	9,42E-03
M4C6	gene20572	AT3G62310	RNA helicase family protein	RNA metabolism	**1,79**	9,42E-03

Table 1a. Cont.

Strawberry gene ID	*F. vesca* Orthologue	*A. thaliana* Orthologue	Gene Description	Relation with Defense/ Biological Function	CC vs. CI	
					Fold Change	FDR qvalue
Direct Defences						
M24B7	gene14817	AT4G16260	Glycosyl hydrolase superfamily protein	Cell wall degradation, PR protein family	**47,54**	0
M16D12	gene02717	AT3G54420	Chitinase class IV	PR protein family	**7,93**	0
EPR5-77	gene32423	AT4G11650	Pathogenesis-related 5 family protein	PR protein family	**7,52**	0
M5B6	gene24296$_{3utr}$	AT5G09360	Laccase	Lignin biosynthesis	**7,48**	0
M23A10	gene07086	AT1G24020	Pathogenesis-related 10 family protein	PR protein family	**7,08**	0
M12C12	gene31975	AT5G14180	Triacylglycerol lipase 2	Lipase, Chemical defenses	**6,60**	0
M6G11	gene26351	AT4G34135	Flavonol 7-O-glucosyltransferase	Secondary metabolism	**4,34**	0
M6B9	gene05185	AT1G24020	Pathogenesis-related 10 family protein	PR protein family	**3,89**	0
EPR5-284	gene32422	AT4G11650	Pathogenesis-related 5 family protein	PR protein family	**3,88**	0
M1F10	gene09812	AT1G20030	Pathogenesis-related 5 family protein	PR protein family	**3,69**	0
M22A10	gene07085	AT1G24020	Pathogenesis-related 10 family protein	PR protein family	**3,20**	0
M24D7*	gene28350	AT5G40150	Peroxidase superfamily protein	Class III peroxidase	**2,76**	7,93E-03
M5G8	gene07082	AT1G24020	Pathogenesis-related 10 family protein	PR protein family	**2,67**	7,93E-03
M10C5	gene00687	AT1G24020	Pathogenesis-related 10 family protein	PR protein family	**2,66**	7,93E-03
M26E5	gene32023	AT5G17000	Zinc-binding dehydrogenase family protein / oxidoreductase	Redox protection	**2,65**	7,93E-03
M4F3	gene27555	AT1G22750	D-serine/D-alanine/glycine transporter	Secondary metabolism	**2,65**	7,93E-03
M25D10	gene07087	AT1G24020	Pathogenesis-related 10 family protein	PR protein family	**2,44**	7,93E-03
M5C8	gene11632	AT4G32320	L-ascorbate peroxidase 6	Antioxidant defences	**2,36**	7,93E-03
M4E10*	gene16110	AT3G52430	Phytoalexin deficient 4, Lipase	Lipase, Chemical defenses, SA pathway regulator	**2,33**	7,93E-03
M23D11	gene20700	AT4G37990	Cinnamyl alcohol dehydrogenase	Lignin biosynthesis	**2,14**	7,93E-03
M29A9	gene21697	AT3G54420	Endochitinase PR4	PR protein family	**2,02**	7,93E-03
M25D11	gene17437	AT3G07320	O-Glycosyl hydrolases family 17, (1->3)-beta-glucanase	Cell wall degradation, PR protein family	**1,98**	7,93E-03
M28C8*	gene12445	AT1G05260	Peroxidase superfamily protein	Class III peroxidase	**1,98**	7,93E-03
M3E6*	gene27591	AT1G71695	Peroxidase superfamily protein (Prx12)	Class III peroxidase	**1,92**	7,93E-03
M10D7	gene07065	AT1G24020	Fra a 2 allergen	PR protein family	**1,86**	7,93E-03
M26G2	gene31048	AT2G30370	CHAL secreted protein	Inhibite stomatal production	**1,79**	7,93E-03
M21G5	gene04724	AT1G69530	Expansin-A1	Stomatal movement	**1,76**	9,42E-03
Hormone-Dependent Pathways						
EDS1-936*	gene09503	AT3G48090	EDS1-specific diacylglycerol lipase alpha	Lipase, SA pathway regulator	**3,82**	0
M12E4	gene32179	AT1G27500	Tetratricopeptide repeat (TPR)-like superfamily protein	Tetratricopeptide repeat	**3,32**	0
M22A6	gene05545	AT1G80360	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	Pyridoxal-phosphate, oxidative stress response	**2,84**	7,93E-03
M14G2	gene31738	AT4G39820	Tetratricopeptide repeat (TPR)-like superfamily protein	Tetratricopeptide repeat	**2,69**	7,93E-03
M8H2	gene09899	AT5G64250	2-nitropropane dioxygenase	JA pathway	**2,67**	7,93E-03
M26D3	gene18908	AT4G01100	Adenine nucleotide transporter 1 (ADNT1)	Purine transporter, Signalling	**2,56**	7,93E-03
M17H1*	gene13547	AT3G56400	WRKY DNA-binding protein 70	Transcription factor, SA-JA crosstalk	**2,53**	7,93E-03
M25B1	gene23034	AT3G13790	Cell wall Invertase 1 (AtcwINV1): Glycosyl hydrolases family 32 protein	Cell wall invertase, signalling	**2,48**	7,93E-03
M9E10	gene03078	AT1G44750	Purine permease 11	Purine transporter, Signalling	**2,44**	7,93E-03
M8H3*	gene13803	AT2G38470	WRKY DNA-binding protein 33	Transcription factor, JA pathway	**2,41**	7,93E-03
M4E10*	gene16110	AT3G52430	Phytoalexin deficient 4, Lipase	Lipase, Chemical defenses, SA pathway regulator	**2,33**	7,93E-03
M12E12*	gene21365	AT3G56400	WRKY DNA-binding protein 70	Transcription factor, SA-JA crosstalk	**2,19**	7,93E-03
M23C11	gene08617	AT1G76180	Dehydrin cold-regulated 47	ABA responsive	**2,15**	7,93E-03
M16H1	gene14094$_{3utr}$	no hit found	Auxin response factor	Auxin responsive	**2,14**	7,93E-03
M9D5	gene29393	AT4G37150	Methyl salicylate (MeSA) esterase 9	SA release from MeSA	**2,03**	7,93E-03
M30F8	gene29769$_{3utr}$	AT1G28480	Glutaredoxin GRX480	SA pathway, REDOX signaling	**1,92**	7,93E-03
M1C12*	gene28174	AT2G38470	WRKY DNA-binding protein 33	Transcription factor, JA pathway	**1,86**	7,93E-03
M28A2	gene15063	AT5G42650	Allene oxide synthase	JA synthesis	**1,75**	9,42E-03
No obviously related to defense response						
M22B1	gene01044	AT2G25660	Embryo defective 2410		8,25	0
M18E11	gene27435	AT1G34550	Embryo defective 2756		6,55	0
M21E9	gene24023	AT2G24960	MRG family protein, chromatin binding		**3,31**	0
M7B12	gene07388	AT2G21170	Triosephosphate isomerase		2,96	7,60E-03
M24C11	gene32086	AT1G64385	Unknown protein, endomembrane system		2,88	7,60E-03
M13A4	gene23331	AT5G13520	Aminopeptidase M1 family protein / Leukotriene A-4 hydrolase		2,39	7,93E-03
M27A2	gene13677[a]	AT1G32060	Phosphoribulokinase		2,08	7,93E-03
M4E4	gene05017[a]	AT5G49930	Embryo defective 1441		2,07	7,93E-03
M25G5	gene06563$_{3utr}$	AT4G13930	Serine hydroxymethyltransferase 4		2,04	7,93E-03
M3F5	gene13777	AT3G08890	Protein of unknown function		**1,97**	7,93E-03
M22G7	gene09933$_{3utr}$	AT5G41835	non-LTR retrotransposon family		1,93	3,64E-02
M4F8	gene15022$_{3utr}$	AT2G25140	Casein lytic proteinase B4/heat shock protein		1,91	7,93E-03

Functional classification

Figure 1, and Tables S2 and S3, show an overview of the strawberry genes with altered expression after *C. acutatum* infection, and their automated functional categorization assignments using their corresponding Arabidopsis orthologs, GO and FunCat association (Ashburner *et al.*, 2000; Ruepp *et al.*, 2004).

Table 1b. Early down-regulated genes by Colletotrichum acutatum in crown tissue of strawberry cultivar Camarosa. Genes were considered as differentially expressed if they fulfilled a FDR < 0.05 after a SAM test analysis and the fold-change was higher that 1.75-fold between the compared conditions. Values represent the ratio of cv. Camarosa mock vs. infected, CC Vs. CI, transformed by: -1/fold-change for better understanding of values. Regulated genes were grouped acordingly to their direct or indirect role in different steps of the defence response against C. acutatum (see Table S4 for associated references). Asterisk marks those genes which take part in more than one unique functional group. Color code of each group of genes is associate with group shaded in Figure 2.

Strawberry gene ID	*F. vesca* Orthologue	*A. thaliana* Orthologue	Gene Description	Relation with Defense/ Biological Function	CC vs. CI	
					Fold Change	FDR qvalue
Invasion sensing						
M6F8	gene29223	AT1G57680	G-Protein coupled receptor 1	G-protein coupled receptor	**-1,99**	3,95E-02
M20C3	gene24345	AT2G32240	Leucine-rich repeat-containing protein	LRR protein, plant receptor	**-1,93**	3,95E-02
Signal Transduction						
M18F3	gene21849	AT5G43010	Regulatory particle AAA-ATPase 4A / Proteasome complex	Regulatory ATPase, Proteasome complex	**-2,02**	3,95E-02
M29G3	gene25430	AT2G22990	Serine carboxypeptidase	Peptidase, Glucosinolate and phenylpropanoid pathway	**-1,88**	3,95E-02
M5E3	gene12921	AT1G74960	Beta-ketoacyl-ACP synthase	Fatty acid biosynthesis	**-1,80**	3,95E-02
M26F4	gene09121	AT5G67090	Subtilisin-like serine endopeptidase	Peptidase	**-1,78**	3,95E-02
M22F5	gene18417	AT5G02310	Protein ubiquitination component of the N-end rule	Ubiquitin ligase, Proteasome complex	**-1,76**	3,95E-02
New Protein Synthesis and Secretion						
M10H10	gene17514	AT2G32700	LEUNIG_homolog transcriptional correpresor	Transcription represor	**-2,39**	3,95E-02
M28F7	gene25662[a]	AT5G02960	40S Ribosomal protein S12/S23	Protein synthesis	**-2,15**	3,95E-02
M22E3	gene12861	AT5G53430	Histone methyltransferase	Indirect transcription regulation	**-1,86**	3,95E-02
M22E11	gene15974$_{3utr}$	AT1G15750	TOPLESS transcriptional correpresor	Transcription represor	**-1,85**	3,95E-02
M22D5	gene31183$_{3utr}$	AT1G22910	RNA-binding (RRM/RBD/RNP motifs) family protein	RNA metabolism	**-1,78**	3,95E-02
M21G2	gene29663	AT1G29170	SCAR family member	Citoesqueleton	**-1,75**	3,95E-02
Direct Defences						
M29H6	gene32347	AT4G22880	Leucoanthocyanidin dioxygenase (LDOX)	Secondary metabolism	**-1,91**	3,95E-02
M21F3	gene11045	AT1G36370	Serine hydroxymetyltransferase	REDOX production	**-1,90**	3,95E-02
M29C12	gene21346	AT5G05270	Chalcone-flavanone isomerase	Secondary metabolism	**-1,89**	3,95E-02
M19C6	gene26641	AT5G15870	Glycosyl hydrolase family 81 protein	Cell wall degradation, PR protein family	**-1,76**	3,95E-02
Hormone-Dependent Pathways						
M18H1	gene14092	AT1G07590	Tetratricopeptide repeat (TPR)-like superfamily protein	Tetratricopeptide repeat	**-1,82**	3,95E-02
M15G5	gene02397	AT4G03550	Glucan / Callose synthase	Negative regulator SA dependent defences	**-1,80**	3,95E-02
No obviously related to defense response						
M8D2	gene14995	AT5G17920	Methionine synthase		**-2,20**	3,95E-02
M9F8	gene16275	AT4G39970	Haloacid dehalogenase-like hydrolase		**-2,02**	3,95E-02
M7B2	gene10408	AT3G03890	Flavin mononucleotide binding		**-1,94**	3,95E-02
M14A10	gene29476	AT5G52820	WD-40 repeat CUL4 RING ubiquitin ligase complex		**-1,94**	3,95E-02
M5B7	gene09169	AT1G48380	DNA binding protein ROOT HAIRLESS 1, component of the topoisomerase VI complex		**-1,92**	3,95E-02
M18D12	gene20804	AT2G22530	Alkaline-phosphatase-like family protein		**-1,83**	3,95E-02
M18A11	gene08921	AT5G47470	Nodulin transporter family protein		**-1,83**	3,95E-02
M28A7	gene15006	AT5G10840	Endomembrane protein 70 protein family		**-1,81**	3,95E-02
M26H5	gene18624	AT1G01090	Pyruvate dehydrogenase alpha		**-1,78**	3,95E-02
M11B2	gene07537	AT3G13990	Kinase-related protein		**-1,76**	3,95E-02

(a) No obvious detection of ortologue gene due to putative fail in *F. vesca* gene prediction are described in detail in Table S1. $_{3utr}$ and $_{5utr}$ labels indicate that the *Fx ananassa* sequence represent untranslated regions of the corresponding *F. vesca* gene (see Table S1 for details).

Figure 1. (next page) Overview of the microarray results. (a, b) Functional categorization of the differentially expressed genes (A, up-regulated; B, down-regulated). Numbers and names have been taken from the functional classification catalogue (FunCat; (Ruepp et al. 2004)). Percentages represent genes that have been annotated within each function with respect to the total of genes analyzed. According to FunCat, description of the found functional categories is as follow: 01 Metabolism, 02 Energy, 10 Cell Cycle and DNA Processing, 11 Transcription, 12 Protein Synthesis, 14 Protein Fate (Folding, Modification, Destination), 16 Protein With Binding Function or Cofactor Requirement (Structural or Catalytic), 18 Regulation of Metabolism and Protein Function, 20 Cellular Transport, Transport Facilities and Transport Routes, 30 Cellular Communication/Signal Transduction Mechanism, 32 Cell Rescue, Defense and Virulence, 34 Interaction with the Environment, 36 Systemic Interaction with the Environment, 40 Cell Fate, 41 Development (Systemic), 42 Biogenesis of Cellular Components, 45 Tissue Differentiation, 47 Organ Differentiation, 70 Subcellular Localization, 99 Unclassified Proteins. Blue rectangle highline those categories with function in defense response (10, 11, 12, 14, 20, 30, 32, 34, 36).

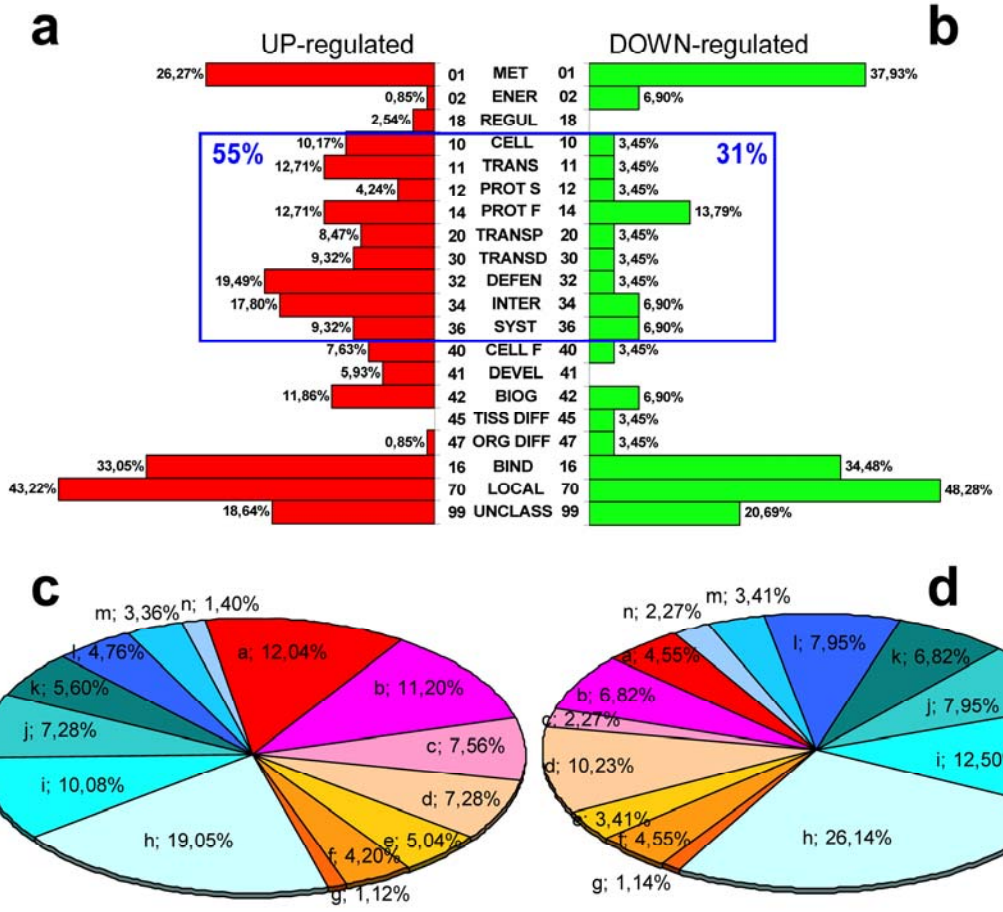


Figure 1. (cont. from previous page) Thus 55% and 31% of up- and down-regulated genes, respectively, have been assigned at least one of these categories. (c, d) Pie chart of gene ontology at the biological process level, UP and DOWN-regulated genes respectively. GO terms shown as follow: a, Response to abiotic or biotic stimulus; b, Response to stress; c, Transport; d, Protein metabolism; e, Signal transduction; f, Transcription, DNA-dependent; g, DNA or RNA metabolism; h, Other metabolic processes; i, Other biological processes; j, Developmental processes; k, Other cellular processes; l, Cell organization and biogenesis; m, Unknown biological processes and n, Electron transport or energy pathways. Terms associated with defense response (a to g) are warm colored. Percentages represent genes that have been associated with each GO term with respect to the total of genes analyzed.

Automated analysis shows that more than 79% of these up- and down-regulated genes were associated to at least one FunCat meaningful functional category (Figure 1A and 1B). 55% of the up-regulated and 31% of the down-regulated set of genes described in Table 1 belong to categories related to plant defense and stress response. Thus, categories 32 (19.49%; cell rescue, defense and virulence), 34 (17.80%; interaction with the environment), and 36 (9.32%; systemic interaction with the environment, fungal specific systemic sensing and response) are among the highest represented. Moreover, categories contributing to an integrative plant response to pathogens, such as trafficking facilities and signal transduction, are well represented (8.47% and 9.32%, respectively). Mechanisms such as transcription control (12.71%), protein fate (12.71%), cell cycle and DNA processing (10.17%), and protein synthesis (4.24%), among others, are also represented, and somehow seem to contribute to the global process of strawberry defense.

Taken together, the FunCat and GO terms automated assignments offer a first overview of the strawberry response against this pathogen. However, to fully understand the specific implication of each strawberry gene into the complex network of defense response to *C. acutatum*, and also when no obvious functional role was annotated within the corresponding orthologue genes, a thoroughly search through the references available in the database from many plant species was performed. Thus, a wider range of the strawberry altered genes could be correlated with defense and biotic stress functions. This study indicated that 89.93% of the up-regulated, and 65.51% of the down-regulated genes were indeed, directly or indirectly related with defense mechanisms. Data showed in Table 1 have been categorized using this information (colored sections), and an extra relation-with-defense/biological-function column has been added, in addition to the gene-description column (see Table S4 for a comprehensive list of associated references reviewed to build up this gene classification). Thus, genes whose expression was modified by infection represented five subsets of molecular functions determining subsequent steps in the strawberry defense response to this pathogen (Figure 2). Thus, in turn, gene functions included from plant receptors to

signal transduction mechanisms under hormonal control (protein modification and degradation), transcriptional changes (transcription factors), new protein synthesis, and secretion of active components of defense (PR proteins, degradative enzymes or chemical defenses). Relevant components implicated in the defense response are discussed below.

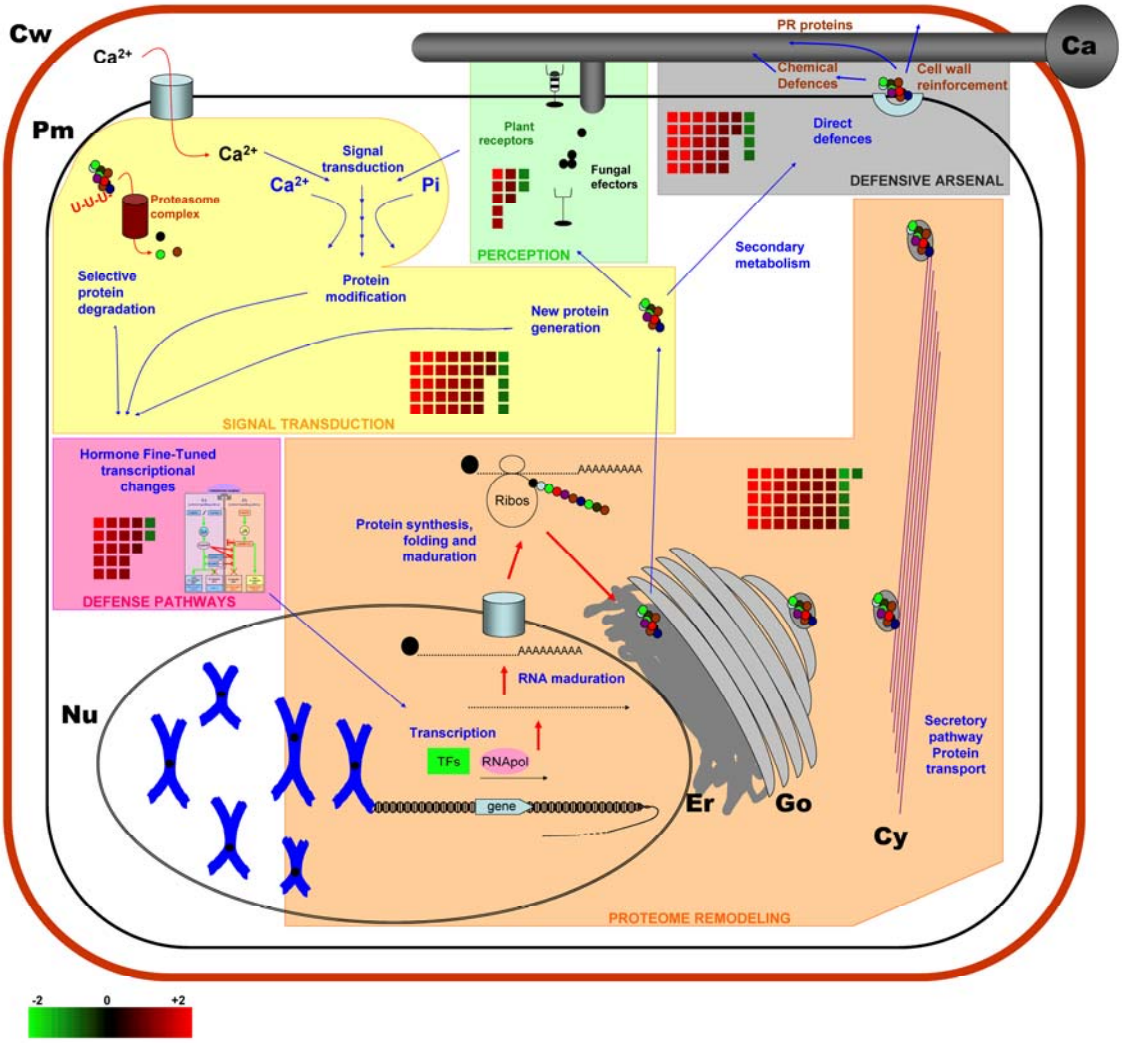


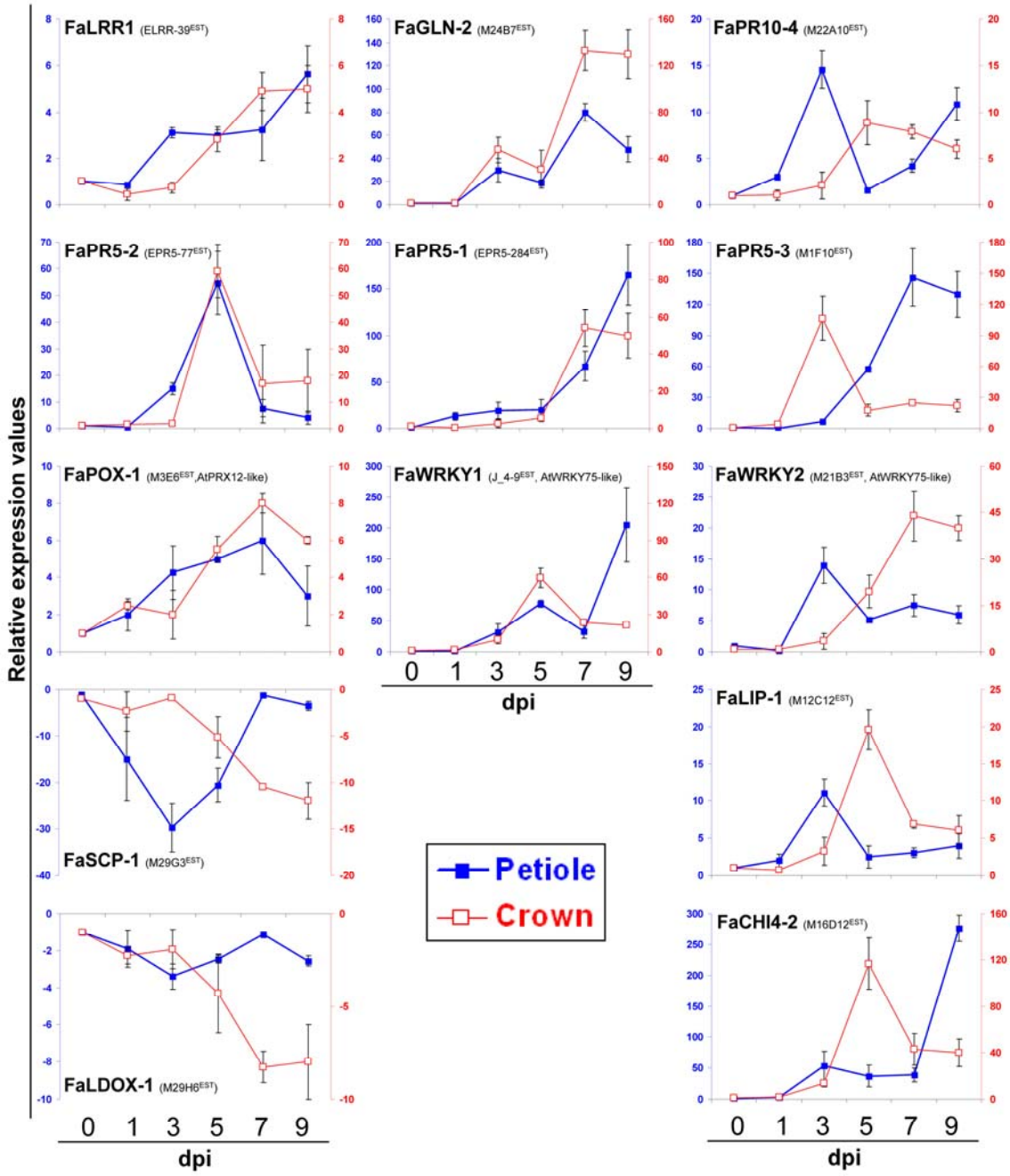
Figure 2. Cellular model for strawberry molecular response to *C. acutatum* infection. Regulated genes have been grouped into five blocs and shaded with colors corresponding to data from functional classification in Table 1. Each gene is represented by a single square and colored according to their respective mean value of LogRatio obtained by the microarray analysis as shown in the colored legend. Cw: cell wall, Ca: Colletotrichum acutatum, Pm: plasma membrane, Nu: nucleus, Er: endomplasmatic reticulum, Go: golgi, Cy: cytosqueletom.

Verification of gene expression changes and extended RTqPCR analysis

Eleven up-regulated genes, and two down-regulated genes, representing the different categories shown in Table 1, were selected to examine the reliability of the microarrays results and to extend the study in a time-course analysis accompanying the progress of infection. The RTqPCR analysis was performed in infected and non-infected crown and petiole tissues, the two main susceptible strawberry tissues to *C. acutatum* attack (Freeman & Katan, 1997). The time points included in the analysis ranged from 1 to 9 days after inoculation to assure a more comprehensive analysis of the early and late gene expression response (Figure 3).

In both strawberry tissues the expression pattern of the analyzed genes after *C. acutatum* inoculation agrees with that obtained by microarray analysis. Although similar pattern and expression level was observed in both crown and petiole for genes FaLRR1 (ELRR-39^{EST}), FaPR5-2 (EPR5-77^{EST}), FaPOX-1 (M3E6^{EST}, AtPRX12-like), slight differences either in timing or gene expression was found for the others when both strawberry tissues were compared. These differences strongly remark the importance of tissue in response to *C. acutatum* infection as previously noted in Casado-Díaz *et al*. (2006). Thus, a similar expression level was detected in both tissues for genes FaPR10-4 (M22A10^{EST}) and FaPR5-3 (M1F10^{EST}) but an earlier increase of gene expression was detected in petiole than in crown in the former gene, and the opposite was found in the latter. Also, differences in timing but similar intensity were found for genes FaWRKY2

Figure 3. (next page) Relative expression values by RTqPCR analysis of relevant strawberry genes during *C. acutatum* infection. Strawberry crown and petiole tissues were harvested 1 to 9 days post treatment (dpi) either with mock or *C. acutatum* spore suspension. At each time point, every inoculated sample was compared with its corresponding mock treated sample. In the graphics, standard value 1 at T0 was added to better illustrate changes. Left and right scales represent relative expression values for petiole and crown tissues respectively. Values obtained for downregulated genes FaSCP-1 and FaLDOX-1 have been represented as 1/2n. AGI locus identifiers for Arabidopsis **orthologue** genes are AT1G71695 (AtPRX12), AT5G13080 (AtWRKY75).



(M21B3^{EST}, AtWRKY75-like), FaLIP-1 (M12C12^{EST}), and FaCHI4-2 (M16D12^{EST}). Thus, earlier gene expression induction was detected in petiole than in crown in genes FaWRKY2 and FaLIP-1, and the opposite was found in gene

FaCHI4-2. Contrastingly, genes FaGLN-2 (M24B7^{EST}), FaPR5-1 (EPR5-284^{EST}), and FaWRKY1 (J_4-9^{EST}) showed similar time expression pattern but FaGLN-2 was strongly induced in crown compared to petiole, and the opposite was found for genes FaPR5-1 and FaWRKY1. Curiously, FaWRKY1 and FaWRKY2, two members of the WRKY family of transcription factors, and both showing high similarity to AtWRKY75, represents conversely behavior in their expression pattern. Also, gene FaSCP-1 (M29G3^{EST}) related with the glucosinolate and phenylpropanoid pathways was repressed earlier and stronger in petiole than in crown, and gene FaLDOX-1 (M29H6^{EST}) encoding a leucoanthocyanidin dioxygenase, was repressed in crown but its expression was practically unchanged in petiole.

Identification of Biological Processes up-regulated after Infection

We have focused on studying the up-regulated set of altered genes to get a closer and comprehensive picture of the strawberry plant defense mechanism, and clues of putative infection strategies of *C. acutatum*.

Important key components of SA-mediated signaling pathway are up-regulated upon challenge with C. acutatum

A comprehensive Singular Enrichment Analysis (SEA) was performed using FATIGO (Al-Shahrour *et al.*, 2004) to identify key processes altered in strawberry after *C. acutatum* attack.

As shown in Figure 4, the cluster of predominantly up-regulated genes is significantly enriched (pvalue < 0.005) in genes belonging to three main subsets: Systemic Acquired Resistance and SA-mediated signaling pathway, responding to bacterium and fungus, and activating the immune response. Strawberry orthologue genes within these enriched categories are: genes FaEDS1 (EDS1-936^{EST}, AtEDS1-like) and FaPAD4 (M4E10^{EST}, AtPAD4-like), already known to be involved in PRR- and R-mediated pathogen-induced SA accumulation in other plants; genes FaWRKY70-1 and FaWRKY70-2 (M17H1^{EST}, and M12E12^{EST},

respectively, two AtWRKY70-like genes); gene FaMeSA1 (M9D5^{EST}, a methyl salicylate esterase); gene FaPBS1 (M13C5^{EST}, a SA-dependent Ser/Thr kinase); and gene FaGRX1 (M30F8^{EST}, similar to a member of the glutaredoxin family which regulates the protein redox state), which are important downstream components of the SA signal transduction pathway, and known to be activators of SA-dependent defense in many plants (see Table S5 for a detailed list of further genes belonging to over-represented functions). These results clearly indicate that SA-signalling pathway is switched on in strawberry upon challenge with *C. acutatum*.

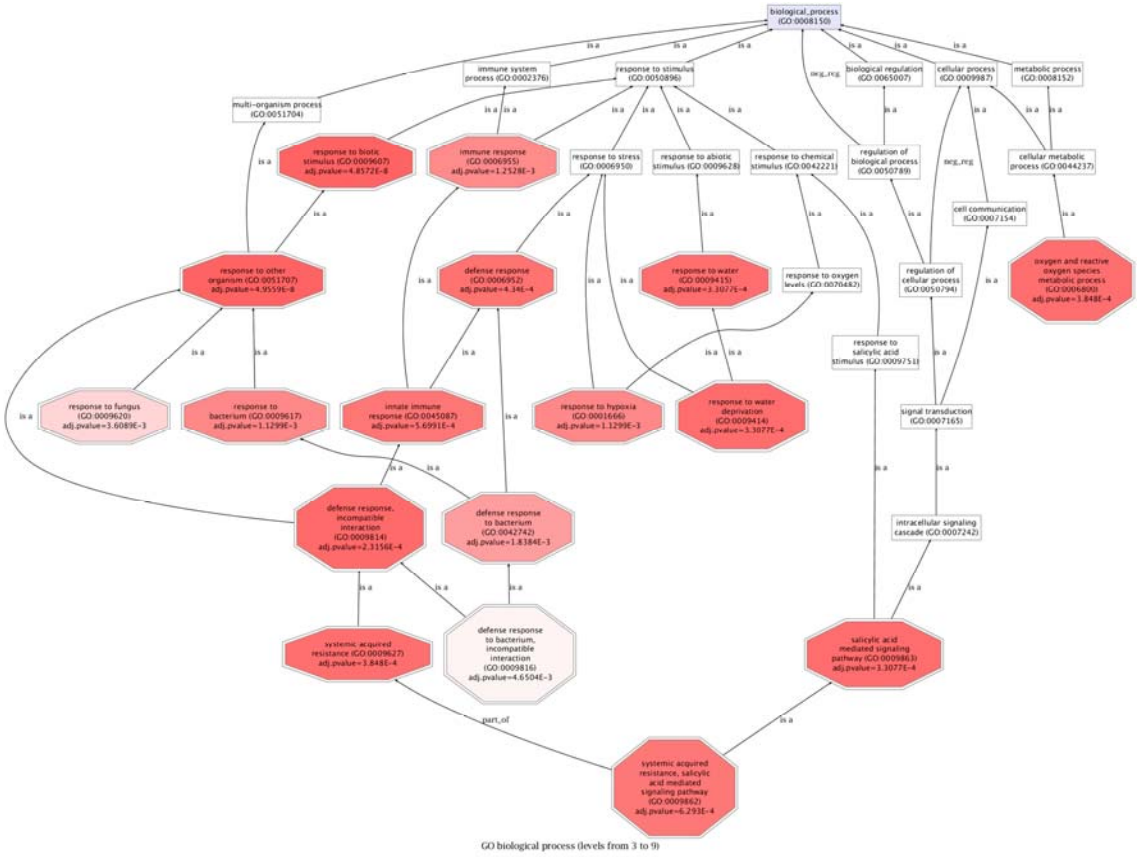


Figure 4. Gene ontology (GO) categories for biological processes (BP) over-represented in strawberry up-regulated genes at pvalue < 0.005 using FatiGO (Al-Shahrour et al. 2004).

Components of Jasmonic acid defense signaling pathway are also induced in strawberry after C. acutatum infection

To verify and extend the expression data, the expression pattern of a representative set of up-regulated genes was analysed in strawberry after SA or MeJA treatments (Figure 5). Importantly, almost all tested genes, which indeed were induced in strawberry by *C. acutatum*, showed significant induction mainly after MeJA treatment. Thus, all the functional identified PR genes such as FaGLN-2 (M24B7^{EST}), FaCHI4-2 (M16D12^{EST}), FaPR5-2 (EPR5-77^{EST}), FaPR5-1 (EPR5-284^{EST}), FaPR5-3 (M1F10^{EST}) and FaPR10-4 (M22A10^{EST}), as well as the WRKY75-like transcription factors (genes FaWRKY1 (J_4-9^{EST}) and FaWRKY2 (M21B3^{EST})) shown to be mainly JA-dependent in strawberry. These results clearly indicate that the JA-dependent defence signaling pathway is also activated in strawberry after *C. acutatum* infection.

Incomplete activation of SA and JA pathways occurs during C. acutatum infection

These results prompted us to investigate whether both SA- and JA- hormone-dependent pathways are fully operative during *C. acutatum* infection. Thus, some well-known components of these signal transduction pathways in other plants were further analyzed in strawberry by RTqPCR. Thus, the expression of strawberry orthologous genes of well studied JA-associated markers such as FaWRKY33-1 (M8H3^{EST}) and FaWRKY33-2 (M1C12^{EST}) (two orthologs to AtWRKY33), FaAOS-1 (M28A2^{EST}, AtAOS ortholog), FaLOX2-1 (AtLOX2 ortholog), FaJAR1 (AtJAR1 ortholog) and FaPDF1 (AtPDF1.2 ortholog), and SA-associated markers such as FaEDS1 (EDS1-936^{EST}, AtEDS1 ortholog), FaPAD4 (M4E10^{EST}, AtPAD4 ortholog), FaGRX1 (M30F8^{EST}, AtGRX480 ortholog), FaWRKY70-1 and FaWRKY70-2 (M17H1^{EST}, and M12E12^{EST}, respectively), FaPR1-1 and FaPR1-2 (AtPR1 ortholog), FaPR2-1 and FaPR2-2 (AtPR2 ortholog), was analyzed in crown and petiole tissues after *C. acutatum* inoculation, and after MeJA or SA exogenous applications (Figures 6 and 7).

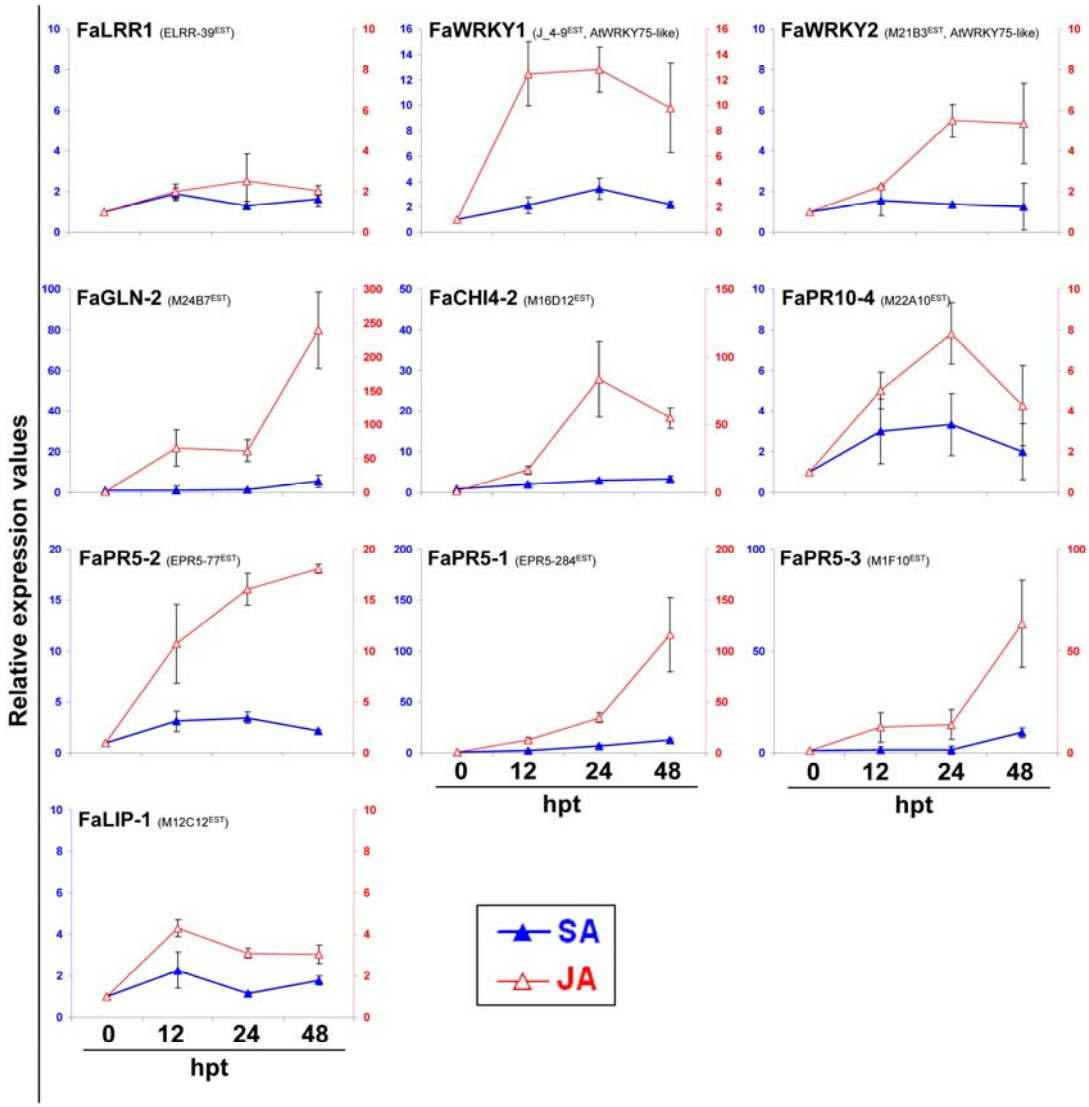
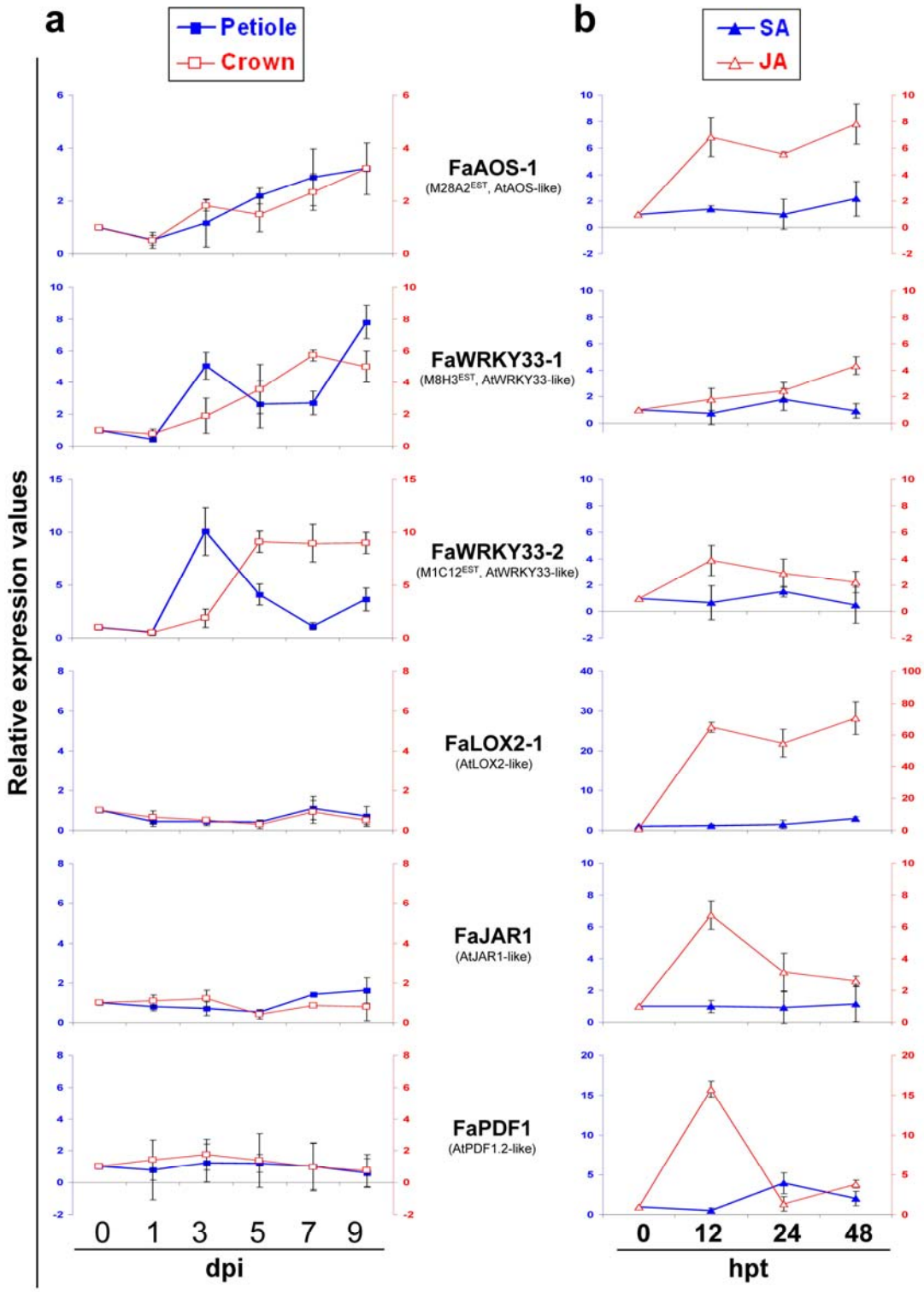


Figure 5. Relative expression values by RTqPCR analysis of ten relevant strawberry genes in response to hormone treatments. Strawberry plants were treated with mock, SA and JA elicitors, and harvested 12 to 48 hours post treatment (htp). At each time point, every elicited sample was compared with its corresponding mock treated sample. In the graphics, standard value 1 at T0 was added to better illustrate changes. Left and right legends represent relative expression values for SA and JA treatments respectively. AGI locus identifiers for Arabidopsis orthologe genes are AT1G71695 (AtPRX12), AT5G13080 (AtWRKY75).

Interestingly, none of the tested strawberry orthologous genes of known markers of JA pathway but genes regulators FaWRKY33 and FaAOS-1 (whose induction was also detected by microarray, Table 1) were induced by infection neither in crown nor in petiole tissues (Figure 6a). On the contrary, the strawberry FaAOS-1, FaWRKY33-1, FaWRKY33-2, FaLOX2-1, FaJAR1, and FaPDF1 were indeed activated in strawberry after MeJA treatment (Figure 6b). As stated before, some differences in their expression pattern were detected when crown and petiole tissues were compared.

On the other hand, all the strawberry SA-pathway associated orthologs but FaPR1-1 were induced after *C. acutatum* infection, accordingly to the results shown in Table 1 (Figure 7). Again, a diversity of expression pattern was detected when crown and petiole was compared. Thus, while the SA pathway regulator orthologs FaEDS1 and FaPAD4, and gene FaPR1-2 (encoding a PR protein) are induced earlier in petiole than in crown, genes FaGRX1 (encoding a glutathione-S-transferase), FaPR2-1 and FaPR2-2 (encoding PRs) are similarly induced in both tissues (Figure 7a). Only significant induction in crown tissue was detected for the two WRKY70-like transcription factors, FaWRKY70-1 and FaWRKY70-2. Interestingly, induction of gene FaPR1-1 (encoding a PR1 protein), a classical SA-pathway-associated marker gene in other plant species, was not detected in strawberry after *C. acutatum* infection. Moreover, all tested strawberry SA-associated orthologous genes were induced by SA treatment but the two classical SA-associated PR orthologous genes, FaPR1-2 and FaPR2-2, which shown to be mainly JA-dependent in strawberry (Figure 7b).

Figure 6. (next page) Relative expression values by RTqPCR analysis of JA-responsive marker genes. a) *C. acutatum* infection in crown and petiole tissues, as previously described in Figure 3. Left and right scales represent expression values for petiole and crown tissues respectively; b) Response to hormone treatments, as described previously in Figure 5. Left and right legends represent expression values for SA and JA treatments, respectively. AGI locus identifiers for Arabidopsis orthologe genes are AT5G42650 (AtAOS), AT2G38470 (AtWRKY33), AT3G45140 (AtLOX2), AT2G46370 (AtJAR1), At5g44420 (AtPDF1.2).


a
Petiole
Crown
b
SA
JA
Relative expression values
FaAOS-1
(M28A2EST, AtAOS-like)
FaWRKY33-1
(M8H3EST, AtWRKY33-like)
FaWRKY33-2
(M1C12EST, AtWRKY33-like)
FaLOX2-1
(AtLOX2-like)
FaJAR1
(AtJAR1-like)
FaPDF1
(AtPDF1.2-like)
0 1 3 5 7 9
dpi
0 12 24 48
hpt

These results strongly suggest that both SA and JA signaling pathways are not fully operative in strawberry during *C. acutatum* infection.

Level of SA and JA during the strawberry/C. acutatum interaction

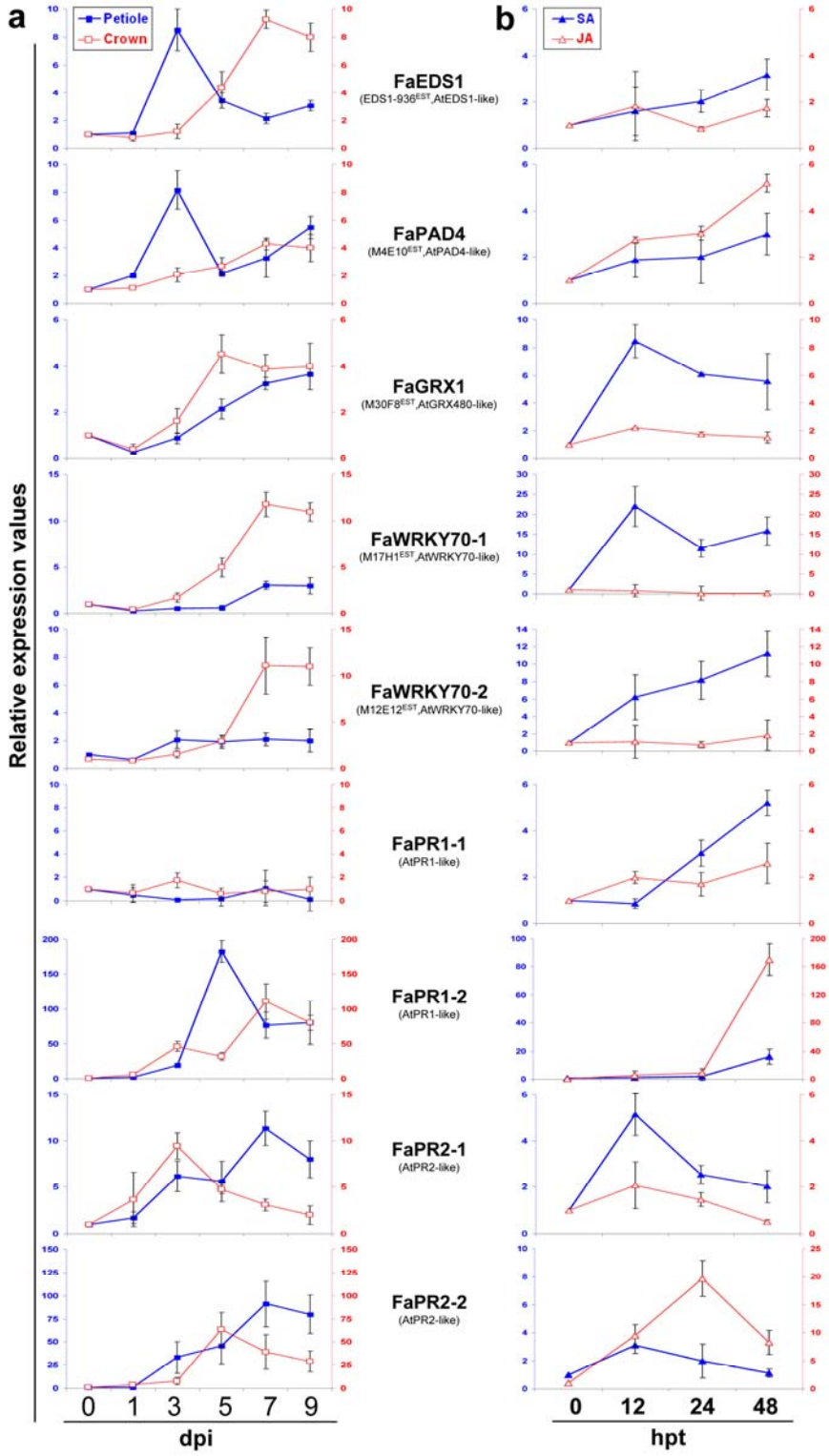
We have measured salicylic acid (SA) and jasmonic acid (JA) content in strawberry plants cv. Camarosa after inoculation with *C. acutatum.* As described in detail in Chapter IV, the very susceptible cultivar Camarosa strongly induced SA production in response to *C. acutatum* infection (see Chapter IV for details). Interestingly, Camarosa also induced JA production in response to *C. acutatum* infection (see Chapter IV for details).

DISCUSSION

Molecular components of the strawberry response to *C. acutatum* identified in this study

The analysis of transcriptomic changes occurred in the strawberry plant upon interaction with *C. acutatum* shows a compendium of responses that this plant can displays in response to this hemibiotrophic pathogen. Many of the identified genes encode proteins with clear known resistance and defense functions, and an important number of strawberry genes encode components that belong to biological steps potentially and sequentially required for successful plant defense.

Figure 7. (next page) Relative expression values by RTqPCR analysis of SA-responsive marker genes. a) *C. acutatum* infection in crown and petiole tissues, as previously described in Figure 3. Left and right scales represent expression values for petiole and crown tissues respectively; b) Response to hormone treatments, as described previously in Figure 5. Left and right legends represent expression values for SA and JA treatments, respectively. AGI locus identifiers for Arabidopsis orthologe genes are AT3G48090 (AtEDS1), AT3G52430 (AtPAD4), AT1G28480 (AtGRX480), AT3G56400 (AtWRKY70), AT2G14610 (AtPR1), AT3G57260 (AtPR2).


a
Petiole
Crown
b
SA
JA
Relative expression values
FaEDS1
(EDS1-936EST,AtEDS1-like)
FaPAD4
(M4E10EST,AtPAD4-like)
FaGRX1
(M30F8EST,AtGRX480-like)
FaWRKY70-1
(M17H1EST,AtWRKY70-like)
FaWRKY70-2
(M12E12EST,AtWRKY70-like)
FaPR1-1
(AtPR1-like)
FaPR1-2
(AtPR1-like)
FaPR2-1
(AtPR2-like)
FaPR2-2
(AtPR2-like)
0 1 3 5 7 9
dpi
0 12 24 48
hpt

Among others, biological steps include members of plant pathogen perception and sensing apparatus, signal transduction machinery, transcriptional factors and regulatory genes, and protein synthesis and secretion mechanisms. Only main components of some of these biological steps are discussed hereafter.

Perception and sensing apparatus: strawberry PRR and R genes

Five genes containing LRR domains, M14D5^{EST} , a proline-, glutamic acid- and leucine-rich protein-1 (Ascencio-Ibáñez *et al.*, 2008); M19F7^{EST} a SLOMO (SLOw MOtion) F-box/LRR-repeat-like protein (Lohmann *et al.*, 2010); M18E3^{EST} and M29F3^{EST}, two CC-NBS-LRR class of R proteins (Meyers *et al.*, 2003; Tan *et al.*, 2007); FaLRR1-ELRR-39^{EST}, a CPR30 LRR protein (Ascencio-Ibáñez *et al.*, 2008; Schenk *et al.*, 2003), were found to be upregulated in strawberry by *C. acutatum*. Leucine-rich repeat proteins (LRR proteins) are 20-29-residue sequence motifs present in a number of proteins with diverse functions in plant including those encoded by defence PRR and R genes (Kobe & Kajava, 2001).

Genes encoding R proteins and PRRs with demonstrated kinase activity in other plants were also upregulated in strawberry upon *C. acutatum* challenge. Thus, gene M13C5^{EST} is similar to a Ser/Thr protein kinase PBS1 described as an R protein with capacity to indirectly recognize the avirulence gene avrPphB monitored by a variable guard protein (Zhang *et al.*, 2010), and gene M2F10^{EST} is similar to a receptor-like protein kinase 4 (RLK4) implicated in pathogen recognition (Du & Chen, 2000), which contains an unusually large number of W-box sequences within its promoter region, suggesting a regulation via WRKY factors. Interestingly, the later Arabidopsis protein is induced by SA treatment or bacterial infection and interacts with E3 ligases (Samuel *et al.*, 2008).

Another upregulated PRR is gene M6C2^{EST}, encoding a protein similar to a member of the highly-divergent family of seven transmembrane receptors (Dunkley *et al.*, 2004). Contrastingly, the strawberry gene M6F8^{EST} encoding

another heptahelical, membrane-spanning G-protein coupled receptor (GPCR) (Gilman, 1987; Gookin *et al.*, 2008), and gene M20C3^{EST} encoding a leucine-rich protein (Kline *et al.*, 2010; Kaplan *et al.*, 2006) were downregulated after *C. acutatum* infection.

Taken together, these results indicate that known components of the sensing apparatus for both basal and R-mediated defence signaling pathways are induced in strawberry during its interaction with *C. acutatum*, and suggest activation of both mechanisms of defense response in this plant.

Signalling transduction pathways: downstream responses against *C. acutatum*

In model plants, one of the big gaps in the understanding of plant immunity is in the signalling pathways that operate immediately downstream of PRR and R protein activation However, partially understood pathways are established (Dodds & Rathjen, 2010). We have found that members of kinase, phosphatase, ubiquitin and calcium gene families related with signal transduction pathways in many plants, were induced in strawberry upon interaction with *C. acutatum* (Figure 2 and Table S4). Importantly, known components of both SA- and JA-dependent defense signalling pathways were also up-regulated.

SA-signalling pathway

Enrichment in specific members of the SA-pathway was detected within the subset of upregulated genes. Thus, the expression of genes FaEDS1 (EDS1-936^{EST}) and FaPAD4 (M4E10^{EST}) is induced by *C. acutatum*. The lipase-like protein EDS1 represents an important node acting upstream of SA molecule in PAMP-triggered immunity (PTI) after stimulation of PRRs and also is required for signalling of all TIR-NB-LRRs tested to date (Wiermer *et al.*, 2005; Heidrich *et al.*, 2011), suggesting that specific effector-triggered immunity (ETI) through TIR domain signaling might be also acting in strawberry against this pathogen. It is known that EDS1 physically interacts with two other positive regulators, PAD4

and SAG101, both of which are putative lipases although hydrolase activity has not been demonstrated for either protein (Wiermer *et al.*, 2005). Interestingly, the expression of Arabidopsis EDS1 is positively regulated by WRKY70 transcription factors (Li *et al.*, 2004) and enrichment in WRKY70 orthologs has also been detected in strawberry (see further below). Moreover, a strawberry PAD4 ortholog (FaPAD4) was also upregulated. PAD4 affect SA accumulation (Wang *et al.*, 2011). Thus, the dissociated forms of EDS1 and PAD4 are fully competent in signalling receptor triggered localized cell death at infection loci (Rustérucci *et al.*, 2001; Aviv *et al.*, 2002) but by contrast, an EDS1–PAD4 complex is necessary for basal resistance involving transcriptional up-regulation of PAD4 itself and mobilization of salicylic acid defences (Rietz *et al.*, 2011).

In many plants, downstream of EDS1 and PAD4 activity, local production of SA trigger defences in the surrounding cells, and, in addition, SAR is activated thorough a systemic signal which primes distal tissues against similar invaders. The SA derivative methyl salicylate (MeSA) is thought to serve as a long-distance phloem-mobile SAR signal in plants (Liu *et al.*, 2011; Dempsey & Klessig, 2012). Once in the distal, uninfected tissue, MeSA must be converted into biologically active SA by esterase activity (Dempsey & Klessig, 2012). Interestingly, induction of the strawberry gene M9D5^{EST} encoding a methyl salicylate (MeSA) esterase similar to the Arabidopsis AtMES9 has been detected (Table 1), which suggests that this signaling mechanism might also be activated in strawberry during *C. acutatum* interaction. Curiously, the Arabidopsis AtMES9 presents in-vitro activity with MeSA, MeJA and MeIAA (Yang *et al.*, 2008) but it showed preference for MeSA as a substrate (Vlot *et al.*, 2008; Dempsey & Klessig, 2012).

Induction of other important genes acting downstream of SA has also been detected during strawberry-*C. acutatum* interaction. Thus, two WRKY70-like genes, FaWRKY70-1 (M17H1$^{EST)}$ and FaWRKY70-2 (M12E12^{EST}), and a glutaredoxin GRX480-like gene, FaGRX1 (M30F8^{EST}), which have been described as essential components for SA-dependent defense activation, was detected in strawberry. In addition, the expression of orthologs to classical SA

marker genes in many plants such as SA-dependent PRs FaPR1-2, FaPR2-1 and FaPR2-2 was highly induced in strawberry after *C. acutatum* infection either in crown or petiole (Figure 7a). Indeed, the FaPR2-2 gene has recently been reported as a good SA-dependent defenses marker in strawberry, as it was induced in this plant by *C. acutatum*, *C. fragariae*, and SA (Zamora *et al.*, 2012). However, although induction was detected in our study for FaPR1-2, FaPR2-1 and FaPR2-2 after SA treatment, both FaPR1-2 and FaPR2-2 turned up to be also highly expressed in strawberry mainly after JA treatment (Figure 7b). Therefore, we propose that these two later genes should not be considered as very selective SA markers in strawberry.

In addition, *C. acutatum* infected strawberry induced expression of gene M8G7^{EST}. The encoded M8G7 protein resembles the HSF-like transcription factor TBF1, a member of a big family of heat responsive proteins (Sanjeev Kumar Baniwal *et al.*, 2004; Ikeda & Ohme-Takagi, 2009) with diversity of functions, including heat stress response (Ikeda *et al.*, 2011; Charng *et al.*, 2007), and plant development (Pernas *et al.*, 2010; ten Hove *et al.*, 2010). Interestingly, the TBF1 protein has recently been shown to be a major molecular switch for plant growth-to-defense transition in Arabidopsis (Pajerowska-Mukhtar *et al.*, 2012). Thus, this transcription factors is a positive regulator of immune responses induced by salicylic acid and PAMPs, and it binds to the TL1 (GAAGAAGAA) *cis* element of NPR1-dependent ER-resident genes required for antimicrobial protein secretion.

JA-signalling pathway

Molecular components unequivocally related to the JA-mediated signalling pathway were also induced in strawberry after *C. acutatum* infection. This is the case of genes FaAOS-1 (M28A2^{EST}), FaWRKY33-1 (M8H3^{EST}), and FaWRKY33-2 (M1C12^{EST}). FaAOS-1 encodes an allene oxide synthase, a member of the cytochrome p450 CYP74 gene family (Song *et al.*, 1993) that functions as a key enzyme in initial steps of the JA biosynthetic pathway (Peña-Cortés *et al.*, 2004; Leon-Reyes *et al.*, 2010), thus generating signalling molecules

which are essential for host immunity and plant development (Bak *et al.*, 2011; Acosta & Farmer, 2010; Gfeller *et al.*, 2010). Interestingly, while only one single copy of AOS gene exists in Arabidopsis (Kubigsteltig *et al.*, 1999), a small AOS gene family with five members can be detected in *F. vesca* genome (unpublished), and three AOS members have been detected in tomato (López-Ráez *et al.*, 2010), suggesting a more complex regulation of this pathway in fruiting plants. In addition, FaWRKY33-1 and FaWRKY33-2 are strawberry orthologs to the well-known WRKY33 transcription factor from Arabidopsis. This important transcription factor acts downstream JA and regulates the expression of classical JA-dependent defense genes such as those encoding glucanases, chitinases, and thaumatin-like proteins, which have been extensively used as JA-asssociated marker genes in other plants. Accordingly, many strawberry orthologs to these JA markers proteins such as FaGLN-2, FaCHI4-2, FaPR10-4, FaPR5-1, FaPR5-2 and FaPR5-3 were strongly induced by *C. acutatum* (Figure 3).

Upregulation of genetic components needed for synthesis of SA and JA in strawberry is also accompanied by a concomitant increase in concentration of such phytohormones in response to infection by *C. acutatum* (see Chapter IV). Therefore, taken together these results clearly demonstrate that both SA and JA defense signalling pathways are activated in strawberry during *C. acutatum* infection.

Evidences that *Colletotrichum acutatum* manipulates the antagonistic effects between immune pathways to promote disease development in strawberry

Extensive cross-talk between SA and JA-dependent signalling pathways fine-tunes the regulation of the plant defence response and both pathways are described mostly antagonistic: elevated biotroph resistance is often correlated with increased necrotroph susceptibility, and elevated necrotroph resistance is often correlated with enhanced susceptibility to biotrophs (Robert-Seilaniantz *et al.*, 2011). More complicated and less understood scenery seems to work in response

to hemibiotrophic pathogens like *C. acutatum* (Münch *et al.*, 2008; Vargas *et al.*, 2012).

Intriguingly, both signalling pathways are activated in strawberry challenged with *C. acutatum* whereas disease is being produced. However, our results also indicate that incomplete activation of both SA- and JA-dependent defense pathways is being produced during this interaction. Thus, very recently, FaPR1-1 was used as a SA dependent marker gene in strawberry and found to be upregulated in cv. Pájaro challenged with the avirulent strain M23 of *C. fragariae* but not after infection with virulent strain M11 of *C. acutatum* (Grellet-Bournonville *et al.*, 2012). Infection with the avirulent strain M23 induced oxidative burst and a temporal SA accumulation in strawberry plants that was accompanied with induction of FaPR1-1 gene expression and protection to a later infection with *C. acutatum*. Interestingly, in our study in spite of many other SA-responsive PRs were upregulated (FaPR1-2, FaPR2-1 and FaPR2-1) no significant induction of FaPR1-1 gene, neither in crown nor in petiole, was detected in cv. Camarosa challenged with *C. acutatum* but indeed this gene responded to SA treatment (Figure 7). Taken together, these results support the hypothesis that *C. acutatum* manipulates a branch of the SA-dependent defense pathway in strawberry which end in the activation of gene FaPR1-1. In addition, the expression of strawberry orthologs to JA-associated defense marker genes in other plant systems and acting downstream of JA, such as FaPDF1, FaLOX2-1 and FaJAR1, also remained unchanged after infection with *C. acutatum,* even though many other components of the JA-mediated signalling pathway were induced (FaAOS-1, FaWRKY33-1, FaWRKY33-2) (Figure 6), which also strongly sustain the hipothesis that *C. acutatum* is able to handle part of the JA-dependent defense pathway in strawberry. All in all, these results indicate that both SA- and JA-dependent defences activated in strawberry during its interaction with *C. acutatum* are not fully operational, which benefits disease development by this pathogen.

How *C. acutatum* is able to interact with specific components of both signaling pathways in strawberry and suppress important plant defenses remains to be

further elucidated but a first integrated model of this complex interacting network can be deduced from our results as shown in Figure 8. Thus, on this biological context, with activation of both SA and JA pathways and increased amount of SA and JA signals, a negative crosstalk between these signals should be expected. Spoel et al. (2007) showed that simultaneous inoculation of *A. thaliana* with a biotrophic and a necrotrophic pathogen resulted in impaired resistance to the necrotrophic pathogen, and demonstrated that the SA pathway that was activated by the biotrophus suppressed the level of JA-dependent resistance against the necrotrophus. Indeed, SA-mediated suppression of JA-responsive gene expression has been reported to be targeted downstream of the JA biosynthesis (Leon-Reyes *et al.*, 2010). Thus, GRX480 is a NPR1 dependent-SA-inducible class III glutaredoxin (Rouhier *et al.*, 2006), (Krinke *et al.*, 2007) specific to land plants (Ziemann *et al.*, 2009), which interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription (Ndamukong *et al.*, 2007; Zander *et al.*, 2011). In addition, WRKY70 also acts downstream of the SA molecule as node of convergence for JA-mediated and SA-mediated signals (Dong, 2004; Li *et al.*, 2004; Wang *et al.*, 2006; Ren *et al.*, 2008), balancing the JA- and SA-dependent responses (Li *et al.*, 2006).

Interestingly, strawberry orthologs FaGRX1, FaWRKY70-1 and FaWRKY70-2 were specifically induced during its interaction with *C. acutatum*, and a negative control on FaPDF1 and other important components of JA-dependent signaling pathway such as genes FaLOX2-1 and FaJAR1, was produced. Moreover, increase in JA synthesis and upregulation of FaAOS-1, the ortholog to Arabidopsis AtAOS, a well known JA-associated marker gene encoding a key enzyme for JA synthesis, was also found after *C. acutatum* infection, supporting that in strawberry repression of JA-responsive genes is targeted downstream of the JA biosynthesis. Indeed, AtAOS has been described as a MeJA-inducible gene but not suppressed by WRKY70 (Li *et al.*, 2006). Very interestingly, the expression of a second group of known JA-responsive genes such as FaGLN-2, FaCHI4-2, FaPR10-4, FaPR5-1, FaPR5-2, FaPR5-3, increased after challenged with this

pathogen, indicating the presence in strawberry of a second GRX480/WRKY70-independent JA-dependent defence branch.

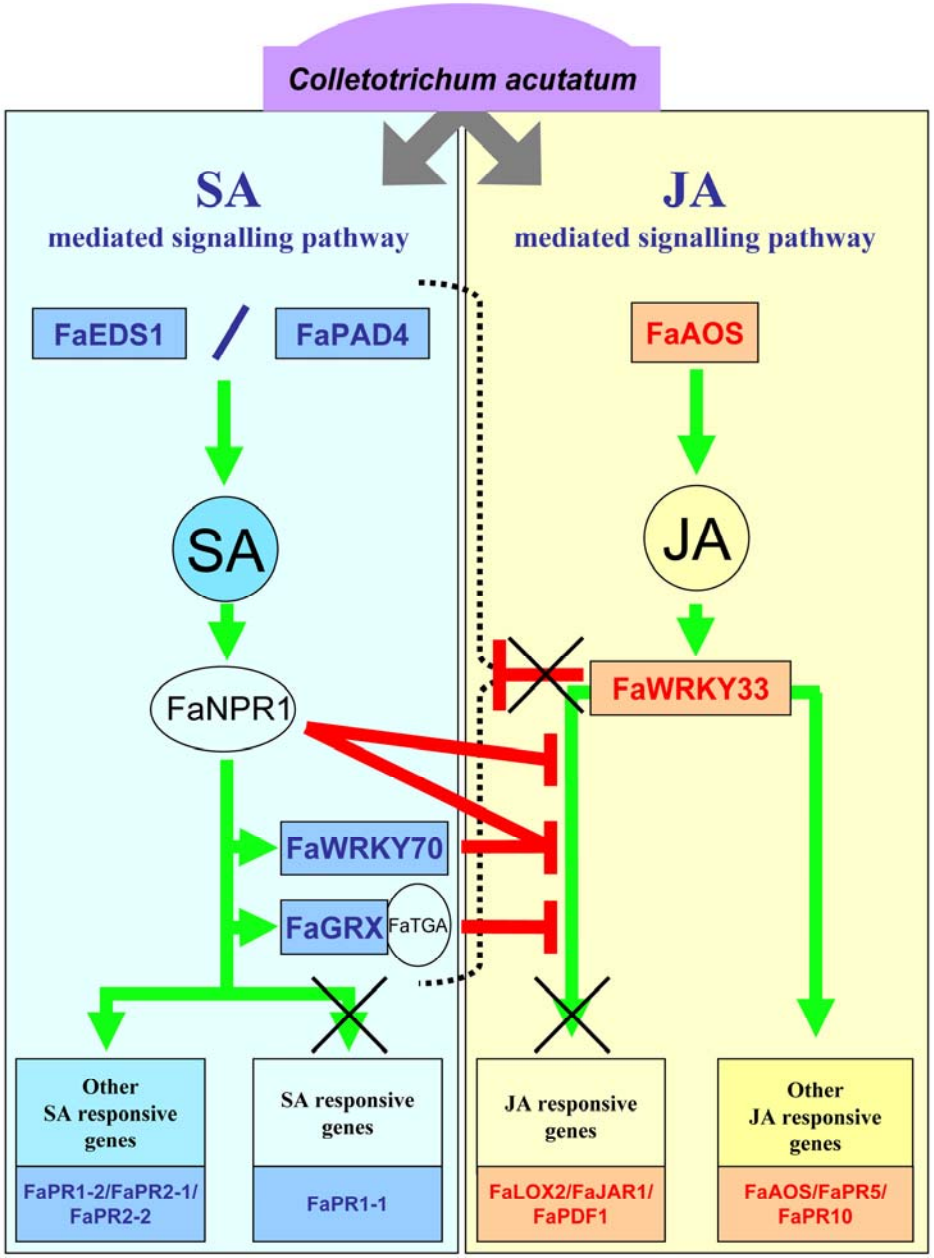


Figure 8. Model for SA-/JA-dependent pathways and crosstalk of defense responses activated in strawberry in response to *C. acutatum*. The strawberry plant activates, at least partially, both SA and JA defense pathways, but negative crosstalk between SA- and JA-mediated defenses prompted by fungal action results in an ineffective defense response against *C. acutatum*.

Importantly, two other JA-dependent AtWRKY33-like genes, FaWRKY33-1 and FaWRKY33-2, were also upregulated in strawberry by *C. acutatum.* The JA-associated component AtWRKY33 has recently been reported as a key transcriptional regulator of defense responses to necrotrophus (Birkenbihl *et al.*, 2012). Indeed, AtWRKY33 acts as a negative regulator of the SA-defense pathway upon pathogen infection and negatively controls the expression of many important genes including those responsible for SA biosynthesis and accumulation, positive regulatory proteins EDS1 and PAD4, and the SA responsive genes PR1, PR2, and PR3. Interestingly, in strawberry, the expression of the SA-dependent orthologous gene FaPR1-1 remained unaltered but very intriguingly, the synthesis of SA and the expression of orthologs to components of SA-mediated signaling pathway acting upstream (FaEDS1 and FaPAD4), and downstream of SA (FaGRX1, FaWRKY70-1, FaWRKY70-2, FaPR1-2, FaPR2-1 and FaPR2-2), was remarkably induced during the infection with *C. acutatum*, in spite of FaWRKY33-1 and FaWRKY33-2 were clearly upregulated. Thus, these results indicate that a repressive control of the entire SA-pathway through these FaWRKY33 transcription factors is not working in strawberry during its interaction with *C. acutatum*, as previously described for AtWRKY33, and highlight a fine strategy of this hemibiotrophic pathogen to spread within this host. In this sense, recent results reported on the tomato-*Botrytis* system (El Oirdi *et al.*, 2011; Rahman *et al.*, 2012) have shown that the exopolysaccharide production by this pathogen (EPS, known as b-(1,3)(1,6)-D-glucan), acted as elicitor of the tomato SA biosynthesis pathway and that inappropriate induction of SA by this pathogen, impaired tomato JA-dependent defences by interrupting the JA signalling pathway downstream of JA production. Consequently, the fungus could gradually spread through tomato plant tissues.

In summary, our results demonstrate that known plant defenses through SA and JA dependent signalling pathways are ineffectively activated in strawberry against *C. acutatum* during its interaction with this pathogen, and support the new emerging paradigm that a key pathogen virulence strategy involves modulation of plant hormone signaling.

To fully understand the molecular basis underlying the response of strawberry to *C. acutatum* is a complex task. However, results from our research will be used to further our understanding of the strawberry immune system to enable future disease control through biotechnological and breeding strategies.

MATERIAL AND METHODS

Plant Materials, Pathogen Inoculation and Hormonal Treatments

Plant culture (*Fragaria* × *ananassa* cultivar Camarosa) and growth conditions, *C. acutatum* (isolate CECT 20240) inoculation, and treatments with chemicals have been previously described (Encinas-Villarejo *et al.*, 2009). Briefly, eight-week-old strawberry plantlets were placed in 20 cm diameter plastic pots containing sterilized peat and grown for a minimum of six additional weeks prior to mock or pathogen inoculation by spraying a spore suspension of 10^4 conidia·ml^{-1}. Crowns and petioles were collected 1, 3, 5, 7 and 9 days after treatment. Under the experimental conditions used these plants looked still healthy and no visible symptom of disease was easily detected even in petioles, crowns or leaves. For treatments and hormonal contents analysis, axenic *in-vitro* plants were aseptically sprayed either with MeJa (2 mM) or SA (5 mM) solutions, or inoculated with *C. acutatum* conidia suspension (10^4 conidia·ml^{-1}), respectively. All samples were immediately frozen in liquid nitrogen and stored at -80 ºC until use.

Total RNA Extraction and Real-time qPCR

Total RNA from strawberry tissues was isolated as described in Casado-Díaz *et al.* (2006), treated with DnaseI (Invitrogen) to remove the residual DNA, and further purified with the RNeasy MinElute Cleanup Kit (QIAGEN). Purified RNA was quantified by NanoDrop 1000 Spectrophotometer (Thermo scientific). RNA integrity was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Deutschland). First-strand cDNA synthesis was carried out using 1µg of purified total RNA as template for a 20 µL reaction (iScript cDNA Synthesis kit (Bio-Rad)). RT reactions were diluted 5-fold with nuclease-free water prior the qPCR.

Specific primer pairs set were designed using Oligo Primer Analysis software version 6.65, tested by dissociation curve analysis, and verified for the absence of non-specific amplification (Table S6). FaGAPDH2 gene was used for normalization (Khan & Shih, 2004). RTqPCR runs were performed with two technical replicates in the same run and three biological replicates in different runs as described previously (Encinas-Villarejo *et al.*, 2009), using SsoAdvanced™ SYBR® Green supermix, and MyIQ v1.004 and iCycler v3.1 real-time PCR systems (Bio-Rad).

Microarray Analysis

For microarray analysis, strawberry samples were collected five days after treatments (spray-infected and mock-treated). Crown from six plants was pooled to make one biological replicate, and total RNA was isolated from three independent biological replicates for hybridization against a proprietary microarray representing approximately 2529 predicted unigenes from *F. vesca* (Shulaev *et al.*, 2011) previously identified from strawberry libraries (Casado-Díaz *et al.*, 2006; and JL Caballero unpublished). Quality control, labeling, hybridization, and scanning were carried out by the SCAI, University of Córdoba (http://www.uco.es/servicios/scai/index.html), following the Genomic Unit guidelines. Microarray images were analysed using GenePix 6.0 software (Molecular Devices). Data were transformed using an intensity-based Lowess function (Yang *et al.*, 2002) with Acuity 4.0 software (Axon Instruments). Genes were considered as differentially expressed if they fulfilled both a FDR < 0.05 after a SAM test analysis (Tusher *et al.*, 2001), and the fold-change (up or down) was above 1.75-fold.

Hormone Determination in Strawberry Tissues

Extraction and purification procedures and chromatographic analysis has been previously described (Durgbanshi *et al.*, 2005). In brief: 3 grams of frozen tissue was lyophilized, and immediately homogenized in 5 mL of ultrapure water. After centrifugation (5000g, 10 min), the pH of the supernatant was adjusted to 2.8 with 15% (v/v) CH3COOH and the supernatant partitioned twice against an equal

volume of diethyl ether. The aqueous phase was discarded and the organic fraction was evaporated in a vacuum at room temperature. The solid residue was resuspended in 1 mL of a 90:10 (v/v) water/methanol solution and then filtered through a cellulose acetate filter (0.22 μm). Then, a 20 μL aliquot of this solution was injected into the high performance liquid chromatography (HPLC) system from Waters, Milford MA (Alliance 2690 system). Aliquots were injected on a Nucleosil ODS reversed-phase column. Phytohormones were eluted with a gradient of methanol and 0.01% CH3COOH in water that started from 10:90 (v/v) and linearly reached 60:40 (v/v) in 10 min. In the following 4 min, the gradient was increased to 80:20 (v/v). Isocratic conditions of 80:20 (v/v) were then retained during the last 2 min of the run. The initial conditions were restored and allowed to equilibrate for 5 min, giving a total time of 21 min per sample. The solvent flow rate was 0.3 mL/min with working pressures around 70-100 bar.

The endogenous contents of plant hormones quoted are mean values from 2 measurements of each of 3 biological replicates. The One-way Analysis of Variance (ANOVA) with a Bonferroni Multiple Comparisons Test was performed using GraphPad InStat3 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) to calculate the significant differences between control and inoculated plants.

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Chapter VI

Others Molecular Mechanisms Altered in Strawberry in Response to *C. acutatum*

ABSTRACT

We have detected alteration in expression of an important number of *F* × *ananassa* genes representing most of the steps which are sequentially required for an efficient defense response. We have detected activation of a variety of signal transduction mechanisms based on calcium, phosphate and ubiquitin binding proteins, and activation of specific hormone-dependent transcriptional factors, which generate a plant growth-to-defense transition and produce a strong impact on synthesis of new proteins and components of secretion to counteract the infection. Table 1 and Figure 2 from Chapter V, shows genes belonging to the different categories mentioned above and a potential model of subsequent steps exhibited in the strawberry cell during the defense response to *C. acutatum*. Thus, new molecular components of defense not mentioned in Chapter V will be further discussed here.

Signal transduction mechanisms altered in response to *C. acutatum*

Protein modification, selective degradation and turnover: Kinase, Phosphatase, ubiquitin and calcium-related signaling components

Our results show that components of catalytic and regulatory PP2A subunits (genes M16B7^{EST} and M27C10^{EST}, respectively) are induced by *C. acutatum*, suggesting that signalling control through this phosphatase might regulate defence in strawberry against this pathogen. PP2A is a major Ser/Thr protein phosphatase that regulates many cellular processes, and consists of multiple subunits with several isoforms, including the catalytic C, the scaffolding A, and the regulatory B subunits (Farkas et al. 2007). The substrate specificity and subcellular localization of PP2A are provided by the variable B subunits (Matre et al. 2009, Ahn et al. 2011). The activity of PP2A is regulated at post-transductional level through ubiquitination of the scaffolding A subunit by CHIP members of E3 ubiquitin ligases (Luo et al. 2006). PP2A controls signal transduction to diverse stresses responses through dephosphorylation of specific target proteins. For example, regulates gene silencing at chromatin level through dephosphorylation of Ser10-phosphorylated histone H3 (histone H3(pSer10)) in response to heat stress (Bíró et al. 2012), activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1 transcription factor (Kim et al. 2011, Tang et al. 2011, Clouse 2011), controls ethylene biosynthesis by differentially regulating the turnover of ACC synthase isoforms (Skottke et al. 2011), and interestingly, a specific B'γ regulatory subunit mediates basal repression of immune reactions preventing unnecessary defence reactions (Trotta et al. 2011b, Trotta et al. 2011a).

Accumulating evidence suggests that ubiquitination modulates signaling mediated by PRRs and is important for the accumulation of NB-LRR type intracellular immune sensors (Furlan et al. 2012). An important example is the case of the fundamental protein NPR1 (Fu et al. 2012). The specificity of substrate ubiquitination is controlled by ubiquitin-protein E3 ligases within a diversity of multi-protein E3 ubiquitin ligase complexes, including the CHIP complex and the Skp, Cullin, F-box containing complex (SCF E3s complex)

(http://www.cellsignal.com/reference/pathway/ubiq_ligase_table.html), and ubiquitin chains promote protein breakdown by the 26S proteasome. Results from the strawberry transcriptome analysis suggest that protein tagging by E3 ligases might be an essential way of control during the strawberry defence response against *C. acutatum*. Thus, we have detected induction of members of this broad SCF E3 ligase complex in strawberry, such as genes M23A6^{EST} (Ubiquitin-like superfamily protein) (Delauré et al. 2008), M27D3^{EST} (E3 ubiquitin-protein ligase) (Delauré et al. 2008), M4F10^{EST} (E3 ubiquitin-protein ligase ARI8) (Mladek et al. 2003, Stone et al. 2005, Kraft et al. 2005), and M1H8^{EST} (E3 Ubiquitin protein ligase SINAT3) (Zhang et al. 2007a, Ryu et al. 2010). In addition, we have detected overexpression of several F-box proteins, such as genes M3D5^{EST} (del Pozo et al. 2002, Xu et al. 2002, Lorenzo and Solano 2005), M21H5^{EST}, and the LRR-repeat-like protein M19F7^{EST}. F-box domains commonly exist in proteins in concert with other protein–protein interaction motifs such as LRR and WD repeats, which are components of the SCF E3s complexes and mediate interactions with SCF substrates (Xiao and Jang 2000, McKinney et al. 2002, Callis and Vierstra 2000). In Arabidopsis F-box proteins and SCF E3s complexes play critical roles in various aspects of plant growth and development, and are implicated in auxin (TIR1) and jasmonate signalling (COI1) (Gagne et al. 2002). Indeed, strawberry protein M3D5^{EST} is similar to a NEDD8-activating enzyme E1 regulatory subunit, known to be necessary for JA and auxin signalling (del Pozo et al. 2002, Xu et al. 2002, Lorenzo and Solano 2005), and to regulate the protein degradation activity of SCF E3s complexes (Dharmasiri et al. 2007, Hotton et al. 2011), (Merlet et al. 2009). Thus, the covalent attachment of ubiquitin is an important determinant for selective protein degradation by the 26S proteasome in plants and animals. Protein selective degradation and turnover is a usual signalling method in plant defence (Delauré et al. 2008), and also this process of regulation is mentioned many times in relation with several of the defence components described on this manuscript. This fact shows the relevance of this regulation in the strawberry defence response.

Contrastingly, other components also implicated in protein degradation are downregulated in strawberry by *C. acutatum*. This is the case of gene M22F5^{EST} that encodes an ubiquitin ligase of the N-end rule pathway with arginine specificity (Garzón et al. 2007), and gene M18F3^{EST}, a putative member of the proteasome complex similar to the proteasoma regulatory particle AAA-ATPase 4A (Glickman et al. 1998, Fu et al. 1999a, Fu et al. 1999b, Fu et al. 2001). The N-end rule pathway has been implicated in diverse functions in plants (Graciet et al. 2009, Licausi et al. 2011).

Calcium signaling mediates a multitude of plant responses to external stimuli and regulates a wide range of physiological processes. We have detected induction of genes M24D7^{EST}, M28C8^{EST}, M3E6^{EST}, and M14H1^{EST}. The first three strawberry genes encode class III peroxidases, known to activate non-selective cation channels so that increase the entrance of Ca^{2+} into plant cell. It is known that the increase in cytosolic Ca^{2+} and ROS are required to activate AOS expression as well as other JA-responsive genes and many other defence responses, including exocitosis and delivery of new membrane material, against fungal infection (Hu et al. 2009, Demidchik and Maathuis 2007). Interestingly, upregulation of components from the secretory pathway has also been detected in strawberry in response to *C. acutatum* (Chapter V Table 1). In addition, gene M14H1^{EST} encodes a protein similar to a vacuolar cation/proton exchanger, which is activated in Arabidopsis by protein kinase SOS2 and modulates Ca^{2+} levels within cells by sequestering Ca^{2+} into the vacuole (Cheng et al. 2004, Cheng et al. 2005, Barkla et al. 2008, Manohar et al. 2011). Intriguingly, the SOS2 strawberry ortholog, gene M8G2^{EST}, is also induced by *C. acutatum* (Chapter V Table 1). The biological meaning of the increased expression detected in genes M14H1^{EST} and M8G2^{EST} in strawberry upon *C. acutatum* interaction remains to be elucidated but it can be either a plant mechanism to back to normal metabolism due to a major release of Ca^{2+} after interaction with the pathogen or a consequence of the pathogen strategy to control Ca^{2+}-dependent defense responses.

In addition, we have also detected induction of genes encoding members of the plant CBL-interacting protein kinases or CIPK gene family such as genes M4C3^{EST} and M8G2^{EST}, indicating that this signal transduction pathway can be active in strawberry challenged with *C. acutatum*. The CIPKs act in calcium signal transduction directly interacting with calcium-binding proteins, like calcineurin B-like (CBL) proteins or other Ca^{2+} sensors (Luan et al. 2002, Hrabak et al. 2003, Kolukisaoglu et al. 2004), and members of this family (i.e. SNF1-related protein kinase/SOS2 like PK5) interacts with and phosphorylate NPR1, a key regulator of the SA pathway acting downstream of SA, so that modulate expression of downstream defence genes (Ferrando et al. 2001, Hrabak et al. 2003, Xie et al. 2010). Interestingly, strawberry M8G2^{EST} encodes a CIPK-SOS2-like protein, whose ortholog is also induced in Arabidopsis during the early interaction with *Golovinomyces cichoracearum* (Fabro et al. 2008).

Lipid Signals

In addition to the well known regulators EDS1 (EDS1-specific diacylglycerol lipase alpha), and PAD4 (phytoalexin deficient 4, lipase), other genes encoding lipid-related proteins connected to defence signal transduction, are upregulated in strawberry challenged with *C. acutatum*. Thus, gene M23A9^{EST} encodes a phospholipase D (PLD), which has emerged as an important enzyme involved in signal transduction of stress responses (Katagiri et al. 2001, McGee et al. 2003), responsible of control hydrogen peroxide mediated cell death (Zhang et al. 2003), and participate in ABA-induced stomatal closure (Uraji et al. 2012).

Two strawberry members of distinct inositol lipid kinase families, genes M8D11^{EST} and M4E6^{EST}, have also been upregulated in strawberry during its interaction with *C. acutatum*. The inositol lipid kinases catalyze the synthesis of phosphoinositides (PIs) from phosphatidylinositol, which play key roles in cell signalling (Mueller-Roeber and Pical 2002) (van Leeuwen et al. 2004). Thus, the M8D11^{EST} ortholog in Arabidopsis encodes a phosphatidylinositol 3-kinase essential for normal plant growth, which meditates ROS production and tolerance to salt stress (Leshem et al. 2007), and regulates protein trafficking through

vacuole reorganization and rearrangement (Whitley et al. 2009). Also, gene M4E6^{EST} encodes a 1-phosphatidylinositol-4-phosphate 5-kinase whose Arabidopsis ortholog controls endomembrane homeostasis including endocytosis, vacuole formation, and vacuolar acidification (Hirano et al. 2011) but it is also implicated in protein transporting (such as auxin transporters) to the vacuole or recycling proteins on the plasma membrane (PM) through the use of endosomes in a variety of eukaryotic cells (Hirano and Sato 2011). Upregulation of genes M8D11^{EST} and M4E6^{EST} suggest that signal mechanisms that control protein trafficking are activated in strawberry during plant defence against *C. acutatum*. Indeed, protein trafficking is a very important process during plant defence (Wang et al. 2012).

Plant cells respond to different biotic and abiotic stresses by producing various uncommon phospholipids that are believed to play key roles in cell signalling (van Leeuwen et al. 2004). Phosphoinositides (PIs) constitute a minor fraction of total cellular lipids in all eukaryotic cells. They fulfil many important functions through interaction with a wide range of cellular proteins. Members of distinct inositol lipid kinase families catalyze the synthesis of these phospholipids from phosphatidylinositol (Mueller-Roeber and Pical 2002). In example, the strawberry gene M8D11^{EST} encoding a Phosphatidylinositol 3-kinase is induced by infection, and has been described in Arabidopsis to be essential for normal plant growth and have been also implicated in diverse physiological functions (Lee et al. 2008a). Thus, meditates ROS production and tolerance to salt stress (Leshem et al. 2007), and take part in forming a complex that regulates protein trafficking, that as commented below, it is a very important process during plant defence (Wang et al. 2012). This protein is essential for vacuole reorganization (Lee et al. 2008b) and have crucial role in modulating the dynamics of vacuolar rearrangement (Whitley et al. 2009). In the same way, gene M4E6^{EST}, and 1-phosphatidylinositol-4-phosphate 5-kinase, functions in vacuole/ lysosome homeostasis and controls endomembrane homeostasis including endocytosis, vacuole formation, and vacuolar acidification in Arabidopsis (Hirano et al. 2011), but also in transporting various proteins (such as auxin transporters) to the vacuole or recycling proteins

on the plasma membrane (PM) through the use of endosomes in a variety of eukaryotic cells (Hirano and Sato 2011). The overexpresion of these two genes in strawberry in response to *C. acutatum* infection manifest the implication of this lipid derived signals during the transduction of the invader signals sensing, and provably shows an specific response based in endomembrane rearrangement trying to prompt an efficient defence response .

Also, gene M7G11^{EST} encoding a sphingoid base hydroxylase 1 (SBH1) was upregulated in strawberry. The Arabidopsis ortholog of M7G11^{EST} protein takes part in sphingolipids synthesis, an important membrane component, and also regulates cell proliferation and apoptosis. Avirulent *Pseudomonas* infection triggers de novo synthesis of these components, and necrotrophic fungi utilize toxins interfering with sphingolipid metabolism of the host plant (Peer et al. 2010). These virulence factors cause apoptotic cell death in various plant species, suggesting that the regulation of specific plant sphingolipids might also be crucial for the outcome of hemibiotrophic plant–pathogen interactions and could be involved in plant pathogen defence processes (Peer et al. 2010).

Strong impact on proteome remodeling

Transcriptional Regulators

Transcriptional reprogramming is needed to produce new proteins and defence-associated changes in plant upon attack of pathogens. Thus, generation of a new set of transcription factors to control the expression of genes encoding new proteins must correlate with modification of many aspects of the RNA and protein metabolism, and will be discussed here.

In addition to the previously mentioned ones, we have detected a wide variety of transcriptional regulators induced in strawberry by *C. acutatum*. Thus, gene M18C5^{EST} encodes a member of the zing-finger family protein C2H2 (Tague and Goodman 1995, Englbrecht et al. 2004) whose ortholog was repressed in Arabidopsis in distal leaf tissue at 72 h (systemic maintenance period) after

inoculation with the necrotrophus *A. brassicicola* (Schenk et al. 2003). Gene M6A9^{EST} encodes a transcription factor MYB44, which belongs to the R2R3 MYB transcription factor family subgroup 22. This TF in Arabidopsis is upregulated by a great variety of phytohormones, elicitors, biotic and abiotic stresses (Kranz et al. 1998, Devoto et al. 2005, Koroleva et al. 2005, Yanhui et al. 2006, Libault et al. 2007, Gadjev et al. 2006, Delessert et al. 2004, Livaja et al. 2008, Gust et al. 2007, Liu et al. 2010, LÜ et al. 2010) and thus, is considered part of common stress response genes (Ma and Bohnert 2007). This last gene is also part of COI1-dependent JA inducible transcription factors, and negatively regulates PAL genes (Wang et al. 2008), while positively regulates ABA sensitivity in stomatal closure through reduced expression of PP2Cs encoding genes which have been reported as negative regulator of ABA signalling (Jung et al. 2008). Intriguingly, constitutive expression of MYB44 in Arabidopsis seems to suppress JA-responsive gene activation of well known jasmonate-responsive genes, including JR2, VSP, LOXII, and AOS (Jung et al. 2010, Shim et al. 2010).

The gene M28B7^{EST} encodes a homeodomain leucine zipper class I protein (HD-ZIP I protein ATHB-6) that is a target of the protein phosphatase ABI1 (a protein phosphatase 2C) and regulates hormone responses (Söderman et al. 1994). Althoug being induced by ABA, it has been described as a negative regulator of the ABA signal pathway, acting downstream of ABI1 (Himmelbach et al. 2002), and ABA negatively regulates ATHB6 protein turnover through Cullin3-based ubiquitin E3 ligase and proteasomal degradation (Lechner et al. 2011).

In addition, we have detected some other upregulated strawberry genes similar to plant genes with no reported direct implication in defence response to pathogens but related to abiotic stress response. Thus, gene M6G7^{EST} encodes a member of the hypoxia-responsive C3HC4-type RING zinc finger protein family of transcription factors (Stone et al. 2005, Kosarev et al. 2002) related to hypoxia response (Gracey et al. 2001). Also, genes M9F6^{EST} and M23C4 ^{EST} encode respectively, a homeobox protein orthopedia, implicated in regulation of transcription in Arabidopsis that is induced by auxin but inhibited by cytokinin

roots (Son et al. 2004), and a AINTEGUMENTA gene-AP2 like transcription factor (APETALA2 domain family) that is required to control cell proliferation and respond to auxin and cytokinin (Alvarez-Buylla et al. 2010, Losa et al. 2010, Holst et al. 2011, Krizek and Eaddy 2012, Krizek 2011, Smith and Long 2010, Krizek et al. 2000).

WRKY Family

WRKY transcription factor family is found only in plants with up to 100 representatives in Arabidopsis and maize. This family of genes appear to be involved in the regulation of various physiological programs that are unique to plants, including pathogen defence, senescence and trichome development (Eulgem et al. 2000, Riechmann et al. 2000, Wei et al. 2012). They physically interact with W-box through their C-terminal DNA-binding domain (Brand et al. 2010). During strawberry-*C. acutatum* interaction, we have detected induction of several members of this WRKY family orthologous to Arabidopsis genes with known function in plant defences such as WRKY75 (FaWRKY1 J49^{EST} and FaWRKY2 M21B3^{EST}), WRKY70 (FaWRKY70-1 M17H1^{EST} and FaWRKY70-2 M12E12^{EST}), and WRKY33 (FaWRKY33-1 M8H3^{EST} and FaWRKY33-2 M1C12^{EST}).

Recently, WRKY75 and FaWRKY1 have been reported to act as positive regulators of defence during compatible and incompatible interactions in Arabidopsis and, very likely, FaWRKY1 was suggested to be an important element mediating defence responses to *C. acutatum* in strawberry (Encinas-Villarejo et al. 2009). Also, WRKY75 is induced by PAMPs (Thilmony et al. 2006), *Pseudomonas* (Zhang et al. 2007b) and *Hyaloperonospora arabidopsidis* (Huibers et al. 2009). In addition, the *Brassica napus* ortholog to WRKY75 is strongly upregulated after infection with *Sclerotinia sclerotiorum,* playing an important role in the defence response to this necrotrophic plant pathogen (Zhao et al. 2007). Therefore, FaWRKY1 seems to be an important element mediating defence responses to *C. acutatum* in strawberry.

Genes WRKY33 and WRKY70 have been extensively studied in plants due to their respective implications in a broad range of stress responses as well as their relevance in the balance between SA- and JA-dependent signalling pathways. Gene WRKY33 belongs to group I of this superfamily of transcription factors (Eulgem et al. 2000). It is upregulated early in plant by a variety of PAMPs and pathogens, including the plant defence elicitor chitin (Wan et al. 2004, Libault et al. 2007, Lippok et al. 2007, Sarowar et al. 2011), as well as a variety of abiotic stresses (Klok et al. 2002, Jiang and Deyholos 2009). WRKY33 induction is dependent on ABA signalling in an SOS-pathway independent manner (Li et al. 2011), and is definitively essential for defence toward the necrotrophic fungus *Botrytis cinerea* (Birkenbihl et al. 2012). Interestingly, induced expression of WRKY33 itself appears to be regulated by WRKY factors including autoregulation by WRKY33 protein (suggesting a potential positive feedback regulatory loop) (Turck et al. 2004, Lippok et al. 2007, Mao et al. 2011). During the last few years, many authors have contributes to increase knowledge about WRKY33 mode of action and regulation at protein level. In the absence of pathogens, Arabidopsis MAP kinase 4 (MPK4) exists in nuclear complexes with the WRKY33 transcription factor and a coupling factor, MKS1 (a MPK4 substrate which negatively regulates defence response against necrotrophus (Fiil and Petersen 2011). Challenge with *Pseudomonas syringae* or flagellin leads to the activation of MPK4 and phosphorylation of MKS1. Subsequently, complexes with MKS1 and WRKY33 are released from MPK4, and thus allowing recruitment of WRKY33 to target PAD3 promoter, which drive the metabolic flow to camalexin production, which is the main phytoalexine in Arabidopsis involved in plant defense, and also has cancer-preventive property (Mezencev et al. 2003, Qiu et al. 2008, Pandey and Somssich 2009, Kishi-Kaboshi et al. 2010, Petersen et al. 2010). It has been shown that expression of pathogen-defence genes such as WRKY33, is mediated by Ca^{2+} signalling pathway, which is associated with AtPep peptides and their receptors (Qi et al. 2010). Also, two sigma factor binding proteins (SIB1 and SIB2) interact with WRKY33 and function as activators of WRKY33 in plant defence against necrotrophic pathogens such as *Botrytis cinerea* (Lai et al. 2011, Birkenbihl et al. 2012), In

conclusion, WRKY33 is a key transcriptional regulator of hormonal and metabolic responses against infection and regulates the antagonistic relationship between defence pathways mediating responses to biotrophic and necrotrophic fungal pathogens. Therefore, loss of WRKY33 function results in inappropriate activation of the salicylic acid (SA)-related host response and elevated SA levels post infection and in down-regulation of jasmonic acid (JA)-associated responses at later stages (Zheng et al. 2006b, Birkenbihl et al. 2012). Accordingly, the expression of two FaWRKY33 genes in strawberry challenged with *C. acutatum* is in agreement with the partial inhibition of the SA-dependent defense pathway, as shown in Chapter V.

On the other hand, WRKY70 belongs to group IIIb of WRKY transcription factor superfamily (Kalde et al. 2003). WRKY70 expression is induced by PAMPs (Libault et al. 2007) and negatively regulated by trimetylation of lysine 4 of histone H3 on its nucleosomes (Alvarez-Venegas et al. 2007, Ndamukong et al. 2010), and by activity of UGT76B1 glucosyltransferase (von Saint Paul et al. 2011). Intriguingly, the strawberry gene M22E3^{EST}, a class III histone methyl transferase (H3-Lys-4) implicated in transcription regulation (Springer et al. 2003), was downregulated after *C. acutatum* infection while the two strawberry WRKY70-like genes, FaWRKY70-1 and FaWRKY70-2, were upregulated. Contrary to WRKY33, WRKY70 acts as negative regulator of defense against necrotrophic *E. amylovora* (Moreau et al. 2012), and mutations in WRKY70 increase susceptibility to biotrophs *Erysiphe cichoracearum* and *Hyaloperonospora parasitica*, while increase resistance to necrotroph *A. brassicicola* (Li et al. 2006, Knoth et al. 2007). Together with WRKY46 and WRKY53, WRKY70 positively regulate basal resistance to *P. syringae,* and they play overlapping and synergetic roles in plant basal defence (Hu et al. 2012). Also WRKY70 controls suppression of JA-signaling together with NPR1 (Li et al. 2006), working downstream from ROS defence reaction, and the biosynthesis of both hormones (Knoth et al. 2007, von Saint Paul et al. 2011). In conclusion, WRKY70 is required for full development of R-dependent and basal defences (Knoth et al. 2007), and it is crucial to control the cross-talk of SA and JA

signalling in plant defence acting as node of convergence for JA-mediated and SA-mediated signals (Dong 2004, Li et al. 2004, Wang et al. 2006, Ren et al. 2008). Accordingly, the expression of two FaWRKY70 genes in strawberry challenged with *C. acutatum* is in agreement with the partial inhibition of the JA-dependent defense pathway shown in Chapter V.

Finally, two strawberry components of the LEUNIG/TOPLESS corepressor complexes described as general repressors of gene transcription in plants (Consortium 2011, Causier et al. 2012, Shyu et al. 2012), genes M22E11^{EST} (TOPLESS transcriptional correpresor protein), and M10H10^{EST} (a LEUNIG_homolog transcriptional correpresor), were downregulated in strawberry upon *C. acutatum* infection. .

RNA Metabolism

Three RNA helicases were upregulated in strawberry by *C. acutatum* infection. Thus, induction of genes M8A6^{EST} (a DEAD-box ATP-dependent RNA helicase 29), M8H8^{EST} (an ATP-dependent RNA helicase DBP10), and M4C6^{EST} (RNA helicase protein family) highlighting the importance of RNA metabolism control during the switch to defence response activation in strawberry. RNA helicases are crucial players in the regulation of gene expression through the rearrangement of ribonucleoprotein (RNP) structure. The majority of RNA helicases can be subdivided into several families including DEAD-box (aminoacids asp-glu-ala-asp) (de la Cruz et al. 1999). Function in plant defences have been demonstrated in rice against biotic and abiotic stresses (Li et al. 2008) and in silencing RNAs against viral infections (Linder and Owttrim 2009).

Also, five members of the RNA-binding family proteins were also regulated in strawberry by *C. acutatum*. Thus, genes M19E4^{EST} (a RNA-binding protein from the RRM/RBD/RNP motifs family), M26G7^{EST} and M18A9^{EST} (RNA-binding KH domain-containing proteins), and M18F1^{EST} (a RNA-binding protein 47C) are induced by *C. acutatum* infection, while gene M22D5^{EST} (a RNA-binding (RRM/RBD/RNP motifs) family protein) was downregulated. These genes

represent a big family of RNA-binding proteins with triple RNA recognition motifs (Peal et al. 2011), which may participate in still undefined steps of pre-mRNA maturation in plant cell nuclei (Lorković et al. 2000). They are regulated by defence signals such as ET and ROS (De Paepe et al. 2004, Pavet et al. 2005), and also are induced by abiotic stresses (Sharma et al. 2007).

In addition, other upregulated genes related with RNA metabolism were M12B6^{EST} (a DNA-directed RNA polymerase TFIIB zinc-binding protein, Bäckström et al. 2007), M7G4^{EST} (a Duplicated homeodomain-like superfamily protein), and M9E2^{EST} (a Pre-mRNA-processing-splicing factor SUS2). The last two proteins are implicated in nuclear mRNA splicing via spliceosome (Schwartz et al. 1994), and mutants in these genes show a similar phenotype to that found in a mutant on valyl-tRNA synthetase gene, valRS (Zhang and Somerville 1997), implicated in protein synthesis (Duchêne et al. 2005). Intriguingly, we have also detected induction on a Valyl-tRNA synthetase gene (M20A3^{EST}) in our experimental conditions.

Further research is needed to clarify the biological function of these genes associated to RNA metabolism during strawberry-*C. acutatum* interaction

Protein Synthesis, Folding and Secretion Machinery

A transcriptional increase on specific components of the protein synthesis machinery has also been detected in strawberry after *C. acutatum* attack. Thus, genes M11C6^{EST} (60S Ribosomal protein L34), M23C7^{EST} (40S ribosomal protein S29), and M7D1^{EST} (60S ribosomal protein L18-2), encode components of the ribosomal complex (Baima et al. 1995, Barakat et al. 2001, Carroll et al. 2008). On the contrary, other ribosomal components such as that encoded by gene M28F7^{EST} (40S Ribosomal protein S12/S23), were downregulated after *C. acutatum* infection. Interestingly, ribosomal components are also negatively regulated in other plants by *Agrobacterium* or geminivirus infection (Ditt et al. 2006, Ascencio-Ibáñez et al. 2008), and are specific targets of patogenic virulent factors (Leh et al. 2000, Rocha et al. 2008).

It is well known that prior to new proteins accumulation, endoplasmic reticulum-resident genes encoding the secretory pathway machinery are coordinately upregulated to ensure proper folding, posttranslational modification, transport and secretion of these antimicrobial peptides (Wang et al. 2005, Kwon et al. 2008, Wang and Dong 2011, Pajerowska-Mukhtar et al. 2012). In this sense, the orthologous product of gene M22D9^{EST} (Armadillo repeat superfamily protein-Hsp70 nucleotide exchange factor fes1) which is induced in strawberry by *C. acutatum*, functions as translocator of proteins to the endoplasmic reticulum in Arabidopsis, and, interestingly, is upregulated by accumulation of unfolded proteins in the endoplasmic reticulum (Kamauchi et al. 2005). Therefore, upregulation of gene M22D9^{EST} may be consequence of a massive synthesis of new proteins produced in strawberry in response to the infection.

Similarly, other genes encoding components implicated in peptide transport are the previously mentioned gene M8D11^{EST}, which encodes a phosphatidylinositol 3-kinase essential for vacuole dynamics and reorganization, and gene M1A2^{EST}, which encodes a coatomer subunit alpha implicated in intracellular protein transport (Bassham et al. 2008). Intriguingly, the M1A2^{EST} orthologous gene was downregulated in Arabidopsis by *Pseudomonas* and *Bradyrhizobium* infection (Cartieaux et al. 2008), while it was induced by *C. acutatum* in strawberry.

In addition, genes encoding proteins with function in the secretory pathway are M5B8^{EST} and M14B5^{EST}. M5B8^{EST} encodes a vacuolar protein sorting-associated protein 24 of the SNF1-related protein kinase family, and a member of the endosomal sorting complex (ESCRT III). This complex consists of two soluble subcomplexes of highly charged coiled-coil proteins, and is required for sorting and/or concentration of multivesicular body cargoes (Winter and Hauser 2006). M14B5^{EST} encodes a peptide transporter PTR1 implicated on proton/oligopeptide cotransport (Chiang et al. 2004) that is regulated by auxins (Goda et al. 2004) and represed by nematode infection (Hammes et al. 2005).

We have detected regulation of members of the strawberry cytoskeleton and motor proteins upon *C. acutatum* infection. Secretory material must be directed to the site of microbial interaction as deposition of membrane proteins, cell wall materials, and presumably secreted proteins are all clearly restricted to the site of pathogen contact. This directional trafficking of the vesicles requires the cytoskeleton and associated motor proteins so that microtubule depolymerization provides a mechanism for the mobilization of the plant defence response against pathogen attacks (Vassileva et al. 2005, Wang and Dong 2011). Accordingly, gene M10C12^{EST} encoding a member of the beta-tubulin family (Snustad et al. 1992) was upregulated in strawberry. Very interestingly, the M10C12^{EST} ortholog is targeted in Arabidopsis by *Pseudomonas* effector HopZ1, a superfamily of type III secreted effector proteins that causes a dramatic destruction of microtubule networks, inhibits protein secretion, and ultimately suppresses cell wall-mediated defence (Lee et al. 2012).

The actin cytoskeleton has also been clearly implicated in plant basal defences and nonhost resistance (reviewed in Day et al. 2011), and also plays a role in race-specific resistance (Skalamera and Heath 1998, Tian et al. 2009). Indeed, nonhost resistance in Arabidopsis-*Colletotrichum* interactions acts at the cell periphery and requires actin filament function (Shimada et al. 2006). In strawberry, we have detected induction of gene M11H4^{EST}, which encodes an actin-11 protein. Actin-11 represents a unique and ancient actin subclass within the complex Arabidopsis actin gene family (Huang et al. 1997), and is negatively regulated by oxylipins (Mueller et al. 2008) but induced by wound-like signals (Guan and Nothnagel 2004). Curiously, we have detected repression on gene M21G2^{EST} encoding a SCAR family protein, which takes part in a complex that acts as a nucleator for actin filaments (Zhang et al. 2008).

In addition, a member of the kinesin superfamily that are microtubule-based motor proteins that transport molecules/organelles along microtubules (Lee and Liu 2004) was also upregulated in strawberry challenged with *C. acutatum*. Thus, gene M3A1^{EST} encodes a kinesin-related protein related with trichome

development (Lu et al. 2005), which is located on Golgi-associated vesicle and is involved in vesicle formation/budding (Wei et al. 2009).

Defensive arsenal

Cell Surface and Deposition of Extracellular Material

It is noteworthy that secretion is required not only for the delivery of antimicrobial molecules, but also for the biogenesis of cell surface sensors to detect microbes and for the deposition of extracellular material important for the resistance (Wang and Dong 2011). Interestingly, gene M3E11^{EST} encodes a plasmodesmata callose-binding endo-1,3-beta-glucosidase protein 3 (PdCB3), a glycosylphosphatidylinositol-anchored protein (X8-GPI family of proteins) localized to the plasmodesmata and was upregulated in strawberry by *C. acutatum*. This gene is predicted to bind callose and regulate cell-to-cell trafficking (Borner et al. 2002, Borner et al. 2003, Simpson et al. 2009). Intriguingly, gene M19C6^{EST}, a beta-glucanase from the glycosyl hydrolase family 81 protein, was repressed upon *C. acutatum* attack, and might indicate a reduction in cell wall components degradation. Also, gene FaCAD1 (M23D11^{EST}), a cinnamyl alcohol dehydrogenase, was upregulated by *C. acutatum*. This strawberry gene has previously been related to lignification, and mainly co-localized with lignin biosynthesis (Blanco-Portales et al. 2002). Curiously, the Arabidopsis ortholog, so-called elicitor inducible 3 (ELI3), seems to be independent of this function (Eudes et al. 2006) and it has an important role in resistance-related aromatic acid-derived metabolism (Somssich et al. 1996). Indeed, it is induced in RPM1-dependent and RPS2-independent ETI activation (Kiedrowski et al. 1992, Boch et al. 1998) and also is positively regulated by SA (Williamson et al. 1995). In addition, gene M5B6^{EST} codes for a laccase enzyme, which has been implicated in lignin production through oxidative polymerization of flavonoids (Pourcel et al. 2005).

The strawberry repressed gene M15G5^{EST} encodes a glucan/callose synthase, which acts in plasmodesmata (Zavaliev et al. 2011) and also produces callose

deposition in response to JA yet ultimately requiring ABA (García-Andrade et al. 2011). Independently of its callose production activity, the glucan/callose synthase contributes to PAMP-induced basal defence, participates in defence signalling, and regulates SA and JA production or signaling (García-Andrade et al. 2011, Wawrzynska et al. 2010). Thus, it is a positive regulator of defences against necrotrophus, and negatively regulates SA-dependent defences (Adie et al. 2007, Wawrzynska et al. 2010). Therefore, downregulation of gene M15G5^{EST} in strawberry upon *C. acutatum* may indicate mechanisms to activate SA-dependent and repress JA-dependent defences.

Stomata

Stomata are essential to prevent establishment and future widespread of pathogens. Although it has been described that *C. acutatum* uses appressoria to penetrate into the strawberry plant (Horowitz et al. 2002), some species also penetrate the host tissues through wounds (Bailey 1992), and stomatal pores (Latunde et al. 1999). Expansins conform a cell wall associated family in plants (Bayer et al. 2006, Cosgrove 2000, Wu et al. 2001, Li et al. 2002) which include members of diverse functions and regulations (Lee et al. 2001). Interestingly, the upregulated strawberry gene M21G5^{EST} encodes an expansin-A1 protein, which has been described to regulate stomatal movement by altering the structure of the guard cell wall (Zhang et al. 2011, Wei et al. 2011) in opposition to ABA-dependent signals (Hu and Ma 2006, Huang et al. 2008). This may suggests activation in strawberry of mechanisms leading to prevent pathogen penetration through stoma. Intriguingly, another upregulated gene, M26G2^{EST,} encodes a CHAL secreted protein, which is an inhibitor of stomatal production (Abrash and Bergmann 2010, Shimada et al. 2011).

Chemical Defences

Apart from the previously described function of genes FaEDS1 and its interacting partner FaPAD4, acting together to promote salicylic acid (SA)-dependent and SA-independent defences (see Chapter V), dissociated forms of PAD4 can control chemical defences against aphids as important modulator of antixenosis (feeding

deterrence) and antibiosis (affect aphid fecundity), and requires neither EDS1 nor SA (Louis et al. 2010a, Louis et al. 2012). Unlike FaPAD4, the Arabidopsis orthologous of the triacylglycerol lipase 2 codifying gene FaLIP-1 ($M12C12^{EST}$) is also induced in response to aphids infestation and ABA but it is not required for antixenosis (Yazaki et al. 2004) and represents an essential component of defence against pests through accumulation of an antibiotic activity that limits its reproduction (Louis et al. 2010b).

In addition, the upregulated gene $M6G11^{EST}$ encoding a flavonol 7-O-glucosyltransferase belongs to a gene family that has been clearly related to plant defence responses in plants. Thus, members of this family are induced by SA and *Pseudomonas* infection (Zhang et al. 2007b), and are considered as part of SA-dependent NPR1-independent immediate early genes (Uquillas et al. 2004, Blanco et al. 2005, Lee et al. 2007). Interestingly, these genes strongly responds to wounding, JA or related molecules (Taki et al. 2005, Guan and Nothnagel 2004), as well as to others plant hormonal compounds (Zhao et al. 2003, Loeffler et al. 2005), and also to diverse abiotic stresses (Rizhsky et al. 2004). This family is considered as part of typical PAMP-induced Arabidopsis genes (Thilmony et al. 2006) and necessary for resistance to *Pseudomonas syringae* pv. tomato in Arabidopsis (Langlois-Meurinne et al. 2005).

Genes $M26E5^{EST}$ and $M4F3^{EST}$ were also upregulated in strawberry. $M26E5^{EST}$ encodes a zinc-binding dehydrogenase protein, which belongs to a very big family of proteins involved in plant protection against REDOX cytotoxicity (Mano et al. 2005). Members of this family are regulated by fungal elicitors, wounding and MeJA (Chivasa et al. 2006, Zheng et al. 2006a). Gene $M4F3^{EST}$ encodes a D-serine/D-alanine/glycine transporter, which participates in secondary metabolism activation and flavonoid biosynthesis, and is positively coregulated by the transcription factor LONG HYPOCOTYL5 (HY5) (Lasserre et al. 2008, Yonekura-Sakakibara et al. 2008).

However, some other genes such as gene M29H6^{EST} and M29C12^{EST} related to phenylpropanoids pathway were negatively regulated is strawberry by *C. acutatum* infection. Thus, gene M29C12^{EST}, a chalcone-flavanone isomerase, is related to the flavonoid biosynthesis pathway (Wei et al. 2006), and gene M29H6^{EST}, a leucoanthocyanidin dioxygenase (LDOX), is a member of the multifunctional dioxygenase family of enzymes (Martens et al. 2010) which is essential for proanthocyanidin synthesis and vacuole development Abrahams et al. 2003), and responds to COI1-dependent JA signalling (Shan et al. 2009, Devoto et al. 2005).

Very interestingly, we have detected induction of the strawberry gene M17E3^{EST}, which encodes an argininosuccinate synthase. This enzyme takes part in the citrulline-nitric oxide cycle to synthesize NO (Tischner et al. 2007), which has been shown to be very important in many plant defence mechanisms (Besson-Bard et al. 2008, Wilson et al. 2008). Indeed, in Arabidopsis, NPR1, the key regulator of the SA-pathway, suffers fine post-translational regulation and changes in its redox status by NO (Tada et al. 2008), and suggests that a similar mechanism might function in strawberry to activate defense against *C. acutatum*.

Pathogenesis Related Proteins (PR proteins)

Many genes belonging to diverse families of pathogenesis-related proteins (van Loon et al. 2006) were altered in strawberry challenged with *C. acutatum*. Thus, the PR2 family is represented by the upregulated genes FaGLN-2 (M24B7^{EST}) (Glycosyl hydrolase superfamily protein), and M25D11^{EST} (O-Glycosyl hydrolases family 17, (1->3)-beta-glucanase), and the down regulated M19C6^{EST} (Glycosyl hydrolase family 81 protein). In strawberry, gene FaGLN-2 has previously been reported to be upregulated upon *C. fragariae* or *C. acutatum* infection both at transcriptomic (Shi et al. 2006, Khan et al. 2003) and proteomic level (Fang et al. 2012), and also by UV-C treatment (Pombo et al. 2011). In other plants, the FaGLN-2 orthologous gene has been localized within cell wall and also identified in the apoplastic fluids of rosettes (Boudart et al. 2005). Also, it responds to abiotic stress (Hammond et al. 2003), and a variety of pathogens,

pests, and hormonal treatments (Mahalingam et al. 2003, Soeno et al. 2010, Pastori et al. 2003, De Paepe et al. 2004, Goda et al. 2004, Kempema et al. 2007, Zhang et al. 2007b, Cartieaux et al. 2008, Lorenzo et al. 2003, Mukherjeea et al. 2010). In addition, the FaGLN-2 may be targeted by a pathogen effector as mode of active suppression of host defences causing successful parasitism by nematodes (Hamamouch et al. 2012).

Some members of the PR3 and PR4 families, such as class IV chitinases FaCHI4-2 (M16D12^{EST}) and M29A9^{EST}, suffered strong up-regulation after *C. acutatum* infection. The FaCHI4-2 Arabidopsis ortholog is categorized as a ubiquitously expressed class IV chitinase (Passarinho et al. 2001, Passarinho and de Vries 2002), regulated after infection (Whitham et al. 2003, de A. Gerhardt et al. 1997) and responding to PAMPs treatments in a NDR1 dependent manner (Qutob et al. 2006, Thilmony et al. 2006,Sato et al. 2007). Also, it is involved in nonhost resistance, and localizes and it is related to plant cell wall biogenesis (Navarro et al. 2004, Borderies et al. 2003). In addition, it is also upregulated by chemical treatment such as gallic acid, JA and ET (Golisz et al. 2008, Devoto et al. 2005) and abiotic stress (Hammond et al. 2003, Oravecz et al. 2006). However, it is downregulated by wounding (Takenaka et al. 2009).

Also, members of the PR5 family were differentially expressed in strawberry by *C. acutatum*. Thus, FaPR5-1 (EPR5-284^{EST}), FaPR5-2 (EPR5-77^{EST}), and FaPR5-3 (M1F10^{EST})) are three members of the thaumatin like family that were strongly upregulated. The former two had been previously reported to respond to *C. acutatum* in strawberry (Casado-Díaz et al. 2006), while the last one has been related to DTI (DAMPs triggered immunity) in strawberry fruit against *Botrytis cinerea* (Osorio et al. 2008). FaPR5-1 and FaPR5-2 orthologue in Arabidopsis was induced after infection by a broad range of pathogens showing different lifestyles (Ditt et al. 2006, Tao et al. 2003, van Wees et al. 2003, Mukherjeea et al. 2010, Mohr and Cahill 2007, Zhang et al. 2007b). In addition, it is induced by JA in a WRKY33 dependent manner (Zheng et al. 2006b).

Four peroxidase proteins belonging to the PR9 family were upregulated in strawberry by *C. acutatum* infection. Thus, gene M5C8^{EST} encodes a L-ascorbate peroxidase 6, which takes part of the ascorbate-glutathione cycle as antioxidant defences in plants (Chew et al. 2003), and is positively regulated by glutaredoxin in tomato (Guo et al. 2010). Genes M28C8^{EST}, M24D7^{EST}, and FaPOX-1 (M3E6^{EST}) encode orthologs to plant class III peroxidases with predicted N-terminal vacuolar signal peptide (Welinder et al. 2002, Valério et al. 2004), which localize in central vacuole (Carter et al. 2004) and cell wall (Bayer et al. 2006, Borderies et al. 2003, Irshad et al. 2008), and are thought to contribute to cell wall remodeling (Andersson-Gunnerås et al. 2006). In particular, the M28C8^{EST} Arabidopsis ortholog, was shown to responds to both biotic and abiotic stresses (Llorente et al. 2002, Ditt et al. 2006), was regulated by classical defence hormones (Goda et al. 2004, Cao et al. 2006, De Paepe et al. 2004), and clearly contributed to ROS production (Kim et al. 2010). Also, the Arabidopsis ortologous to gene M24D7^{EST}, was downregulated by DELLA (Cao et al. 2006), while the ortologous to gene FaPOX-1 (M3E6^{EST}) was induced in distal leaf tissue at 72 h (systemic maintenance period) after inoculation with *A. brassicicola* (Schenk et al. 2003).

In addition, seven genes (M23A10^{EST}, M6B9^{EST}, M22A10^{EST}, M5G8^{EST}, M10C5^{EST}, M25D10^{EST}, and M10D7^{EST}) encoding members of the plant PR10 protein family with ribonuclease like properties were overexpresed in strawberry after *C. acutatum* infection. .The PR10 is a multigene family with low intraspecific variation and higher interspecific variation (Kim 2011). Some members of this family are induced in plants upon a broad range of interactions such as pest and pathogen attack (Little et al. 2007, Guidarelli et al. 2011), abiotic stress (Abercrombie et al. 2008) and SA treatment (Rajjou et al. 2006), but strongly downregulated by the obligate biotrophic protist *Plasmodiophora brassicae* in Arabidopsis (Siemens et al. 2006). Molecular function of this family is related with binding a variety of ligands, especially hydrophobic lipids (Radauer et al. 2008, Mogensen et al. 2002, Marković-Housley et al. 2003). Also in strawberry, members of this family of proteins have demonstrated allergenic

properties (Karlsson et al. 2004, Hjernø et al. 2006, Musidlowska-Persson et al. 2007) and also have been related to flavonoid biosynthesis and pigment formation in fruit (Muñoz et al. 2010). However, a direct relation between this family of proteins and an increase in defence capacities still remains undiscovered.

Finally, it is know that members of the serine hydroxymetyltransferase family play a critical role in controlling ROS production and pathogen-induced cell death (Moreno et al. 2005). Interestingly, downregulation of gene M21F3^{EST}, which encodes a serine hydroxymetyltransferase in strawberry, has also been detected.

Other strawberry altered genes

Induction of genes such as M23C11^{EST} (Dehydrin cold-regulated 47) and M16H1^{EST} (auxin response factor), may indicate activation of ABA and auxin pathways in the strawberry defence response to *C. acutatum*. Thus, gene M23C11^{EST} has been described in other plants being induced by ABA and a variety of abiotic stresses (Nylander et al. 2001, BRAY 2002, Kovacs et al. 2008, Kline et al. 2010). SA and auxin signalling pathways interact, for the most part, antagonistically, thus elevated auxin correlates with increased susceptibility to biotrophic pathogens. Auxin can also interact with the JA signalling pathway, although reports are conflicting (Llorente et al. 2008, Robert-Seilaniantz et al. 2011).

Activation of defensive responses through signalling molecules such as purines (ATP and ADP) and carbohydrate derivatives has also been reported in plants (Demidchik and Maathuis 2007) (Smeekens 2000, Gibson 2005). In strawberry, transporters of purine such as gene M26D3^{EST} (adenine nucleotide transporter 1 (ADNT1)) (Palmieri et al. 2008), or purine derivatives such gene M9E10^{EST} (purine permease 11) (Gillissen et al. 2000) were upregulated upon *C. acutatum* infection. In addition, increasing evident support that sucrose and hexoses play major roles as metabolic signals, regulating plant physiology by affecting expression of different classes of genes. Thus, cell wall invertases have been also

implicated in defence responses (Roitsch et al. 2003) and regulated by biotic and abiotic stimuli (Ehness et al. 1997, Quilliam et al. 2006, Ascencio-Ibáñez et al. 2008). Accordingly, a strawberry invertase gene M25B1^{EST} (cell wall Invertase 1 (AtcwINV1): Glycosyl hydrolases family 32 protein), is induced upon *C. acutatum*. Its ortologous gene shows the highest expression level of the six Arabidopsis cell-wall-type hydrolases (Sherson et al. 2003) and it is further induced after fungal infection in Arabidopsis (Fotopoulos et al. 2003) and tomato (Verhaest et al. 2005).

Finally, the specific function of two upregulated genes, M12E4^{EST} and M14G2^{EST}, and the downregulated gene M18H1^{EST}, all members of the tetratricopeptide repeat (TPR)-like superfamily protein remain still unknown. However, this domain that facilitates specific interactions with a partner protein (Blatch and Lässle 1999), have been identified in transcriptional repressors of ETI (Kwon et al. 2009) and in disease resistance regulation by ubiquitin-mediated proteolysis (Tör et al. 2002), suggesting their active implication in the strawberry defense response against *C. acutatum*.

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Chapter VII

Dissecting the Genetic Basis of the Plant Defense Response Against *Colletotrichum acutatum* in two Strawberry (*Fragaria* × *ananassa*) Cultivars with Different Susceptibility to this Pathogen

ABSTRACT

To gain insights into the molecular mechanisms related with differential susceptibility exhibited by two strawberry (*Fragaria* × *ananassa*) cultivars, Camarosa and Andana, a comparison of their transcriptional profiling before and after infection with *Colletotrichum acutatum* was performed. Interestingly, gene overexpression appears as the main genetic regulation in both strawberry cultivars under this biotic stress (80% of the modified genes in both cultivars). Significantly, biological functions activated in Andana as consequence of infection were clearly related with defense responses, but contrary to what we have previously detected in Camarosa cultivar, known salicylic acid mediated defenses were not significantly activated in Andana challenged with *C. acutatum*. This result agrees with those previously obtained about changes in the hormonal balance of the strawberry plant challenged with the pathogen, as Andana did not increase its SA contents after *C. acutatum* infection. In addition, an important number of cultivar dependent differentially expressed genes have been identified, which could explain differences in the susceptibility to *C. acutatum* exhibited by these two strawberry cultivars. However, further analysis is needed to clearly associate molecular components here identified to differences in susceptibility to this pathogen.

INTRODUCTION

This Chapter reports the use of a specific *F* × ananassa microarray to examine the expression profiles of selected genes in two strawberry cultivars exhibiting different susceptibility to *C. acutatum*. Crown tissue, the site of natural infection, was used to analyze the transcriptome responses of these cultivars challenged with *C. acutatum*. Similarities and differences in the molecular response between cultivars have been assessed.

Result from cv. Camarosa mock vs. cv. Camarosa infected (experiment (a), CC vs. CI) analysis was deeply described in a previous chapter (Chapter V), so here we will focus exclusively on the rest of comparisons to highlight the differential response exhibited in a cultivar-dependent manner.

RESULTS AND DISCUSSION

Comparative Transcriptomic Analysis: Experimental Design

The two *F* × *ananassa* cultivars used to analyze their transcriptomes in response to infection have been previously described as showing different behavior in terms of susceptibility to anthracnose caused by *C. acutatum* (cv. Camarosa, very high susceptible, and cv. Andana, moderately susceptible) and a good adaptability to Spanish climatic conditions (Casado-Díaz et al. 2006). In all infected plants, the pathogen was re-aisled to ensure disease establishment. However, during the first 9 days post infection, plants looked still healthy and visible symptoms were rarely detected (data not shown), thus senescence and necrotic mechanisms are expected to be absent in the analyzed samples, and main changes consequence of disease are avoided to centre the analysis in the early defense response mechanism during the beginning of the pathogen colonization. As consequence, fungal progress was still localized in some few cells, and not yet extended through overall in all plant tissues.

For the microarray hybridization, total RNA from crown tissue harvested at 5 days post treatment was extracted from both, Camarosa and Andana cultivars, and from both, mock treated and fungal-inoculated plants, in a total of 3 biological replicates per stage (6 independent plants were pooled to make one biological replicate). So, in total, 12 RNA samples were used to be transcriptomically analyzed. Figure 1 summarizes the experimental design here described. Four microarray experiments were conducted per triplicate, comparing all four samples as following: (a) cv. Camarosa mock vs. cv. Camarosa infected, CC vs. CI; (b) cv. Andana mock vs. cv. Andana infected, AC vs. AI; (c) cv. Camarosa mock vs. cv. Andana mock, CC vs. AC; (d) cv. Camarosa infected vs. cv. Andana infected, CI vs. AI. Reciprocal hybridizations (dye swaps) were utilized for all comparisons to avoid dye bias.

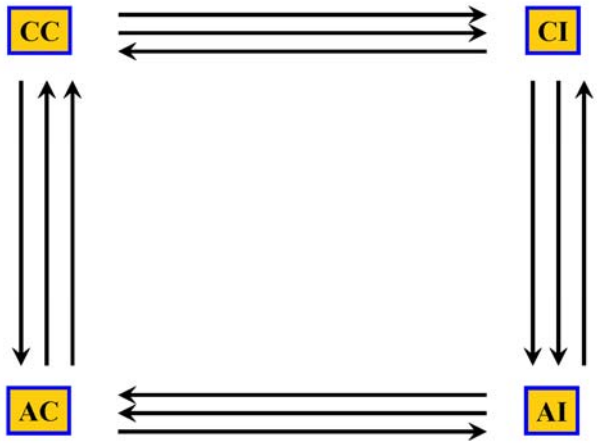

Figure 1. Experimental design. Four microarray experiments were conducted, comparing all four samples as following: Camarosa Control vs. Camarosa Infected (CC vs. CI); Andana Control vs. Andana Infected (AC vs. AI); Camarosa Control vs. Andana Control (CC vs. AC); Camarosa Infected vs. Andana Infected (CI vs. AI). For each sample, three biological replicates were performed in a total of 12 microarray hybridizations. Reciprocal hybridizations (dye-swaps) were utilized for all comparisons to avoid dye bias.

Identification of Defence Related Genes Differentially Regulated to *C. acutatum*

Genes were considered as differentially expressed if they fulfilled a FDR ≤ 0.05 after a SAM test analysis, and the fold-change was higher that 1.75-fold. According to this criteria, a total of 110 genes were differentially expressed in Andana (86 genes induced and 24 repressed, See Tables 1 and 2). According to what it has been described for Camarosa cultivar in Chapter V (with 118 upregulated and 29 downregulated genes), gene overexpression (nearly 80% of the altered genes) also seems to be the main genetic regulation in Andana under this biotic stress.

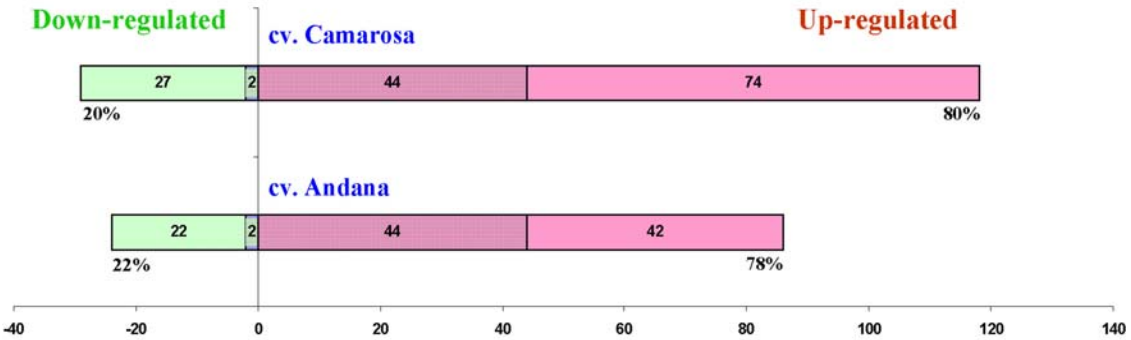

Figure 2. Differentially regulated genes in Camarosa and Andana cultivars by *Colletotrichum acutatum* infection. Genes were considered as differentially expressed if they fulfilled a FDR ≤ 0.05 after a SAM test analysis, and the fold-change was higher that 1.75-fold. Although 25% minus regulated genes have been detected in Andana, conservation between up- and down-regulated ratios is detected in both cultivars with a non-negligible predominance in overexpression of around 80% of the genes. Genes co-regulated in both cultivars are represented as stripped bar.

Figures 2 and 3, represents a comparison between differentially regulated genes in both Camarosa and Andana cultivars after *C. acutatum* infection. This shows a group of cultivar-independent regulated genes, regulated in the same manner in both tested cultivars (represented in Figure 2 by stripped bars). Thus, forty-four genes were induced, and two genes repressed in both cultivars after the infection. Interestingly, only in two cases an inverse regulation has been detected

between both cultivars (marked with asterisk in Table 2). Interestingly, 38 out of 161 of the cultivar-specific genes present, in fact, fold-change values between 1.5 and 1.75 (or, alternatively, between 0.65 and 0.57) in the other cultivar, and therefore, they could be considered as cultivar-independent regulated genes. These data reveal that at least 60% of the differentially expressed genes (125 genes) were definitely induced (81 genes) or repressed (37 genes) in one cultivar but not in the other, or were inversely regulated (7 genes).

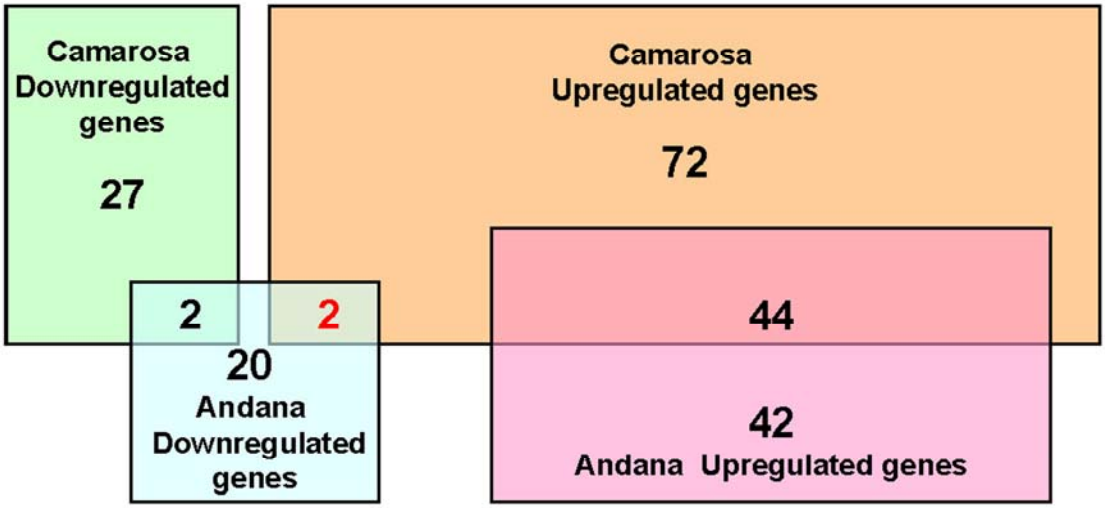

Figure 3. Intersection between the strawberries regulated genes by infection in both cultivars (Camarosa and Andana). According to the criteria of differentially expressed, some of these genes are regulated by *Colletotrichum acutatum* specifically in one cultivar but not in the other one. However an important amount of genes are equally regulated in both. Interestingly only in two cases an inverse regulation have been detected between both cultivars.

Identification of Biological Processes Implicated in Andana Defense Response

A comprehensive functional analysis was set up by the Singular Enrichment Analysis (SEA) tool FATIGO (Al-Shahrour et al. 2004) to determine which biological processes were significantly over-represented. Thus, enrichment in some defensive biological processes, such as categories “defence response” (GO:0006952, adj. pvalue 4.08E-2), “response to biotic stimulus” (GO:0009607,

Table 1. Up-regulated genes in crown tissue of strawberry cultivar Andana after *Colletotrichum acutatum* infection. Genes were considered as differentially expressed if they fulfilled a FDR ≤ 0.05 after a SAM test analysis, and the fold-change was higher that 1.75-fold in the compared conditions. Marked in bold those genes upregulated similarly in cultivar Camarosa. Values represent the ratio of cv. Andana mock vs. infected, AC vs. AI.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous	*A. thaliana* Ortologous	AC vs. AI	
				Fold Change	FDR (qvalue)
M24B7	Glycosyl hydrolase superfamily protein	gene14817	AT4G16260	**8,40**	0,00
M5B6	Laccase	gene24296	AT5G09360	**6,18**	0,00
M8H8	ATP-dependent RNA helicase DBP10	gene10702	AT4G17960	**4,95**	0,00
M6B9	Pathogenesis-related 10 family protein	gene05185	AT1G24020	**4,57**	0,00
M12C12	Triacylglycerol lipase 2	gene31975	AT5G14180	**3,92**	0,00
M16D12	Chitinase class IV	gene02717	AT3G54420	**3,90**	0,00
M28G6	hypothetical protein	gene28516	AT1G49600	**3,65**	0,00
M27A2	Phosphoribulokinase	gene13677	AT1G32060	**3,59**	0,00
M23C2	no hit found	no hit found	no hit found	**3,50**	0,00
M6G7	Hypoxia-responsive Zinc finger (C3HC4-type RING finger) family	gene32154	AT3G48030	**3,33**	0,00
M16B7	Serine/threonine-protein phosphatase PP2A catalytic subunit	gene00744	AT1G69960	**3,14**	0,00
M12E6	Transcription initiation factor TFIID subunit 2	gene26210	AT1G73960	**3,07**	0,00
M27D3	RING/U-box E3 ubiquitin-protein ligase	gene18784	AT5G01160	**3,04**	0,00
M6G11	Flavonol 7-O-glucosyltransferase	gene26351	AT4G34135	**2,93**	0,01
M13C5	Serine/threonine-protein kinase PBS1	gene07245	AT5G13160	**2,90**	0,00
EDS1-936	EDS1-specific diacylglycerol lipase alpha	gene09503	AT3G48090	**2,88**	0,00
M26E5	Zinc-binding dehydrogenase family protein / oxidoreductase	gene32023	AT5G17000	**2,85**	0,00
M12E4	Tetratricopeptide repeat (TPR)-like superfamily protein	gene32179	AT1G27500	**2,81**	0,00
M11F8	Phospho-2-dehydro-3-deoxyheptonate aldolase 2, chloroplastic,	gene16261	AT4G39980	**2,80**	0,00
M26G7	RNA binding KH domain-containing protein	gene31909	AT2G25970	**2,77**	0,01
M18A9	RNA-binding KH domain-containing protein	gene30367	AT5G46190	**2,67**	0,01
M25F7	Queuine tRNA-ribosyltransferase	gene11939	AT5G65310	**2,67**	0,01
M13A4	Aminopeptidase M1 family protein / Leukotriene A-4 hydrolase	gene23331	AT5G13520	**2,60**	0,00
M25E9	Elongation factor 1-alpha (EF-1-alpha)	gene28639	AT1G07940	**2,60**	0,00
M2B1	Endochitinase 1	gene22465	AT1G05850	**2,59**	0,00
M1A2	Coatomer subunit alpha	gene24354	AT1G62020	**2,58**	0,01
M18E11	Embryo defective 2756	gene27435	AT1G34550	**2,58**	0,00
M23C11	Dehydrin cold-regulated 47	gene08617	AT1G76180	**2,57**	0,00
M23A9	Phospholipase D delta	gene14522	AT4G35790	**2,50**	0,00
M21G9	Protein SCAR3 (AtSCAR3)	gene29663	AT1G29170	**2,43**	0,01
M14E9	Cleavage and polyadenylation specificity factor subunit 2 (CPSF 100	gene13255	AT4G33410	**2,35**	0,01
M15H8	Actin-related protein 2	gene01351	AT3G27000	**2,29**	0,00
M19D11	Casein Serine/threonine-protein kinase	gene10418	AT3G03940	**2,29**	0,01
M3G1	Inositol oxygenase 2 (MI oxygenase 2)	gene11353	AT1G14520	**2,28**	0,00
M23A10	Pathogenesis-related 10 family protein	gene07086	AT1G24020	**2,28**	0,01
M22B1	Embryo defective 2410	gene01044	AT2G25660	**2,23**	0,00
EDR1	EDR1-Serine/threonine-protein kinase CTR1	gene16465	AT1G08720	**2,22**	0,00
ELRR-39	CPR30 Leucine-rich repeat (LRR) family protein	gene25524	AT5G21090	**2,20**	0,00
M1D6	Protein NUCLEAR FUSION DEFECTIVE 5, mitochondrial	gene21983	AT1G19520	**2,20**	0,00
M14B6	alpha/beta-hydrolase-like protein	gene06032	AT1G80280	**2,18**	0,01
M11C6	60S Ribosomal protein L34	gene03828	AT1G69620	**2,11**	0,01
M23C5	Peroxisomal biogenesis factor 6	gene24919	AT1G03000	**2,11**	0,00
M9F6	Homeobox protein orthopedia	gene29752	AT1G28420	**2,10**	0,00
M17D4	Transmembrane protein 208	gene08443	AT4G30500	**2,04**	0,01
M21B3	WRKY DNA-binding protein 75	gene01340	AT5G13080	**2,02**	0,01
M15G8	Putative oxidoreductase GLYR1	gene22501	AT3G05430	**2,02**	0,01
M3C1	Cellulose synthase A catalytic subunit 2 [UDP-forming] (AtCesA2)	gene08114	AT2G21770	**1,98**	0,01
M11B8	40S ribosomal protein S5-1	gene18014	AT3G11940	**1,97**	0,01
M26E8	Auxin-responsive protein IAA9	gene05555	AT2G22670	**1,96**	0,01
M7G11	Sphingoid base hydroxylase 1 (SBH1)	gene04753	AT1G69640	**1,95**	0,01
M11A12	Histone deacetylase HDT1 (HD2a)	gene14356	AT5G22650	**1,95**	0,01
M5C8	L-ascorbate peroxidase 6	gene11632	AT4G32320	**1,94**	0,01
M10H5	Cellulose synthase A catalytic subunit 3 [UDP-forming] (AtCesA3)	gene26807	AT5G05170	**1,94**	0,01
M3D5	NEDD8-activating enzyme E1 regulatory subunit / AXR1 (Auxin	gene23778	AT1G05180	**1,93**	0,00
M3F5	Protein of unknown function	gene13777	AT3G08890	**1,93**	0,01
M5H11	F-box protein At4g12560	gene07749	no hit found	**1,92**	0,01
M10D4	Tetratricopeptide repeat protein 7B (TPR repeat protein 7B)	gene02575	AT1G27460	**1,91**	0,00
M27C10	Serine/threonine protein phosphatase 2A regulatory B subunit	gene30942	AT5G25510	**1,91**	0,01

Table 1. Cont.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous	*A. thaliana* Ortologous	AC vs. AI	
				Fold Change	FDR (qvalue)
M11G2	HIPL1 protein	gene09553	AT1G74790	**1,91**	0,01
M2E4	hypothetical protein	gene02111	AT3G29300	**1,90**	0,01
J_4-9	WRKY DNA-binding protein 75	gene07210	AT5G13080	**1,90**	0,01
M6C2	Lung seven transmembrane receptor family protein	gene01890	AT5G42090	**1,89**	0,01
M8D11	Phosphatidylinositol 3-kinase	gene06214	AT1G60490	**1,89**	0,01
M20B5	tRNA-dihydrouridine synthase 3-like (AtC3H50)	gene01681	AT4G38890	**1,88**	0,01
M29F3	LRR and NB-ARC domains-containing disease resistance protein	gene16731	AT3G14460	**1,87**	0,01
M27D4	Transcription factor bHLH68 (bHLH 68)	gene25821	AT2G20100	**1,86**	0,01
M4H3	Serine/threonine-protein kinase haspin	gene22924	AT1G09450	**1,85**	0,01
M23C4	AINTEGUMENTA gene - AP2 like transcription factor	gene02623	AT4G37750	**1,85**	0,01
M25B1	Cell wall Invertase 1 (AtcwINV1): Glycosyl hydrolases family 32	gene23034	AT3G13790	**1,85**	0,01
M4C3	CIPK-Serine/threonine-protein kinase 5	gene15015	AT5G10930	**1,84**	0,01
M11G4	BTB/POZ domain-containing protein At5g48800	gene01554	AT5G67385	**1,83**	0,01
M10C5	Pathogenesis-related 10 family protein	gene00687	AT1G24020	**1,82**	0,01
M12E2	Photosystem II 10 kDa polypeptide, chloroplastic (PII10)	gene10470	AT1G79040	**1,82**	0,01
M20B8	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	gene07254	AT5G50850	**1,82**	0,01
M17H5	Chlorophyll a-b binding protein 40, chloroplastic (LHCP)	gene34432	AT1G29910	**1,79**	0,01
M6A9	Transcription factor MYB44	gene00185	AT5G67300	**1,78**	0,01
M14F9	UPF0667 protein C1orf55 homolog	gene19200	AT4G01000	**1,78**	0,01
M7A5	Probable E3 ubiquitin-protein ligase ARI2	gene19662	AT2G16090	**1,78**	0,01
M23F8	60S ribosomal protein L8-3	gene17157	AT2G18020	**1,77**	0,01
M9H9	Tetratricopeptide repeat protein 7B (TPR repeat protein 7B)	gene02575	AT1G27460	**1,76**	0,01
M1A1	Sn1-specific diacylglycerol lipase alpha (DGL-alpha)	gene09503	AT3G48090	**1,76**	0,01
M14E3	no hit found	no hit found	no hit found	**1,76**	0,01
M11B5	Mediator of RNA polymerase II transcription subunit 12	gene30526	AT5G37710	**1,76**	0,01
M15B8	Protein LIM1	gene17455	AT5G48485	**1,75**	0,01
M10F4	Protein vip1	gene13763	AT5G46870	**1,75**	0,01
M24D7	Peroxidase superfamily protein	gene28350	AT5G40150	**1,75**	0,01

Table 2. Down-regulated genes in crown tissue of strawberry cultivar Andana after Colletotrichum acutatum infection. Genes were considered as differentially expressed if they fulfilled a FDR <0.05 after a SAM test analysis, and the fold-change was lower that 0,57-fold in the compared conditions. Marked in bold and with an asterisk those genes downregulated similarly or oppositely in cultivar Camarosa, respectively. Values represent the ratio of cv. Andana mock vs. infected, AC vs. AI.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	AC vs. AI	
				Fold Change	FDR (qvalue)
M24F9	GTP cyclohydrolase-2	gene16232	AT2G22450	**0,39**	0,04
M22G7 *	non-LTR retrotransposon family	gene09933	AT5G41835	**0,42**	0,03
M16B12	Photosystem II 5 kDa protein, chloroplastic (PSII-T)	gene14692	AT1G51400	**0,45**	0,01
M21B2	Ubiquitin-conjugating enzyme 15	gene15203	AT1G75440	**0,46**	0,01
M21H5 *	Actin-related protein 8	gene01441	AT5G56180	**0,47**	0,01
M4D1	Extended synaptotagmin-1 (E-Syt1)	gene08549	AT2G20990	**0,50**	0,01
M21H4	Probable aquaporin PIP-type 7a	gene20927	AT1G01620	**0,50**	0,01
M5F7	26S protease regulatory subunit S10B (CADp44)	gene21849	AT1G45000	**0,52**	0,01
M17H2	E3 ubiquitin-protein ligase KEG	gene15725	AT4G32250	**0,53**	0,01
M24D11	Cyclin-dependent kinase E-1 (CDKE)	gene24158	AT5G63610	**0,53**	0,01
M19H9	RNA-binding protein 25 (RED120)	gene00656	AT1G60200	**0,53**	0,01
M4E2	60S ribosomal protein L2, mitochondrial	gene03327	AT2G44065	**0,54**	0,01
M10C3	Asparagine-rich protein (Protein ARP)	gene11676	AT3G15680	**0,54**	0,01
M13G5	Thioredoxin F-type 2, chloroplastic (Trx-F2)	gene16819	AT5G16400	**0,54**	0,05
M6F8	G-Protein coupled receptor 1	gene29223	AT1G57680	**0,55**	0,01
M27B2	hypothetical protein	gene10111	AT4G30720	**0,55**	0,01
M14G3	Protein SDS23	gene15737	AT1G15330	**0,55**	0,01
M25C8	Phenylalanyl-tRNA synthetase beta chain (PheRS)	gene19753	AT4G22320	**0,56**	0,01
M5A10	U5 small nuclear ribonucleoprotein 200 kDa helicase (U5-200KD)	gene10687	AT1G20960	**0,56**	0,01
M11A10	Pentatricopeptide repeat-containing protein At5g48910	gene31026	AT5G56310	**0,57**	0,01
M20F3	UPF0636 protein C4orf41 homolog	gene13006	AT5G65950	**0,57**	0,01
M10F9	70 kDa peptidyl-prolyl isomerase (PPIase)	gene02602	AT3G25230	**0,57**	0,01
M19E8	Probable protein phosphatase 2C 27 (AtPP2C27)	gene31511	AT2G33700	**0,57**	0,01
M18F3	Regulatory particle AAA-ATPase 4A / Proteasome complex	gene21849	AT5G43010	**0,57**	0,01

adj. pvalue 2.35E-2), “response to other organism” (GO:0051707, adj. pvalue 4.08E-2) and “response to bacterium” (GO:0009617, adj. pvalue 4.88E-2) was detected within the Andana upregulated set of genes (Table S1, Figure 4). Curiously, components of SA-dependent defenses, which were identified in Camarosa defense response (M4E10, M17H1, M12E12, M9D5 and M30F8; see Chapter V) were not detected in Andana, neither within the up or the down regulated gene collections identified after infection. However, this result agrees with that of changes in the hormonal balance of the strawberry plant after infection, as previously described in Chapter IV for Andana cultivar. Thus, no increase in SA content was detected in Andana after infection.

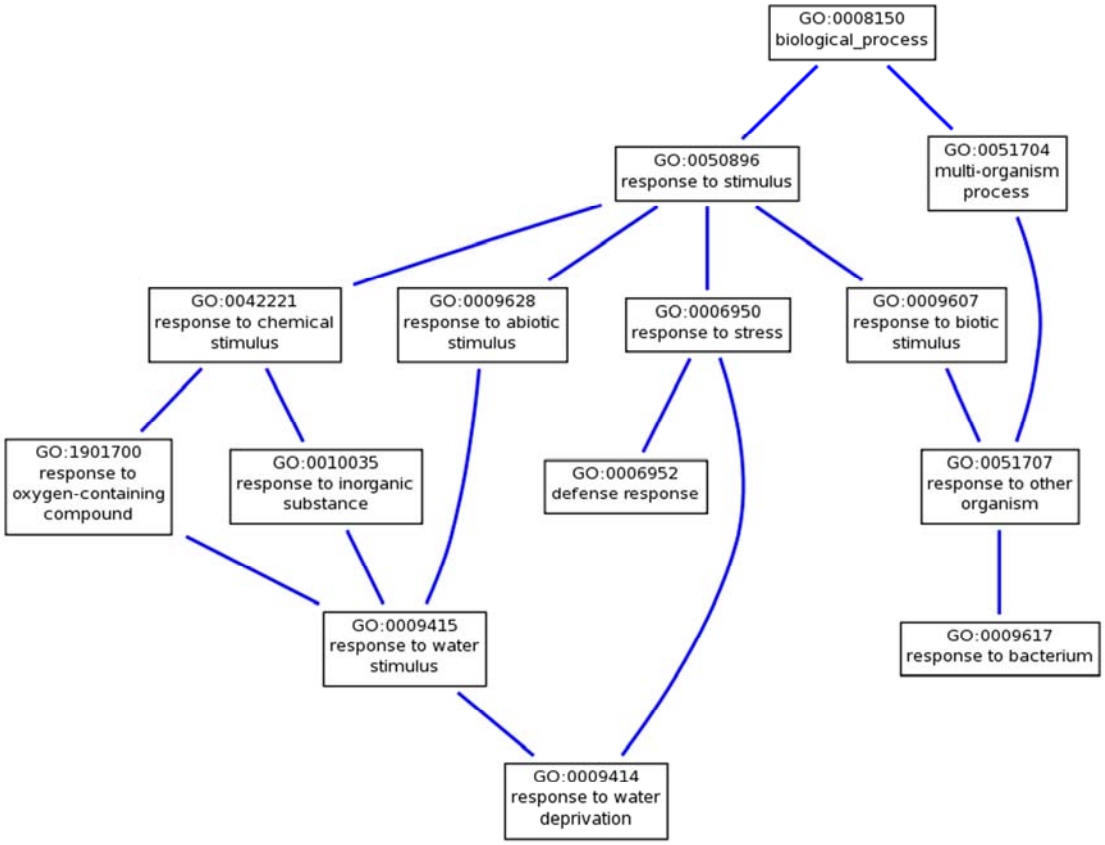

Figure 4. Biological processes significantly over-represented in Andana defense response to *C. acutatum* by Singular Enrichment Analysis (SEA, FATIGO).

Curiously, proteasome components of the AAA-type ATPase regulatory particles (genes M18F3 and M5F7, representing IPR005937 term and ath03050 pathway, with FatiGO ajd. pvalue of 6.09E-2 and 8.43E-2, respectively) were found enriched within the Andana downregulated set of genes in response to infection (Fu et al. 2001). As commented in Chapter VI, protein selective degradation and turnover is a usual signalling method in plant defense (Delauré et al. 2008). Figure 5 shows a schematic view of proteasome pathway and the localization of these regulatory components on such complex.

Additional functional information was obtained by FunCat and associated KEGG pathways (Ruepp et al. 2004). Thus, an important number of components was identified in Andana within the upregulated set of genes, for categories related to defense response such as “Cell, Rescue, Defense and Virulence” (Category 32, 16 entries), “Cellular Communication/Signal Transduction Mechanism” (Category 30, 12 entries), “Cellular Sensing and Response to External Stimulus” (Category 34.11, 11 entries) and “Plant/Fungal Specific Systemic Sensing and Response” (Category 36.20, 8 entries). Interestingly, components for the category “Immune Response to wounding” (Category 36.25.16.08) was detected within the downregulated set of genes. Tables S2 and S3, show a complete list of up- and down-regulated genes, respectively.

Curiously, KEGG pathways such as “Biosynthesis of secondary metabolites” (ath01110) and “Plant-pathogen interaction” (ath04626), were detected within the upregulated set of genes while others, such as “Spliceosome” (ath03040), “Proteasome” (ath03050), and “Ubiquitin mediated proteolysis” (ath04120), were representatives within the downregulated set of genes.

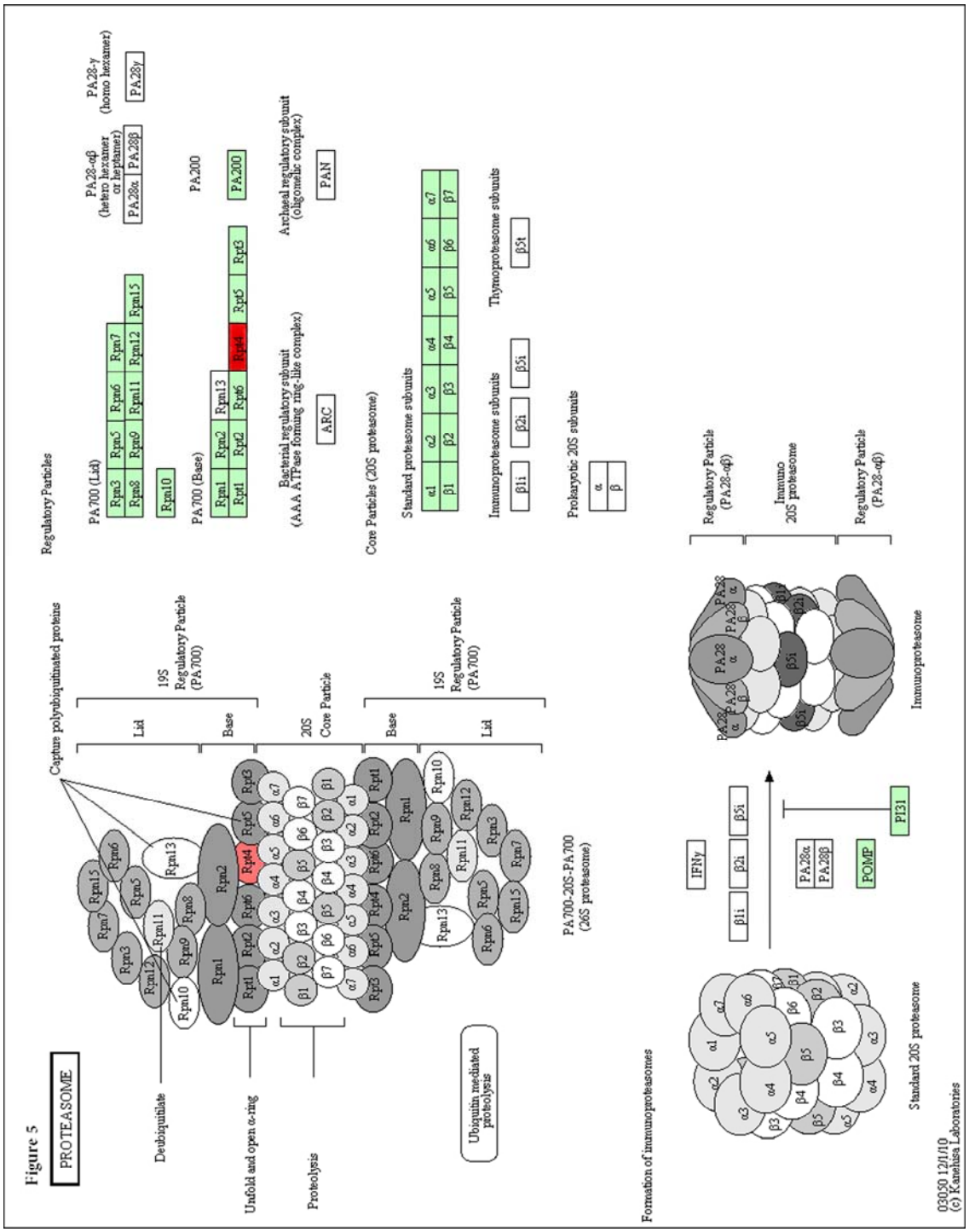

Figure 5. Proteasome complex and localization of AAA-type ATPase regulatory particles Rpt4.

Identification of Defense Related Genes with Different Expression Level in both Cultivars

In order to identify putative cultivar-dependent determinants of tolerance, additional comparisons were set up using Andana and Camarosa transcriptomes (experiments described above as (c) and (d)).

Following the same criteria as described above, 332 genes were identified as differentially more abundant in one cultivar than in the other when mock treatments of both cultivars were compared (235 genes more abundant in CC than in AC and 97 genes more in AC than in CC) (Tables 3 and 4). When cultivars were compared after infection, 333 genes were detected more abundant in one cultivar than in the other (132 genes more abundant in CI than in AI, and 201 genes more abundant in AI than in CI). Combined results of both experiments ((c) and (d)) show a subset of treatment independent genes, which are specifically more abundant in Camarosa (67 genes) and in Andana (69 genes) (Tables 3 and 4, respectively, and Figure 6). Interestingly, only 19 out of the 67 more abundant genes in Camarosa but none of the more abundant genes in Andana, shown to be regulated by infection (16 genes were upregulated, and 3 genes were downregulated) (See Table 1 in Chapter V for a list of Camarosa genes which expression was altered by infection).

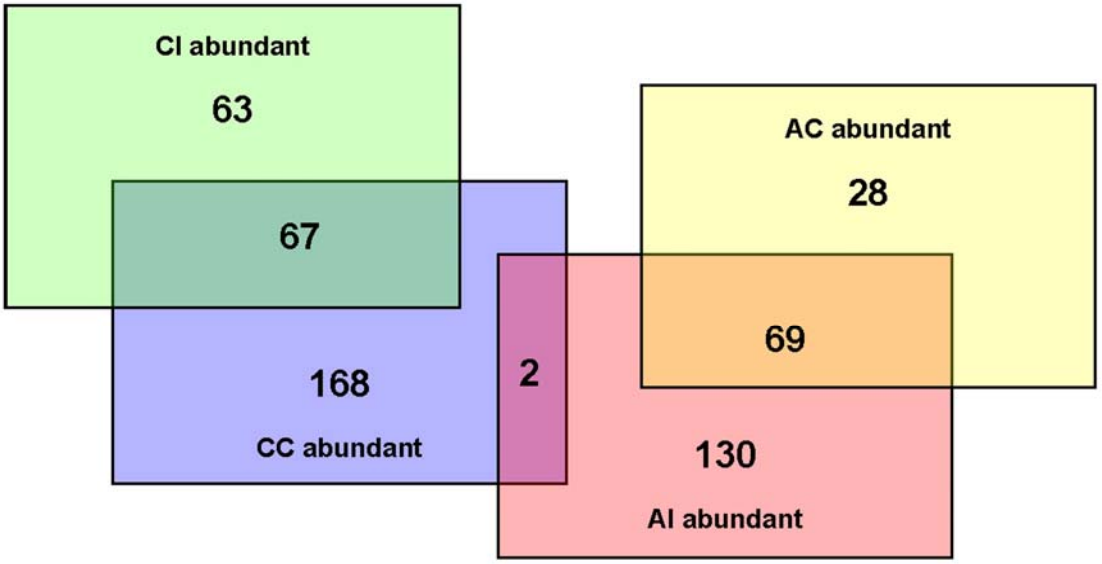

Figure 6. Intersection between the intercultivar comparisons: CC vs. AC, CI vs. AI. Accordingly to the evaluation criteria some of these genes are more abundant in one cultivar than in the other one when mock treated or pathogen inoculation tissue is examined. However an important number of genes are more abundant in one cultivar than in the other independently of treatments. Interestingly only in two cases an inverse regulation have been detected between both cultivars, being more abundant in Camarosa than Andana in mock treatment, but getting higher level in Andana than Camarosa when infected by the pathogen.

Identification of Cultivar-Specific Biological Processes

To identify biological processes associated specifically with a single cultivar, FATIGO and FunCat analysis was carried out as described previously. Thus, when the Camarosa set of most abundant genes was analyzed, enrichment in categories “response to biotic stimulus” (GO:0009607, adj. pvalue 2.62E-3) and “response to other organism” (GO:0051707, adj. pvalue 6.44E-3) was detected, (Figure 7). However, none category was enriched when the Andana set of most abundant genes was analyzed. In addition, FunCat categories such as “response to

Table 3. Genes identified as more abundant in Camarosa than in Andana cultivar from the inter-cultivar transcriptomic comparisons. Genes were considered as differentially expressed if they fulfilled a FDR <0.05 after a SAM test analysis, and the fold-change was higher that 1.75-fold in the compared conditions. Marked in bold data fulfilling these conditions. Values represent the ratio of cv. Camarosa mock vs. cv. Andana mock, CC vs. AC, and cv. Camarosa infected vs. cv. Andana infected, CI vs. AI.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	CC vs. AC		CI vs. AI	
				Fold Change	FDR qvalue	Fold Change	FDR qvalue
M28G6	hypothetical protein	gene28516	AT1G49600	**7,76**	0,00	1,37	0,01
M7B5	Pentatricopeptide repeat-containing protein At4g21190	gene13176	AT4G21190	**6,19**	0,02	1,49	0,00
M17H5	Chlorophyll a-b binding protein 40, chloroplastic (LHCP)	gene34432	AT1G29910	**6,14**	0,00	1,70	0,00
M23F1	No hit found	No hit found	No hit found	**5,18**	0,02	1,67	0,00
M12E6	Transcription initiation factor TFIID subunit 2	gene26210	AT1G73960	**4,94**	0,02	1,56	0,00
M1D6	Protein NUCLEAR FUSION DEFECTIVE 5, mitochondrial	gene21983	AT1G19520	**4,88**	0,02	1,66	0,00
M12E2	Photosystem II 10 kDa polypeptide, chloroplastic (PII10)	gene10470	AT1G79040	**4,72**	0,02	1,20	0,01
M1D3	GATA transcription factor 16	gene05175	AT5G49300	**4,67**	0,02	1,69	0,00
M4A4	Probable cellulose synthase A catalytic subunit 5 [UDP-forming]	gene29320	AT4G39350	**4,57**	0,02	1,55	0,00
M23C2	No hit found	No hit found	No hit found	**4,10**	0,02	1,27	0,01
M18C12	60S ribosomal protein L11	gene29695	AT4G18730	**4,00**	0,02	1,51	0,00
M13E12	SNAP25 homologous protein SNAP33 (AtSNAP33)	gene26076	AT5G61210	**3,97**	0,02	1,05	0,19
M7F3	Inner membrane protein yfgF	gene10263	No hit found	**3,57**	0,02	1,55	0,00
M20D10	3-dehydroquinate synthase	gene14571	AT5G66120	**3,55**	0,02	m.s.	m.s.
M17D4	Transmembrane protein 208	gene08443	AT4G30500	**3,45**	0,02	1,14	0,06
M11B8	40S ribosomal protein S5-1	gene18014	AT3G11940	**3,40**	0,02	1,22	0,01
M11B2	Kinase-related protein	gene07537	AT3G13990	**3,40**	0,02	m.s.	m.s.
M12E8	Upstream stimulatory factor (USF)	gene12097	AT4G24610	**3,37**	0,02	m.s.	m.s.
M7D11	Ubiquinone biosynthesis protein coq-8	gene12393	No hit found	**3,35**	0,02	m.s.	m.s.
M18G5	Auxin-induced protein 5NG4	gene11153	AT4G08290	**3,33**	0,02	1,37	0,00
M3F6	tRNA pseudouridine synthase B (Psi55 synthase)	gene13772	AT5G01590	**3,31**	0,02	1,19	0,04
M27D4	Transcription factor bHLH68 (bHLH 68)	gene25821	AT2G20100	**3,28**	0,02	1,34	0,01
M11D9	Serine hydroxymethyltransferase 1 (SHMT 1)	gene06563	AT4G13930	**3,28**	0,02	1,61	0,00
M19C6	Glycosyl hydrolase family 81 protein	gene26641	AT5G15870	**3,27**	0,02	1,10	0,04
M2F11	Protein TRANSPARENT TESTA 12	gene19270	AT4G00340	**3,25**	0,02	1,24	0,01
M14E9	Cleavage and polyadenylation specificity factor subunit 2 (CPSF 100 kDa subunit)	gene13255	AT4G33410	**3,18**	0,02	1,21	0,01
M11G2	HIPL1 protein	gene09553	AT1G74790	**2,99**	0,02	1,36	0,00
M21E5	Transcriptional corepressor LEUNIG	gene30949	AT4G32551	**2,97**	0,02	1,71	0,00
M9D6	Eukaryotic translation initiation factor 3 subunit G (eIF3g)	gene01938	AT3G11400	**2,91**	0,02	1,19	0,01
M23F8	60S ribosomal protein L8-3	gene17157	AT2G18020	**2,91**	0,02	1,65	0,00
M9H7	Nuclear-interacting partner of ALK (mNIPA)	gene30383	AT1G17210	**2,86**	0,02	1,09	0,07
M13G1	Anthocyanidin 3-O-glucosyltransferase	gene12684	No hit found	**2,82**	0,02	1,54	0,00
M28B4	Cell division control protein 2 homolog	gene31613	AT3G48750	**2,80**	0,02	1,68	0,00
M9F2	Thromboxane-A synthase (TXA synthase)	gene02708	AT2G26170	**2,79**	0,02	1,44	0,00
M2A7	Staphylococcal nuclease domain-containing protein 1	gene01810	AT5G07350	**2,77**	0,02	1,40	0,00
M11F7	No hit found	No hit found	No hit found	**2,72**	0,02	1,32	0,00
M22B6	SKP1-like protein 1B	gene08563	AT1G75950	**2,70**	0,02	1,34	0,00
M16H5	Calcium-dependent protein kinase 4 (CDPK 4)	gene17341	AT2G17290	**2,68**	0,02	1,37	0,00
M24D1	LRR receptor-like serine/threonine-protein kinase ERECTA	gene15491	AT2G26330	**2,67**	0,02	1,43	0,00
M18H11	Ubiquitin-conjugating enzyme 15	gene15203	AT1G75440	**2,65**	0,02	1,21	0,09
M24A7	Protein CWC15 homolog	gene22649	AT3G13200	**2,65**	0,02	1,42	0,00
M11G4	BTB/POZ domain-containing protein At5g48800	gene01554	AT5G67385	**2,59**	0,02	1,18	0,02
M30D1	Tubulin beta-6 chain	gene08531	AT1G75780	**2,56**	0,02	1,02	0,26
M10D8	Zinc finger CCCH domain-containing protein 6 (AtC3H6)	gene08487	No hit found	**2,55**	0,02	1,18	0,01
M1D9	Tubulin alpha chain	gene01798	AT4G14960	**2,48**	0,02	1,06	0,26
M18F3	Regulatory particle AAA-ATPase 4A / Proteasome complex	gene21849	AT5G43010	**2,46**	0,02	1,42	0,00
M23A12	No hit found	No hit found	No hit found	**2,46**	0,02	1,38	0,00
M20A6	Nuclear-interacting partner of ALK (mNIPA)	gene30383	AT1G17210	**2,46**	0,02	1,46	0,00
M10C6	Sirohydrochlorin ferrochelatase	gene30604	No hit found	**2,44**	0,02	1,19	0,02
M9B2	No hit found	No hit found	No hit found	**2,41**	0,02	1,64	0,00
M24A10	Probable leucine-rich repeat receptor-like protein kinase At1g35710	gene04649	AT5G61240	**2,39**	0,02	1,12	0,09
M9D9	Putative phagocytic receptor 1b	gene09316	AT5G37310	**2,39**	0,02	1,48	0,00
M5H4	Thioredoxin H-type (Trx-H)	gene15211	AT5G42980	**2,37**	0,02	1,37	0,00
M22C11	DEAD-box ATP-dependent RNA helicase 28	gene08545	AT4G16630	**2,35**	0,02	1,18	0,04
M4D6	Pyruvate kinase, cytosolic isozyme (PK)	gene00768	AT5G08570	**2,34**	0,02	m.s.	m.s.
M21G2	SCAR family member	gene29663	AT1G29170	**2,34**	0,02	1,06	0,12
M4H3	Serine/threonine-protein kinase haspin	gene22924	AT1G09450	**2,32**	0,02	1,54	0,00
M15F6	ATP synthase subunit delta (F-ATPase subunit delta)	gene23174	No hit found	**2,30**	0,02	1,07	0,09
M16C8	Putative clathrin assembly protein At5g35200	gene31116	AT5G35200	**2,30**	0,02	1,38	0,00
M14B11	No hit found	No hit found	No hit found	**2,30**	0,02	1,67	0,00
M11H9	Glutathione reductase, cytosolic (GRase)	gene29906	AT3G24170	**2,26**	0,02	1,26	0,02
M24B5	SUMO-conjugating enzyme UBC9	gene13119	AT5G53300	**2,26**	0,02	1,50	0,00
M27F10	Probable NAD(P)H-dependent oxidoreductase 1	gene09318	No hit found	**2,25**	0,02	1,45	0,00
M1B8	No hit found	No hit found	No hit found	**2,24**	0,02	1,45	0,00
M1B4	DEAD-box ATP-dependent RNA helicase 56	gene11642	AT5G11170	**2,22**	0,02	1,51	0,01
M9B4	Transcription factor bHLH13 (bHLH 13)	gene19143	AT4G16430	**2,20**	0,02	1,34	0,00
M7E4	Pumilio homolog 2 (Pumilio-2)	gene26337	AT2G29200	**2,19**	0,02	1,27	0,01
M23C5	No hit found	No hit found	No hit found	**2,19**	0,02	1,65	0,00
M3H9	hypothetical protein	gene00808	No hit found	**2,19**	0,02	1,02	0,24
M4G9	Magnesium-chelatase subunit H	gene02502	AT5G13630	**2,19**	0,02	1,14	0,02
M20F6	No hit found	No hit found	No hit found	**2,19**	0,02	1,40	0,00
M25F10	No hit found	No hit found	No hit found	**2,17**	0,02	1,37	0,01
M10C2	No hit found	No hit found	No hit found	**2,16**	0,02	1,60	0,00
M20B11	Acetolactate synthase small subunit (AHAS)	gene29713	AT5G16290	**2,16**	0,02	1,50	0,00
M17B2	Phosphoglucan, water dikinase, chloroplastic	gene16902	AT5G26570	**2,16**	0,02	1,16	0,01
M4A11	ADP,ATP carrier protein 1, mitochondrial (ANT 1)	gene26994	AT5G13490	**2,14**	0,02	1,42	0,00
M2F8	GEM-like protein 1 (FH-interacting protein 1)	gene12067	AT1G28200	**2,14**	0,02	1,57	0,00
M11E5	ZF-HD homeobox protein At4g24660 (AtHB-22)	gene14755	AT2G18350	**2,14**	0,02	1,51	0,00
M14D8	No hit found	No hit found	No hit found	**2,14**	0,02	1,34	0,00
M14E10	Translation initiation factor IF-2	gene16588	No hit found	**2,12**	0,02	1,49	0,00
M8D1	Splicing factor, arginine/serine-rich 12 (SRrp86)	gene02340	AT3G14450	**2,12**	0,02	1,51	0,00
M17F6	Vacuolar protein sorting-associated protein 54	gene16757	AT4G19490	**2,11**	0,02	1,33	0,00
M20B8	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (PDHE1-B)	gene07254	AT5G50850	**2,10**	0,02	1,73	0,00
M24F9	GTP cyclohydrolase-2	gene16232	AT2G22450	**2,09**	0,02	1,68	0,20
M6E4	Erythrocyte-binding antigen 175 (EBA-175)	gene09052	No hit found	**2,08**	0,02	1,16	0,02
M28E2	Luminal-binding protein 2 (BiP2)	gene25105	AT5G42020	**2,08**	0,02	1,02	0,19
M11H3	No hit found	No hit found	No hit found	**2,08**	0,02	1,27	0,01
M6E6	Ferredoxin--nitrite reductase, chloroplastic	gene28260	AT2G15620	**2,08**	0,02	1,56	0,00
M9H9	No hit found	No hit found	No hit found	**2,07**	0,02	1,64	0,00
M1A12	Trafficking protein particle complex subunit 9	gene10991	AT5G11040	**2,07**	0,02	1,01	0,19

Table 3. Cont.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	CC vs. AC Fold Change	CC vs. AC FDR qvalue	CI vs. AI Fold Change	CI vs. AI FDR qvalue
M9C8	hypothetical protein	gene24921	No hit found	**2,06**	0,02	1,39	0,00
M13B11	L-ascorbate oxidase homolog	gene29389	AT5G66920	**2,06**	0,02	1,50	0,00
M22G4	No hit found	No hit found	No hit found	**2,06**	0,02	1,40	0,00
M14A5	UPF0546 membrane protein	gene11869	AT5G19570	**2,05**	0,02	1,35	0,01
M14D3	Histidine-rich glycoprotein	gene04882	No hit found	**2,04**	0,02	1,48	0,00
M23D6	No hit found	No hit found	No hit found	**2,04**	0,02	1,60	0,00
M23C11	Dehydrin cold-regulated 47	gene08617	AT1G76180	**2,03**	0,02	1,72	0,00
M19A11	RING-H2 finger protein ATL1E	gene10865	No hit found	**2,03**	0,02	1,46	0,01
M11E7	Pistil-specific extensin-like protein (PELP)	gene02380	No hit found	**2,03**	0,02	m.s.	m.s.
M1H12	No hit found	No hit found	No hit found	**2,03**	0,02	1,26	0,01
M17D11	DNA ligase	gene13773	No hit found	**2,02**	0,02	1,41	0,00
M1C1	Serine hydroxymethyltransferase (Serine methylase)	gene05088	No hit found	**2,01**	0,02	1,53	0,00
M23D7	No hit found	No hit found	No hit found	**2,01**	0,02	1,54	0,00
M25A5	FK506-binding protein 2-1 (PPIase)	gene17435	AT5G48580	**2,00**	0,02	m.s.	m.s.
M8B5	60S ribosomal protein L6	gene26958	AT1G74050	**2,00**	0,02	1,14	0,02
M2C10	Cleft lip and palate transmembrane protein 1 homolog	gene12346	AT5G08500	**1,99**	0,02	1,45	0,01
M8C12	Olfactomedin-like protein 2A	gene09715	No hit found	**1,99**	0,02	1,41	0,01
M28F9	No hit found	No hit found	No hit found	**1,99**	0,02	1,00	0,19
M2A9	50S ribosomal protein L2, chloroplastic	gene09706	No hit found	**1,99**	0,02	1,33	0,00
M23C3	Pattern formation protein EMB30	gene26324	AT1G13980	**1,98**	0,02	1,20	0,01
M5B11	TRAF-type zinc finger domain-containing protein 1	gene26588	AT1G09920	**1,97**	0,02	1,52	0,00
M3D11	Transmembrane protein 93	gene05035	AT5G49540	**1,97**	0,02	1,41	0,00
M4A7	4-coumarate--CoA ligase 2 (4CL 2)	gene15877	AT1G51680	**1,96**	0,02	1,03	0,19
M11B5	Mediator of RNA polymerase II transcription subunit 12	gene30526	AT5G37710	**1,96**	0,02	1,19	0,02
M24F11	Formamidopyrimidine-DNA glycosylase (Fapy-DNA glycosylase)	gene03241	No hit found	**1,95**	0,02	1,36	0,02
M1A3	Chaperonin CPN60-2, mitochondrial	gene02729	AT3G23990	**1,95**	0,02	1,29	0,00
M3G1	Inositol oxygenase 2 (MI oxygenase 2)	gene11353	AT1G14520	**1,95**	0,02	1,58	0,00
M12A10	Actin-7	gene18570	AT5G09810	**1,94**	0,02	1,09	0,09
M9C7	Ankyrin repeat domain-containing protein 2 (AtAKR2)	gene17130	AT4G35450	**1,94**	0,02	1,24	0,00
M21D6	60S ribosomal protein L27-1	gene00414	AT3G22230	**1,93**	0,02	1,24	0,00
M23F10	mRNA-decapping enzyme-like protein	gene15633	AT1G08370	**1,93**	0,02	1,48	0,00
M5B1	Basic leucine zipper and W2 domain-containing protein 2	gene16812	AT5G36230	**1,93**	0,02	1,24	0,01
M8C9	Endoplasmin homolog	gene08540	AT4G24190	**1,93**	0,02	1,53	0,00
M6A7	No hit found	No hit found	No hit found	**1,92**	0,02	1,60	0,00
M22G7	non-LTR retrotransposon family	gene09933	AT5G41835	**1,92**	0,02	1,71	0,20
M10D4	Tetratricopeptide repeat protein 7B (TPR repeat protein 7B)	gene02575	AT1G27460	**1,91**	0,02	1,18	0,02
M11B7	Transcription factor UNE12 (bHLH 59)	gene08188	AT4G02590	**1,90**	0,02	1,17	0,01
M17H11	Sporulation-specific protein 15	gene32044	No hit found	**1,90**	0,02	1,06	0,14
M6H11	Proteasome subunit alpha type-7	gene14583	AT3G51260	**1,90**	0,02	1,22	0,02
M9G9	No hit found	No hit found	No hit found	**1,90**	0,02	1,28	0,00
M11E1	Metallothionein-like protein 1 (MT-1)	gene00310	No hit found	**1,89**	0,02	1,15	0,20
M24A1	Protein RUPTURED POLLEN GRAIN 1	gene24944	No hit found	**1,89**	0,02	1,09	0,04
M28D4	60S ribosomal protein L7-2	gene05665	AT2G01250	**1,88**	0,02	1,21	0,00
M11E10	Centrosomal protein of 290 kDa (Cep290)	gene17709	AT4G28300	**1,88**	0,02	1,20	0,01
M4D2	Glucan endo-1,3-beta-glucosidase 8 ((1->3)-beta-glucanase 8)	gene21072	AT1G64760	**1,88**	0,02	1,50	0,01
M7A5	No hit found	No hit found	No hit found	**1,86**	0,02	1,44	0,00
M6G4	Tubulin alpha chain	gene01798	AT4G14960	**1,86**	0,02	1,57	0,00
M6F3	3-oxoacyl-[acyl-carrier-protein] synthase III, chloroplastic (KAS III)	gene32240	AT1G62640	**1,86**	0,02	1,21	0,02
M13A4	Aminopeptidase M1 family protein / Leukotriene A-4 hydrolase	gene23331	AT5G13520	**1,86**	0,02	1,41	0,00
M10E12	Trans-cinnamate 4-monooxygenase (CA4H)	gene28093	AT2G30490	**1,86**	0,02	1,66	0,00
M19F8	Serrate RNA effector molecule	gene07673	AT2G27100	**1,85**	0,02	1,73	0,00
M10H5	Cellulose synthase A catalytic subunit 3 [UDP-forming] (AtCesA3)	gene26807	AT5G05170	**1,84**	0,02	1,25	0,04
M17F1	Histone H2A	gene14169	AT1G51060	**1,84**	0,02	1,05	0,20
M12D8	Serine/threonine-protein kinase PBS1	gene23053	AT4G02010	**1,84**	0,02	1,47	0,00
M6H7	ABC transporter F family member 3 (ABC transporter ABCF.3)	gene25103	AT1G64550	**1,83**	0,02	1,05	0,14
M3A12	General transcription factor 3C polypeptide 2	gene14199	AT1G19485	**1,83**	0,02	1,11	0,05
M8D10	Protein TRANSPARENT TESTA 1	gene20598	AT3G57670	**1,82**	0,02	1,19	0,02
M12F12	Aquaporin PIP1-3 (AtPIP1)	gene19301	AT4G23400	**1,82**	0,02	1,64	0,00
M8C4	Histone H3.3	gene25489	AT5G65360	**1,81**	0,02	1,35	0,01
M10F3	Probable galactinol--sucrose galactosyltransferase 5	gene28489	AT5G40390	**1,81**	0,02	1,13	0,03
M21H11	Protease 2	gene29438	AT5G66960	**1,80**	0,02	1,47	0,00
M23A1	No hit found	No hit found	No hit found	**1,80**	0,02	1,25	0,01
M7H2	RNA polymerase II C-terminal domain phosphatase-like 1 (FCP-like 1)	gene30412	AT4G21670	**1,80**	0,02	1,20	0,01
M18F12	Glucose-6-phosphate 1-dehydrogenase, cytoplasmic isoform 2 (G6PD6)	gene26419	AT5G40760	**1,78**	0,02	1,60	0,00
M29B11	Casein kinase I isoform delta-like (CKI-delta)	gene08271	AT5G44100	**1,78**	0,02	1,30	0,01
M24D2	Kinesin-2	gene12992	AT5G65930	**1,78**	0,02	1,21	0,01
M10B8	No hit found	No hit found	No hit found	**1,78**	0,02	1,19	0,01
M2B1	Endochitinase 1	gene22465	AT1G05850	**1,78**	0,02	1,35	0,01
M16E10	Glucan endo-1,3-beta-glucosidase-like protein 3	gene25805	AT1G18650	**1,77**	0,02	1,28	0,00
M30E10	Elongation factor 2 (EF-2)	gene30053	AT1G56070	**1,77**	0,02	1,21	0,01
M7F6	No hit found	No hit found	No hit found	**1,77**	0,02	1,50	0,01
M9A9	Salivary glue protein Sgs-3	gene25166	AT1G68490	**1,77**	0,02	1,59	0,00
M5E5	No hit found	No hit found	No hit found	**1,76**	0,02	1,46	0,00
M14B6	Probable potassium transporter 17	gene06032	AT1G80280	**1,76**	0,02	m.s.	m.s.
M10C8	Tetratricopeptide repeat protein 7B (TPR repeat protein 7B)	gene02575	AT1G27460	**1,76**	0,02	1,22	0,01
M24H9	No hit found	No hit found	No hit found	**1,74**	0,02	1,20	0,09
M15E2	Indole-3-acetate beta-glucosyltransferase 1	gene06602	AT4G15550	**1,74**	0,02	1,45	0,00
M8A2	Leucoanthocyanidin reductase (LAR)	gene24665	No hit found	**1,74**	0,02	1,07	0,09
M21B2	Beta-galactosidase (Beta-gal)	gene33082	No hit found	**7,51**	0,00	**10,97**	0,00
M27F8	No hit found	No hit found	No hit found	**7,12**	0,00	**2,07**	0,00
M11F8	Phospho-2-dehydro-3-deoxyheptonate aldolase 2, chloroplastic	gene16261	AT4G39980	**6,51**	0,00	**3,06**	0,00
M27A2	Phosphoribulokinase	gene13677a	AT1G32060	**5,99**	0,00	**3,67**	0,00
M28F7	40S Ribosomal protein S12/S23	gene25662a	AT5G02960	**5,61**	0,00	**2,30**	0,00
M20B5	tRNA-dihydrouridine synthase 3-like (AtC3H50)	gene01681	AT4G38890	**5,55**	0,00	**1,97**	0,00
M10F6	Translocase of chloroplast 159, chloroplastic (AtToc159)	gene29826	No hit found	**5,38**	0,00	**1,92**	0,00
M11A12	Histone deacetylase HDT1 (HD2a)	gene14356	AT5G22650	**4,96**	0,00	**2,26**	0,00
M15H8	Actin-related protein 2	gene01351	AT3G27000	**4,68**	0,02	**3,56**	0,00
M21G9	Protein SCAR3 (AtSCAR3)	gene29663	AT1G29170	**4,63**	0,02	**1,83**	0,00
M17B3	Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase (GlcNAcT-I)	gene17328	AT4G38240	**4,51**	0,02	**4,09**	0,00
M9E2	Pre-mRNA-processing-splicing factor SUS2	gene15731	AT1G80070	**4,49**	0,02	**2,94**	0,00
M2C6	Heterogeneous nuclear ribonucleoprotein A3 (hnRNP A3)	gene29831	No hit found	**4,44**	0,02	**2,37**	0,00
LTP46	No hit found	No hit found	No hit found	**4,44**	0,02	**2,46**	0,00
M25E9	Elongation factor 1-alpha (EF-1-alpha)	gene28639	AT1G07940	**4,21**	0,02	**3,42**	0,00

Table 3. Cont.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	CC vs. AC Fold Change	CC vs. AC FDR qvalue	CI vs. AI Fold Change	CI vs. AI FDR qvalue
M14E2	Ocs element-binding factor 1 (OCSBF-1)	gene04187	AT5G38800	**4,20**	0,02	**2,85**	0,00
M1E6	UBA domain-containing protein 7	gene11248	AT1G21660	**4,01**	0,02	**1,96**	0,00
M16C3	Protein kinase G11A	gene22007	AT2G44830	**3,79**	0,02	**2,04**	0,00
M28G1	Cytochrome P450 71A26	gene22676	No hit found	**3,64**	0,02	**2,89**	0,00
M7D10	Shaggy-related protein kinase kappa (AtK-1)	gene29593	AT1G09840	**3,61**	0,02	**2,02**	0,00
M19G6	Probable ubiquitin carrier protein E2 25	gene17712	AT4G31670	**3,48**	0,02	**2,18**	0,00
M9F11	No hit found	No hit found	No hit found	**3,47**	0,02	**2,12**	0,00
M5G11	Phenylalanine ammonia-lyase 1	gene09753	AT2G37040	**3,43**	0,02	**2,30**	0,00
M4D11	No hit found	No hit found	No hit found	**3,42**	0,02	**1,82**	0,00
M19C7	Ferrochelatase-2, chloroplastic	gene02670	AT5G26030	**3,36**	0,02	**2,81**	0,00
M17G9	Mitochondrial import receptor subunit TOM40 homolog 1	gene22468	AT3G20000	**3,27**	0,02	**2,31**	0,00
M19G2	hypothetical protein	gene02684	AT5G11840	**3,14**	0,02	**3,64**	0,00
M3D7	Iron-sulfur cluster assembly enzyme ISCU, mitochondrial (NifU-like protein)	gene32190	AT4G22220	**3,10**	0,02	**1,86**	0,00
M26E8	Auxin-responsive protein IAA9	gene05555	AT2G22670	**2,98**	0,02	**1,81**	0,00
M27D1	Methylthioribose-1-phosphate isomerase (MTR-1-P isomerase)	gene12526	AT2G05830	**2,82**	0,02	**1,84**	0,00
M6G2	Protein TRANSPARENT TESTA 12	gene15073	AT1G47530	**2,76**	0,02	**1,84**	0,00
M14E3	Beta-galactosidase (Beta-gal)	gene33082	No hit found	**2,74**	0,02	**2,44**	0,00
M2E4	hypothetical protein	gene02111	AT3G29300	**2,73**	0,02	**1,81**	0,00
M18A9	RNA-binding KH domain-containing protein	gene30367	AT5G46190	**2,69**	0,02	**2,64**	0,00
M18B2	Flavonoid 3'-monooxygenase	gene25801	No hit found	**2,66**	0,02	**2,54**	0,00
M1A2	Coatomer subunit alpha	gene24354	AT1G62020	**2,55**	0,02	**2,78**	0,00
M26F5	UPF0496 protein 4	gene11838	No hit found	**2,52**	0,02	**2,04**	0,00
M21A6	MOSC domain-containing protein 2, mitochondrial	gene19953	AT1G30910	**2,52**	0,02	**1,83**	0,00
M8H8	ATP-dependent RNA helicase DBP10	gene10702	AT4G17960	**2,41**	0,02	**2,20**	0,00
M19F12	No hit found	No hit found	No hit found	**2,30**	0,02	**1,81**	0,00
M15G5	Glucan / Callose synthase	gene02397	AT4G03550	**2,28**	0,02	**1,86**	0,00
M14G7	Probable mitochondrial 2-oxoglutarate/malate carrier protein (OGCP)	gene20791	AT4G24570	**2,27**	0,02	**4,52**	0,00
M18A11	Nodulin transporter family protein	gene08921	AT5G47470	**2,26**	0,02	**1,90**	0,00
M19E4	RNA-binding (RRM/RBD/RNP motifs) family protein	gene05323	AT2G44710	**2,24**	0,02	**2,78**	0,00
M3C1	Cellulose synthase A catalytic subunit 2 [UDP-forming] (AtCesA2)	gene08114	AT2G21770	**2,20**	0,02	**1,92**	0,00
M21B10	hypothetical protein	gene19493	AT4G22120	**2,19**	0,02	**1,78**	0,00
M12E4	Tetratricopeptide repeat (TPR)-like superfamily protein	gene32179	AT1G27500	**2,16**	0,02	**2,00**	0,00
M19G4	Serine/threonine-protein kinase SAPK1	gene16244	AT4G33950	**2,15**	0,02	**2,08**	0,00
M12C12	Triacylglycerol lipase 2	gene31975	AT5G14180	**2,15**	0,02	**3,03**	0,00
M13G3	Uridine-cytidine kinase-like 1	gene25903	AT5G40870	**2,12**	0,02	**2,65**	0,00
M10E3	No hit found	No hit found	No hit found	**2,07**	0,02	**3,41**	0,00
M6B9	Pathogenesis-related 10 family protein	gene05185	AT1G24020	**2,05**	0,02	**1,87**	0,00
M22G5	Major allergen Pru ar 1	gene07077	No hit found	**2,05**	0,02	**1,89**	0,01
M9H4	Tetratricopeptide repeat protein 7B (TPR repeat protein 7B)	gene02575	AT1G27460	**2,04**	0,02	**2,48**	0,00
M11C12	Putative endo-1,4-beta-xylanase (Xylanase)	gene13162	No hit found	**2,03**	0,02	**1,80**	0,00
M9D11	Signal peptide peptidase-like 2B (Protein SPP-like 2B)	gene19307	AT1G01650	**1,98**	0,02	**2,55**	0,00
EDS1-936	EDS1-specific diacylglycerol lipase alpha	gene09503	AT3G48090	**1,97**	0,02	**2,11**	0,00
M5B6	Laccase	gene24296	AT5G09360	**1,93**	0,02	**2,64**	0,00
M6G11	Flavonol 7-O-glucosyltransferase	gene26351	AT4G34135	**1,91**	0,02	**2,24**	0,00
M6G7	Hypoxia-responsive Zinc finger (C3HC4-type RING finger) family protein	gene32154	AT3G48030	**1,89**	0,02	**2,15**	0,00
M3E8	50S ribosomal protein L1, chloroplastic	gene29496	AT3G63490	**1,86**	0,02	**2,49**	0,00
M4F10	E3 ubiquitin-protein ligase ARI8	gene21532	AT1G65430	**1,84**	0,02	**2,47**	0,00
M24D9	Probable salt tolerance-like protein At1g78600	gene14875	AT2G21320	**1,83**	0,02	**1,76**	0,00
M14D5	Proline-, glutamic acid- and leucine-rich protein 1	gene13911	AT1G30240	**1,82**	0,02	**2,00**	0,00
M9H8	Tetratricopeptide repeat protein 7B (TPR repeat protein 7B)	gene02575	AT1G27460	**1,80**	0,02	**1,88**	0,00
M3F3	Putative L-ascorbate peroxidase 6 (AtAPx08)	gene11632	AT2G25480	**1,78**	0,02	**1,85**	0,00
M14H1	Vacuolar cation/proton exchanger 3	gene07894	AT3G51860	**1,75**	0,02	**2,43**	0,00
EPR5-77	Pathogenesis-related 5 family protein	gene32423	AT4G11650	m.s.	m.s.	**9,69**	0,00
M23A6	Ubiquitin-like superfamily protein	gene32391	AT4G11740	1,57	0,02	**4,00**	0,00
M28H1	No hit found	No hit found	No hit found	m.s.	m.s.	**3,84**	0,00
M16B7	Serine/threonine-protein phosphatase PP2A catalytic subunit	gene00744	AT1G69960	1,69	0,02	**3,19**	0,00
M16H1	Auxin response factor	gene14094	no hit found	1,53	0,02	**3,12**	0,00
M19D11	Casein Serine/threonine-protein kinase	gene10418a	AT3G03940	1,60	0,02	**3,07**	0,00
M24B7	Glycosyl hydrolase superfamily protein	gene14817	AT4G16260	m.s.	m.s.	**2,71**	0,00
M26G7	RNA binding KH domain-containing protein	gene31909	AT2G25970	1,17	0,15	**2,67**	0,00
M14G2	Tetratricopeptide repeat (TPR)-like superfamily protein	gene31738	AT4G39820	1,52	0,02	**2,59**	0,00
M12E12	WRKY DNA-binding protein 70	gene21365	AT3G56400	m.s.	m.s.	**2,56**	0,00
M22A10	Pathogenesis-related 10 family protein	gene07085	AT1G24020	1,44	0,02	**2,53**	0,00
M25E3	Histone-lysine N-methyltransferase ATX4 (TRX-homolog protein 4)	gene12861	AT5G53430	1,61	0,02	**2,48**	0,00
M11C6	60S Ribosomal protein L34	gene03828	AT1G69620	1,19	0,13	**2,48**	0,00
M21E9	MRG family protein, chromatin binding	gene24023	AT2G24960	1,19	0,12	**2,45**	0,00
M26A7	No hit found	No hit found	No hit found	1,15	0,18	**2,35**	0,00
M10A10	No hit found	No hit found	No hit found	1,22	0,22	**2,34**	0,00
M17F5	Elongation factor 1-alpha (EF-1-alpha)	gene28639	AT1G07940	1,53	0,02	**2,33**	0,00
M26B12	No hit found	No hit found	No hit found	1,36	0,03	**2,32**	0,00
M13C5	Serine/threonine-protein kinase PBS1	gene07245	AT5G13160	1,57	0,02	**2,31**	0,00
M27D3	RING/U-box E3 ubiquitin-protein ligase	gene18784	AT5G01160	1,05	0,40	**2,31**	0,00
M22H2	No hit found	No hit found	No hit found	1,66	0,02	**2,26**	0,00
M6G3	Pyrophosphate-energized vacuolar membrane proton pump 1 (H(+)-PPase 1)	gene31580	AT1G15690	m.s.	m.s.	**2,26**	0,00
M21F1	Polyadenylate-binding protein 2 (Poly(A)-binding protein 2)	gene26422	AT1G49760	1,56	0,02	**2,24**	0,00
M8D2	Methionine synthase	gene14995	AT5G17920	0,53	0,02	**2,21**	0,00
M3D5	NEDD8-activating enzyme E1 regulatory subunit / AXR1 (Auxin resistant 1) NAD(P)-binding F	gene23778	AT1G05180	1,63	0,02	**2,19**	0,00
M1F10	Pathogenesis-related 5 family protein	gene09812	AT1G20030	1,42	0,02	**2,19**	0,00
M27F3	F-box/kelch-repeat protein At5g48980	gene24889	No hit found	1,39	0,02	**2,16**	0,00
M24C11	Unknown protein, endomembrane system	gene32086	AT1G64385	m.s.	m.s.	**2,15**	0,00
M25E7	Bromodomain-containing protein	gene01516	AT1G15780	m.s.	m.s.	**2,15**	0,00
M19F7	SLOMO (SLOw MOtion) F-box/LRR-repeat protein	gene15497	AT4G33210	m.s.	m.s.	**2,14**	0,00
M20G3	Putative vacuolar protein sorting-associated protein 13A	gene23008	AT1G48090	m.s.	m.s.	**2,11**	0,00
M18F1	RNA-binding protein 47C	gene09051	AT1G47490	1,17	0,15	**2,10**	0,00
M22B1	Embryo defective 2410	gene01044	AT2G25660	1,29	0,10	**2,09**	0,00
M27C10	Serine/threonine protein phosphatase 2A regulatory B subunit	gene30942	AT5G25510	1,14	0,20	**2,06**	0,00
M16D12	Chitinase class IV	gene02717	AT3G54420	1,48	0,02	**2,04**	0,00
M19G7	No hit found	No hit found	No hit found	1,41	0,02	**2,03**	0,00
M27C7	Exostosin-2	gene20928	AT3G55830	1,53	0,02	**2,00**	0,00
M13H7	Putative vesicle-associated membrane protein 726 (AtVAMP726)	gene20060	AT2G32670	1,70	0,02	**1,98**	0,00
M2H2	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial (MCCase subunit alpha)	gene20329	AT1G03090	1,74	0,02	**1,98**	0,00
M1D1	Clathrin heavy chain 1	gene20994	AT3G08530	1,61	0,02	**1,98**	0,00
M21E8	Ankyrin repeat-containing protein At3g12360	gene08896	No hit found	1,36	0,03	**1,96**	0,00

Table 3. Cont.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	CC vs. AC Fold Change	CC vs. AC FDR qvalue	CI vs. AI Fold Change	CI vs. AI FDR qvalue
M14G3	Protein SDS23	gene15737	AT1G15330	1,32	0,05	**1,96**	0,00
M11G11	No hit found	No hit found	No hit found	1,42	0,02	**1,94**	0,00
M23D11	Cinnamyl alcohol dehydrogenase	gene20700	AT4G37990	1,23	0,09	**1,94**	0,00
M8E1	Protein kinase APK1B, chloroplastic	gene26595	No hit found	1,09	0,26	**1,91**	0,00
M23E8	No hit found	No hit found	No hit found	m.s.	m.s.	**1,91**	0,00
M23A9	Phospholipase D delta	gene14522	AT4G35790	1,34	0,07	**1,91**	0,00
M13G2	Zinc finger protein 1	gene15032	AT5G25160	1,37	0,04	**1,88**	0,00
M2B12	Peroxisomal acyl-coenzyme A oxidase 1 (AOX 1)	gene08871	AT2G35690	m.s.	m.s.	**1,88**	0,00
M1D7	NifU-like protein 2, chloroplastic (AtCNfu2)	gene29355	AT5G49940	1,41	0,02	**1,87**	0,00
M22H5	No hit found	No hit found	No hit found	0,54	0,02	**1,86**	0,02
M17D10	Probable inactive receptor kinase At4g23740	gene13121	AT5G53320	1,39	0,03	**1,83**	0,00
M29B5	Eukaryotic translation initiation factor 5A-2 (eIF-5A-2)	gene10075	AT1G69410	1,11	0,24	**1,82**	0,00
M8H3	WRKY DNA-binding protein 33	gene13803	AT2G38470	m.s.	m.s.	**1,81**	0,00
M10E2	NPGR1-No pollen germination related 1	gene02575	AT1G27460	1,08	0,34	**1,81**	0,00
M1C6	No hit found	No hit found	No hit found	1,52	0,02	**1,80**	0,00
M10F12	Protein sfi1	gene05759	AT5G67540	m.s.	m.s.	**1,80**	0,00
M17G1	B2 protein	gene32150	AT5G42050	1,33	0,04	**1,80**	0,00
M18E11	Embryo defective 2756	gene27435	AT1G34550	m.s.	m.s.	**1,80**	0,00
M8G2	CIPK-Serine/threonine-protein kinase 6	gene10067	AT4G30960	1,06	0,32	**1,80**	0,00
M3C8	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2, 6-diaminopimelate ligase	gene32500	AT1G63680	1,35	0,03	**1,79**	0,00
M30B5	hypothetical protein	gene13232	No hit found	m.s.	m.s.	**1,79**	0,00
M2E11	Centrosomal protein of 164 kDa (Cep164)	gene07259	No hit found	m.s.	m.s.	**1,78**	0,00
M14F10	Endochitinase 1	gene22465	AT1G05850	1,65	0,02	**1,76**	0,00
M4F3	D-serine/D-alanine/glycine transporter	gene27555	AT1G22750	1,37	0,03	**1,75**	0,00

m.s.: mised data. After an initial step of discarding low quality spot signals, only high cuality data from the microarray are conserved and so, some raw data were mised in the final re

Table 4. Genes identified as more abundant in Andana than in Camarosa cultivar from the inter-cultivar transcriptomic comparisons. Genes were considered as differentially expressed if they fulfilled a FDR <0.05 after a SAM test analysis, and the fold-change was higher that 1.75-fold in the compared conditions. Marked in bold data fulfilling these conditions. Values represent the ratio of cv. Camarosa mock vs. cv. Andana mock, CC vs. AC, and cv. Camarosa infected vs. cv. Andana infected, CI vs. AI.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	AC Vs. CC Fold Change	AC Vs. CC FDR qvalue	AI Vs. CI Fold Change	AI Vs. CI FDR qvalue
M24D11	Cyclin-dependent kinase E-1 (CDKE)	gene24158	AT5G63610	**10,99**	0,00	m.d.	m.d.
M8H11	V-type proton ATPase subunit H (V-ATPase subunit H)	gene12559	AT3G42050	**2,56**	0,01	1,71	0,01
M24D5	No hit found	No hit found	No hit found	**2,36**	0,01	1,54	0,00
M8F11	Elongation factor 1-delta 1 (EF-1-delta 1)	gene26965	AT2G18110	**2,24**	0,01	1,07	0,19
M19B2	Ribonuclease 2	gene17135	No hit found	**2,20**	0,01	m.d.	m.d.
M22C9	hypothetical protein	gene16038	No hit found	**2,18**	0,01	m.d.	m.d.
M14F2	Auxin-induced protein 22D	gene08191	AT4G14560	**2,18**	0,01	1,61	0,00
M24A11	Actin-7	gene01836	AT3G12110	**2,16**	0,01	1,13	0,03
M14F3	No hit found	No hit found	No hit found	**2,11**	0,01	1,21	0,01
M21E11	1-acyl-sn-glycerol-3-phosphate acyltransferase (1-AGP acyltransferase)	gene22829	No hit found	**2,08**	0,01	1,44	0,00
M16H2	Putative clathrin assembly protein At2g01600	gene06205	AT1G14910	**2,07**	0,01	1,15	0,04
M26B8	No hit found	No hit found	No hit found	**2,07**	0,01	1,30	0,04
M30D6	No hit found	No hit found	No hit found	**2,07**	0,01	1,06	0,20
M18D3	Receptor-type tyrosine-protein phosphatase epsilon (Protein-tyrosine phosphatase epsilon)	gene26020	AT1G71860	**2,05**	0,01	1,59	0,00
M24D6	NADH-cytochrome b5 reductase 1	gene10215	AT5G17770	**2,00**	0,01	1,41	0,00
M18B7	No hit found	No hit found	No hit found	**1,93**	0,01	m.d.	m.d.
M24F8	No hit found	No hit found	No hit found	**1,89**	0,01	m.d.	m.d.
M9A8	Root phototropism protein 2	gene28060	AT2G30520	**1,88**	0,01	1,08	0,06
M10C7	No hit found	No hit found	No hit found	**1,88**	0,01	1,14	0,15
M14H7	No hit found	No hit found	No hit found	**1,88**	0,01	1,22	0,01
M30G7	42 kDa peptidyl-prolyl isomerase (PPIase)	gene29368	AT3G21640	**1,85**	0,01	m.d.	m.d.
M6C1	Pectinesterase 1 (PE 1)	gene25980	No hit found	**1,84**	0,01	1,66	0,00
M30B6	No hit found	No hit found	No hit found	**1,80**	0,01	m.d.	m.d.
M4E2	No hit found	No hit found	No hit found	**1,78**	0,01	1,13	0,12
M21G5	Expansin-A1	gene04724	AT1G69530	**1,77**	0,01	1,10	0,04
M15C2	ATPase family AAA domain-containing protein 1-A	gene06348	AT4G24860	**1,76**	0,01	m.d.	m.d.
M8A5	Protein PPLZ12	gene04018	AT1G69840	**1,75**	0,01	1,09	0,17
M19F9	Aldose reductase (AR)	gene18366	AT2G37790	**1,75**	0,01	m.d.	m.d.
M28G7	Protein TRANSPORT INHIBITOR RESPONSE 1	gene24947	AT3G62980	**2,72**	0,01	**5,16**	0,00
M28H3	Capsid protein	gene07494	No hit found	**2,60**	0,01	**5,04**	0,00
M7A2	60S ribosomal protein L27a-3	gene22275	AT1G70600	**2,59**	0,01	**3,61**	0,00
M8H5	RING finger protein 139	gene22198	No hit found	**2,54**	0,01	**3,90**	0,00
M12C10	Anthocyanidin 3-O-glucosyltransferase 2	gene12591	No hit found	**2,53**	0,01	**1,92**	0,00
M20H8	Probable LRR receptor-like serine/threonine-protein kinase At4g37250	gene24030	AT5G67270	**2,40**	0,01	**5,93**	0,00
M19B11	No hit found	No hit found	No hit found	**2,32**	0,01	**4,18**	0,00
M4C2	ABC transporter D family member 1 (ABC transporter ABCD.1)	gene12355	AT4G39850	**2,25**	0,01	**4,96**	0,00
M9D10	Zinc finger protein CONSTANS-LIKE 2	gene12862	AT4G27900	**2,24**	0,01	**4,60**	0,00
M8F8	Probable histone H2AXb	gene22354	AT1G08880	**2,18**	0,01	**5,49**	0,00
M29F6	Pentatricopeptide repeat-containing protein At3g46610	gene09207	AT3G46610	**2,18**	0,01	**3,54**	0,00
M2E9	Pentatricopeptide repeat-containing protein At2g15690	gene28582	No hit found	**2,17**	0,01	**4,31**	0,00
M20E6	Transmembrane protein 121	gene22050	AT2G27590	**2,17**	0,01	**6,19**	0,00
M19H4	No hit found	No hit found	No hit found	**2,14**	0,01	**4,47**	0,00
M14E11	Anthranilate phosphoribosyltransferase	gene21093	AT5G09580	**2,13**	0,01	**4,87**	0,00
M2D1	Paired amphipathic helix protein Sin3 (AtSin3)	gene01324	No hit found	**2,12**	0,01	**4,56**	0,00
M25E6	Cytokinin-N-glucosyltransferase 2	gene32078	No hit found	**2,11**	0,01	**5,84**	0,00
M18A10	hypothetical protein	gene18779	AT2G38000	**2,10**	0,01	**3,30**	0,00
M25B4	Acyl-CoA-binding domain-containing protein 4 (Acyl-CoA binding protein 4)	gene23551	AT5G04420	**2,10**	0,01	**4,30**	0,00
M29G2	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)	gene11421	AT2G19590	**2,09**	0,01	**5,17**	0,00
M29F10	Scarecrow-like protein 8 (AtSCL8)	gene13212	No hit found	**2,09**	0,01	**3,94**	0,00
M15H3	Aspartate aminotransferase, chloroplastic	gene08794	AT5G19550	**2,09**	0,01	**5,86**	0,00
M1D2	Target of rapamycin	gene01794	AT1G50030	**2,08**	0,01	**2,78**	0,00
M13H11	Calcium-binding mitochondrial carrier protein SCaMC-1	gene30020	AT1G78180	**2,08**	0,01	**2,89**	0,00
M1H10	Cell division cycle protein 48 homolog (VCP)	gene09618	AT5G03340	**2,08**	0,01	**4,82**	0,00
M20C7	Inhibitor of growth protein 4	gene10354	AT1G54390	**2,06**	0,01	**2,67**	0,00
M1A4	LIM domain and RING finger protein C1223.01	gene13921	AT2G47090	**2,06**	0,01	**4,75**	0,00
M10B9	Laccase-4	gene18812	AT5G01190	**2,06**	0,01	**6,51**	0,00
M29A10	Adenylate cyclase, terminal-differentiation specific	gene25311	AT5G02970	**2,06**	0,01	**5,78**	0,00
M20G10	No hit found	No hit found	No hit found	**2,05**	0,01	**5,57**	0,00
M16D4	No hit found	No hit found	No hit found	**2,05**	0,01	**5,03**	0,00
M2F1	hypothetical protein	gene13744	No hit found	**2,04**	0,01	**3,41**	0,00
M30E12	Valyl-tRNA synthetase (ValRS)	gene13331	AT1G14610	**2,02**	0,01	**5,90**	0,00

Table 4. Cont.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	AC Vs. CC Fold Change	AC Vs. CC FDR qvalue	AI Vs. CI Fold Change	AI Vs. CI FDR qvalue
M29G8	Calcium release-activated calcium channel protein 1	gene33612	No hit found	**2,02**	0,01	**3,56**	0,00
M21E6	No hit found	No hit found	No hit found	**2,01**	0,01	**1,96**	0,00
M24H4	No hit found	No hit found	No hit found	**2,00**	0,01	**3,92**	0,00
M3E1	Probable protein ABIL1	gene18995	AT2G46225	**2,00**	0,01	**2,61**	0,00
M29E11	No hit found	No hit found	No hit found	**1,99**	0,01	**4,82**	0,00
M3D2	40S ribosomal protein S3-3	gene12732	AT5G35530	**1,98**	0,01	**3,18**	0,00
M30D10	Protein HOTHEAD	gene23054	AT1G72970	**1,98**	0,01	**5,94**	0,00
M21E1	Sugar transporter ERD6-like 7	gene27745	No hit found	**1,97**	0,01	**6,19**	0,00
M7A4	No hit found	No hit found	No hit found	**1,96**	0,01	**3,63**	0,00
M22G8	Calnexin homolog	gene01809	AT5G61790	**1,95**	0,01	**3,90**	0,00
M23H1	Importin-13 (Imp13)	gene01415	AT1G12930	**1,92**	0,01	**4,95**	0,00
M25C4	No hit found	No hit found	No hit found	**1,91**	0,01	**2,90**	0,00
M4E1	Vacuolar-processing enzyme (VPE)	gene02665	AT4G32940	**1,90**	0,01	**2,52**	0,00
M29D12	No hit found	No hit found	No hit found	**1,90**	0,01	**3,81**	0,00
M22E9	No hit found	No hit found	No hit found	**1,89**	0,01	**3,64**	0,00
M16H10	60S ribosomal protein L5	gene11461	AT3G25520	**1,89**	0,01	**2,72**	0,00
M1E1	G2/mitotic-specific cyclin S13-6	gene22742	AT4G37490	**1,89**	0,01	**1,84**	0,00
M26B7	Probable 26S proteasome non-ATPase regulatory subunit 8	gene25118	AT5G42040	**1,88**	0,01	**4,12**	0,00
M29E10	Pre-mRNA polyadenylation factor fip1	gene25369	AT1G17870	**1,88**	0,01	**3,88**	0,00
M18H2	Probable disease resistance protein At5g66900	gene24118	No hit found	**1,88**	0,01	**3,08**	0,00
M30A1	Probable ethanolamine kinase A	gene31007	AT2G26830	**1,87**	0,01	**3,45**	0,00
M23G1	Proteasome activator complex subunit 4 (Protein TEMO)	gene01456	AT3G13330	**1,86**	0,01	**6,49**	0,00
M6D4	No hit found	No hit found	No hit found	**1,85**	0,01	**3,15**	0,00
M21B9	No hit found	No hit found	No hit found	**1,84**	0,01	**3,67**	0,00
M24B3	Putative disease resistance protein At3g14460	gene16731	No hit found	**1,81**	0,01	**2,60**	0,00
M20C12	No hit found	No hit found	No hit found	**1,80**	0,01	**4,35**	0,01
M1E9	Fasciclin-like arabinogalactan protein 17	gene18252	AT2G35860	**1,79**	0,01	**3,50**	0,00
M22D12	WD-40 repeat-containing protein MSI4	gene12638	AT2G19520	**1,78**	0,01	**2,80**	0,00
M29B9	No hit found	No hit found	No hit found	**1,78**	0,01	**4,33**	0,00
M5A1	No hit found	No hit found	No hit found	**1,77**	0,01	**2,58**	0,01
M25B5	Adenosine kinase 2 (AK 2)	gene09755	AT3G09820	**1,76**	0,02	**3,82**	0,00
M18D10	Pistil-specific extensin-like protein (PELP)	gene02380	No hit found	**1,76**	0,01	**1,90**	0,00
M29D9	Putative F-box/LRR-repeat protein 19	gene07874	No hit found	**1,75**	0,02	**2,76**	0,01
M25A11	No hit found	No hit found	No hit found	**1,75**	0,01	**2,65**	0,02
M27B6	AP-1 complex subunit mu-1	gene31794	AT1G60780	**1,75**	0,01	**3,39**	0,00
M29G5	No hit found	No hit found	No hit found	**1,75**	0,01	**2,39**	0,00
M30A4	MADS-box protein SOC1	gene19425	No hit found	1,65	0,02	**4,69**	0,00
M16C12	No hit found	No hit found	No hit found	1,68	0,02	**4,68**	0,00
M24C8	Ubiquitin carboxyl-terminal hydrolase 15 (AtUBP15)	gene31345	AT1G17110	1,73	0,01	**4,37**	0,00
M22A1	Cell division cycle and apoptosis regulator protein 1 (CARP-1)	gene04716	No hit found	1,69	0,02	**4,37**	0,00
M30D5	No hit found	No hit found	No hit found	1,70	0,02	**4,33**	0,00
M25G7	No hit found	No hit found	No hit found	1,48	0,04	**4,23**	0,00
M26C1	hypothetical protein	gene30572	No hit found	1,69	0,02	**4,17**	0,00
M26F6	CCR4-NOT transcription complex subunit 10	gene31111	No hit found	1,69	0,02	**4,14**	0,00
M30A8	No hit found	No hit found	No hit found	1,60	0,02	**4,11**	0,00
M22F3	Probable U3 small nucleolar RNA-associated protein 7 (U3 snoRNA-associated protein 7)	gene09215	AT3G10530	1,44	0,05	**3,89**	0,00
M25B9	Cullin-1	gene10866	AT4G02570	1,68	0,02	**3,83**	0,00
M13C1	No hit found	No hit found	No hit found	1,49	0,04	**3,78**	0,00
M6A3	Glycosyltransferase QUASIMODO1	gene17381	AT3G25140	1,71	0,01	**3,73**	0,00
M25E1	No hit found	No hit found	No hit found	1,56	0,02	**3,62**	0,00
M21F2	Tubulin alpha chain	gene03851	AT1G50010	m.d.	m.d.	**3,52**	0,00
M25B2	No hit found	No hit found	No hit found	1,71	0,01	**3,46**	0,00
M9C12	Novel plant SNARE 13 (AtNPSN13)	gene22814	AT3G17440	1,71	0,01	**3,34**	0,00
M3E9	Serine-rich adhesin for platelets	gene07537	AT3G13990	1,47	0,04	**3,32**	0,00
M29C6	Enolase	gene07865	AT2G29560	1,72	0,01	**3,29**	0,00
M30F10	Activator of 90 kDa heat shock protein ATPase homolog	gene17995	AT3G12050	1,42	0,06	**3,22**	0,00
M17C12	No hit found	No hit found	No hit found	1,45	0,04	**3,20**	0,00
M16C4	Serine/threonine-protein kinase phg2	gene13578	No hit found	1,39	0,07	**3,10**	0,00
M4G12	Malonyl CoA-acyl carrier protein transacylase, mitochondrial (MCT)	gene28189	AT2G30200	1,33	0,07	**3,09**	0,00
M25B12	No hit found	No hit found	No hit found	1,64	0,02	**2,96**	0,00
M22G2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6	gene25170	AT3G18410	1,57	0,02	**2,92**	0,01
M28F1	Dihydrolipoyllysine-residue acetyltransferase component of acetoin cleaving system (FMP)	gene30278	AT1G74280	1,64	0,02	**2,89**	0,00
M24F2	60S ribosomal protein L4	gene18103	AT3G09630	1,37	0,06	**2,86**	0,00
M26A5	Protein transport protein Sec24-like At4g32640	gene02736	AT3G44340	1,59	0,02	**2,86**	0,00
M18H6	Syntaxin-21 (AtSYP21)	gene12569	AT5G16800	1,67	0,02	**2,86**	0,00
M16G11	MADS-box protein JOINTLESS	gene12120	AT4G24540	1,62	0,02	**2,77**	0,01
M6A12	60S ribosomal protein L4	gene17076	AT3G09630	1,51	0,03	**2,77**	0,00
M30B1	Acidic leucine-rich nuclear phosphoprotein 32-related protein	gene29586	AT3G50690	1,23	0,16	**2,77**	0,01
M27E4	Protein FRIGIDA	gene17484	AT5G48385	1,29	0,12	**2,74**	0,00
M2C7	Pentatricopeptide repeat-containing protein At5g61990, mitochondrial	gene32224	No hit found	1,41	0,05	**2,74**	0,00
M28H7	NAC domain-containing protein 29 (ANAC029)	gene07251	AT5G13180	1,68	0,02	**2,73**	0,00
M22E7	MMS19 nucleotide excision repair protein homolog	gene25728	AT5G48120	1,49	0,03	**2,70**	0,00
M29F7	No hit found	No hit found	No hit found	1,51	0,03	**2,69**	0,00
M25E11	B2 protein	gene25869	AT3G27090	1,44	0,06	**2,66**	0,01
M21C9	Tubulin alpha chain	gene26908	AT1G50010	1,64	0,02	**2,61**	0,00
M29H4	Pentatricopeptide repeat-containing protein At1g51965, mitochondrial	gene25700	AT1G51965	1,55	0,02	**2,61**	0,00
M27G6	Chaperone protein dnaJ 10 (AtJ10)	gene21835	AT4G39150	1,23	0,15	**2,59**	0,00
M22C12	DnaJ homolog subfamily B member 11 (ABBP-2)	gene02321	AT3G62600	1,37	0,06	**2,54**	0,00
M11E9	LIM domain-containing protein A	gene24341	No hit found	1,42	0,03	**2,53**	0,01
M13A2	U1 small nuclear ribonucleoprotein 70 kDa (U1 snRNP 70 kDa)	gene14787	AT3G50670	1,39	0,06	**2,51**	0,00
M1D5	Cell cycle checkpoint protein RAD17 (AtRAD17)	gene14575	AT5G66130	1,54	0,03	**2,50**	0,00
M29F8	No hit found	No hit found	No hit found	1,17	0,22	**2,50**	0,01
M19A1	No hit found	No hit found	No hit found	1,37	0,07	**2,48**	0,00
M19A8	Histidine-rich glycoprotein	gene10820	AT4G17520	1,43	0,05	**2,44**	0,00
M22E6	Serine carboxypeptidase-like 45	gene07283	AT2G33530	1,38	0,06	**2,43**	0,00
M11B11	No hit found	No hit found	No hit found	1,10	0,34	**2,43**	0,00
M6B11	hypothetical protein	gene06611	No hit found	1,08	0,36	**2,41**	0,01
M26A4	No hit found	No hit found	No hit found	1,37	0,07	**2,38**	0,00
M14A6	3-oxoacyl-[acyl-carrier-protein] reductase, chloroplastic	gene07112	AT1G24360	1,58	0,02	**2,35**	0,01
M29H10	Cingulin	gene25917	AT5G43230	1,47	0,04	**2,34**	0,00
M6A8	NHP2-like protein 1	gene31016	AT4G22380	1,53	0,03	**2,34**	0,00
M29D6	No hit found	No hit found	No hit found	1,49	0,03	**2,27**	0,00
M30E3	Adenylyl-sulfate kinase	gene08497	AT1G19920	1,30	0,10	**2,26**	0,00

Table 4. Cont.

Strawberry Unigene	Gene Annotation/ Description	*F. vesca* Ortologous/ Homologous	*A. thaliana* Ortologous/ Homologous	AC Vs. CC Fold Change	AC Vs. CC FDR qvalue	AI Vs. CI Fold Change	AI Vs. CI FDR qvalue
M8D6	Transmembrane protein 165	gene16802	AT5G36290	1,12	0,31	**2,25**	0,00
M23B5	No hit found	No hit found	No hit found	1,47	0,04	**2,23**	0,00
M20A10	No hit found	No hit found	No hit found	1,10	0,32	**2,23**	0,01
M8D2	Methionine synthase	gene14995	AT5G17920	0,53	0,02	**2,21**	0,00
M29E5	Probable pectate lyase P59	gene01266	AT1G04680	1,71	0,02	**2,18**	0,00
M22D4	Aldehyde dehydrogenase family 3 member I1, chloroplastic (AtALDH3)	gene24172	AT4G34240	1,63	0,02	**2,17**	0,00
M19B8	Calcium-transporting ATPase 1, chloroplastic	gene02564	AT4G37640	1,72	0,02	**2,16**	0,00
M14G11	Histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH1 (Su(var)3-9 homolog protein	gene02482	AT5G04940	1,70	0,02	**2,15**	0,00
M16E1	No hit found	No hit found	No hit found	m.d.	m.d.	**2,15**	0,01
M21G1	Xylem serine proteinase 1 (AtXSP1)	gene18968	AT4G00230	1,23	0,18	**2,14**	0,00
M26A1	hypothetical protein	gene18041	AT3G12010	1,53	0,03	**2,13**	0,00
M24A9	No hit found	No hit found	No hit found	1,62	0,02	**2,13**	0,00
M5G6	Adenosylhomocysteinase (AdoHcyase)	gene06564	AT4G13940	1,33	0,07	**2,12**	0,00
M27A6	NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial	gene15989	AT4G02580	1,47	0,03	**2,10**	0,01
M29H6	Leucoanthocyanidin dioxygenase (LDOX)	gene32347	AT4G22880	1,13	0,22	**2,09**	0,00
M29H11	No hit found	No hit found	No hit found	1,30	0,12	**2,09**	0,00
M16B1	Polar tube protein 1 (Major PTP)	gene28017	AT3G01720	1,49	0,03	**2,09**	0,00
M29D4	hypothetical protein	gene18708	No hit found	1,37	0,07	**2,08**	0,01
M27D10	Pentatricopeptide repeat-containing protein At1g26460, mitochondrial	gene04619	No hit found	1,66	0,02	**2,06**	0,00
M29C4	Probable dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucosyltransferase	gene11817	No hit found	m.d.	m.d.	**2,06**	0,01
M24E5	DNA-directed RNA polymerases I, II, and III subunit RPABC1 (RNA polymerases I, II, and III	gene21892	No hit found	1,69	0,02	**2,06**	0,00
M18B8	LRR receptor-like serine/threonine-protein kinase HSL2	gene05604	AT1G04820	1,61	0,02	**2,04**	0,00
M29E2	DNA damage-inducible protein 1	gene01475	AT3G13235	1,68	0,02	**2,04**	0,00
M9H12	F-box/LRR-repeat protein At3g48880	gene13557	No hit found	1,36	0,07	**2,04**	0,01
M9C5	DNA repair helicase XPB1 (AtXPB1)	gene31804	AT5G41370	1,08	0,36	**2,03**	0,00
M17C8	No hit found	No hit found	No hit found	1,56	0,03	**2,03**	0,01
M25D2	Ran-binding protein 1 homolog a	gene07941	AT1G07140	1,50	0,04	**2,02**	0,00
M29G4	Lipase (ROL)	gene10824	No hit found	1,54	0,03	**2,02**	0,00
M27C9	No hit found	No hit found	No hit found	1,15	0,27	**2,02**	0,01
M5E3	Beta-ketoacyl-ACP synthase	gene12921	AT1G74960	1,61	0,02	**2,01**	0,00
M21D11	Ubiquitin-like protein SMT3	gene03443	AT4G26840	1,64	0,02	**2,00**	0,00
M26F2	No hit found	No hit found	No hit found	1,24	0,15	**2,00**	0,01
M19E5	Auxin-induced protein 5NG4	gene27911	AT5G07050	1,13	0,22	**2,00**	0,00
M29D3	Probable serine/threonine-protein kinase NAK	gene18422	AT2G28930	1,67	0,02	**1,99**	0,00
M25A7	Glutamate receptor 3.6	gene13028	AT4G35290	1,38	0,06	**1,99**	0,01
M3G3	Serine/threonine-protein kinase LMTK2	gene07773	No hit found	1,23	0,15	**1,97**	0,00
M30E2	No hit found	No hit found	No hit found	1,40	0,05	**1,96**	0,01
M29C12	Chalcone-flavanone isomerase	gene21346	AT5G05270	1,69	0,02	**1,96**	0,00
M8H9	Agglutinin-like protein ALA1	gene14339	No hit found	1,70	0,01	**1,96**	0,00
M24D4	Serine/threonine-protein phosphatase PP2A-2 catalytic subunit	gene09527	AT1G69960	1,25	0,15	**1,96**	0,00
M29G7	Transcription factor bHLH106 (bHLH 106)	gene14814	No hit found	1,59	0,02	**1,96**	0,00
M3H8	Protein transport protein Sec16B (RGPR-p117)	gene03967	AT5G47490	1,31	0,10	**1,95**	0,00
M22G10	Cell division protease ftsH homolog	gene17893	AT5G64580	1,17	0,22	**1,94**	0,03
M19B4	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1- carboxylate synthase (SEPHCHC synth	gene04506	AT1G68890	1,55	0,02	**1,94**	0,00
M8A8	Myosin-11	gene29975	AT3G22790	1,62	0,02	**1,94**	0,00
M17B1	Peptide transporter PTR2	gene00604	AT3G54140	1,45	0,04	**1,94**	0,00
M21C10	hypothetical protein	gene12656	AT3G47560	1,55	0,03	**1,94**	0,01
M24E4	Photosystem II stability/assembly factor HCF136, chloroplastic	gene00815	AT5G23120	m.d.	m.d.	**1,93**	0,01
M16B8	Reticuline oxidase-like protein	gene20166	AT1G26420	1,43	0,02	**1,91**	0,00
M23E4	No hit found	No hit found	No hit found	1,32	0,09	**1,89**	0,00
M13C11	Serine/threonine-protein kinase WNK8 (AtWNK8)	gene23978	AT5G41990	1,17	0,15	**1,88**	0,01
M23H11	Endochitinase 1	gene22465	AT1G05850	1,29	0,12	**1,88**	0,04
M3F5	Protein of unknown function	gene13777	AT3G08890	1,24	0,15	**1,87**	0,00
M7F4	Glutamyl-tRNA(Gln) amidotransferase subunit A (Glu-ADT subunit A)	gene24503	No hit found	1,07	0,37	**1,86**	0,00
M22H5	No hit found	No hit found	No hit found	0,54	0,02	**1,86**	0,02
M20B9	Protein RUPTURED POLLEN GRAIN 1	gene06839	No hit found	1,73	0,01	**1,86**	0,00
M25H8	Eukaryotic initiation factor iso-4F subunit p82-34 (eIF-(iso)4F p82-34)	gene10104	AT5G57870	1,39	0,06	**1,85**	0,00
M13F9	Cation transport regulator-like protein 2	gene08748	AT4G31290	1,28	0,12	**1,84**	0,00
M11G8	conserved hypothetical protein	gene03733	No hit found	1,11	0,32	**1,84**	0,00
M13D10	Serine/threonine-protein kinase PKH3	gene30910	AT2G20130	1,05	0,32	**1,84**	0,01
M28H12	No hit found	No hit found	No hit found	1,53	0,03	**1,82**	0,01
M30F4	Cullin-3 (CUL-3)	gene03848	AT1G26830	1,21	0,16	**1,81**	0,00
M7G9	Protein BPS1, chloroplastic	gene19237	No hit found	1,36	0,09	**1,80**	0,00
M20H7	Ribulose bisphosphate carboxylase small chain, chloroplastic (RuBisCO small subunit)	gene17686	AT1G67090	1,29	0,12	**1,80**	0,01
M7A12	Protein kinase C (PKC)	gene27257	AT2G42610	1,31	0,10	**1,80**	0,00
M15H11	Probable serine/threonine-protein kinase At5g41260	gene07775	AT5G59010	1,59	0,02	**1,80**	0,00
M29D10	No hit found	No hit found	No hit found	1,42	0,05	**1,80**	0,00
M28B5	No hit found	No hit found	No hit found	m.d.	m.d.	**1,78**	0,00
M6D5	Protein FD (AtbZIP14)	gene08566	No hit found	1,59	0,02	**1,78**	0,00
M26B10	Processed angiotensin-converting enzyme 2	gene14807	No hit found	1,32	0,07	**1,77**	0,00
M13A1	Stamen-specific protein FIL1	gene17454	No hit found	1,07	0,36	**1,75**	0,00
M15H2	Trafficking protein particle complex subunit 3	gene03183	AT5G54750	1,55	0,03	**1,75**	0,00
M29E9	hypothetical protein	gene08631	AT1G42430	m.d.	m.d.	**1,75**	0,00
M27F7	No hit found	No hit found	No hit found	1,27	0,12	**1,75**	0,00
M26D10	F-box/LRR-repeat protein 14	gene30797	AT1G15740	1,00	0,43	**1,74**	0,03

m.s.: mised data. After an initial step of discarding low quality spot signals, only high cuality data from the microarray are conserved and so, some raw data were mised in the final results.

biotic stimulus" (category 34.11.10), "systemic interaction with the environment" (category 36) and "plant/fungal specific systemic sensing and response" (category 36.20), as well as KEGG pathway "Biosynthesis of secondary metabolites" (ath01110), were found within the Camarosa set of most abundant genes (Table

S4). Categories such as “transport routes” (category 20.09) and “plant hair cell (trichome)” (category 43.02.05.02), and KEGG pathway “Biosynthesis of secondary metabolites” (ath01110), were found within the Andana set of most abundant genes (Table S5).

Results here obtained represent the first approach to identify putative molecular components which might be responsible for differences in susceptibility to *C. acutatum* exhibited by strawberry cultivars but further molecular analysis must be carried out to get clues of these phenotypic differences.

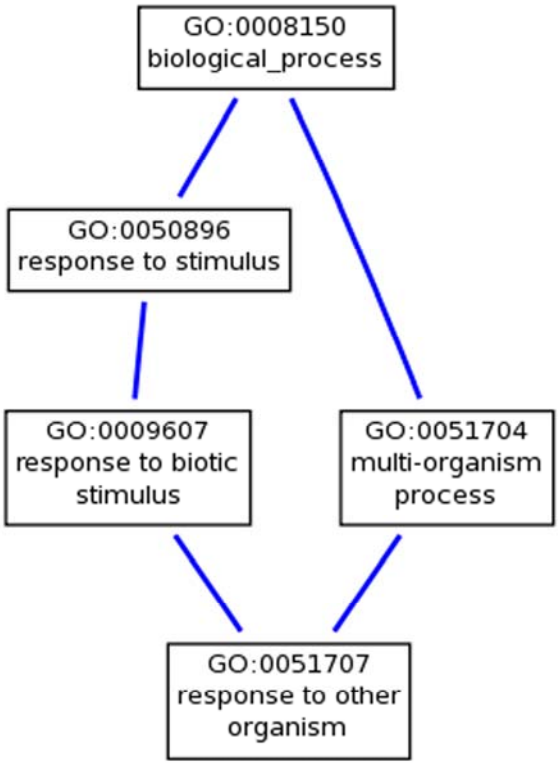

Figure 7. Biological processes significantly over-represented in Camarosa compared with Andana cultivar by Singular Enrichment Analysis (SEA, FATIGO).

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Capítulo VIII

Identificación, Clonación y Caracterización de los Miembros de la Familia NPR1-like en Fresa (Fragaria × ananassa)

Abreviaturas:

PFU: partículas formadoras de fago (phage forming unit)
NPR1: non-expressor of PR1
ADNc: ADN copia

ABSTRACT

Effective strawberry crop protection against pathogens needs a thorough knowledge of the innate defense mechanisms that this plant can display under attack. This will help to develop alternative strategies to the use of chemical in order to protect this crop. Furthermore, signaling pathways and regulatory elements leading to defense responses have to be properly characterized in this species.

We have identified five members of the NPR1-like family in strawberry. Members of the NPR1-like family are key players in salicylic acid (SA)-mediated resistance to pathogens in Arabidopsis and other plants. Very interestingly, overexpression of a control gene such as *NPR1* in Arabidopsis and other species (i.e. rice, tobacco, grapevine) has been described to increase the innate defense system in these plants. Therefore, modulation of the expression level of NPR1-like genes offers an attractive alternative to increase strawberry resistance. Thus, molecular and functional studies are being conducted to unravel the putative implication of FaNPR1, FaNPR31, FaNPR32, FaNPR33 and FaNPR5 in the strawberry plant physiology.

To get knowledge of the biological role of the FaNPR31 protein we have carried out functional characterization studies of the seven different FaNPR31 alleles found in strawberry in both heterologous and homologous systems (Arabidopsis and *F* × *ananassa* cv. Camarosa, respectively). Thus, by using gateway technology, we are conducting complementation analyses in three single-gene knock-out Arabidopsis mutants (npr1-1, npr3-1, and npr4-3), and into the double mutant npr3.1/npr4.3. While Atnpr1-1 is a SAR defective mutant, AtNPR3, and AtNPR4 proteins seem to act as negative regulators of SAR. Also, we have overexpressed *FaNPR31* in strawberry (cv. Camarosa), and in wild-type Arabidopsis (At Col-0) plants. In addition, by using RNAi technology and pFRN binary vectors, we have used a 407-bp conserved region among all the *FaNPR31* alleles to transform strawberry cv. Camarosa and At Col-0. Currently, we are analyzing the corresponding transgenic lines.

INTRODUCCIÓN

El ácido salicílico actúa como una fitohormona endógena en el desarrollo de la resistencia sistémica adquirida (SAR), una respuesta inmune de amplio espectro y a larga distancia que se activa por el reconocimiento de patógenos avirulentos (Vlot et al. 2009). Para la activación de esa respuesta de defensa se requiere el reclutamiento de un "enhanceosome" transcripcionalmente dependiente de SA (Rochon et al. 2006). Este "enhanceosome" está formado por miembros de la familia de factores de transcripción TGA2 (Zhang et al. 2003) y el coactivador transcripcional NPR1 (Rochon et al. 2006), el regulador central de SAR y de la activación génica dependiente de SA (Ryals et al. 1997, Cao et al. 1997). NPR1 es, además, mediador del "crosstalk" que existe entre las dos rutas de defensa principales conocidas mediadas por SA y JA (Spoel et al. 2007, Leon-Reyes et al. 2009). Además, otros miembros de la familia NPR1-like, NPR3 y NPR4, son también componentes fundamentales en la regulación de la respuesta de defensa (Liu et al. 2005, Zhang et al. 2006, Shi et al. 2012). Muy recientemente se han descrito que NPR3 y NPR4 son los verdaderos receptores de la molécula de SA en la planta (Fu et al. 2012). Sin embargo, aún existen discrepancias sobre qué miembro o miembros de la familia de NPR1-like tienen esa función. Así, mientras Wu et al. (2012) (Wu et al. 2012) defienden que NPR1 es el receptor de la hormona de SA, Fu et al. (2012) (Fu et al. 2012) sostienen que esta función recae sobre NPR3 y NPR4, ya que en sus análisis NPR1 no presentó una actividad "SA-binding" considerable. Finalmente, NPR5 y NPR6 no parecen participar en el control de defensas en plantas sino que, principalmente, están involucrados en la regulación de distintos aspectos del desarrollo de las mismas (Hepworth et al. 2005, Jun et al. 2010).

Aunque existen gran cantidad de estudios sobre los miembros de la familia NPR1-like y se conoce una buena parte de la regulación y función biológica que desempeñan, la mayoría de estos estudios se han llevado a cabo en sistemas de plantas modelo como Arabidopsis. Sin embargo, no se conoce qué función pueden ejercer estos componentes en la regulación de la respuesta de defensa en plantas de cultivo como la fresa y cómo lo hacen.

RESULTADOS Y DISCUSIÓN

Sección I: Selección del gen *FaNPR31* como un componente clave en la respuesta de defensa en fresa

A partir de la información obtenida tras el escrutinio de las genotecas de ESTs y el análisis de secuencias que se ha descrito en el Capítulo II de este trabajo de Tesis, se identificaron algunos componentes moleculares que pudieran ser clave en la regulación de la respuesta de defensa en la planta de fresa. Este fue el caso del clon CUI5_T_396, que fue aislado a partir de la genoteca sustractiva enriquecida en genes de respuesta a elicitores de defensa (UT, descrita en Capítulo II), y que proporcionó una secuencia (una vez curada) de 491bp, muy similar a miembros de la familia NPR1-like de otras especies (Figure 1).

Con objeto de aislar el clon genómico correspondiente a la EST CUI5_T_396, se procedió a escrutar una genoteca de ADN genómico de fresa (*Fragaria* × *ananassa* cv. Chandler) generada mediante el sistema Lambda Fix® II/ XhoI Partial Fill-In Vector Kit (Stratagene). La sonda empleada en el escrutinio fue la EST CUI5_T_396 completa, de 491pb de longitud, marcada radiactivamente (^{32P}dCTP) con el kit Megaprime DNA Labelling System (Amersham, GE). Se realizó un escrutinio primario, en condiciones de hibridación y lavado de alta astringencia, de un total de $2x10^5$ fagos recombinantes (descripción detallada en *Material y Métodos*). Se aislaron 51 fagos recombinantes posiblemente portadores del gen *FaNPR31*, y 25 de ellos fueron analizados de nuevo mediante escrutinio secundario (también por hibridación con sonda radiactiva). Por último, 7 de ellos fueron totalmente aislados mediante un escrutinio terciario utilizando la técnica de PCR (con oligos específicos diseñados sobre la secuencia CUI5_T_396^{EST}, 396A y 396B, ver *Material y Métodos*). Una vez estos fagos recombinantes positivos fueron identificados y aislados, se procedió a extraer y purificar el ADN genómico (Qiagen® Lambda Midikit (Qiagen)) de dos de ellos (λ42361 y λ19381). La Figura 2 muestra el resultado de subsecuentes escrutinios primarios y secundarios (con sonda radiactiva), y terciarios (por PCR) de dos de los fagos positivos, así como electroforesis del DNA purificado a partir de los dos fagos aislados.

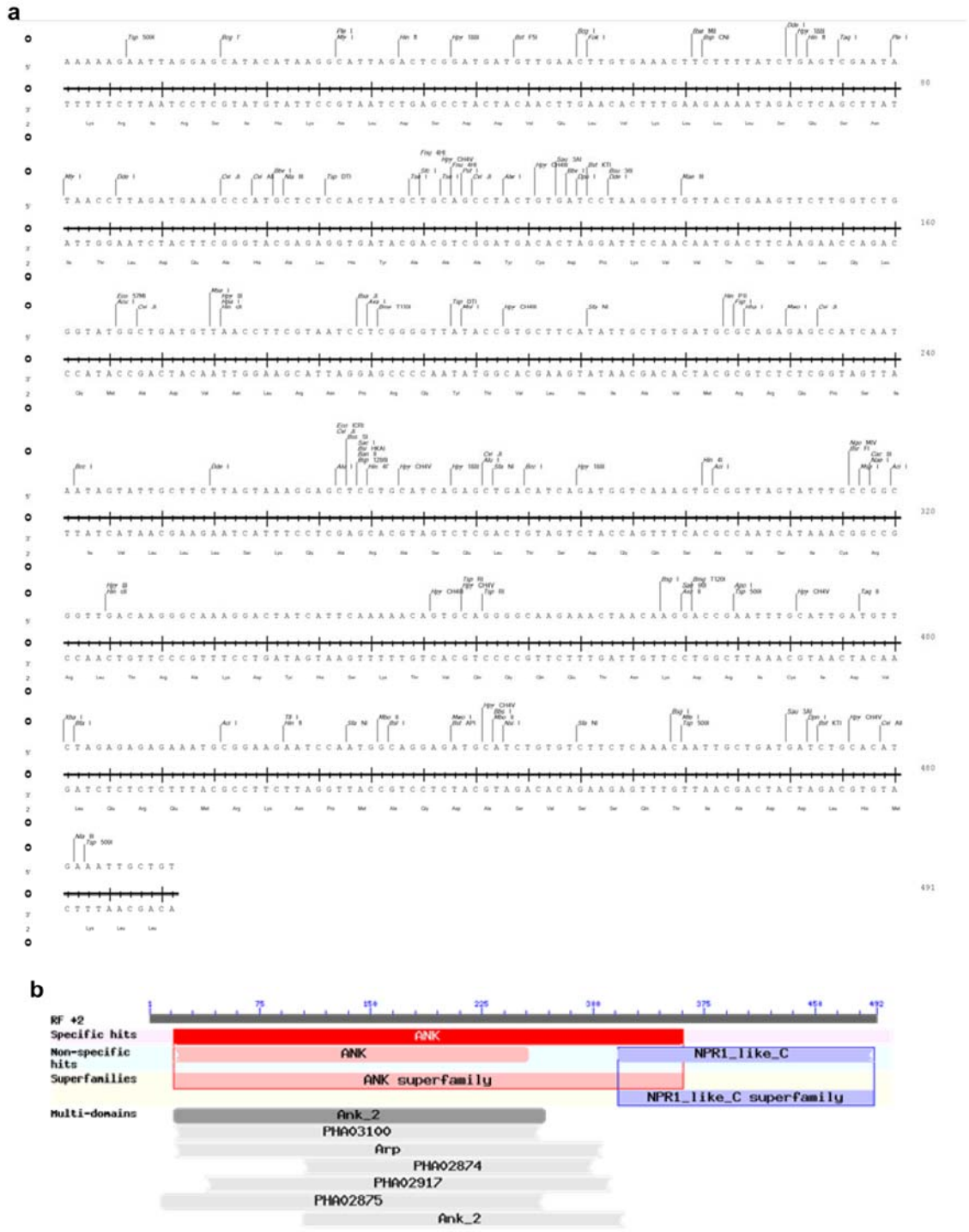


Figura 1. Secuencia nucleotídica del clon CUI5_T_396. Este clon fue aislado a partir de la genoteca sustractiva enriquecida en genes de respuesta a elicitores de defensa (UT en Capítulo II) y su secuencia obtenida (CUI5_T_396^{EST}) presentó una elevada homología con secuencias de miembros de la familia NPR1-like de otras especies. (a) Secuencia de la EST donde se identifican los sitios de corte para enzimas de restricción que presenta dicha secuencia, así como los aminoácidos resultantes de su traducción. (b) Resultado de blastx contra la base de datos nr en NCBI (dominios conservados).

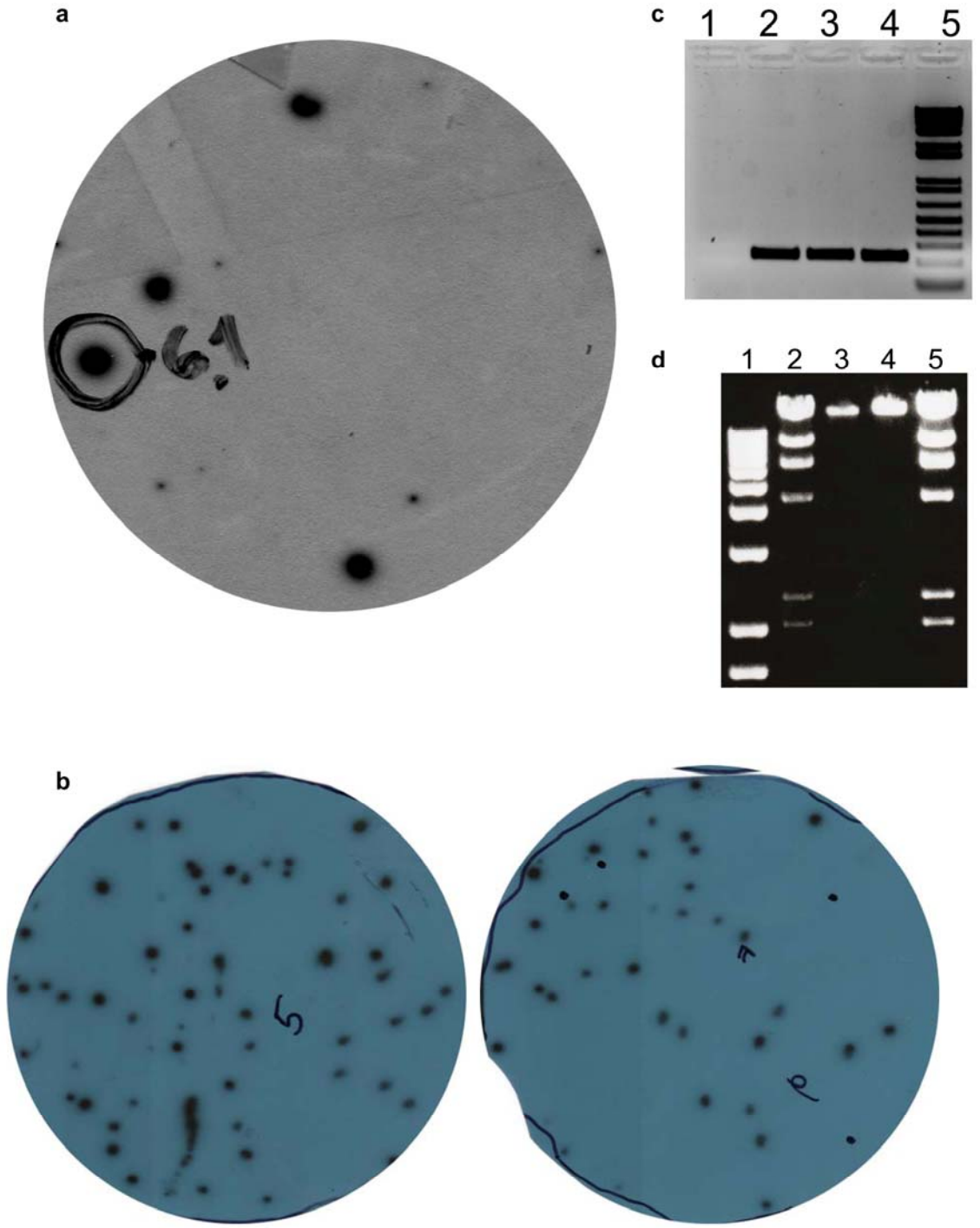
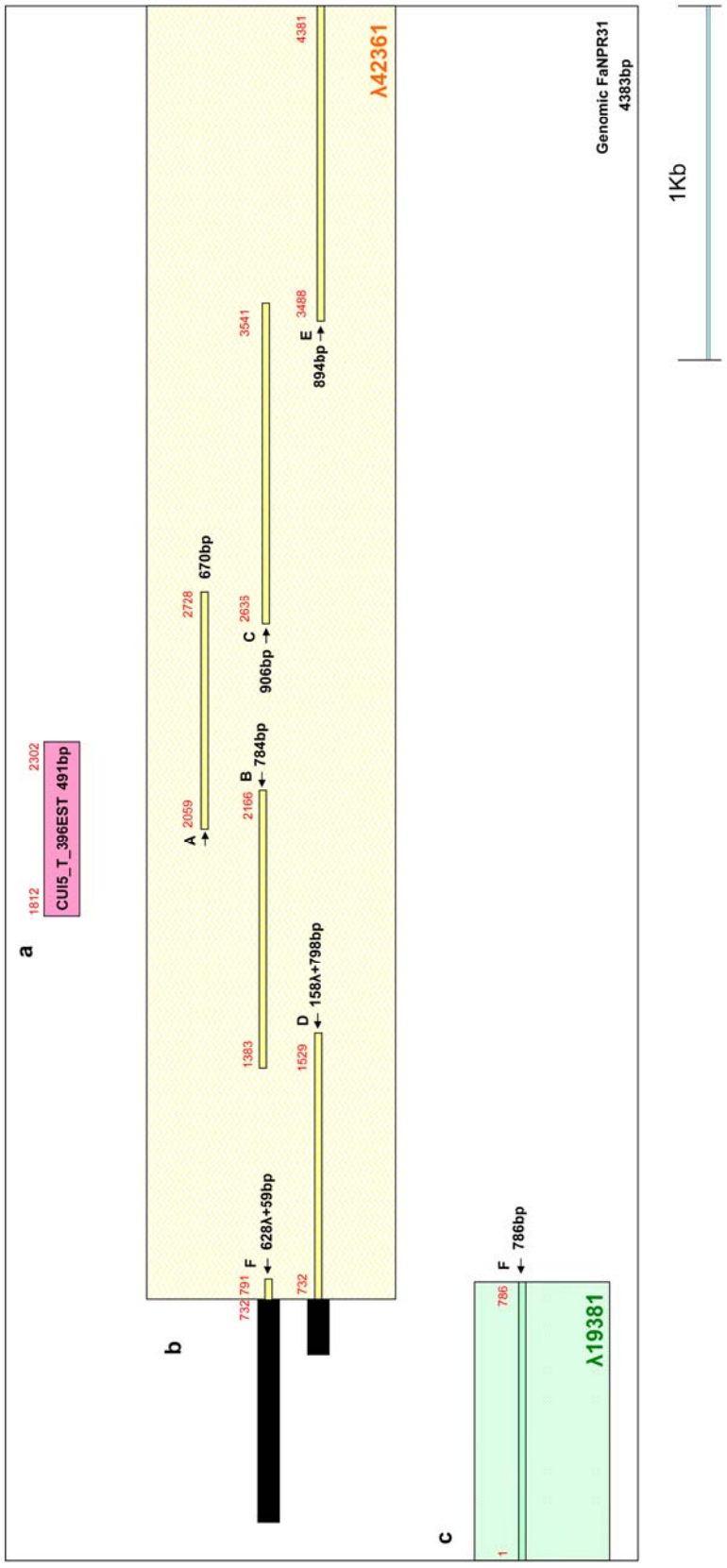


Figura 2. Resultado de subsecuentes escrutinios de la genoteca genómica en el sistema Lambda Fix® II/ XhoI Partial Fill-In, utilizando como sonda CUI5_T_396^{EST}. (a) Escrutinios primario y (b) secundario con sonda marcada con 32PdCTP. (c) Escrutinio terciario mediante PCR de dos fagos positivos (1: control negativo, 2: Fago1, 3: Fago2, 4: control positivo-EST, 5: 1Kb plus DNA ladder (Invitrogen). (d) Electroforesis del ADN purificado de los dos fagos aislados (1: 1Kb plus DNA ladder (Invitrogen) 600ng, 2: λ DNA ladder 300ng, 3: Fago1 20ng, 4: Fago2 100ng, 5: λ DNA ladder 600ng).

Una vez que el DNA del fago λ42361 fue purificado, se llevaron a cabo secuenciaciones sucesivas solapadas, usando la EST CUI5_T_396 para el diseño de los primeros oligos y avanzando sucesivamente desde la posición de la EST hacia ambos extremos 5´ y 3´. En la Figura 3 se muestra el progreso de las sucesivas secuenciaciones de este fago, hasta conseguir llegar al "stop codon" predicho de la proteína FaNPR31, hacia la zona del brazo izquierdo del vector fago lambda, y a la zona cercana a la amino terminal de la proteína, orientada hacia al brazo derecho de dicho vector (representado como barra negra en la Figura 3). Lamentablemente, este fago recombinante no pudo proporcionar la secuencia completa del gen *FaNPR31*, ya que contenía una secuencia incompleta de la región genómica de dicho gen.

Con objeto de obtener la secuencia completa del gen, se procedió, por tanto, a secuenciar el segundo fago λ19381. El consenso de las secuencias obtenidas de ambos fagos proporcionó la región codificante completa del gen *FaNPR31*, desde el codón de inicio (ATG), al codón de término (Stop: TAG), incluyendo además 581 nucleótidos de la región "downstream" de corte y poliadenilazión, y 525 nucleótidos de la región "upstream" reguladora (Figura 4). El codón de inicio de la traducción (posición 525) se ajusta a la secuencia consenso descrita en plantas, con una guanina conservada en la posición +4 (Kozak 1989, Gallie 1993). Así mismo, mediante el algoritmo de predicción POLYAH (http://linux1.softberry.com) se ha identificado una posible secuencia de corte y poliadenilación (con resultado de precisión: LDF-2.60) situada entre los nucleótidos 4277 y 4282, que se ajusta a la secuencia consenso NUE (near upstream element) propuesta para plantas superiores "AATAAA". También se identificó un elemento FUE (far upstream element, "TTTGTT"), en posiciones 4145 a 4150 (Gallie 1993, Loke et al. 2005).

Figura 3. (página siguiente) Esquema a escala mostrando las subsecuentes secuenciaciones de los dos fagos (λ42361 y λ19381) llevadas a cabo hasta obtener la secuencia completa del gen *FaNPR31*. a) Secuencia originalmente obtenida de la EST. b) Secuencias solapantes obtenidas de las secuenciaciones del fago λ42361, hasta obtener el stop codon en 3´, y el brazo derecho del fago (representado como barra negra) y, por lo tanto, quedando el gen incompleto en la zona 5´. c) Secuencia obtenida del fago λ19381 de la zona 5´. Con la información combinada de ambos fagos obtuvimos la secuencia completa del gen *FaNPR31*.


a
1812
CUI5_T_396EST 491bp
2302
b
A 2059
2728
670bp
B 784bp
2166
1383
C 906bp
2638
3541
D 158λ+798bp
1529
732
E 894bp
3488
4381
λ42361
F 628λ+59bp
732 791
c
F 786bp
1
786
λ19381
Genomic FaNPR31
4383bp
1Kb


Dof
Myc/Myb
Myc
Dof
Wrky
Myb
Caat
Wrky
Dof
Circadian
bZip
light
Caat
Dof
Myb
Light
Dof
Wrky
TATA
TATA
Myc/Myb
Dof
-300 Dof
polyA

Adicionalmente, se realizó una predicción de posibles elementos *"cis"* en la secuencia de 524 nucleótidos corriente arriba del codon de inicio AUG (frecuencia de nucleótidos: A - 0.24, G - 0.22, T - 0.41, C - 0.13) correspondiente a la región reguladora del gen *FaNPR31*. Para este análisis se utilizaron dos herramientas predictivas basadas en la información depositada en las bases de datos públicas: "*PLACE: A Database of Plant Cis-acting Regulatory DNA Elements"* y *"PlantCARE: A Database of Plant Promoters and their Cis-acting Regulatory Elements*". Este estudio reveló la presencia de posibles secuencias o elementos *"cis"* reguladores descritos para otros genes de plantas (Tabla S1). Los elementos reguladores más relevantes identificados por ambos sistemas se muestran en las Tablas 1 y 2 (resultados PLACE y PlantCARE, respectivamente) y algunos han sido incluidos en la Figura 4.

Cabe destacar la identificación de sitios de unión para factores de transcripción de tipo MYB, MYC, DOF y, especialmente WRKY. Estos últimos, claramente identificados en el promotor del gen ortólogo de Arabidopsis *AtNPR1*. Addicionalmente, se identificaron varios sitios de respuesta a control hormonal (ABA, SA, GA), y de regulación por luz y de control circadiano, lo que podría significar una relación entre la respuesta de defensa en fresa, regulada por FaNPR31, y la regulación por luz, como ya se ha descrito en otras plantas (Wang et al. 2011). Igualmente, se identificaron las posibles cajas TATA y CAAT, elementos típicos estructurales de promotores (Tablas 1 y 2).

Figura 4. (página anterior) Secuencia nucleotídica del gen *FaNPR31*. Se muestran 4381 nucleótidos de la secuencia genómica de este gen, que comprenden parte de su región reguladora y su región estructural. La región codificante y la secuencia aminoacídica de la proteína deducida figuran en mayúscula y sombreada en amarillo. Los codones de inicio y terminación se han señalado en rojo. Las secuencias de corte y poliadenilación (NUE, "aataaa"; FUE, "tttgtt") predichas se muestra encuadrada en azul. Algunos de los elementos estructurales y reguladores predichos en la zona promotora se muestran sobre la secuencia (ver detalle en Tablas 1 y 2).

Table 1. Selección de los elementos cis reguladores más relevantes predichos en el promotor del gen FaNPR31 por "PLACE: A Database of Plant Cis-acting Regulatory DNA Elements". Los elementos se han ordenado de acuerdo a su coordenada en la secuencia de promotor. Sombreados en gris, elementos estructurales del promotor; en amarillo, sitios de reconocimiento para MYC y MYB; en rosa, sitios de reconocimiento para DOF; en violeta, sitios de regulación circadiana o por luz; en verde, sitios de reconocimiento de WRKY y sitios de respuesta a SA y ABA.

Factor or Site Name	Loc. (Str.)	Signal Sequence	SITE #	Description
DOFCOREZM site	7 (+) 137 (+) 219 (-) 295 (-) 347 (-) 497 (-) 514 (+)	AAAG	S000265	Core site required for binding of Dof proteins.
MYBCORE site	93 (+) 318 (+) 473 (+)	CNGTTR	S000176	Binding site for two plant MYB, proteins ATMYB1 and ATMYB2.
MYCCONSENSUSAT site	93 (+) 113 (+) 473 (+)	CANNTG	S000407	MYC recognition site of ATMYC2, transcriptional activators in abscisic acid signaling.
BOXLCOREDCPAL site	169 (-)	ACCWWCC	S000492	Consensus of the putative "core" sequences of DcMYB1, which acts as a transcriptional activator of the carrot phenylalanine ammonia-lyase gene (DcPAL1) in response to elicitor treatment.
MYBPZM site	169 (-)	CCWACC	S000179	Core of maize myb homolog binding site, and directly activate a flavonoid biosynthetic gene subset.
WBOXATNPR1 site	162 (+)	TTGAC	S000390	W-box" found in promoter of Arabidopsis thaliana (A.t.) NPR1 gene; Located between +70 and +79 in tandem; They were recognized specifically by salicylic acid (SA)-induced WRKY DNA bindingproteins; See S000142 (SQ=TTGACC); See S000310 (SQ=TTTGACY); A cluster of WRKY binding sites act as negative regulatory elementsfor the inducible expression of AtWRKY18 (Chena and Chen, 2002)*.
WRKY71OS site	163 (+) 208 (-) 378 (-)	TGAC	S000447	A core of TGAC-containing W-box.
WBOXNTERF3 site	378 (+)	TGACY	S000457	"W box" found in the promoter region of a transcriptional repressor ERF3 gene in tobacco; May be involved in activation of ERF3 gene by wounding.
DPBFCOREDCDC3 site	244 (-)	ACACNNG	S000292	bZIP transcription factors binding core sequence, induced by
CIACADIANLELHC site	229 (-) 233 (-)	CAANNNNATC	S000252	Region necessary for circadian expression.
GT1CONSENSUS site	265 (-) 342 (-)	GRWAAW	S000198	Consensus GT-1 binding site in many light-regulated genes through direct interaction between TFIIA and GT-1; Binding of GT-1-like factors to the PR-1a promoter influences the level of SA-inducible gene expression.
CAATBOX1 site	201 (-) 230 (-)	CAAT	S000028	CAAT promoter consensus sequence.
CCAATBOX1 site	283 (-)	CCAAT	S000030	Common sequence found in the 5'-non-coding regions of eukaryotic genes.
-300CORE site	511 (+)	TGTAAAG	S000001	"TGTAAAG core motif" in "-300 elements"

***Chen, C. and Chen, Z. (2002)** Potentiation of Developmentally Regulated Plant Defense Response by AtWRKY18, a Pathogen-Induced Arabidopsis Transcription Factor. Plant Physiology, 129, 706-716.

Table 2. Selección de los elementos cis reguladores más relevantes predichos en el promotor del gen FaNPR31 por "PlantCARE: A Database of Plant Promoters and their Cis-acting Regulatory Elements". Los elementos se han ordenado de acuerdo a su coordenada en la secuencia de promotor. Sombreados en gris, elementos estructurales del promotor; en amarillo, sitios de reconocimiento para MYC y MYB; en violeta, sitios de regulación circadiana o por luz.

Site Name	Organism	Position	Strand	Matrix score.	Sequence	Function
TATA-box	Arabidopsis thaliana	20	-	9	ccTATAAAaa	core promoter element around
	Lycopersicon esculentum	63	+	5	TTTTA	-30 of transcription start
	Helianthus annuus	182	-	6	TATACA	
	Oryza sativa	335	-	8	TATAAGAA	
	Lycopersicon esculentum	405	+	5	TTTTA	
	Arabidopsis thaliana	419	-	5	TATAA	
CAAT-box	Brassica rapa	101	-	5	CAAAT	common cis-acting element in
	Arabidopsis thaliana	143	+	5	CCAAT	promoter and enhancer regions
	Hordeum vulgare	187	-	4	CAAT	
	Brassica rapa	220	-	5	CAAAT	
	Arabidopsis thaliana	269	-	5	CCAAT	
	Brassica rapa	345	+	5	CAAAT	
MBS	Arabidopsis thaliana	80	-	6	CAACTG	MYB binding site involved in
	Arabidopsis thaliana	456	-	6	CAACTG	drought-inducibility
GARE-motif	Brassica oleracea	303	+	7	TCTGTTG	gibberellin-responsive element
circadian	Lycopersicon esculentum	219	-	6	CAANNNNATC	cis-acting regulatory element involved in circadian control
Sp1	Zea mays	319	-	5.5	CC(G/A)CCC	light responsive element
ATCT-motif	Pisum sativum	342	+	9	AATCTAATCC	part of a conserved DNA module involved in light responsiveness

Obtención de la secuencia codificante completa del gen *FaNPR31*

Con objeto de identificar y clonar la secuencia codificante completa del gen *FaNPR31*, se diseñaron oligos específicos (FaNPR31Fw y FaNPR31Rv) en las zonas próximas a los codones de inicio y fin de lectura usando como molde la secuencia genómica obtenida anteriormente. El producto de PCR obtenido fue una banda única de 1.8Kb de tamaño aproximado, que se clonó en el vector pGemTEasy (Promega) (*descrito en Material y Métodos,* Figura 5).

Se seleccionaron un total de 10 colonias transformantes independientes y se obtuvieron las secuencias completas de los insertos que portaban. Tras el análisis de las secuencias obtenidas, se identificaron un total de 7 secuencias diferentes que presentaban escasos cambios nucleotídicos, y que, por tanto, correspondían a 7 alelos diferentes del gen

FaNPR31 presentes en *F* × *ananassa.* En la Figura 6 se muestra el alineamiento de las distintas secuencias obtenidas para los 7 alelos. Curiosamente, la secuencia correspondiente al Alelo 2 presentó una identidad del 100% con la secuencia correspondiente a cuatro fragmentos espaciados de la secuencia genómica deducida de los fagos λ42361 y λ19381 (Figura 4) lo que proporcionó la arquitectura génica de este gen: 4 exones separados por 3 intrones (Figura 7a). Así, la región codificante incluye un marco abierto de lectura de 1.764 pb, incluyendo el codón de terminación, que codifica un polipéptido de 587 aminoácidos, con un peso molecular de 65,27kDa y un punto isoeléctrico de 6,7 (valores calculados por el programa EditSeq del paquete informático DNASTAR). Por otro lado, el Alelo 1 presentó una inserción de una base, que le genera un cambio de la fase de lectura (este hecho se comprobó al secuenciar en ambos sentidos esa zona en cada clon) y, por tanto, una proteína truncada. Las correspondientes secuencias aminoacídicas deducidas de las 6 isoformas de la proteína codificada FaNPR31 (Alelos 2 al 7) y de la proteína truncada que es codificada por el Alelo 1, se muestran en la Figura 7b.

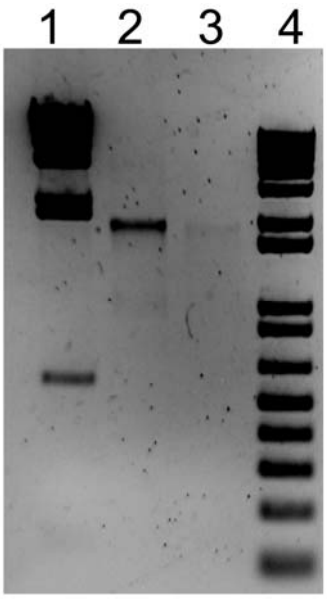


Figura 5. Producto de PCR obtenido como resultado de amplificar DNA copia de corona de fresa cv. Camarosa con los oligos que flanquean el gen *FaNPR31*, (1:Lambda DNA marker; 2: PCR FaNPR31 a 50ºC de anillamiento; 3: a 55ºC de anillamiento; 4: 1Kb Plus DNA ladder).

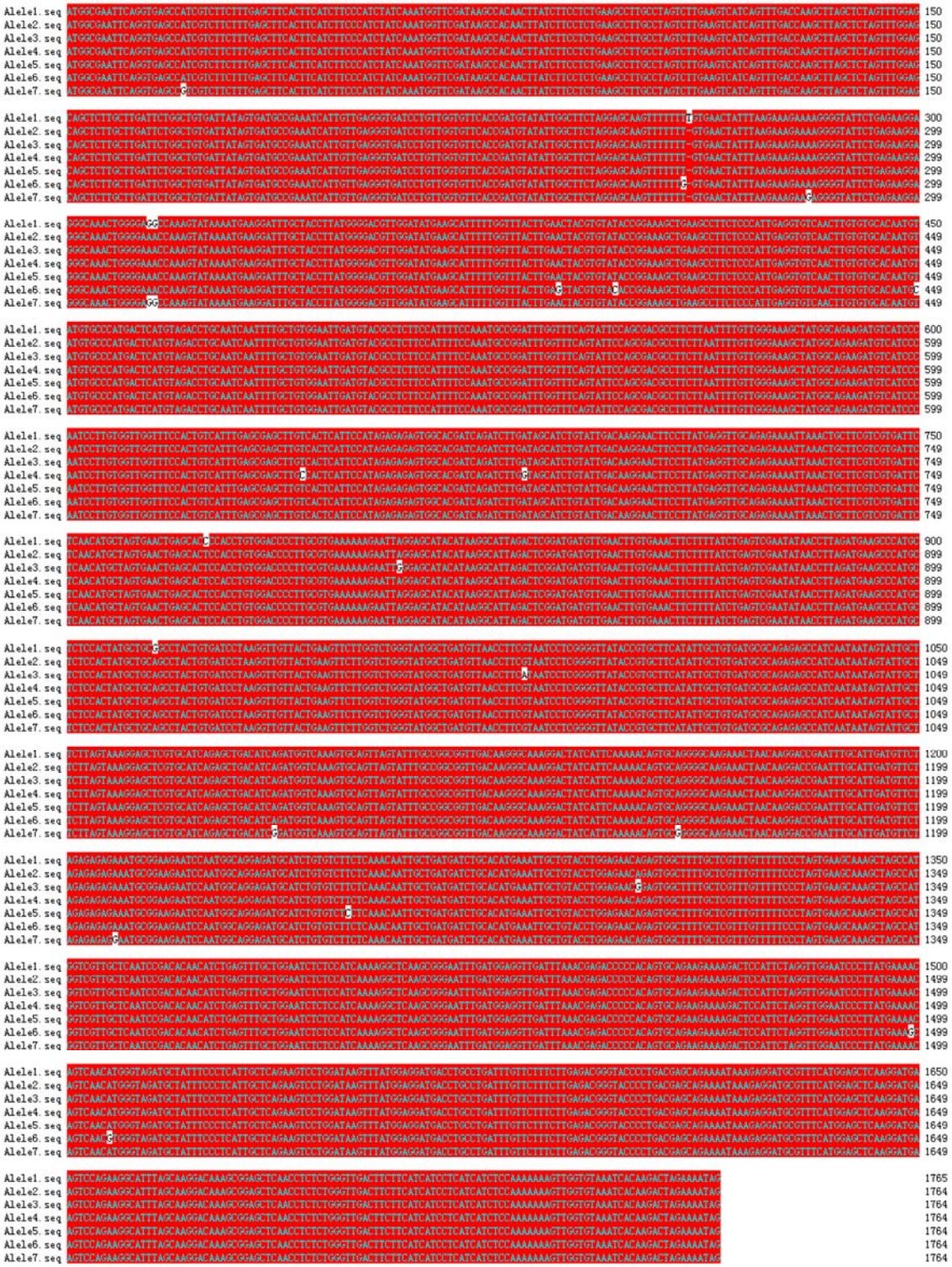


Figura 6. Alineamiento múltiple de las 7 secuencias nucleotídicas diferentes obtenidas (alelos) del gen *FaNPR31*. En fondo blanco se muestran los cambios nucleotídicos puntuales. El algoritmo ClustalW se implementó en el módulo Megalign del software DNAStar (v 7.1.0; LaserGene, Madison, WI, USA).

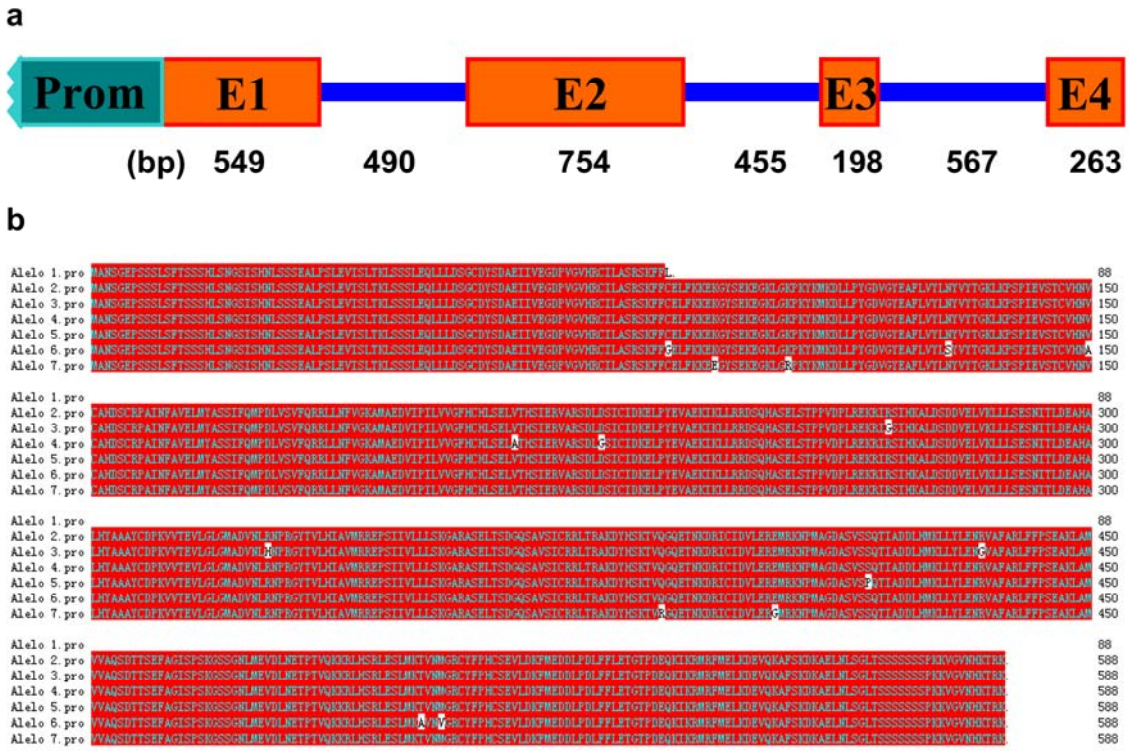


Figura 7. Arquitectura génica de *FaNPR31* obtenida al comparar las secuencias de la región codificante con la secuencia genómica obtenida previamente de los fagos λ42361 y λ19381. Alineamiento múltiple de las 7 secuencias aminoacídicas deducidas de los 7 alelos identificados. En fondo blanco se muestran los cambios aminoacídicos puntuales. El algoritmo ClustalW se implementó en el módulo Megalign del software DNAStar (v 7.1.0; LaserGene, Madison, WI, USA).

Caracterización estructural de la proteína FaNPR31 de fresa

La secuencia deducida de la proteína FaNPR31-Alelo 2 se comparó con otras existentes en la base de datos NCBI (Nacional Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov), utilizando el programa BlastP contra la colección de proteínas nr (no redundante) (Altschul et al. 1990; Altschul et al. 1997). El análisis y comparación de la secuencia proteica reveló una elevada similitud e identidad de secuencia de esta proteína de fresa con proteínas pertenecientes a la familia de reguladores NPR1-like en diversas especies. La Figura 8a muestra un alineamiento de la proteína FaNPR31 con las proteínas más similares presentes en las bases de datos públicas.

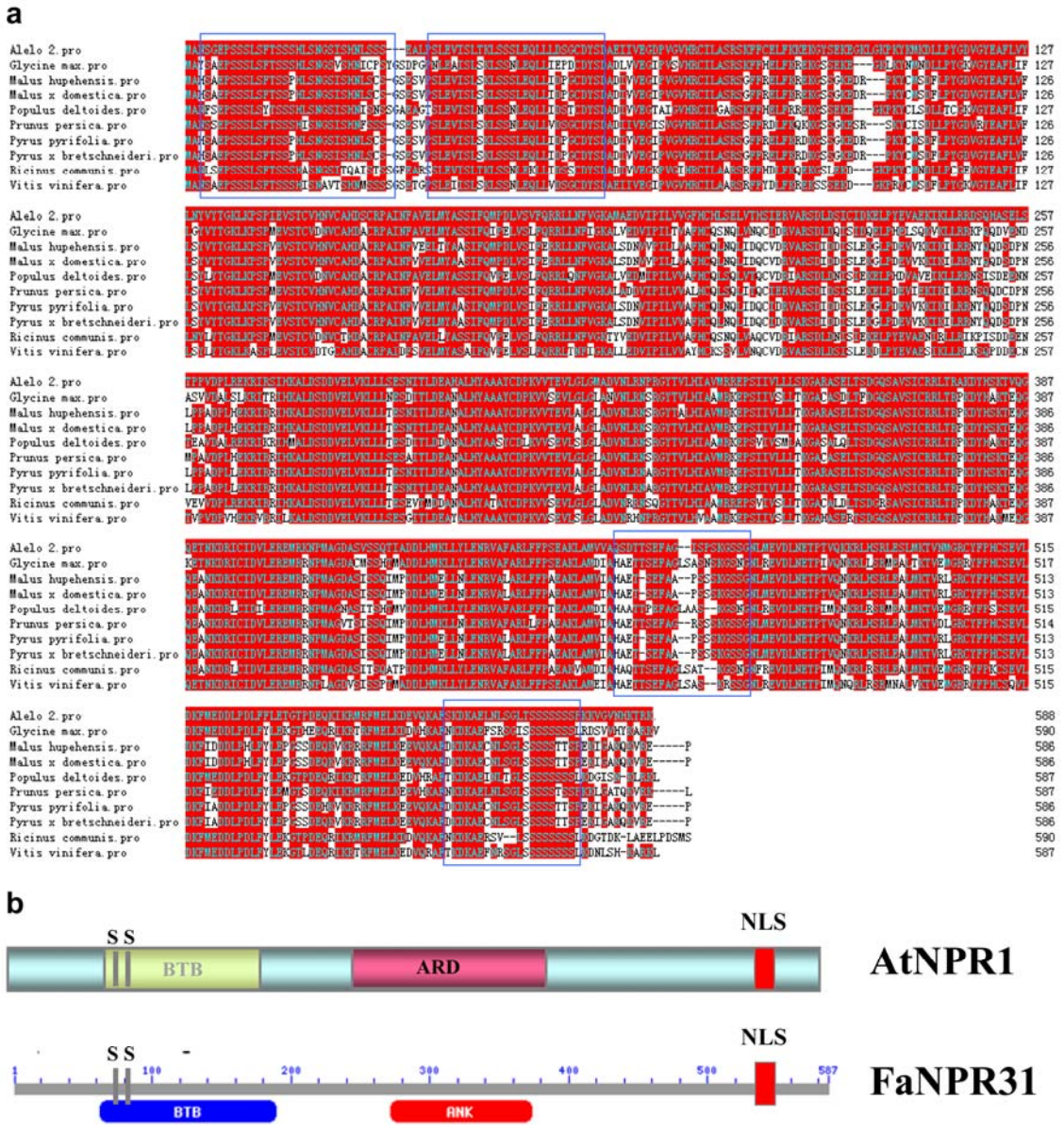
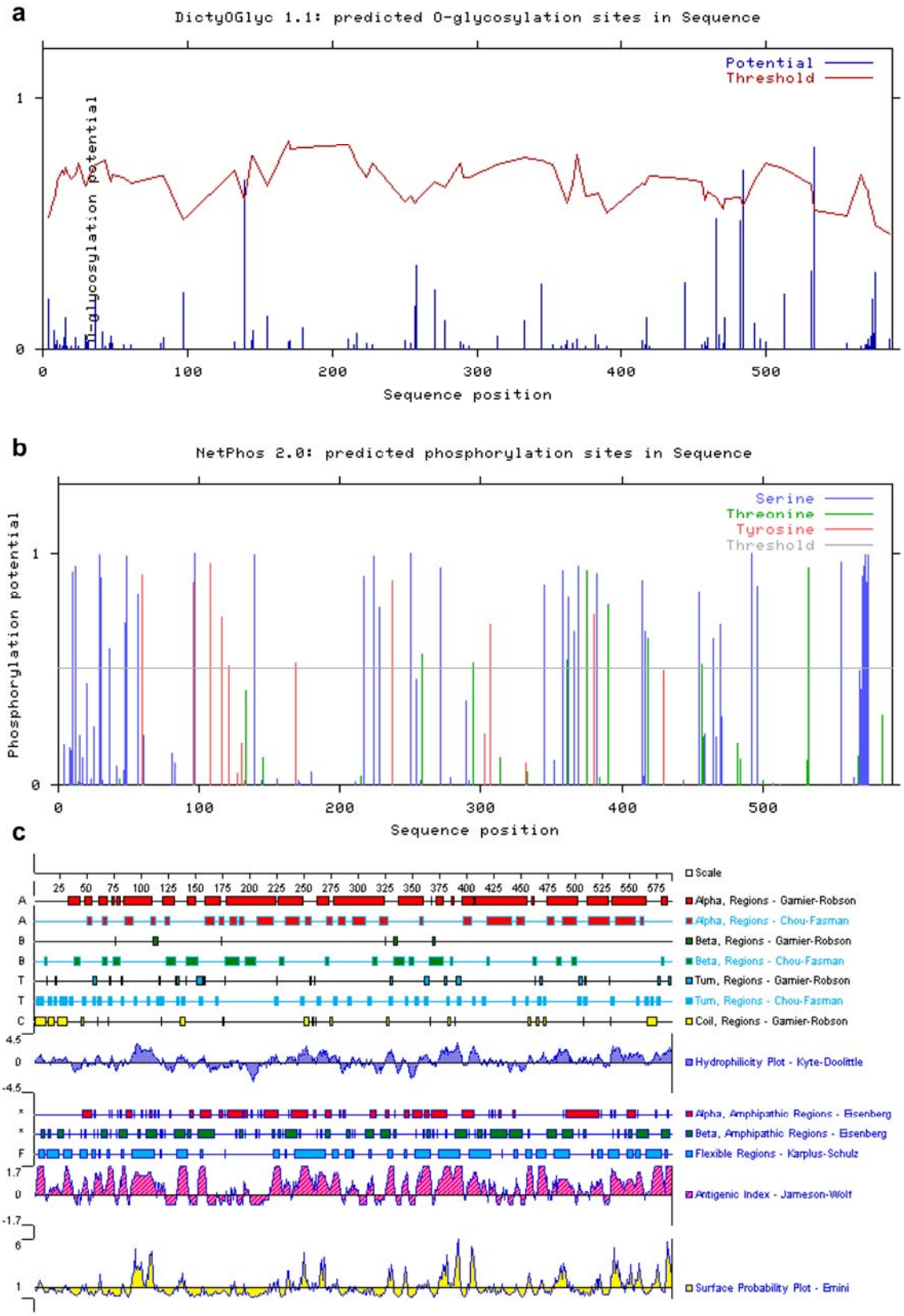


Figura 8. Características estructurales de la proteína FaNPR31. (a) Alineamiento múltiple de la proteína FaNPR31 codificada por el Alelo 2, y otras proteínas pertenecientes a la familia de reguladores NPR1-like en diversas especies. Se han enmarcado en azul cuatro zonas muy enriquecidas en Ser, y con posibilidad de ser susceptibles de modificación post-traduccional, por ejemplo por fosforilación. El algoritmo ClustalW se implementó en el módulo Megalign del software DNAStar (v 7.1.0; LaserGene, Madison, WI, USA). (b) Esquema representando los dominios conservados descritos para esta familia de proteínas en Arabidopsis y que también han sido identificados en la proteína FaNPR31 de fresa.

Curiosamente, se han identificado cuatro zonas muy ricas en residuos Serina, conservadas entre especies, que pudieran ser susceptibles de modificaciones post-traduccionales para regular la función molecular de estas proteínas. Además, se han identificado dos dominios conservados de interacción proteína proteína (BTB y ANK), así como una señal de localización nuclear (NLS) (Figura 8b).

La existencia de otros sitios diana para modificaciones post-traduccionales en la proteína FaNPR31 se determinó mediante el uso de diversas aplicaciones de software. DictyOGlyc 1.1 (http://www.cbs.dtu.dk/services/DictyOGlyc-1.1/) fue usado para predecir los sitios de O-glicosilación (Tabla S2, Figura 9a). Así, se identificaron 3 residuos con alta probabilidad de ser glicosilados: Ser139, Thr484 y Thr533. El programa NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos-2.0/) se utilizó para predecir los posibles sitios susceptibles de ser fosforilados, y se encontraron un gran número de residuos con probabilidad alta de ser dianas de esta modificación (34Ser, 8Thr, 9Tyr; Tabla S3 y Figura 9b). Para determinar los posibles sitios de sumoilación se usaron dos programas (SUMOsp 2.0: http://sumosp.biocuckoo.org/online.php y SUMOplot™ Analysis Program (Abgent): http://www.abgent.com/tools) que coincidieron en determinar dos sitios con muy alta probabilidad de ser sumoilados (Tabla S4): K548 y K91, ambos del mismo tipo (Type I: Ψ-K-X-E). También se predijo que esta proteína no presenta péptido señal (SignalP-4.1, Figura S1). Por último, mediante el programa PROTEAN del soporte informático Lasergene Navigator se realizó la predicción de la estructura secundaria de la proteína deducida de la secuencia del Alelo 2 (Figura 9c).

Figura 9. (página siguiente) Predicción de los posibles sitios de: (a) O-glicosilación (DictyOGlyc), (b) fosforilación (NetPhos). (c) Predicción de la estructura secundaria de la proteína deducida de FaNPR31 Alelo 2 realizada con el programa PROTEAN del soporte informático Lasergene Navigator.


a
DictyOGlyc 1.1: predicted O-glycosylation sites in Sequence
Potential
Threshold
O-glycosylation potential
Sequence position
b
NetPhos 2.0: predicted phosphorylation sites in Sequence
Serine
Threonine
Tyrosine
Threshold
Phosphorylation potential
Sequence position
c
Scale
Alpha, Regions - Gamier-Robson
Alpha, Regions - Chou-Fasman
Beta, Regions - Gamier-Robson
Beta, Regions - Chou-Fasman
Turn, Regions - Gamier-Robson
Turn, Regions - Chou-Fasman
Coil, Regions - Gamier-Robson
Hydrophilicity Plot - Kyte-Doolittle
Alpha, Amphipathic Regions - Eisenberg
Beta, Amphipathic Regions - Eisenberg
Flexible Regions - Karplus-Schulz
Antigenic Index - Jameson-Wolf
Surface Probability Plot - Emini

Análisis filogenético de *FaNPR31* con la familia de parálogos NPR1-like descrita en la planta modelo *Arabidopsis thaliana*

Para tratar de identificar el posible ortólogo de FaNPR31 en el proteoma de Arabidopsis, y así poder disponer de la vasta información molecular y funcional que hay disponible para esta especie modelo, se realizó una comparación con los seis parálogos que se han identificado en Arabidopsis (Figura 10a). El resultado indicó que el gen *FaNPR31* es mucho más próximo a los genes AtNPR3 y AtNPR4 de Arabidopsis, que han sido muy recientemente identificados como los receptores de SA en la planta (Fu et al. 2012) y reguladores negativos de la respuesta de defensa (Shi et al. 2012). Además, se realizó un alineamiento múltiple de la secuencia aminoacídica de *FaNPR31* Alelo 2 y las 6 proteínas de Arabidopsis. De nuevo el gen *FaNPR31* fue más similar a las proteínas NPR3 y NPR4, que incluso mantienen alta similitud en las zonas ricas en residuos Ser mencionadas anteriormente (Figura 10b).

Descripción de los patrones de expresión de *FaNPR31* regulado en situaciones experimentales relacionadas con la respuesta de defensa de la planta de fresa

Con el objeto de obtener información sobre la posible función biológica que el gen *FaNPR31* pudiera desempeñar en la planta de fresa, se procedió a caracterizar su regulación transcripcional ante diversas situaciones experimentales relacionadas con el estrés y la respuesta de defensa de la planta de fresa (todas estas situaciones experimentales, y los métodos usados para este análisis, se han descrito anteriormente en el Capítulo III). Así, este gen mostró una expresión prácticamente constante en tejidos reproductivos, tanto en distintos estadios de maduración, como en el posterior decaimiento y senescencia del fruto (Figura 11a). Del mismo modo, la expresión de *FaNPR31* no se alteró en frutos sometidos a un experimento de infección fúngica con *C. acutatum* (Figura 11b). Sin embargo, el gen *FaNPR31* sufrió una leve y transitoria sobreexpresión cuando tejidos vegetativos (corona y peciolo) del cultivar Camarosa se infectaron con *C. acutatum* (Figura 12a, b), aunque permaneció inalterado en el cultivar Andana igualmente infectado (Figura 12c).

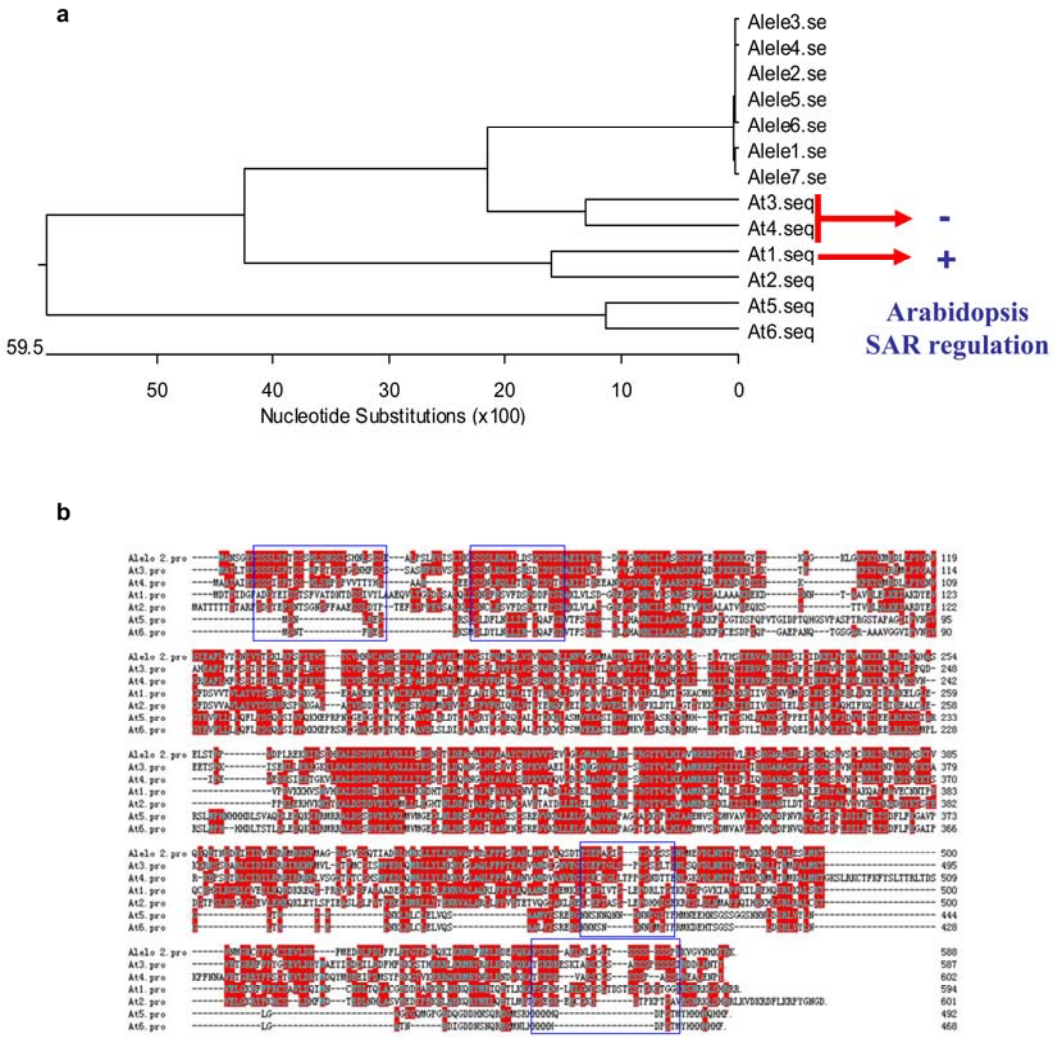


Figura 10. Análisis filogenético de las secuencias nucleotídicas de los 7 alelos de *FaNPR31* identificados en fresa (a), y alineamiento múltiple de las secuencias aminoacídicas del alelo 2 (b), con los 6 parálogos NPR1-like descritos en la planta modelo Arabidopsis. Las zonas especialmente ricas en Ser se han enmarcado en recuadro azul. El algoritmo ClustalW se implementó en el módulo Megalign del software DNAStar (v 7.1.0; LaserGene, Madison, WI, USA).

Para complementar esta información, se desarrolló un experimento de tratamiento de plantas de fresa con los elicitores químicos de la respuesta de defensa en plantas, SA y MeJA. El gen *FaNPR31* mostró regulación positiva en plantas de Camarosa por aplicación de SA y muy fuerte regulación negativa por aplicación de MeJA (Figura 13a). Sin embargo, cuando estos tratamientos se aplicaron a suspensiones celulares de fresa del cultivar Chandler, el nivel de inducción fue aún mayor tras aplicación de SA, y la respuesta a aplicación de MeJA fue, aunque menos intensa, positiva en lugar de sufrir una intensa represión, como ocurrió en las plantas de Camarosa (Figura 13b).

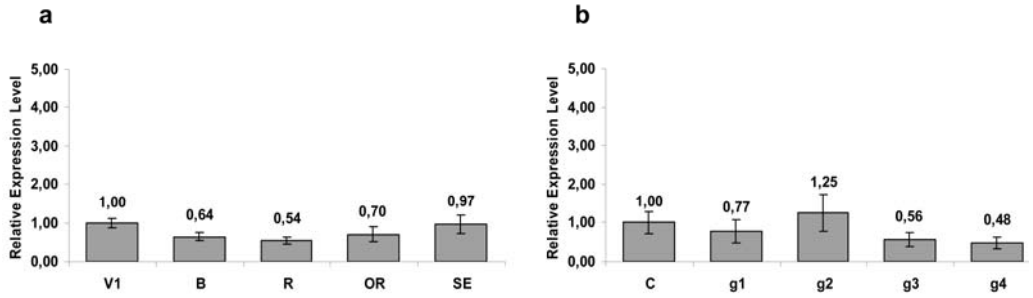


Figura 11. Niveles de expresión relativa del gen *FaNPR31* en tejidos reproductivos de fresa. (a) Patrón de expresión durante la maduración del fruto de fresa (V1: fruto verde, B: blanco, R: rojo, OR: sobre-maduro, SE: senescente). (b) Patrón de expresión en fruto de fresa con síntomas crecientes de antracnosis (C: fruto no infectado, g1 a g4: grados de menor a mayor sintomatología).

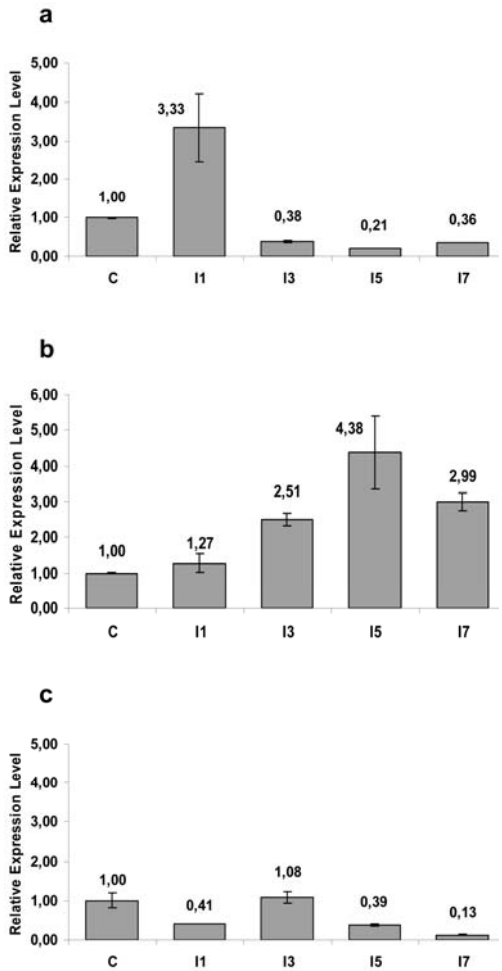


Figura 12. Niveles relativos de expresión del gen *FaNPR31* en tejidos vegetativos de fresa infectados con *C. acutatum*. (a) Corona de cultivar Camarosa, (b) Peciolo de cultivar Camarosa, (c) Peciolo de cultivar Andana. C: tejido no infectado, I1 a I7: tejidos infectados y recolectados 1, 3, 5 y 7 días tras la inoculación.

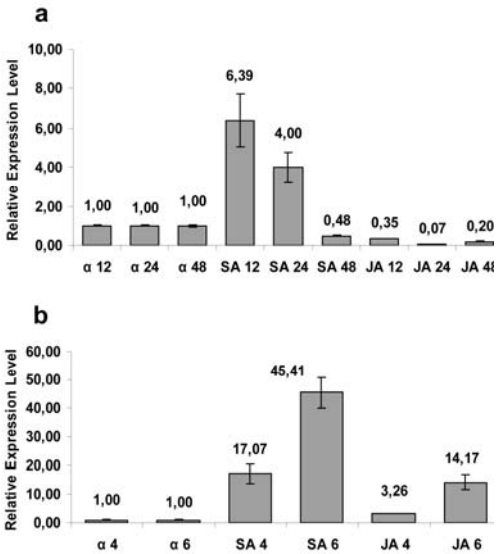


Figura 13. Niveles relativos de expresión del gen *FaNPR31* en fresa elicitada químicamente con SA y MeJA. (a) Patrón de expresión de plantas de Camarosa en respuesta a estas hormonas, (b) Patrón de expresión de suspensiones celulares de Chandler tratados con estas hormonas. α: Agua, SA: Ácido Salicílico, JA: Metil-Jasmonato; los números junto al tratamiento indican horas tras la aplicación.

Estudios funcionales del gen *FaNPR31*

Sistema homólogo: F × ananassa cv. Camarosa

Para determinar la función del gen *FaNPR31* en la planta de fresa se procedió a sobreexpresar y silenciar el mismo mediante tecnología de ARN interferente (Wesley et al. 2001, Waterhouse and Helliwell 2003), en plantas del cultivar Camarosa. Para llevar a cabo estas estrategias se usaron vectores adecuados con tecnología Gateway (Hartley et al. 2000).

Se generaron dos construcciones de sobreexpresión en el vector pK7WG2.0 (Karimi et al. 2002, Figura 14a) con los alelos 1 y 2 del gen *FaNPR31* descritos anteriormente. Estos dos alelos fueron seleccionados debido a que el alelo 2 es idéntico a la secuencia genómica obtenida de los fagos, mientras que el alelo 1, al tener una inserción de una base, da lugar a una proteína muy corta, truncada y no funcional, y lo utilizaremos como control interno. Así, con esta última construcción podremos identificar un incremento de la expresión del gen en las plantas modificadas, aunque en ausencia de producción de la proteína FaNPR31.

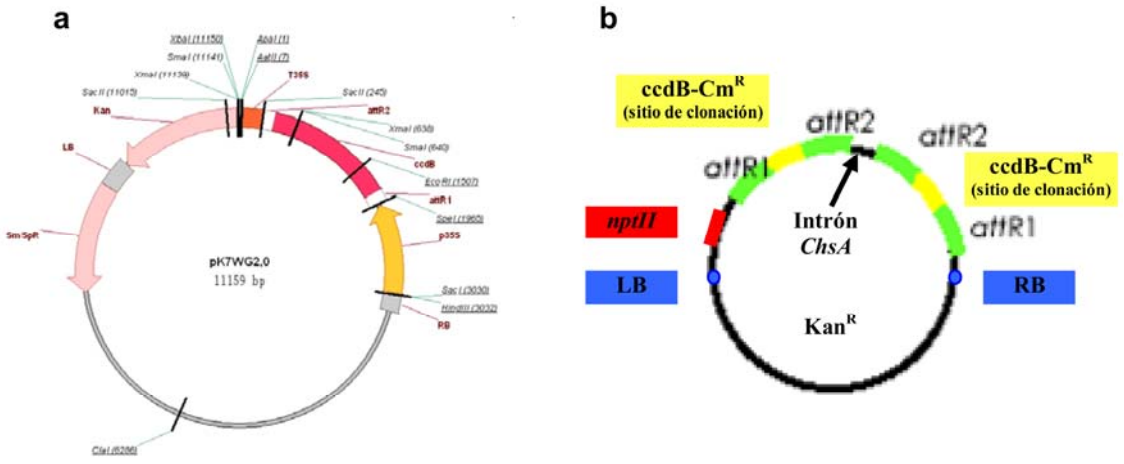
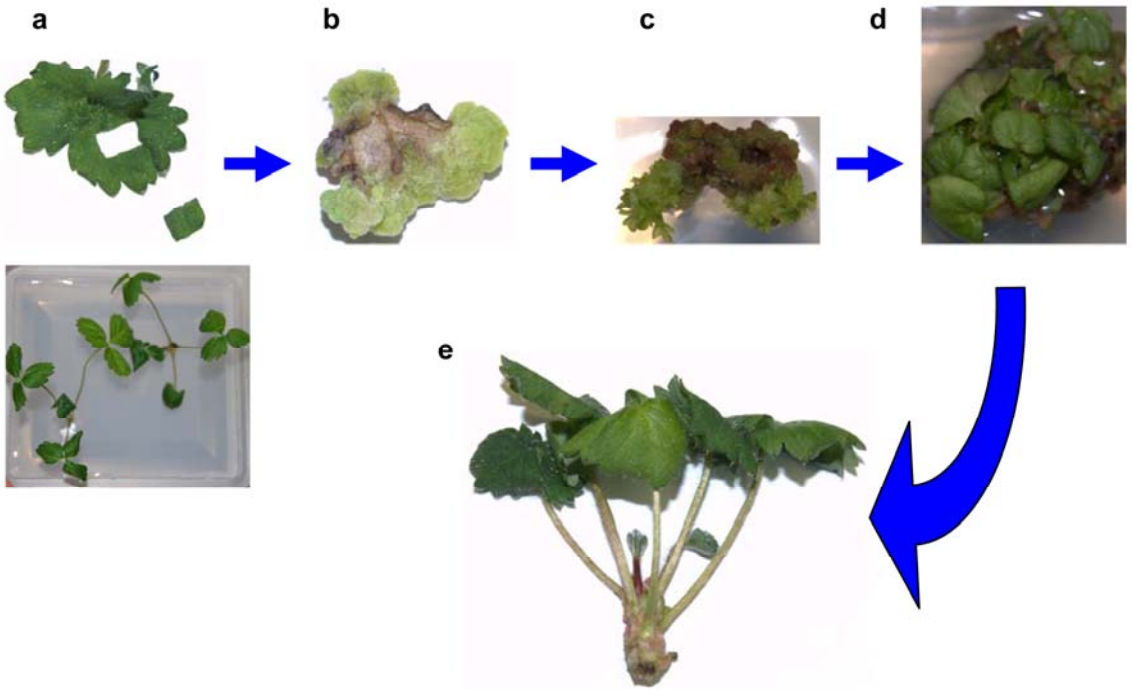


Figura 14. Mapa de los vectores gateway compatibles pK7WG2.0 (a) y pFRN (b) (sobreexpresión y silenciamiento por RNA interferente, respectivamente).

Una vez comprobada, por secuenciación, la integridad de ambas construcciones, se transformaron células competentes de *Agrobacterium tumefaciens* GV3101pMP90 y, tras la selección de transformantes positivos, se llevó a cabo la transformación de 100 explantos de hoja de fresa cultivar Camarosa por cada construcción generada. Tras 4 semanas en medio selectivo adecuado, se observaron las primeras zonas con proliferación celular indiferenciada, la mayoría con coloración verde, en los explantos infectados. A partir de las 8 semanas de regeneración en medio selectivo se observó la aparición de las primeras plántulas completamente desarrolladas. Tras un período de 6-10 meses desde el inicio de la transformación y selección "in vitro" en presencia de kanamicina 50µg/ml, se obtuvieron un total de 10 líneas transgénicas independientes de plántulas pK71, y 30 líneas transgénicas independientes de plántulas pK72, correspondientes a las líneas transformadas con las construcciones pK7WG2.0+FaNPR31.Alelo1 y pK7WG2.0+FaNPR31.Alelo2, respectivamente. Estos resultados suponen un 10% y 30% de eficiencia de transformación y regeneración, respectivamente. La Figura 15 describe el proceso de generación de las plantas transgénicas de fresa, desde la extracción de los explantos de hoja y la generación de callos, hasta la obtención final de las líneas independientes transformadas.



Por otro lado, se realizó una tercera construcción para poder silenciar el gen FaNPR31 mediante ARN interferente. Así, utilizando metodología Gateway, un fragmento de 407bp se clonó en el vector pCR8/GW/TOPO (Invitrogen), que se utilizó para una reacción LR Clonasa (Invitrogen) con el vector de expresión binario pFRN (derivado de pFGC5941), el cual posee dos regiones de clonación delimitadas por extremos attR para la inserción del ADN de interés en las dos orientaciones opuestas (sentido y antisentido) y separadas por un intrón. Una vez comprobada por secuenciación la integridad de la construcción, se transformaron células competentes de *A. tumefaciens* LBA4404 con dicha construcción y con el vector pFRN vacío, utilizado como control negativo. Siguiendo el mismo proceso que se ha comentado anteriormente (Figura 15) para la construcción de sobreexpresión, se transformaron 100 explantos de hoja de fresa cultivar Camarosa con la construcción de silenciamiento y otros 100 explantos con el vector pFRN vacío. Así, se obtuvieron 5 líneas transgénicas independientes transformadas con la construcción pFRN+FaNPR31(RNAi), y 10 líneas transgénicas independientes transformadas con el vector vacío, lo que supuso un 5% y 10% de eficiencia de transformación y regeneración, respectivamente.

Para asegurar que las líneas transgénicas independientes que se generaron portaban el transgén correcto en su genoma, se procedió a extraer y purificar ADN genómico de las mismas, utilizando el "Quantum prep Aquapure Genomic DNA Kit" (BioRad), que se utilizó como molde, en cada línea, para una amplificación específica del transgén mediante PCR, que incluía parte de la región promotora 35S y parte del gen *FaNPR31*. Todas las líneas derivadas de las construcciones de sobreexpresión que se analizaron contenían el transgén en su genoma, sin embargo, solo dos (pFi-1 y pFi-5) de las 5 líneas que debían portar la construcción de silenciamiento (RNA interferente) fueron positivas para este test, por lo que las otras tres se descartaron y no se prosiguió con su análisis (Figura 16).

Figura 15. (página anterior) Subsecuentes etapas del proceso de transformación y regeneración de plantas transgénicas de *F* x *ananassa* cv. Camarosa para la sobreexpresión y silenciamiento del gen *FaNPR31*. (a) Explanto estraído de un foliolo, (b) callo indiferenciado con color verde a las 6 semanas de la transformación, (c) primeras plántulas a las 8 semanas de la transformación, (d) pequeñas plantas ya formadas a las 24 semanas, (e) planta totalmente desarrollada tras 30-40 semanas.

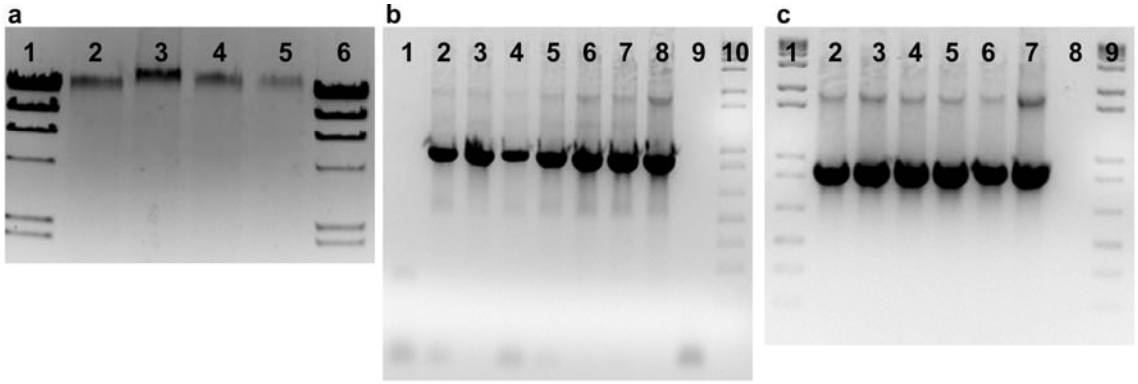


Figura 16. Electroforesis en gel de agarosa de (a) DNA genómico extraído y purificado de las líneas de fresa transformadas con pK7WG2.0+FaNPR31.Alelos 1 y 2 (1: Lambda DNA ladder, 2: pK71-1, 3: pK71-1, 4: pK72-1, 5: pK72-2 y 6: Lambda DNA ladder); (b y c) Test por PCR para detectar la presencia del transgén en los genomas analizados (b1: Camarosa no transformada, b2: pK71-1, b3: pK71-2, b4: pK71-3, b5: pK71-6, b6; pK71-8, b7: pK71-9, b8: Control positivo PCR (plásmido pK7WG2.0+FaNPR31.Alelo2), b9: Control negativo PCR (agua), b10: 1Kb Plus DNA ladder, c1: 1Kb Plus DNA ladder, c2: pK72-1, c3: pK72-2, c4: pK72-3, c5: pK72-4, c6: pK72-6, c7: Control positivo PCR (plásmido pK7WG2.0+FaNPR31.Alelo2), c8: Control negativo PCR (agua) y c9: 1Kb Plus DNA ladder).

Para confirmar la posible sobreexpresión del gen *FaNPR31* en las diferentes líneas transgénicas de fresa obtenidas, se procedió a la cuantificación del transcrito de este gen utilizando la técnica de RTqPCR. Así, se realizó la extracción de ARN a partir de hojas de las líneas transgénicas (pK71-14, pK71-9, pK72-1, pK72-4, pK72-26, pK72-3, pK72-6, pK72-23, pK72-13, pK72-24 y pK72-28), en las cuales ya se había detectado la presencia del transgen por PCR, mediante el "Aurum^{TM} Total RNA mini kit" (BioRad). Se comprobó que todas las muestras de ARN tenían calidad apropiada para estudios de RTqPCR, y a partir de 1μg de ARN extraído de hojas de cada una de las líneas transgénicas seleccionadas se realizó una reacción de retrotranscripción utilizando "iScript^{TM} cDNA Síntesis Kit" (BioRad). Se utilizaron oligos capaces de diferenciar las especies moleculares endógenas de FaNPR31 (oligo Rv anclando en zona 3´UTR, iQutrFaNPR31Fw e iQutrFaNPR31Rv, ver *Material y Métodos*), e igualmente, capaces de amplificar específicamente los transcritos derivados de la construcción (oligo Rv anclando en zona específica del vector pK7, iQpk7FaNPR31Rv, ver *Material y Métodos*). Los valores de expresión relativos se calcularon normalizándolos con respecto a los valores de expresión del gen de referencia FaGAPDH2.

La Figura 17a muestra los niveles de *FaNPR31* exógeno detectados en plantas transformadas con las dos construcciones de sobreexpresión (Alelos 1 y 2), relativo al nivel de gen endógeno en plantas de Camarosa no transformadas. Se obtuvieron valores que oscilaban entre 44 y 478 veces más transcrito derivado de la construcción, que los niveles de transcrito endógenos en planta no transformada, lo que indicó que ambas construcciones habían sido efectivas en incrementar de manera muy importante el nivel de expresión del gen *FaNPR31* en las plantas de Camarosa transformadas. Por otro lado, la cuantificación específica de los niveles de transcrito endógeno (no derivado de la construcción), permitió identificar tres líneas de tipo pK72 (Alelo 2, líneas 3, 6 y 26) en las que la expresión del propio gen endógeno se vio afectada positivamente (Figura 17b). Esto podría sugerir que la expresión de la proteína FaNPR31 ectópica podría activar la transcripción del propio gen endógeno (feed-back positivo), siempre y cuando los niveles de proteína ectópica alcanzados en estas líneas fuesen superiores al de las otras líneas de mayor expresión génica. Para demostrar este hecho será necesario llevar a cabo estudios mediante western blot.

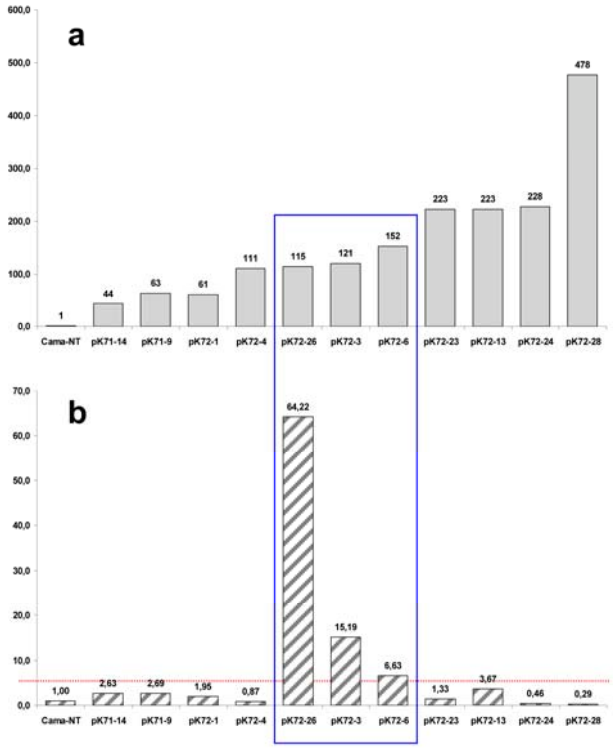


Figura 17. Cuantificación de los niveles de transcrito de *FaNPR31* en plantas de fresa transformadas con las construcciones de sobreexpresión. (a) Niveles de transgén relativo al nivel de gen endógeno en plantas de Camarosa no transformadas. (b) Modificación en los niveles de gen endógeno como consecuencia de la transformación.

Con la intención de identificar aquel subset de genes de defensa que pudiera estar bajo el control transcripcional de FaNPR31, se realizó un estudio transcriptómico en las plantas modificadas con las dos construcciones de sobreexpresión de 6 genes de la familia PR (FaPR1-2, FaPR2-1, FaPR5-1, FaPR5-2, FaPR5-3 y FaPR10-4) considerados marcadores clásicos de rutas de defensa conocidas en otras plantas y descritos en el Capítulo V (Figura 18).

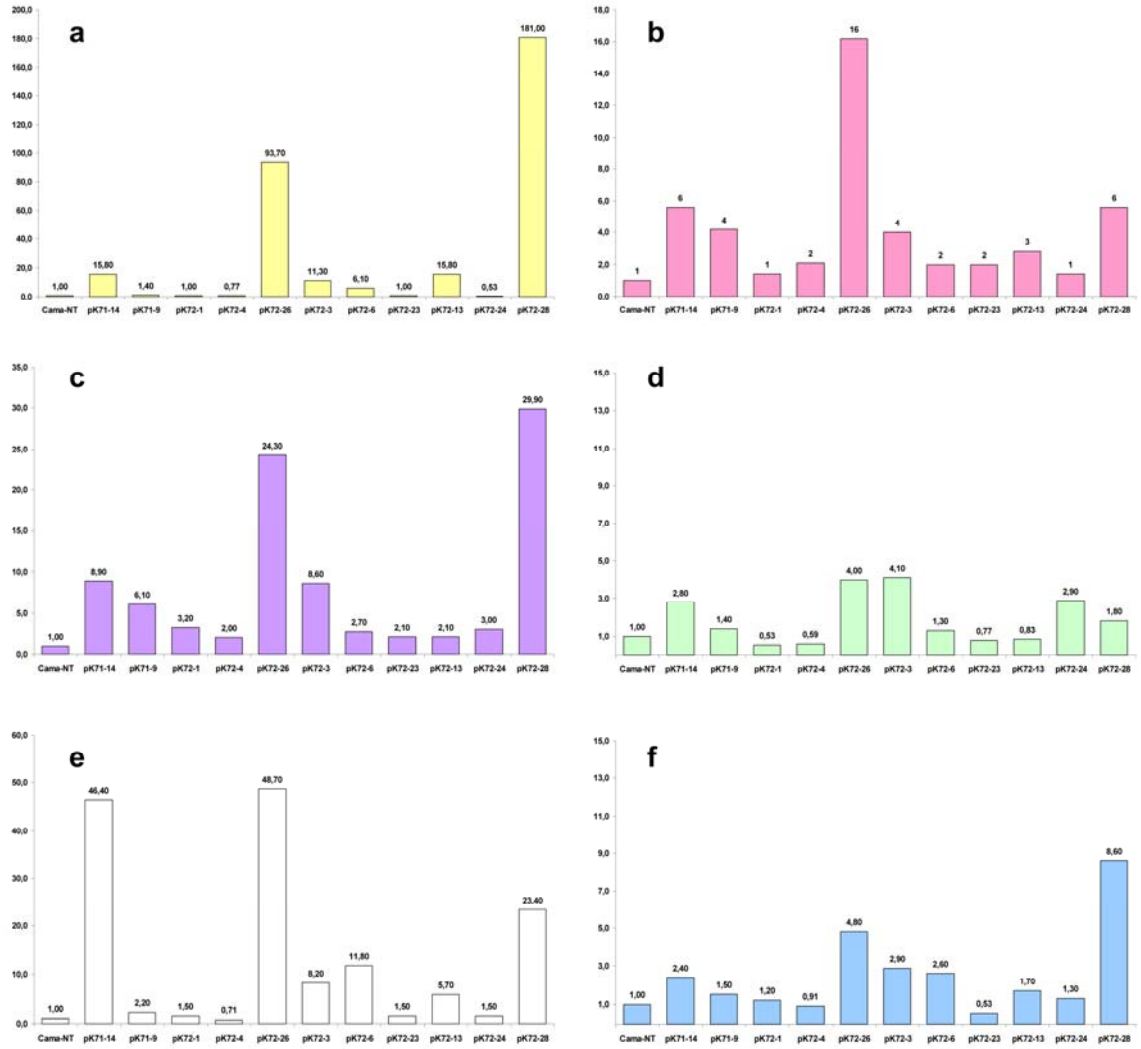


Figura 18. Analisis transcriptómico de los genes de defensa (PRs) que, tal como se ha descrito en el Capítulo V, responden a la infección por *C. acutatum* en Camarosa. (a) FaPR1-2, (b) FaPR2-1, (c) FaPR5-1, (d) FaPR5-2, (e) FaPR5-3, (f) FaPR10-4.

Cinco líneas, (pK71-14, pK72-3, pK72-13, pK72-26 y pK72-28) exhibieron niveles de expresión de genes de defensa superiores a la línea control, particularmente dos de estas líneas, pK72-26 y pK72-28, que coinciden con los mayores niveles detectados de FaNPR31 endógeno y exógeno, respectivamente. Curiosamente, una de las líneas que sobreexpresa el alelo truncado (pK71-14), también presentó niveles elevados de estos genes de defensa.

Para conocer si las lineas transgénicas eran más o menos susceptibles a *C. acutatum*, se realizó un experimento de inoculación de hojas de estas plantas con una suspensión de conidias del patógeno. Así, cinco líneas (Camarosa no transformada, pK72-3, pK72-28, pFi-1 y pFi-5) se inocularon con aprox. 2500 conidias (3ul). A los 8 días de la inoculación se tomaron fotografías de las mismas y las plantas completas se almacenaron a -80ºC, para su procesamiento en posteriores estudios transcriptómicos.
La Figura 19 muestra un ejemplo de los síntomas observados en cada una de las líneas analizadas tras la infección con *C. acutatum*.

Sistema heterólogo: A. thaliana

Los 7 alelos de *FaNPR31* identificados se utilizaron para sobreexpresar este gen en el sistema heterólogo Arabidopsis usando el vector gateway pAMpAT35SSGW (Tabla 3) para transformar plantas de Arabidopsis Col-0 (experimento de sobreexpresión en fondo genético silvestre), así como cuatro líneas mutantes knock-out para los genes NPR1, NPR3, NPR4 y el doble mutante NPR3/NPR4 (experimento de complementación de mutantes). Además, la construcción utilizada (pFi) en esta tesis para silenciamiento de FaNPR31 mediante RNA interferente, en fresa, se usó para transformar Arabidopsis Col-0 wild-type (experimento de silenciamiento heterólogo). De todas estas transformaciones se obtuvieron semillas transgénicas, que fueron seleccionadas por su resistencia a fosfinotricina (Figura 20).

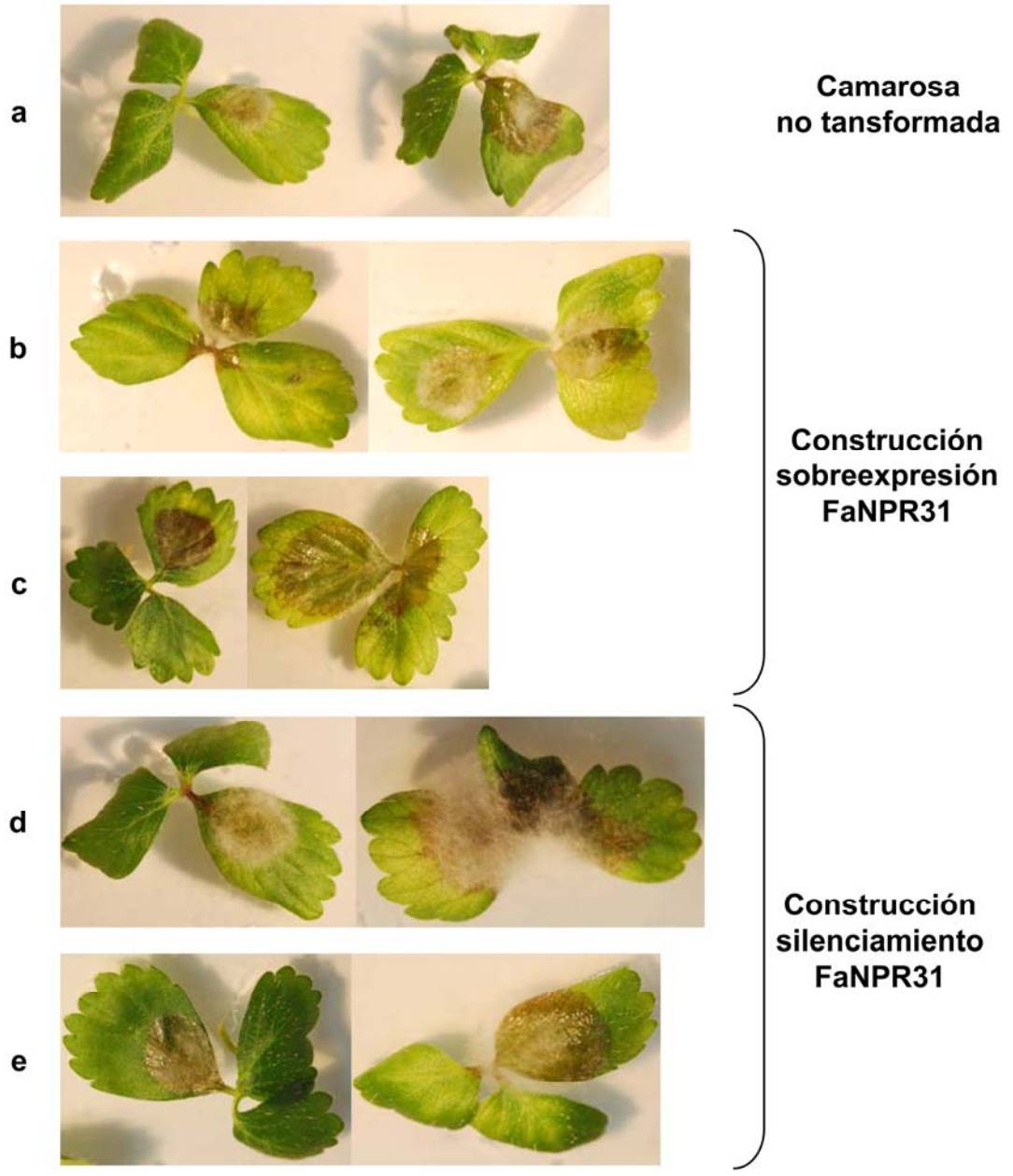


Figura 19. Síntomas observados en cada una de las líneas transgénicas analizadas ((a) Camarosa no transformada, (b) pK72-3, (c) pK72-28, (d) pFi-1 y (e) pFi-5) 8 días tras la infección con *C. acutatum.* Las imágenes representan el trifollio con menor y mayor sintomatología identificados entre las 6 plantas inoculadas de cada línea.

Tabla 3. Transformación de FaNPR31 en sistema heterólogo (sobreexpresión, complementación de mutantes y silenciamiento).

Genotipo Arabidopsis	**Vector**	***FaNPR31***
Sobreexpresión		
Col-0	pAMpAT35SSGW	vector vacío
Col-0	pAMpAT35SSGW	Alelo1
Col-0	pAMpAT35SSGW	Alelo2
Col-0	pAMpAT35SSGW	Alelo3
Col-0	pAMpAT35SSGW	Alelo4
Col-0	pAMpAT35SSGW	Alelo5
Col-0	pAMpAT35SSGW	Alelo6
Col-0	pAMpAT35SSGW	Alelo7
Complementación mutantes nockout en genotipo Col-0		
npr1_1	pAMpAT35SSGW	Alelos 1 a 7 y vector vacío
npr3_1	pAMpAT35SSGW	Alelos 1 a 7 y vector vacío
npr4_3	pAMpAT35SSGW	Alelos 1 a 7 y vector vacío
npr3_1/npr4_3	pAMpAT35SSGW	Alelos 1 a 7 y vector vacío
Silenciamiento RNA interferente		
Col-0	pFRN	vector vacío
Col-0	pFRN	fragmento RNAi

En esta tesis nos concentramos en caracterizar primero aquellas líneas transgénicas control pK70 (vector vacío), pK71 y pK72 que portaban los transgenes FaNPR31-Alelo-1 y FaNPR31-Alelo-2, respectivamente. Las líneas se autofecundaron y seleccionaron hasta la cuarta generación, para asegurar que todas eran homocigotas para la inserción del transgén. Mediante PCR con oligos específicos sobre ADN genómico, se comprobó que las líneas transgénicas contenían el transgén FaNPR31 insertado en su genoma (Figura 21). De esta manera se identificaron entre 7 y 15 líneas independientes transformadas para cada evento de transformación.

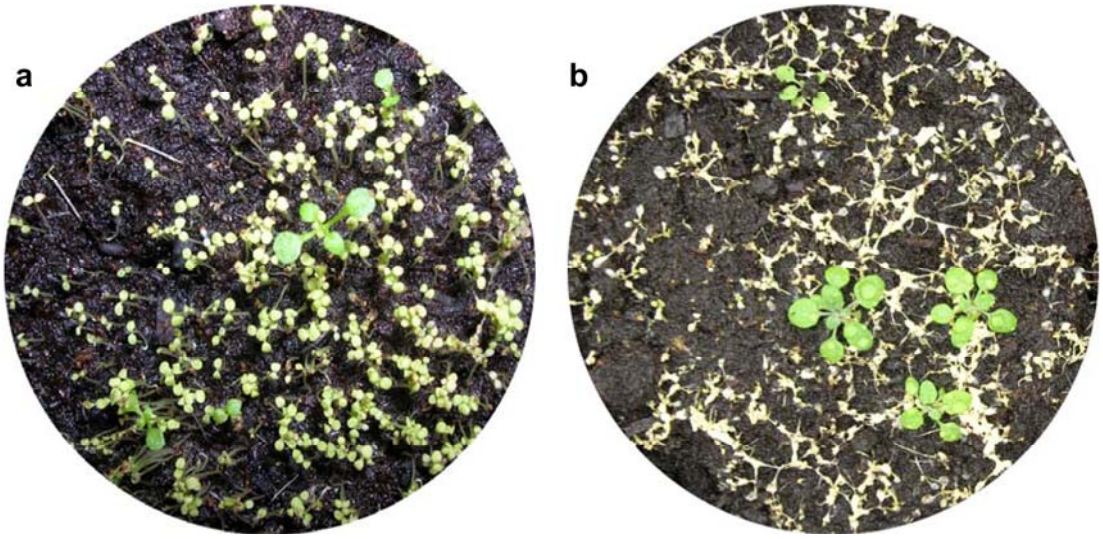


Figura 20. Selección de plantas de Arabidopsis transformadas por aplicación de 300µM de fosfinotricina. (a) 15 días tras la primera aplicación de fosfinotricina, las plantas no transformadas comienzan a amarillear, y (b) 20 días tras la primera aplicación, las plantas no transformadas han muerto, y las líneas transgénicas son fácilmente reconocibles.

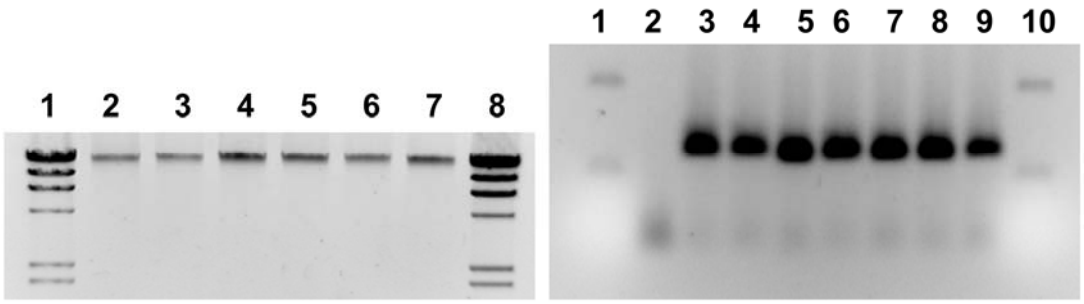


Figura 21. Purificación de DNA genómico de las líneas independientes de Arabidopsis Col-0 silvestre transformadas con *FaNPR31*-Alelo2 (a) (1: Lambda DNA ladder, 2: Línea 1, 3: Línea 2, 4: Línea 3, 5: Línea 4, 6: Línea 5, 7: Línea 6, 8: Lambda DNA ladder), e identificación del transgén en esas líneas por PCR con oligos específicos (b) (1: bandas de 100bp y 200 bp de 1Kb Plus DNA ladder, 2: control negativo PCR, 3: Línea 1, 4: Línea 2, 5: Línea 3, 6: Línea 4, 7: Línea 5, 8: Línea 6, 9: control positivo PCR (pDNA), 10: bandas de 100bp y 200 bp de 1Kb Plus DNA ladder).

Ya que se ha demostrado que las plantas mutantes en el gen *npr1* son muy sensibles a SA exógeno y, contrariamente, tanto las plantas silvestres como las mutantes en el gen *npr4* son insensibles a este compuesto (Liu et al. 2005), se realizó un experimento de sensibilidad a SA (germinación en medio MS envenenado con SA) con las líneas transgénicas obtenidas.

Tabla 4. Diseño experimental de test sensibilidad a aplicación de SA exógeno de los genotipos de Arabidopsis transformados.

Experimento	*FaNPR31*	Línea	Medio MS sin SA	Medio MS con 0.4mM de SA
Sobreexpresión en Col-0 silvestre	No transform.	Wt		
	Alelo 1	1.5.1.1		
	Alelo 1	2.5.1.1		
	Alelo 1	3.1.1.1		
	Alelo 2	1.5.1.1		
	Alelo 2	4.6.1.1		
	Alelo 2	6.2.1.1		
Complementación de mutante npr1_1	No transform.	npr1_1		
	Alelo 1	1.5.1.1		
	Alelo 1	2.5.3.1		
	Alelo 1	3.4.2.1		
	Alelo 2	1.3.1.1		
	Alelo 2	2.4.1.1		
	Alelo 2	3.6.1.1		
Complementación del doble mutante npr3_1/npr4_3	No transform.	doble		
	Alelo 1	1.1.1.1		
	Alelo 1	2.3.1.1		
	Alelo 2	2.5.1.1		
	Alelo 2	3.1.1.1		
	Alelo 2	4.5.1.1		
Silenciamiento Col-0 silvestre	pFRN vector	1.2.1.1		
	pFRN vector	3.1.1.1		
	RNAi	3homo		
	RNAi	4homo		
	RNAi	11homo		

Así, las 25 líneas descritas en la Tabla 4 se expusieron a 0.4mM de SA para evaluar si el gen *Fanpr31* de fresa era capaz de revertir la sensibilidad en las plantas npr1_1 mutantes, o bien presentaban algún otro fenotipo en respuesta a este compuesto (ver Tabla 4 y Figura 22).

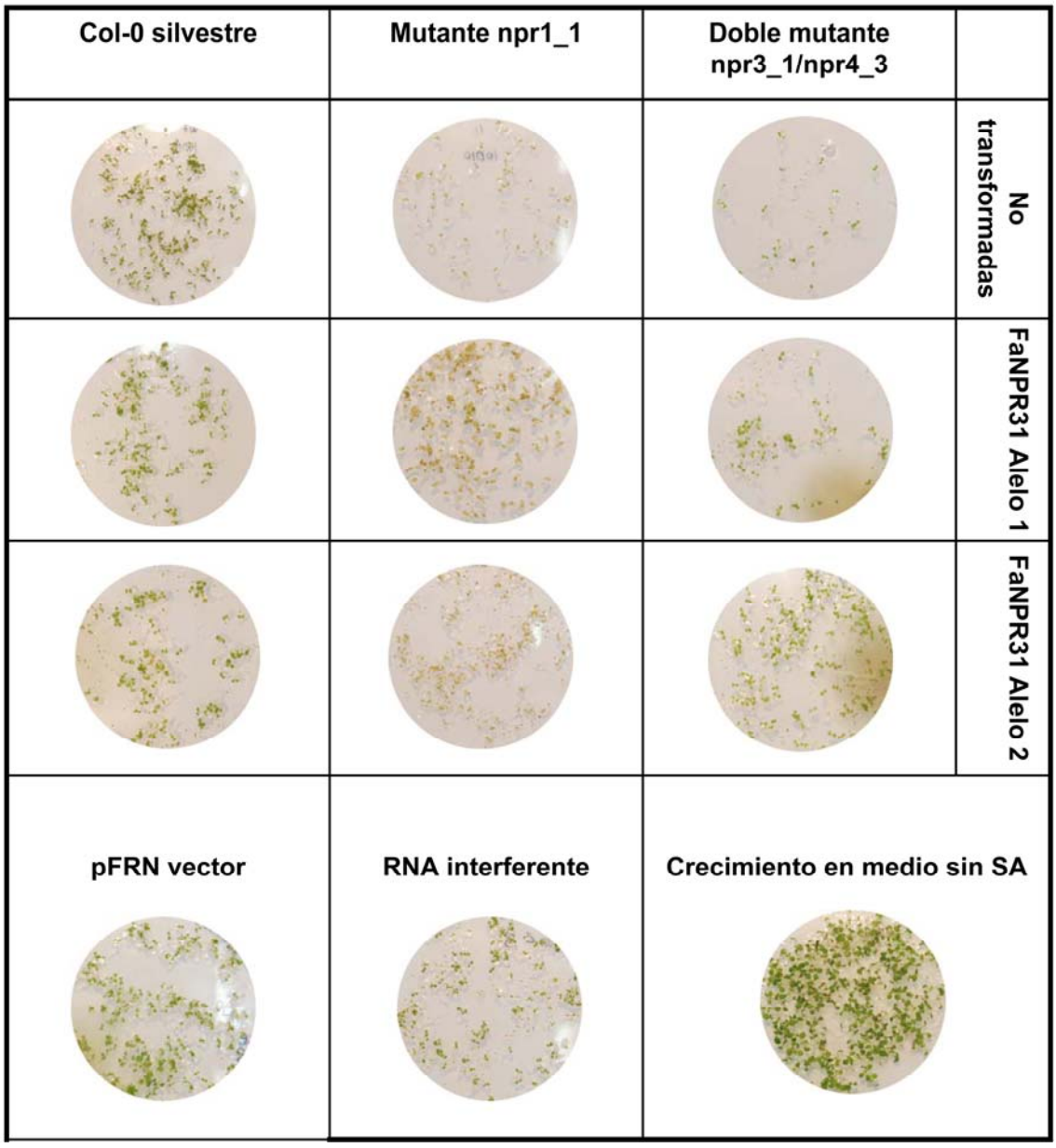


Figura 22. Sensibilidad a SA de las distintas líneas de Arabidopsis transformadas con *FaNPR31*. Se muestra una imagen como ejemplo de los resultados obtenidos para todas las líneas testadas de cada transformación (ver Tabla 4).

La concentración de 0.4mM de SA en el medio MS se había determinado previamente como la concentración más adecuada para identificar las diferencias entre Col-0 wild-type y el doble mutante npr3_1/npr4_3 (fueron insensibles) y el mutante npr1_1 (fue altamente sensible). El resultado obtenido mostró que FaNPR31 no logró complementar la mutación npr1_1, ya que las líneas transgénicas transformadas con el gen *FaNPR31* mostraron la misma sensibilidad al SA exógeno que las líneas control npr1_1 sin transformar. Además, tanto en las otras líneas transgénicas de sobreexpresión o complementación utilizadas, como en aquellas silenciadas, no se apreció ningún cambio fenotípico relacionado con diferencias en la sensibilidad a SA. En conjunto, todos estos resultados sugieren que, pese a presentar similitud de secuencia con AtNPR1, AtNPR3 y AtNPR4, el gen *FaNPR31* debe ser el ortólogo en fresa bien del gen *AtNPR3* o del *AtNPR4*, pero no del *AtNPR1*.

Expresión de la proteína FaNPR31 y caracterización molecular del gen *FaNPR31* mediante otras aproximaciones moleculares

Se ha intentado expresar la proteína FaNPR31 en *E. coli*. Así, mediante el uso del vector pET28a+ (Novagen) y las células *E. coli* Rossetta Gami 2 (DE3) se procedió a expresar un fragmento que codificaba para los primeros 250aa de la proteína FaNPR31. Esta región de FaNPR31 contiene el dominio de interacción proteína-proteína de tipo BTB, que se ha descrito como el responsable de la polimerización de NPR1 en Arabidopsis. Así, se realizaron construcciones con los alelos 2, 4, 6 y 7. También se realizaron construcciones con los tres genes de Arabidopsis (*NPR1, NPR3* y *NPR4*) que se han descrito están relacionados con el control de la SAR. Una vez que la proteína parcial fue expresada y purificada, una alícuota fue incubada en presencia de DTT (agente reductor), y se procedió realizar un western blot con anticuerpos policlonales anti-AtNPR1 (Figura 23). Los anticuerpos policlonales anti-AtNPR1 reconocieron la proteína de fresa y se comprobó como, al igual que ocurre con la proteína NPR1 de Arabidopsis, FaNPR31 puede formar dímeros y polímeros, y monomeriza en ambiente reductor.

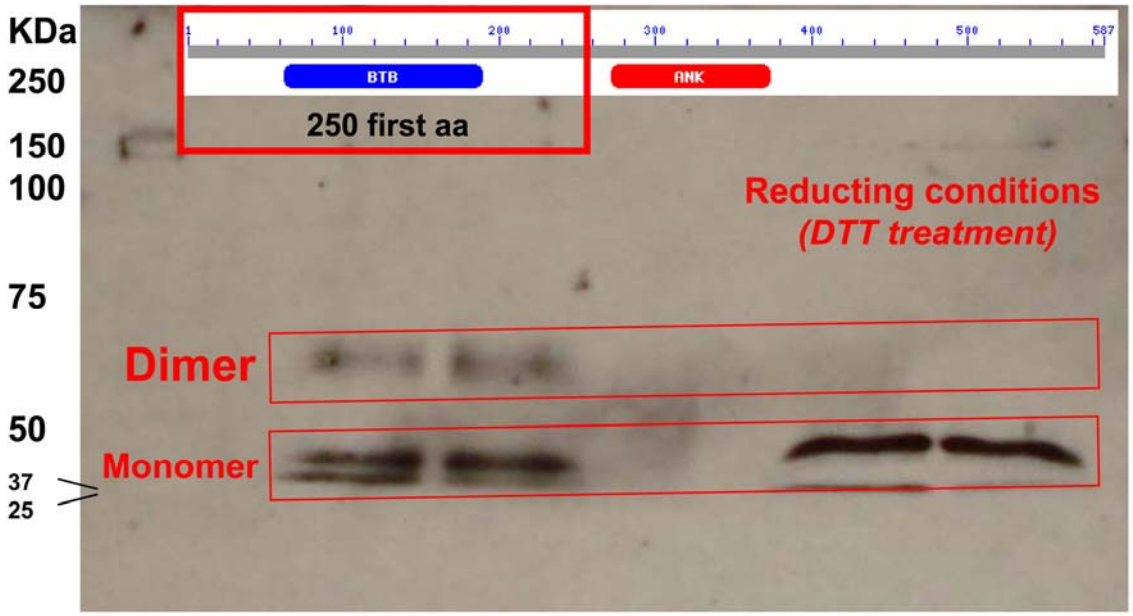


Figura 23. Expresión de la proteína parcial FaNPR31/dominio BTB. Los anticuerpos policlonales anti-AtNPR1 reconocieron la proteína de fresa. Al igual que ocurre con la proteína de Arabidopsis, FaNPR31 puede formar dímeros y polímeros, y monomeriza en ambiente reductor.

Por otro lado, se están llevando a cabo otras aproximaciones para obtener más información acerca de la posible función gen *FaNPR31*. Así, ya que NPR1 se localiza normalmente en citoplasma en forma de polímero y en respuesta a SA se monomeriza y es transportado hasta el núcleo, donde realiza funciones de regulación de la transcripción, se ha generado una construcción del gen FaNPR31-Alelo-2 fusionado a GFP (vectores pK7FWG2.0 y pK7WGF2.0) con objeto de visualizar la localización de esta proteína de fresa en los espacios celulares. Además, se están generando construcciones para realizar ensayos de doble híbrido de levadura, con los vectores pGBKT7 y pGADT7, para demostrar las posibles interacciones de los dominios identificados en la proteína FaNPR31 con los factores de transcripción de tipo TGA que interaccionan con NPR1 en Arabidopsis, y un factor TGA identificado en nuestra colección de secuencias de fresa (Figura 24).

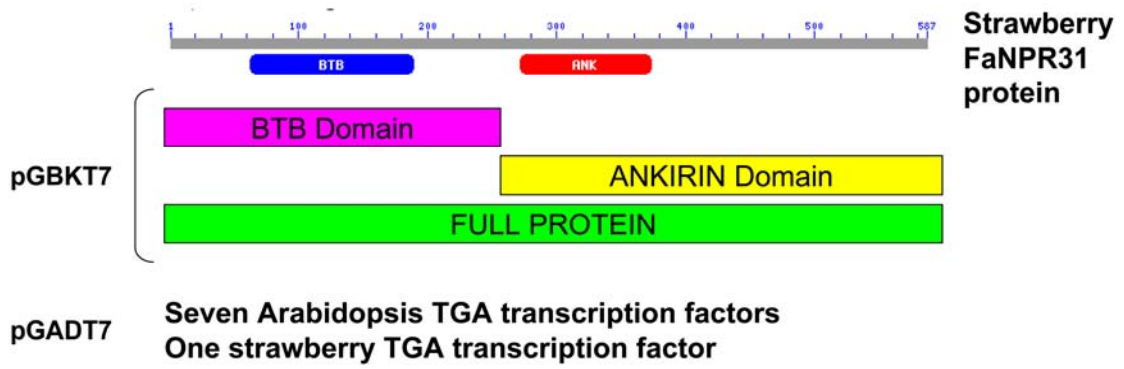


Figura 24. Esquema de las construcciones que se están generando para realizar los ensayos de doble híbrido en levadura. La secuencia completa de *FaNPR31*, así como las regiones que contienen los dominios BTB y Ankirin independientemente (como se muestra en la figura) se han clonado en el vector pGBKT7. Los posibles candidatos a interaccionar con los dominos mencionados antes (los 7 TGAs de Arabidopsis y uno de fresa) se han clonado en el vector pGADT7.

Conclusión

Los resultados obtenidos sugieren que el gen de fresa *FaNPR31* podría ser bien el ortólogo del gen NPR3 o del NPR4 de Arabidopsis, con los que comparte un nivel de semejanza elevado a nivel de secuencia. Además, también se regula formando estructuras poliméricas en el citoplasma, para monomerizar en respuesta a una señal redox.

Sección II: Identificación y caracterización parcial de los restantes componentes de la familia NPR1-like en fresa: genes *FaNPR1, FaNPR32, FaNPR33* y *FaNPR5*

La secuenciación y reciente publicación del genoma de la especie silvestre de fresa (*F. vesca*, Shulaev et al. 2011), nos ha permitido la búsqueda de otros componentes de la familia NPR1-like en esta planta. Así, utilizando la secuencia del gen *FaNPR31*, que hemos obtenido en la sección anterior, se llevó a cabo una búsqueda, mediante tBlastx, entre la colección de transcritos predichos para la especie *F. vesca*. Siete secuencias de *F. vesca* resultaron muy similares a *FaNPR31*, de las cuales, 5 estaban anotadas como NPR1-like (ver resultado tBlastx en Tabla S5, Figura 25). Una comparación por alineamiento múltiple y filogenético de los 5 miembros de la familia NPR1-like mostró que se pueden identificar tres grupos, por su similitud con los correspondientes ortólogos de Arabidopsis (Figura 25c). Así, el grupo 1, compuesto por el gen *FvNPR1* (gene12668) presentó alta semejanza con *AtNPR1* y *AtNPR2*; el grupo 2, compuesto por los genes *FvNPR31* (gene20070), *FvNPR32* (gene28770) y *FvNPR33* (gene28768), fue muy similar a *AtNPR3* y *AtNPR4*; y el grupo 3, compuesto por *FvNPR5* (gene21905), fue muy similar a *AtNPR5* y *AtNPR6*. Curiosamente, dos de los miembros del grupo 2 (*FvNPR32* (gene28770) y *FvNPR33* (gene28768)) se encuentran separados por una pequeña zona de 2.43Kb en el genoma de *F. vesca*. Esta proximidad entre ambos genes, además de su alta identidad de secuencia (76.6% de identidad, Figura S2), podría indicar que se trata de una duplicación génica en tándem.

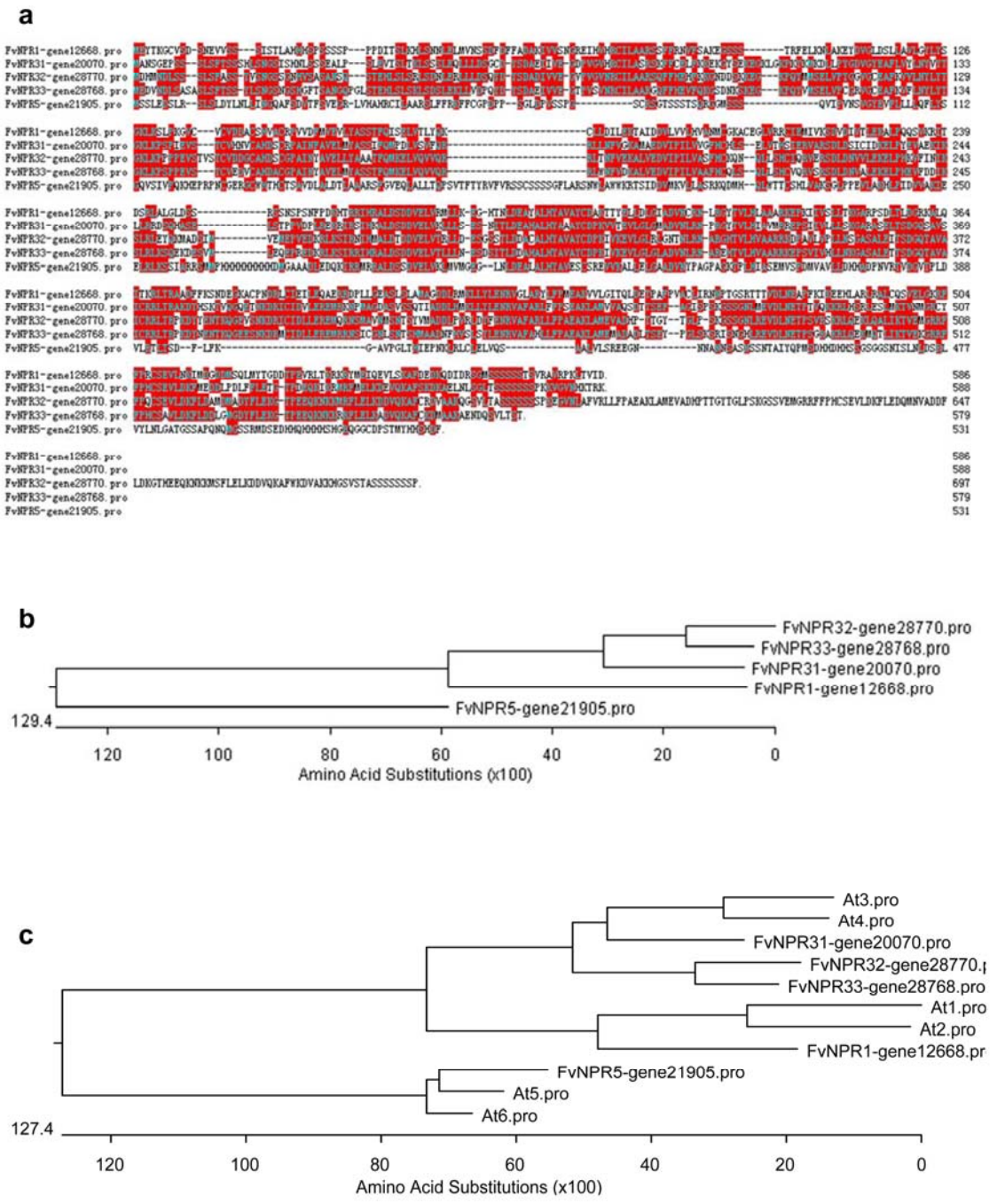


Figura 25. Alineamiento múltiple (a) y phenograma (b) de los 5 miembros de la familia NPR1 identificados en *F. vesca*. (c) Phenograma de los 5 miembros de *F. vesca* y los 6 miembros de Arabidopsis. La comparación se realizó utilizando el programa MEGALIGN ("ClustalW Method") del software Lasergene Navigator.

Clonación de los genes *FaNPR1, FaNPR32, FaNPR33* y *FaNPR5* de *F* × *ananassa* cv. Camarosa

Con esta información se diseñaron oligos (FaNPR32Fw, FaNPR32Rv, FaNPR33Fw, FaNPR33Rv, FaNPR1Fw, FaNPR1Rv, FaNPR5Fw, FaNPR5Rv; ver *Material y Métodos*) flanqueando las regiones codificantes de los 4 nuevos miembros de la familia NPR1-like, que se utilizaron para amplificar ADNc a partir de ARN aislado del cultivar Camarosa. Los productos de amplificación se clonaron, posteriormente en vectores de almacenamiento tipo pGemTEasy (Promega).

En el caso de *FaNPR1*, se han identificado diferencias de secuencia en 6 bases, que se traducen en 6 aminoácidos distintos, con respecto a la secuencia de su ortólogo en la fresa silvestre, *FvNPR1* (Figura 26ab). La comparación de FaNPR1 con su ortólogo en Arabidopsis mostró la conservación de dos regiones ricas en Ser, así como de los resíduos Cys implicados en la oligomerización, y la regulación por NO descrita para AtNPR1 (Figura 26c).

La clonación y secuenciación de *FaNPR32* permitió identificar diferencias puntuales de secuencia además de la inclusión de un triplete completo en la misma, con respecto a la correspondiente secuencia del ortólogo de *F. vesca* (Figura 27ab). Sin embargo, la mayor diferencia detectada entre ambos genes radica en el extremo carboxilo, de manera que FvNPR32 presenta una región extra adicional que se asemeja a una repetición de los exones E3 y E4 (Figura 27cd). Así, la proteína FvNPR32 de *F. vesca* podría tener una estructura muy distinta a la de su ortóloga de FaNPR32 de *F* × *ananassa*. Alternativamente, esta estructura podría ser la consecuencia de un error de ensamblaje o predicción en el genoma de *F. vesca*. Por otro lado, caso de no serlo, la estructura génica podría indicar que en *F. vesca*, el gen *FvNPR32* podría estar regulado vía "splicing" alternativo, dando lugar a dos proteínas distintas en función de qué zona se mantiene en el extremo carboxilo terminal.

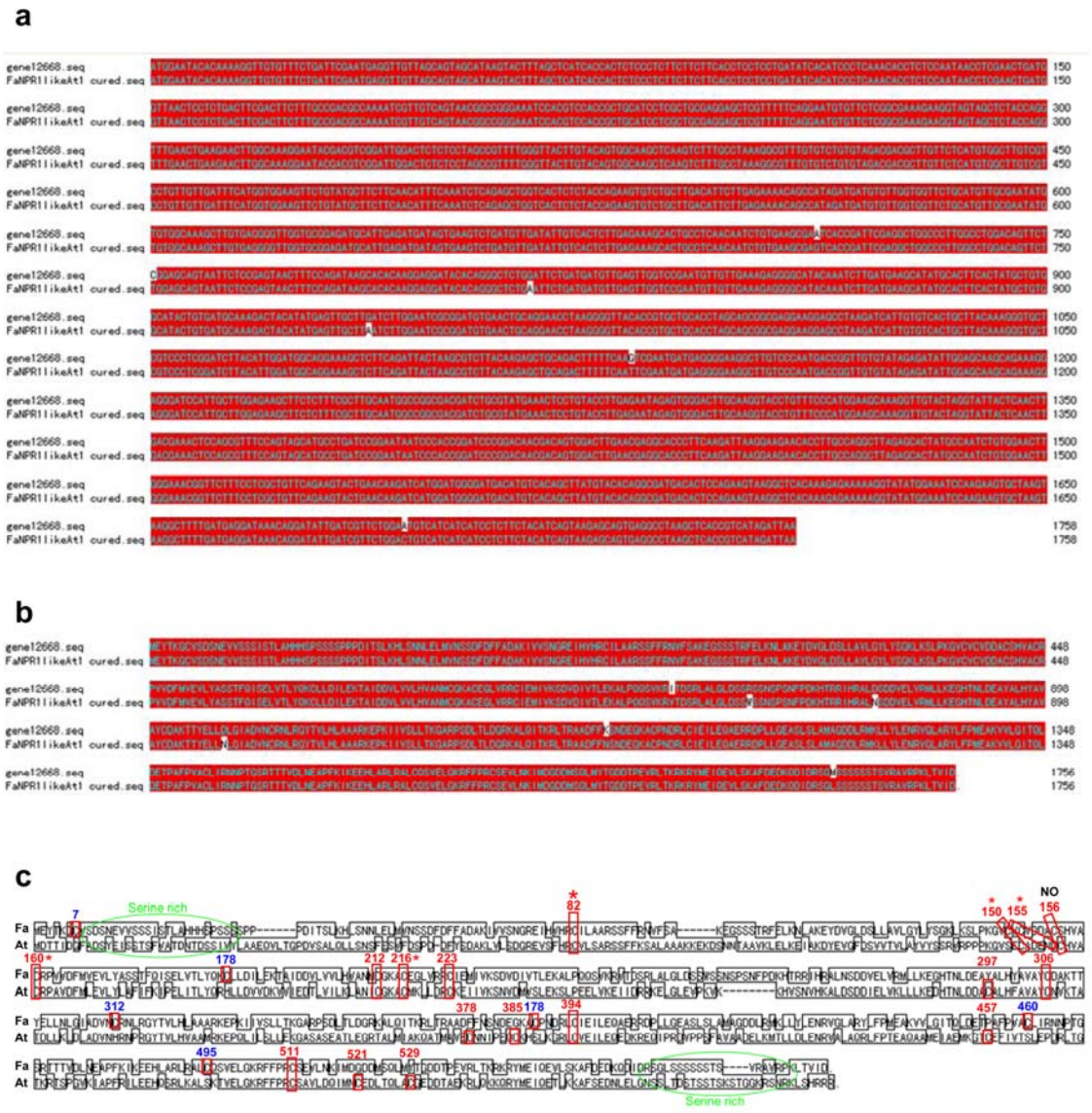


Figura 26. Alineamiento de los genes *FaNPR1* y *FvNPR1*: (a) secuencias nucleotídicas, (b) secuencias aminoacídicas deducidas. Localización de los residuos conservados en FaNPR1 descritos en la regulación postraduccional de AtNPR1 (c). Se enmarcan en verde dos zonas ricas en Ser, y en rojo los residuos de Cys. Marcados con asterisco los residuos de Cys implicados en la oligomerización, y "NO" indica donde se produce la regulación por óxido nítrico, tal como se han descrito en Arabidopsis. En azul, coordenada respecto FaNPR1, y en rojo, coordenada respecto AtNPR1.

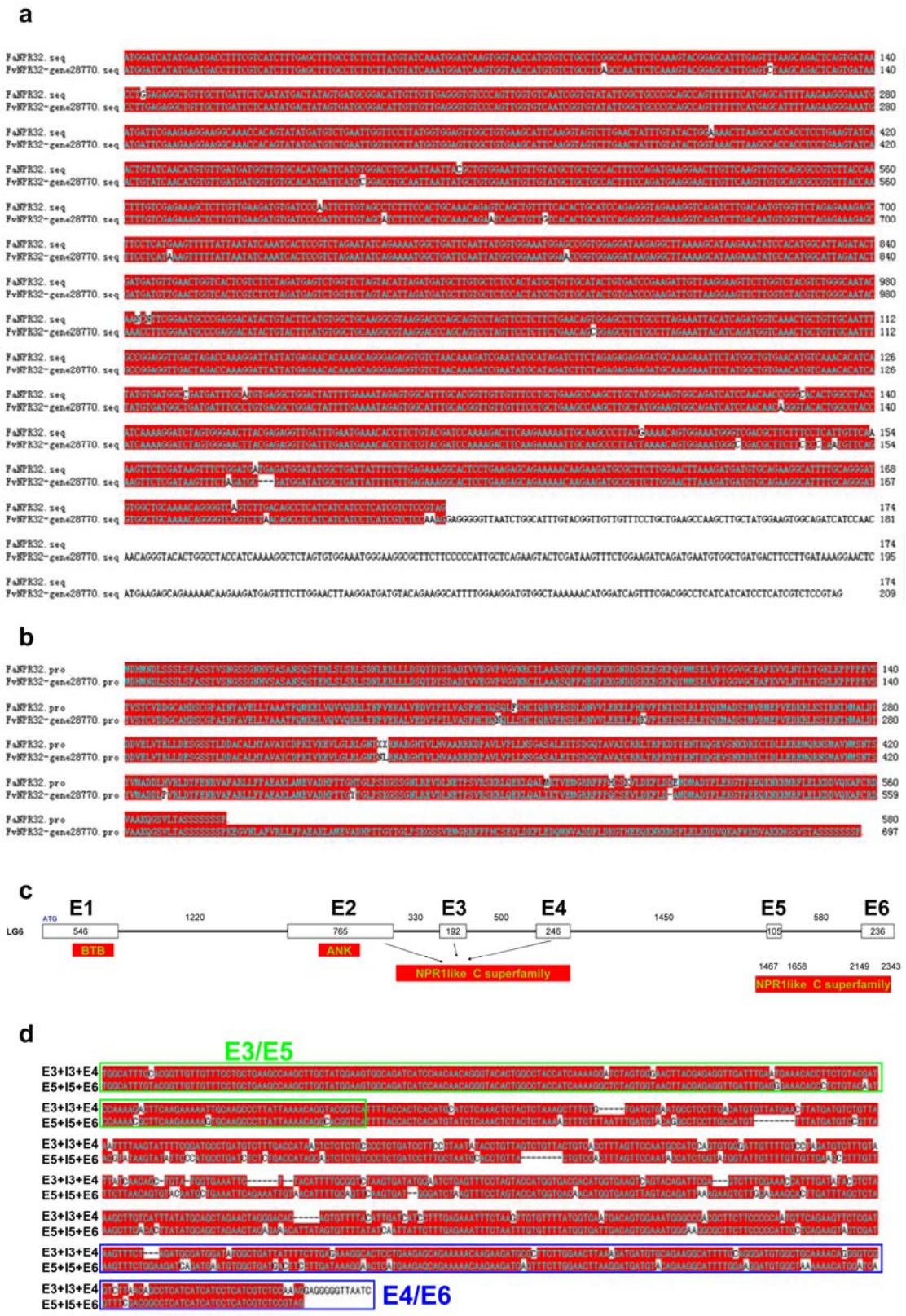


Figura 27. Alineamiento de los genes *FaNPR32* y *FvNPR32*: (a) secuencias nucleotídicas, (b) secuencias aminoacídicas deducidas. Arquitectura génica de *FvNPR32* (c). Se indican los dominios BTB, Ankirin y NPR1like conservados en la proteína y los exones que los codifican, y se muestra la similitud los exones 3 y 4 con los exones 5 y 6 (d).

Por último, *FaNPR5* también presentó algunas diferencias de secuencia puntuales con respecto a *FvNPR5*. Además, *FaNPR5* carece de un fragmento concreto localizado en la parte central de *FvNPR5* (Figura 28a). Curiosamente, este fragmento de ADN lleva información que codifica una región de aminoácidos rica en Ser, que como ya se ha comentado antes, son regiones muy presentes en todos los miembros de esta familia susceptibles de ser modificadas post-traduccionalmente (Figura 28b). Esta diferencia en la estructura de las proteínas NPR5 de las dos especies de fresa podría indicar distinta regulación en algunas funciones relacionadas con la producción de meristemo floral o morfología de las hojas, en las que se ha descrito que participa este factor (Hepworth et al. 2005, Ha et al. 2007).

La clonación y secuenciación de *FaNPR33* se está llevando a cabo actualmente.

Actualmente se está procediendo a realizar una caracterización exhaustiva del comportamiento transcriptómico de todos estos miembros de la familia NPR1-like en fresa en respuesta a distintos tratamientos bióticos y abióticos.

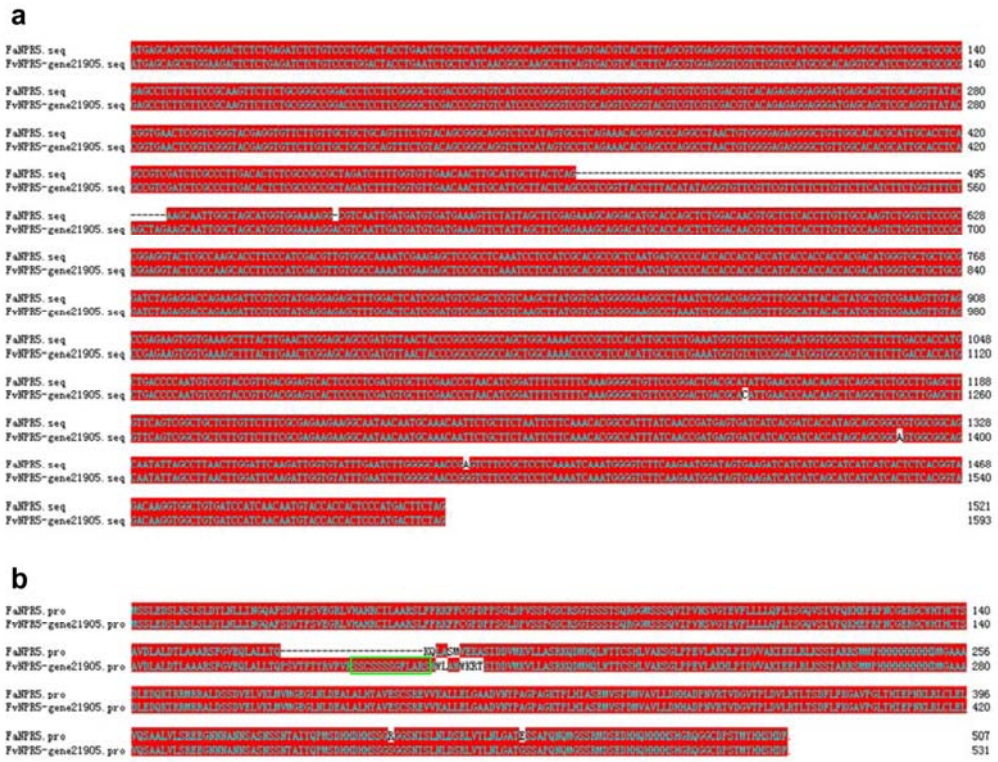


Figura 28. Alineamiento de los genes *FaNPR5* y *FvNPR5*: (a) secuencias nucleotídicas, (b) secuencias aminoacídicas deducidas. Se ha encuadrado la zona rica en Ser exclusiva de la proteína FvNPR5.

MATERIAL Y MÉTODOS

Escrutinio de genoteca genómica (Lambda Fix® II/ XhoI Partial Fill-In, Stratagene) con sonda radiactiva (escrutinios primario y secundario)

Los medios de cultivo, cepas bacterianas y condiciones de infección e incubación de las infecciones se realizaron como se describe en el manual comercial (http://www.genomics.agilent.com/files/Manual/248211.pdf). Para el escrutinio primario, se usaron 10 placas de petri (150mm diámetro) conteniendo 2×10^4 pfu/placa de la genoteca original. Se transfirieron los fagos a una membrana de nylon Hybond^{TM}N+ (Amersham), que se trató para desnaturalizar las partículas de fago y fijar el ADN a la misma con luz UV (Stratalinker, Stratagene) siguiendo el proceso descrito en el manual comercial. Las 10 membranas se pre-hibridaron 2 horas a 42ºC en un horno giratorio con 20ml de solución ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambión). Transcurrido este tiempo, se añadió la sonda marcada radiactivamente (como se describe a continuación) y se hibridó durante 14 horas a la misma temperatura.

El marcaje de la sonda se llevó a cabo usando el fragmento completo de la EST CUI5_T_396, extraído del vector (pGEMTEasy) por digestión (EcoRI), y purificado a partir de gel de agarosa (QIAquick Gel Extraction Kit, Qiagen). El ^{32P}dCTP se incorporó a 25ng de sonda usando el kit Megaprime DNA Labelling System (Amersham, GE), y siguiendo las recomendaciones del manual (https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314807262343/litdoc25006068AD_20110831205841.pdf). Una vez terminada la reacción de marcaje, la sonda se purificó por columna (Bio-Spin Columns P-30 in Tris Buffer, Bio-Rad) para eliminar los restos de radiación no incorporados, primers, enzima y sales de la reacción. La sonda marcada y purificada se desnaturalizó a 100ºC, 5 minutos y se mantuvo en hielo hasta su uso inmediato.

Para eliminar el exceso de radiactividad unida a la membrana de forma inespecífica, después de retirar la solución de hibridación, las membranas se lavaron tres veces con una solución de 2xSSC/0.1%SDS (100ml a 65ºC) y dos veces más con una solución de

mayor astringencia (0.1xSSC/0.1%SDS). A continuación, las membranas se envolvieron en plástico transparente y se expusieron a películas de revelado de rayos X (Kodak Scientific Imaging Film, BIOMAX) y se dejaron impresionar durante 1-2 semanas. La manipulación de las películas de rayos X se realizó siempre en una cámara oscura para evitar su velado. Para el revelado de las películas se sumergieron en líquido revelador (2 minutos) y fijador (5 minutos) AGFA, y posteriormente se lavaron en agua destilada (15 minutos), para finalmente dejarlas secar al aire.

Con las películas impresionadas por los fagos positivos que han hibridado, se procedió a localizarlos en las placas que originalmente se utilizaron para generar las membranas. Una vez localizados, los fagos positivos se extrajeron de la placa y se almacenaron individualmente en tubos eppendorf conteniendo 1 ml de tampón SM (Tris-HCl (50mM pH 7,5), NaCl (100mM), $MgSO_4$ (10mM), Gelatina (0,1% p/v).

El mismo procedimiento se desempeñó para el escrutinio secundario, en donde usaron los fagos obtenidos en el escrutinio primario que se sembraron a una menor densidad (2000 pfu/placa).

Escrutinio de genoteca genómica por PCR (escrutinio terciario). Purificación y secuenciación de ADN de fagos recombinantes

Para determinar qué fagos recombinantes seleccionados en el escrutinio secundario portaban el gen de interés, se sembraron a muy baja densidad (20-40 pfu/placa). De cada muestra de fago recombinantes seleccionados en el escrutinio secundario se aislaron entre 10 y 15 halos terciarios bien aislados del resto. Alícuota de 1µl de cada uno de los fagos terciarios así aislados se utilizaron para realizar una PCR, con los oligos específicos 396A y 396B (Tabla 5). Los fagos recombinantes que dieron positivos se guardaron a 4ºC.

Para purificar el ADN genómico de los fagos positivos se utilizó el kit Qiagen® Lambda Midikit (Qiagen), siguiendo estrictamente las indicaciones del manual comercial (http://www.ebiotrade.com/buyf/productsf/qiagen/1011142_lambda.pdf). El

ADN del fago recombinante así obtenido, se utilizó para obtener la secuencia del gen mediante secuenciación secuencial con oligos diseñados a partir de las secuencias previas (unidad genómica de SCAI, University of Córdoba (http://www.uco.es/servicios/scai/index.html).

Tabla 5. Secuencia de los oligos usados en este capítulo.

ID	Secuencia Orientada 5´ - 3´	Cadena	Función
396A	TCATATTGCTGTGATGCGCAGAGAGCCA	sentido	Escrutino terciario genoteca genómica
396B	CGGTCCTTGTTAGTTTCTTGCCCCTGCAC	antisentido	
FaNPR31Fw	ACTGTAAAGTAGATTAATGGCGA	sentido	Clonar secuencia codificante completa
FaNPR31Rv	TACAATTTACATGCCTAAACTAT	antisentido	
FaNPR32Fw	ATGGATCATATGAATGACCTTTCGTCATCTTTGA	sentido	
FaNPR32Rv	CAGCTTGGCCTTTTCCTAACCTTACGAT	antisentido	
FaNPR33Fw	ATGGAGGATGTGAATGATCTGTCTGCTTCGGCT	sentido	
FaNPR33Rv	TCATGTGGATGTCAAAACAGACTGGTCATTTTCA	antisentido	
FaNPR1Fw	ATGGAATACACAAAAGGTTGTGTTTCTG	sentido	
FaNPR1Rv	TTAATCTATGACGGTGAGCTTAGGCCTC	antisentido	
FaNPR5Fw	ATGAGCAGCCTGGAAGACTCTCTGA	sentido	
FaNPR5Rv	CTAGAAGTCATGGGAGTGGTGGTACATT	antisentido	
iQFaNPR31Fw	ATAAGTTTATGGAGGATGACCTGCCT	sentido	Cuantificar expresión génica por RTqPCR
iQFaNPR31Rv	CTATTTTCTAGTCTTGTGATTTACAC	antisentido	
iQutrFaNPR31Fw	AAATAGTGTGGCTGTCGTCTGTAATATC	sentido	
iQutrFaNPR31Rv	AGAGCGCAAATTGATTATGTATGAGTAT	antisentido	
iQpk7FaNPR31Rv	CACCACTTTGTACAAGAAAGCTGGGT	antisentido	
FaN31pENT-Fw	CACCATGGCGAATTCAGGTGAGCC	sentido	Construcción Gateway en pENT
FaN31pENT-Rv	TTTCTAGTCTTGTGATTTACAC	antisentido	
RNAiFaN31Fw	AGGCATTAGACTCGGATGATG	sentido	Construcción silenciamiento RNAi
RNAiFaN31Rv	CATTGGATTCTTCCGCATT	antisentido	

Obtención de la secuencia codificante completa de los 5 miembros de la familia NPR1-like de fresa

A partir de una muestra de ARN total obtenida del tejido de corona de *F* × *ananassa* cv. Camarosa tras 5 días de infección con el hongo *C. acutatum*, se realizó una reacción de RT-PCR utilizando para la reacción de amplificación los cebadores específicos diseñados para amplificar la zona codificante completa (Tabla 5), así como una Taq Polimerasa de "alta fidelidad" (Platinum® Taq DNA Polymerase High Fidelity, Invitrogen). El producto de la amplificación se fraccionó en un gel de agarosa y la banda de ADN de tamaño correspondiente a los fragmentos de ADNc amplificados se

purificó a partir del gel. Los ADNc se subclonaron en el vector pGEM-T Easy y las construcciones resultantes se utilizaron para transformar células de *E. coli* DH5α. Los diferentes clones positivos se seleccionaron sembrando estas últimas en placas de petri con medio LB-Amp-IPTG-XGal. Se seleccionaron al azar clones positivos y se obtuvo la secuencia de sus insertos de ADNc utilizando los cebadores universales T7 y SP6.

Obtención de construcciones gateway y transformación de plantas de fresa y Arabidopsis

Los 7 alelos del gen *FaNPR31* se amplificaron (KOD Hot Start DNA Polymerase, Novagen) usando oligos específicos que permitieron el clonaje direccional en el vector pENTR/D/TOPO (Invitrogen) gateway compatible, utilizando los primers FaN31pENT-Fw y FaN31pENT-Rv (Tabla 5) y siguiendo el protocolo descrito en el manual (http://tools.invitrogen.com/content/sfs/manuals/pentr_dtopo_man.pdf). Una vez comprobadas por secuenciación, se realizaron las reacciones pertinentes de LR Clonase (Invitrogen) para cada alelo, siguiendo el manual comercial (http://tools.invitrogen.com/content/sfs/manuals/lr_clonase_man.pdf), para generar la construcción en los vectores pK7WG2.0 (para transformar fresa) y pAMpAT35SSGW-AY436765 (para transformar Arabidopsis). Estas construcciones fueron transferidas finalmente a las cepas de *Agrobacterium tumefaciens* GV3101pMP90 (pK7WG2.0) y GV3101pMP90RK (pAMpAT35SSGW).

Para generar la construcción de silenciamiento basada en RNA interferente, un fragmento de 407bp de *FaNPR31* (flanqueado por los oligos RNAiFaN31Fw y RNAiFaN31Rv, Tabla 5) se clonó en el vector pCR8/GW/TOPO (Invitrogen) siguiendo el manual comercial (http://tools.invitrogen.com/content/sfs/manuals/pcr8gwtopo_man.pdf). Posteriormente, esta construcción se utilizó para realizar la reacción LR Clonasa (Invitrogen) con el vector de expresión binario pFRN (derivado de pFGC5941), el cual posee dos regiones de clonación delimitadas por extremos attR para la inserción del ADN de interés en las dos orientaciones opuestas (sentido y antisentido) y separadas por un intrón. Una vez comprobado por secuenciación que la construcción y el inserto eran

correctos, se transformaron células competentes de *A. tumefaciens* LBA4404. Una transformación con el vector pFRN vacío se utilizó como control negativo.

Las cepas de Agrobacterium portadoras de todas las construcciones fueron utilizadas para transformar los diferentes genotipos mutantes de Arabidopsis utilizados (Tabla 3) siguiendo las instrucciones descritas en Clough and Bent (1998) (Clough and Bent 1998), o plantas de fresa cv. Camarosa, con la metodología descrita por Barceló et al. (1998) (Barceló et al. 1998).

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Conclusions

- **1.** A high proportion of genes related with defense response to *Colletotrichum acutatum* are present in our strawberry EST collection.

- **2.** *FaRIB413*, *FaACTIN, FaEF1α* and *FaGAPDH2* are strongly recommended as superior reference genes for relative quantification of gene expression in strawberry (*Fragaria* × *ananassa*) challenged with *C. acutatum*, as well as in SA and MeJA plant treatments, and fruit ripening and senescence studies.

- **3.** A higher basal level of SA in strawberry might produce a lower susceptibility to *C. acutatum*.

- **4.** Strawberry (*F* × *ananassa*) cv Camarosa is able to increase free SA and JA levels, and activate known components of both SA and JA plant signaling defense pathways upon *C. acutatum* infection.

- **5.** Negative crosstalk between SA and JA signaling defense pathways benefit the spread of this pathogen in strawberry.

- **6.** Important orthologous WRKY transcription factors such as *FaWRKY70* and *FaWRKY33*, might mediate the crosstalk between SA and JA signaling pathways as seen in model plants, and act as important key factors to control defense response in strawberry.

- **7.** At least five members of the NPR1-like family of plant genes are present in the strawberry (*F* × *ananassa*) genome.

Conclusiones

- **1.** Una alta proporción de genes relacionados con los mecanismos de de la respuesta de defensa frente a *C. acutatum* están presentes en nuestra colección de ESTs.

- **2.** *FaRIB413*, *FaACTIN, FaEF1α* y *FaGAPDH2* son fuertemente recomendados como genes de referencia óptimos para la cuantificación relativa de expresión génica en fresa (*Fragaria* × *ananassa*) inoculada con *C. acutatum*, así como en plantas tratadas con SA y MeJA, y estudios de maduración y senescencia de fruto.

- **3.** Un mayor nivel basal de SA en fresa podría producir una menor susceptibilidad a *C. acutatum*.

- **4.** El cultivar de fresa (*F* × *ananassa*) Camarosa es capaz de incrementar los niveles de SA y JA libre, y activar conocidos componentes de rutas de señalización de defensa dependientes de ambos, SA y JA, en respuesta a infección por *C. acutatum*.

- **5.** La regulación mutua negativa entre las rutas de señalización mediadas por SA y JA beneficia la dispersión de este patógeno en fresa.

- **6.** Los genes *FaWRKY70* y *FaWRKY33*, ortólogos a importantes factores de transcripción de tipo WRKY, podrían mediar en la regulación mutua entre las rutas de señalización dependientes de SA y JA, como se ha mostrado en otras plantas modelo, y actuar como importantes factores clave en el control de la respuesta de defensa en fresa.

- **7.** Al menos cinco miembros de la familia de genes de plantas *NPR1-like* están presentes en el genoma de la fresa (*F* × *ananassa*).