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Molecular aspects in pathogen-fruit interactions: Virulence and resistance

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ABSTRACT

Fruit losses during postharvest storage and handling due to pathogen infections are one of the major problems in the global food chain supply. The application of chemical fungicides to control diseases is currently limited by legislation in some countries and also raises concerns about food and environmental safety. Exploring molecular aspects of pathogen-fruit interactions therefore has biological and economic significance as a means to help develop rational alternatives for disease control. In this review we present the current knowledge of molecular aspects in pathogen-fruit interactions, addressing the following topics: the application of new "omics" technologies for studying these interactions; the molecular mechanisms of fungal pathogen attack; the regulation of virulence by exogenous factors; and, finally, fruit defense mechanisms.

1. Introduction

Postharvest diseases caused by fungal pathogens lead to huge economic losses worldwide every year. Currently, the use of synthetic fungicides constitutes the main means to control these diseases. However, the development of resistance in fungal pathogens to fungicides and the growing public concern over the health and environmental risks associated with high levels of pesticides in fruits have resulted in significant interest in developing alternative non-chemical methods of disease control (Mari et al., 2014). Furthermore, despite the application of fungicides and the increased use of new alternative strategies, fruits continue to be exposed to high infection pressure during production and commercial processing. In order to increase the arsenal of tools available to fight fruit decay we need to develop new approaches. In this context, the study of fruit-host interactions has gained increasing interest.

In the present review we have followed the nomenclature adopted by the American Phytopathological Society to define the terms pathogenicity and virulence (Sacristán and García-Arenal, 2008). Pathogenicity denotes the ability of a pathogen to cause disease on a particular host (a qualitative trait), whereas virulence is the degree of damage caused to the host (a quantitative trait). Virulence factors may be defined as those pathogen components that are non-essential for *in vitro* growth in a culture medium but contribute to disease. When one of these factors is required for pathogenicity it can be considered a pathogenicity factor. As virulence factors are important for infection, preventing pathogens from producing them constitutes an interesting alternative strategy for disease control. This strategy has been termed "antivirulence therapy" (Cegelski et al., 2008). As this therapy aims to disarm the pathogen rather than kill it or halt its growth, it is presumed that antivirulence compounds will generate much less selection pressure than traditional antibiotics in the pathogen to regain resistance, a problem faced with commonly used fungicides. However, before any antivirulence therapy can be developed there is a need to identify the virulence factors. In a broad sense, these factors can be as diverse as plant cell wall degrading enzymes (CWDEs), effectors (molecules that modify the physiology of the host in order to allow pathogen infection), or mechanisms that permit the rapid adaptation of the pathogen to the host environment. The regulatory systems that govern all these processes can also be considered as virulence factors. Most of the research on antivirulence factors has been conducted with human pathogenic bacteria, although research on preventing

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fungal infections by *Candida albicans* and *Aspergillus fumigatus* has already been actively explored (Cui et al., 2015).

It is well documented that in many instances the differences in the outcome of a host-pathogen interaction (whether it is a compatible interaction, *i.e.*, where disease occurs, or an incompatible/non-host interaction, *i.e.*, where disease does not occur) depend on a rapid and efficient deployment of defense responses (Ferreira et al., 2006). These defenses are complex and constitute a multilevel series of structural and biochemical barriers that are either constitutive, preformed, and/or inducible. A first line of defense is common against all potential pathogens and is triggered by pathogen-associated molecular patterns (PAMPs). When a successful pathogen is able to escape this defense, then a second defense line is deployed by the host, the so called effector-triggered immunity. Plants respond to pathogen attack even in compatible interactions, although this response is insufficient to avoid infection progress. One of the earliest responses detected in many incompatible interactions (*i.e.*, where the plant is resistant to the pathogen) is the activation of an oxidative burst characterized by an increase in the levels of reactive oxygen species (ROS) (Pitzschke et al., 2006). The oxidative burst precedes the synthesis of antimicrobial compounds like phytoalexins. This burst leads up to alterations in the synthesis of cell-wall structural proteins and the transcriptional activation of specific genes leading to the synthesis of pathogenesis-related (PR) proteins such as chitinases, β-1,3-glucanases and peroxidases (POD). Although these defense factors in host tissues have been extensively studied, the mechanisms that regulate these responses have not been elucidated.

The application of molecular genetics techniques has significantly changed the study of plant-pathogen interactions, enhancing scientists' ability to test hypotheses and thus provide new information on the biochemical mechanisms underlying these interactions, *i.e.*, pathogen virulence and host resistance (An et al., 2016; Frías et al., 2011; Schouten et al., 2008). Cloning and the characterization of crucial genes in several fungal pathogens that are related to virulence/pathogenicity are likely to lead to a deeper understanding of the molecular mechanisms underlying fruit disease susceptibility/resistance. The above mentioned facts justify the need to increase our knowledge of the mechanisms of pathogen virulence and host fruit defense. This knowledge may lead to the rational design of new and safer control strategies.

2. New technologies for studying host-pathogen interactions in postharvest fruit systems

During the last ten years we have witnessed an "omics" revolution that has involved all fields of biological sciences. This change is reflected in the way researchers now go about the study of biological processes, from a single gene/enzyme level to a holistic approach through which the researchers try to examine problems from a global point of view. Sequencing technology and its applications have expanded from the sequencing of individual genes to the assembly of complete genomes, from the analysis of the expression of a few genes to global transcriptomic analyses, and so on. However, the implementation of these new technologies in the field of postharvest pathology is lagging behind their uses in other closely related fields. "Omics" technologies are being used to investigate many fruit crops from other points of view, and the information gathered from those studies can be used in postharvest pathology. Fruit crop genomes that have been sequenced include grape (Jaillon et al., 2007); apple (Velasco et al., 2010); banana (D'Hont et al., 2012); citrus (Xu et al., 2013); peach (Verde et al., 2013); and pear (Chagné et al., 2014). The genomes of many fungal plant pathogens have also been sequenced in recent years. The fungal sequencing genome projects contained in MycoCosm (Grigoriev et al., 2013) (http://genome.jgi.doe.gov/pages/fungi-1000-projects.jsf) provide a picture of the rapid progress being made in this field. The genomes of several postharvest pathogens are now available, including *Botrytis cinerea* (Amselem et al., 2011) and several different species of *Alternaria* (Dang et al., 2015) and *Colletotrichum* (Gan et al., 2013). The genomes of the following specific postharvest fruit pathogens from the genus *Penicillium* have also been recently sequenced: *P. digitatum* (Marcet-Houben et al., 2012; Sun et al., 2011); *P. griseofulvum* (Banani et al., 2016); and *P. expansum* and *P. italicum* (Ballester et al., 2015; Li et al., 2015). There are also ongoing genome sequencing projects for other relevant postharvest fungi, such as *Monilinia fructicola* and *M. laxa*.

The genetic information gathered in these projects provides a foundation for an in-depth analysis of the virulence factors of these important postharvest pathogens. Data are available on the whole set of putative genes present in these pathogens and on how many of these genes are secreted, which ones are candidate effectors and the arsenal of carbohydrate-active enzymes (CAZymes). There is also data on the array of proteases that each fungus can produce. In order to decipher which genes are relevant to pathogenesis and virulence, however, more studies are needed. Genes involved in pathogenicity and virulence are actively expressed during the colonization of the host. A transcriptomic analysis of the pathogen-fruit interaction will thus reveal these genes, as well as other genes that are necessary for the normal growth of the fungus and are not necessarily related to pathogenicity/virulence. One advantage of directly analyzing the host/pathogen interaction is that information on host defense responses deployed in response to the fungal attack can also be obtained. The first approach used to analyze the fruit-pathogen interactome at the transcription level was based on sequencing ESTs (expressed sequence tags) (González-Candelas et al., 2010a) and on the identification of differentially expressed genes (Sánchez-Torres and González-Candelas, 2003). Other approaches that allow us to identify genes from either the fruit or the pathogen that are overexpressed during their interaction are based on the analysis of cDNA libraries using suppression subtractive hybridization (SSH) (Casado-Diaz et al., 2006; González-Candelas et al., 2010b; López-Pérez et al., 2015) or microarray hybridization (Guidarelli et al., 2011; Vilanova et al., 2014b). One advantage of the SSH approach is that it does not require previous knowledge of either the host or the pathogen, whereas a microarray can only be prepared once a large set of cDNAs from either interacting partners has been isolated. Recent advances in high-throughput sequencing technologies and decreasing costs have enabled small labs to conduct complex transcriptomic analyses using a whole transcriptome shot-gun sequencing approach (RNA-Seq). RNA-Seq, with its capability of producing millions of sequences from complex RNA samples, is displacing microarray analysis as the preferred tool for conducting global transcriptomic studies. Researchers have only recently begun to apply RNA-Seq in fruit-pathogen studies. Blanco-Ulate et al. (2014) investigated the expression of CAZymes-encoding genes in *B. cinerea* during its interaction with lettuce leaves, tomato fruit and grape berries, highlighting host-specific commonalities and differences. The interaction between apple fruit and *P. expansum* has also been recently studied using RNA-Seq to analyze fungal genes expressed during colonization at 24, 48 and 72 h post-inoculation (hpi) (Ballester et al., 2015). Several genes classically related to virulence were induced, such as proteases, CWDEs and oxidoreductases. These types of enzymes are typically encoded by genes that are part of large gene families. By sequencing the global RNA population, one can determine which genes are actually induced. It was thus found that an aspergillopepsin-encoding gene showed the highest expression level at just 24 hpi, and that different pectinase-encoding genes exhibited different patterns of regulation during the course of infection. RNA-Seq also made it possible to discover genes that may represent putative virulence/