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MINI-REVIEW

Scientifically advanced solutions for chestnut ink disease

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Abstract On the north regions of Portugal and Spain, the *Castanea sativa* Mill. culture is extremely important. The biggest productivity and yield break occurs due to the ink disease, the causal agent being the oomycete *Phytophthora cinnamomi*. This oomycete is also responsible for the decline of many other plant species in Europe and worldwide. *P. cinnamomi* and *Phytophthora cambivora* are considered, by the generality of the authors, as the *C. sativa* ink disease causal agents. Most *Phytophthora* species secrete large amounts of elicitins, a group of unique highly conserved proteins that are able to induce hypersensitive response (HR) and enhances plant defense responses in a systemic acquired resistance (SAR) manner against infection by different pathogens. Some other proteins involved in mechanisms of infection by *P. cinnamomi* were identified by our group: endo-1,3-beta-glucanase (complete cds); exo-glucanase (partial cds) responsible by adhesion, penetration, and colonization of host tissues; glucanase inhibitor protein (GIP) (complete cds) responsible by the suppression of host defense responses; necrosis-inducing *Phytophthora* protein 1 (NPP1) (partial cds); and transglutaminase (partial cds) which inducts defense responses and disease-like symptoms. In this mini-review, we present some scientifically advanced solutions that can contribute to the resolution of ink disease.

Keywords *Castanea sativa* · *Phytophthora cinnamomi* · Ink disease · Elicitins · Defense responses

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Introduction

The culture of the chestnut tree is extremely important in the northern region of Portugal and Spain, occupying a significant proportion of useful agricultural area. New plantation areas have increased in the last few decades. However, the ink disease caused by the oomycete *Phytophthora cinnamomi* has damage and killed many trees, and up to now, no solutions have been offered to control the illness. As a consequence, the disease propagation in the orchards of chestnut trees have been causing severe productivity and yield breaks. In addition to the economical losses, the importance of sociological and landscape aspects for the region cannot be neglected. Characterizing environmentally triggered gene expression changes provide insights into when and where each gene is expressed and offer a glimpse at the physiological response of the cells to changes in their surroundings. The critical question of how cells coordinate their gene expression with varying environments has only been answered for a limited number of pathways. Some of them are essential virulence factors that critically influence pathogenesis and contribute to the establishment of the fungal disease. Understanding the cellular response, the type of triggering stimuli, and the degree of crosstalk that exists among them is important to infer its role in plant diseases. Consequently, the focus of this systems biology-embedded project is on multidisciplinary research that will integrate a wide variety of biological data and develop and apply system biology approaches to understand basic biological processes relevant to plant pathogens at all levels of cellular organization and metabolism. The two main goals pursued are (a) to develop new models of oomycete infection and (b) to elucidate the function of genes and gene products involved in infection mechanisms and their interactions in complex networks. The oomycetes form a phylogenetically distinct group of eukaryotic microorganisms that includes some of the most notorious pathogens of plants. Among these, members of the genus *Phytophthora* cause

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enormous economic losses on crop species as well as environmental damage in natural ecosystems. *P. cinnamomi* is the most widely distributed *Phytophthora* species, with nearly 1,000 host species (Erwin and Ribeiro 1996; Judelson and Blanco 2005; Moreira and Martins 2005). Although they have a filamentous growth habit, oomycetes are distantly related to fungi and possess distinct mechanisms for pathogenicity. Consequently, fungicides rarely control them, and the few anti-oomycete products are often overcome by resistant pathogen variants. There are no eradication methods available to combat those species (Hardham 2005; Kamoun 2003).

Pathogenic processes in oomycetes

Oomycetes species can manipulate biochemical and physiological processes in their host plants through a diverse array of virulence or avirulence molecules known as effectors. In susceptible plants, these effectors promote infection by suppressing defense responses, enhancing susceptibility, or inducing disease symptoms. Alternatively, in resistant plants, effectors are recognized by the products of plant resistance genes, resulting in host cell death and effective defense responses known as the hypersensitive response (HR) (de Wit 2007; Kamoun 2007; Oliva et al. 2010; Schornack et al. 2009).

Suppression of host defenses can occur through the production of inhibitory proteins that target host enzymes. Oomycetes synthesize a family of endo-1,3-beta-glucanase inhibitor proteins, which bind to endo-1,3-beta-glucanases produced by their plant hosts, thereby suppressing the degradation of glucans in the oomycete cell wall and the release of oligoglucoside elicitors. These proteins, termed glucanase inhibitor proteins (GIPs), show significant structural similarity to the trypsin class of serine proteases but bear mutated catalytic residues and are proteolytically nonfunctional as a consequence. Glucanase inhibitor proteins are thought to function as counter defensive molecules that inhibit the degradation of beta(1,3) and (1,6)glucans in the pathogen cell wall and/or the release of defense-eliciting oligosaccharides by host endo-1,3-beta-glucanases (Bishop et al. 2005; Damasceno et al. 2008; Day and Graham 2007; Duclos et al. 1998; Horta et al. 2009; McLeod et al. 2003; Rose et al. 2002; Valueva and Mosolov 2004; York et al. 2004).

Our group have identified and characterized some proteins involved in mechanisms of infection by *P. cinnamomi*: endo-1,3-beta-glucanase GenBank: AM259651.1 (complete cds) (Meirinho et al. 2010), endo-glucanase GenBank: AJ964942.1 (partial cds) (unpublished results), glucanase inhibitor protein (GIP) GenBank: AM259384.1 (complete cds) (Martins et al. 2014), necrosis-inducing *Phytophthora* protein 1 (NPP1) GenBank: AM403130.1 (complete cds) (unpublished results) (Gijzen and Nurnberger 2006; Yu et al. 2008), and transglutaminase GenBank: AM403129.1 (unpublished results).

Gene expression in *Phytophthora*

Fundamental insights into the pathogenesis of fungal surface infections and the strategy of fungal invasion of a host can be achieved by identifying the genes of *Phytophthora infestans* and *P. cinnamomi* that are expressed during standard lab conditions and during infection. This would help to establish the genes involved in the pathogenic processes. The environmental changes associated with invasion and spread in the host demand specific adaptations. Indeed, it has been shown that *P. infestans* and *P. cinnamomi* express specific genes at certain stages of infection. It is unlikely that a single pathogenicity program would be sufficient to account for the range of infections that can be caused by these pathogens. A permanent interplay exists between the host and the pathogen that regulates the transcriptional profile of *P. infestans* and *P. cinnamomi*; in this scenario, gene expression of *P. infestans* and *P. cinnamomi* is regulated by dynamic changes in relation to the environment. Determining which genes are expressed during the different stages and types of infections is of essential importance in understanding the biological and physiological responses of those fungal pathogens to their environment.

Several technologies, such as real-time PCR and in vivo expression technology, have been used to study the expression of selected genes from fungi during infection. Much broader genome-wide approaches, including differential display, cDNA subtractive hybridization, antibody-based strategies, and DNA microarrays, have been used to identify infection-associated genes. In vivo transcriptional profiling protocols face a number of technical challenges. For example, most methods have in common the limitation that they are based on the transcript profile of population of cells. This is likely to be less of a problem in in vitro studies of cultures or in more simple models that mimic infections. To understand the true principles of host-fungus interactions, one ultimately needs to investigate the gene expression of cells in the same microenvironment or even on a single-cell basis. Furthermore, we need to look simultaneously at expression profiles of the host, as infections with *P. infestans* and *P. cinnamomi* are the result of an equilibrium between counteracting genetic programs of the host and the pathogen.

P. infestans and *P. cinnamomi* are highly adaptable pathogens that can adapt to several host environments. Recent findings suggest that the fungus senses local environmental changes and rapidly responds to these changes by modifying its transcriptional profile. Furthermore, they use transcriptional programs associated with the hypha penetration to maintain attachment, to invade tissue, and to further promote survival. Transcriptional regulation may not only help the fungi to adapt to the host environment but also to actively change the local microenvironment. In vivo transcriptional profiling in its infancy and future work will shed further light on fungal strategies

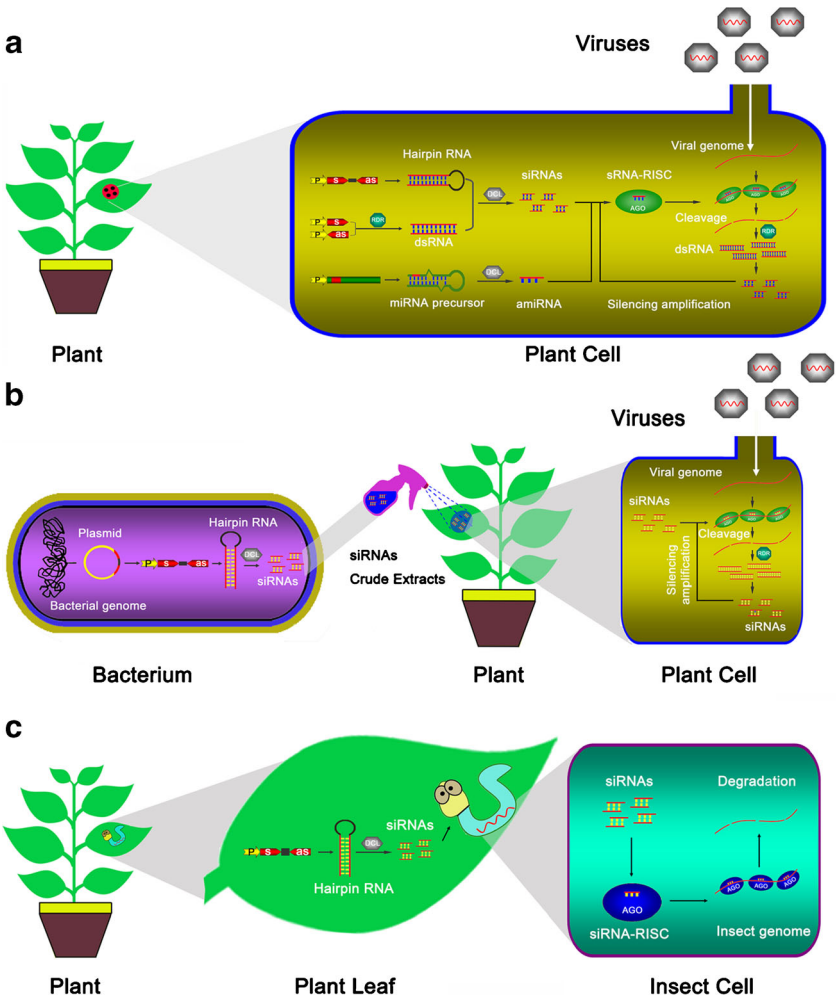

Fig. 1 Distinct approaches of the application of RNA silencing to plant disease resistance. **a** Expression of viral small RNA in host plants triggers antiviral silencing. **b** Sprayed bacterium processed siRNAs confers resistance against virus. **c** Feeding on transgenic plants that carry RNAi constructs confers resistance against insect. *As* antisense, *P* promoter, *s* sense. Figure reprinted from (Duan et al. 2012)

and adaptations. DNA microarrays are firmly established as a standard tool in biomedical research and particularly have become routine tools for the study of *P. infestans*.

In the studies of expression by RT-PCR, the genes isolated by our group in *P. cinnamomi*, in vivo infection, with cell lines of *Castanea sativa*, reveal the intimate relationship between plants and phytopathogens that has led to the coevolution of a number of complex strategies for attack and defense (Martins et al. 2014).

Biological solutions for ink disease

In the case of chestnut, rhizosphere microorganisms associated with the culture are not studied, although it has been reported that microorganisms are able to inhibit *P. cinnamomi*. The real possibility of performing effectively biocontrol of chestnut ink by the use of antagonistic microorganisms or metabolites produced by these are still not very well-explored. Whether the reduced disease is due to decreased pathogen activity or to induced resistance in the plant, or whether there are other mechanisms operating to suppress the pathogen, is still not fully understood. A number of study shows that antagonistic microbes are potential as biocontrol agent against plant pathogen (Hoopen et al. 2003; Lourenço et al. 2004; Okamoto et al. 2000; Rajkumar et al. 2005; Sid et al. 2003). Other studies on pot trials also indicate some potential biocontrol agents against *Phytophthora*. Aryantha and co-workers were able to identify antagonistic microorganisms isolated from composted manures that strongly suppress the root rot caused by *P. cinnamomi* (Aryantha et al. 2000). The role of those antagonistic microorganisms was latter assessed providing an explanation for the activity of composted manures and their potential for biological control of *P. cinnamomi* (Aryantha and Guest 2006). Most of the interactions were observed between *P. cinnamomi* and the antagonists *Trichoderma* sp., *Gliocladium* sp., and *Pseudomonas* sp. (Aryantha and Guest 2006). Further studies are required to evaluate the potential use of these antagonistic and mycoparasitic isolates in biological control and to determine the most active isolates or combinations, application frequency, and amounts.

Repeated biodisinfection for the control of root and crown rot in protected pepper crops located in temperate climate

regions can improve soil quality and suppressiveness, as well as allow for a reduction in the dose of organic amendment needed for biodisinfection. Among the studied organic amendments, the semi-composted amendment was the best option in terms of reduction in disease incidence (Nuñez-Zofio et al. 2012). Also, morphological and phenological studies of cultivars of sweet chestnut (*C. sativa* Miller), in order to find cultivars with improved characteristics and resistance, are very important (Furones-Pérez and Fernández-López 2009).

To reduce the losses caused by plant pathogens, plant biologists have adopted numerous methods to engineer resistant plants. Among them, RNA silencing-based resistance has been a powerful tool that has been used to engineer resistant crops during the last two decades. Based on this mechanism, diverse approaches were developed (Duan et al. 2012).

A better understanding of RNA silencing pathways has also contributed to the development of this technique. The RNA silencing-mediated approach is now a powerful tool in antiviral research. HIGS-mediated antifungal and anti-insect pathogens are also being developed. Although RNA silencing has been successful, there are still many limitations in utilizing this strategy. RNA silencing-mediated resistance and the silencing efficacy are the results of interaction between many factors, including sequence similarity, target selection, pathogen titer, and environmental temperature (Szittya et al. 2003). Thus, it is difficult to accurately predict the resistance efficacy. Moreover, to our knowledge, most of the successful examples were obtained in greenhouses. Considering that mixed infections are common in nature, it is still a challenge to obtain resistant plants. Therefore, further scientific research is required to uncover the factors affecting RNA silencing-mediated resistance in specific cases and to test the resistance efficacy in the field (Duan et al. 2012) (Fig. 1). Transient gene silencing can be used to generate detectable phenotypes in *P. infestans* and should provide a high-throughput tool for *P. infestans* functional genomics (Whisson et al. 2005). A strategy that integrates these biological and molecular techniques will be essential for elucidating the function of virulent genes in *P. cinnamomi* and consequently contribute to the resolution of the chestnut ink disease.

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