EXPRESSION PROFILING OF CANDIDATE GENES OF RESISTANCE TO *PHYTOPHTHORA CINNAMOMI* DETERMINED IN DIFERENTE GENOTYPES OF *CASTANEA* SPP

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Phytophthora cinnamomi is a hemibiotrophic that causes root rot, also known as ink disease. Little information has been acquired in chestnut on the molecular defense strategies against this pathogen. The expression of eight candidate genes potentially involved in the defense to *P. cinnamomi* was quantified by digital PCR in *Castanea* genotypes showing different susceptibility to the pathogen. Seven of the eight candidate genes displayed differentially expressed levels depending on genotype and time-point after inoculation. Cast_Gnk2-like revealed to be the most expressed gene across all experiments and the one that best discriminates between susceptible and resistant genotypes. Our data suggest that the pre-formed defenses are crucial for the resistance of *Castanea crenata* to *P. cinnamomi*. A lower and delayed expression of the eight studied genes was found in the susceptible *Castanea sativa*, which may be related with the establishment and spread of the disease in this species. A working model integrating the obtained results is presented.

INTRODUCTION

The aim of this study was to evaluate the early expression of candidate resistance genes to *P. cinnamomi* infection (0, 24, and 48 h) in *C. sativa* and a *C. crenata*, as well as in four hybrids (three *C. sativa* × *C. crenata* genotypes and a *C. sativa* × *C. mollissima*) with different susceptiblities to *P. cinnamomi*, produced from the breeding program on course, for resistance to *P. cinnamomi*, the causal agent of root rot, to understanding of the molecular mechanisms underlying the resistance that Asian species (*C. crenata* and *C. mollissima*) show to this pathogen. Among the different methods available to quantify gene expression in plants, digital PCR (dPCR) is emerging as an absolute quantification method with high precision, sensitivity and specificity (Majumdar et al. 2015). This new technology has been mainly used for biomedicine research, however, some studies in plant science using dPCR have also been recently released (Bahder et al. 2016; Ge et al. 2016; Stevanato and Biscarini 2016).

MATERIAL AND METHODS

Six chestnut genotypes showing different levels of resistance after inoculation with pathogen were used in this work. *C. crenata* (resistant), *C. sativa* (susceptible) and four hybrid genotypes, selected from the on-going chestnut breeding program (Costa et al. 2011): three *C. sativa* × *C. crenata* hybrids (SC55 – resistant, SC914 – intermediate, and SC903 – susceptible) and one *C. sativa* × *C. mollissima* hybrid (SM904), selected as a resistance control. All plant material used in this study was multiplied by micropropagation. *P. cinnamomi* root inoculation was performed 80 days after plant acclimatization, under controlled conditions according to Santos et al. (2015). Genes were selected from the 283 *C. crenata* differentially expressed genes (DEGs), previously identified by Serrazina et al. (2015). In this study, gene selection parameters were: (1) DEGs with the log2 of the ratio between *C. crenata* inoculated (Cci) and non-inoculated (Ccn) reads higher than 1.5(Log2Cci/Ccn >1.5); (2) The correspondent

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DEGs in *C. sativa* transcriptomes with Log2Csi/Csn <1.5 or absent; (3) DEGs not involved in general biological processes, such as oxidative, metabolic and transporter activities; (4) DEGs involved in defense response and categorized in pathogen recognition which usually triggers resistance signalling pathways, anti-pathogen proteins, cell wall modification proteins, and transcription factors involved in the regulation of other defense related processes. QS3D digital PCR System (Life Technologies) was used to quantify gene expression of eight *P. cinnamomi* resistance candidate genes in the roots of the six chestnut genotypes under study.

RESULTS AND DISCUSSION

Physical and Chemical Barriers to *P. cinnamomi* Infection

Using the gene selection parameters defined, eight candidate genes were identified. These genes codify proteins potentially involved in the three layers of defense to *P. cinnamomi* infection, previously described (Freeman and Beattie 2008) two pathogen recognition proteins (Cast_LRR-RLK and Cast_C2CD) which trigger resistance signaling pathways; three transcription factors (Cast_WRKY 31, Cast_ABR1, and Cast_Myb4) involved in the regulation of other defense processes; a ubiquitination regulator (Cast_RNF5); a cell wall modification enzyme (Cast_PE-2) and an antifungal protein (Cast_Gnk2-like). All genes selected were up-regulated after inoculation in *C. crenata* root transcriptomes (Serrazina et al. 2015). The *P. cinnamomi* resistance candidate genes, their respective contig name (Serrazina et al., 2015), primers and TaqManR - Probes sequences are listed in Santos et al. (2017).

Before inoculation there is a clear differentiation in gene expression between *C. sativa* and *C. crenata*. Except for Cast_ABR1 and Cast_RINF5, the pre-inoculated expression of all other genes is significantly higher in *C. crenata*. This pattern, with some variation, holds for the two most resistant hybrids tested (SM904 and SC55). The secretion of toxic compounds is an effective defense mechanism against pathogens in plants (Montesinos 2007, Wittstock and Gershenzon 2002). Ginkbilobin-2 (Gnk2) is a protein secreted by *Ginkgo biloba* seeds that exhibits an antifungal activity (Sawano et al. 2007, Wang and Ng 2000). Gnk2 has a plant-specific cysteine-rich motif DUF26 (domain of unknown function 26, also known as stress-antifungal domain: PF01657) which belongs to cysteine-rich receptor-like kinases (CRKs) (Miyakawa et al. 2014) not showing any similarity with other known antimicrobial proteins (Miyakawa et al. 2014, Sawano et al. 2007). It was recently shown that Gnk2 can also activate actin-dependent cell death (Gao et al. 2016). Therefore, Cast_Gnk2-like may prevent pathogen growth either by its chemical properties or by inducing HR-related cell death. The highest Cast_Gnk2-like expression registered in non-inoculation conditions suggests that *C. crenata* root surroundings may be a hostile environment for fungal and fungal-like pathogens, such as *P. cinnamomi*. On the other hand, *C. sativa* showed a very low Cast_Gnk2-like expression level, even after pathogen inoculation. Considering the whole experiment, Cast_Gnk2-like was the most expressed gene and that best discriminates between susceptible and resistant genotypes (Santos et al. 2017). The isolation and purification of Cast_Gnk2- like protein may have biotechnological applications, such as the development of an antimicrobial phytopharmaceutical against *P. cinnamomi*.

A crucial constitutive defense is the formation of wall appositions that comprise a physical barrier to pathogen growth (Hardham and Blackman 2010). The reinforcement of plant cell walls by calcium-pectate gel apposition with the involvement of pectinesterases have been shown to confer resistance to *Phytophthora* species (Kieffer 2000, Wiethölter et al. 2003). In this study, expression levels of Cast_PE-2 show that this enzyme may have a role on *P. cinnamomi* resistance in chestnut. Compared with *C. sativa*, *C. crenata* exhibited higher Cast_PE-2 expression levels in all time points, mainly in the non-inoculated samples (about 10x more), suggesting that their

cell walls may be more resistant to pathogen penetration. After the first pathogen contact, Cast_PE-2 expression increases, suggesting a possible continuing apposition of pectates in cell walls, probably to inhibit further colonization. This seems to be more important in a late stage of infection (48 hpi) except for the *C. sativa* × *C. mollissima* hybrid. Possibly, other resistance mechanisms may be activated earlier in this hybrid and control the infection.

Pathogen Recognition and Successive Host Response Regulation

Generally, during pathogen infection, PAMPs are recognized by pattern-recognition receptors (PRRs) at the plant's cell surface. The best-studied class of plant PRRs are receptor-like kinases (RLKs), which have an ectodomain of leucine-rich repeats (LRRs) involved in PAMP perception (Boller and Felix 2009; Jones and Dangl 2006; tenHove et al. 2011). Resistance related LRR proteins have been shown to be differentially expressed in global transcript profiling studies in *Phytophthora* spp. infection response (Ballvora et al. 2002, Boava et al. 2011, Coelho et al. 2011, Gao et al. 2005, Mahomed and Berg 2011, van der Vossen et al. 2003). Contrasting to *C. sativa*, *C. crenata* has a much higher (about 10x more) Cast_LRR-RLK expression before inoculation (Santos et al. 2017), which may mediate a fast and effective response against *P. cinnamomi*, suggesting that this earlier recognition is part of the resistance phenotype. Furthermore, Cast_LRRRLK expression increased after *P. cinnamomi* inoculation for all *Castanea* genotypes. Considering the previous studies on LRR biological functions in Fagaceae, Cast_LRR-RLK may recognize and interact with PAMPs molecules, secreted by *P. cinnamomi*, activating downstream signaling responses (Coelho et al. 2011).

Cast_WRKY 31 may have a role in the response of chestnut to *P. cinnamomi* infection, since its expression increased in inoculated samples when compared with non-inoculated ones, probably regulating SA-responsive genes expression. This increase seems more consistent in the more resistant hybrids.

The overexpression of WRKY 31 in rice seedlings after treatment with a hemibiotrophic fungus (*Magnaporthe grisea*) was associated with blockade of pathogen invasion (Zhang et al. 2008). The balance between SA and other phytohormones is increasingly recognized as central to the outcome of plant– pathogen interactions (de Torres-Zabala et al. 2009). Abcisic acid (ABA) disrupts SA-mediated response and suppresses the expression of many defense-related genes. The ethylene responsive transcription factor ABR1 is a negative regulator of ABA signaling pathway in *Arabidopsis thaliana* (Pandey et al. 2005) and its expression allows SA and lignin accumulation (Boatwright and Pajerowska-Mukhtar 2013; de Torres-Zabala et al. 2009; Mohr and Cahill 2007). Cast_ABR1 expression was triggered after *P. cinnamomi* inoculation, earlier in the more resistant genotypes, suggesting that ABA may be repressed after pathogen perception. In the resistant *C. crenata* genotype the relatively low increase of Cast_ABR1 expression may be due to the efficiency of other resistant mechanisms that avoid pathogen colonization, or by independence of ABA suppression for SA signalling activation. Genes of the MYB transcription factor family are involved in the control of specific processes including responses to biotic stresses (Dubos et al. 2010). The expression balance of Cast_Myb4 in *Castanea* genotypes may regulate SA accumulation vs. synthesis of phenylpropanoids. The ratio of Cast_Myb4 expression between 24/48 hpi decreased progressively from the resistant *C. crenata*, to *C. sativa* × *C. crenata* hybrids (the most resistant to the most susceptible) to the susceptible *C. sativa*. This indicates that SA signaling may be faster (24 hpi) in resistant genotypes than in susceptible ones. As mentioned before, elevated concentrations of endogenous SA will induce expression of Cast_Gnk2-like and Cast_WRKY31. For resistant genotypes, (*C. crenata* and SC55) after a probable early induction of SA pathways, expression of Cast_Myb4 decreases at 48 hpi, which may allow the synthesis of lignin and other defense molecules.

Hypothetical *P. cinnamomi* Response Mechanism in *Castanea*

The expression profiles obtained suggest that susceptible and resistant plants may share the same response mechanisms. Despite, resistant plants show a much higher constitutive expression of the tested candidate genes before inoculation. A working model describing part of the molecular interaction of *Castanea* spp. to *P. cinnamomi* infection was presented in Santos et al. (2017): Physiochemical barriers, antifungal proteins secretion (Cast_Gnk2-like) and stronger cell walls (by action of Cast_PE-2, Cast_ABR1) respectively, may inhibit *P. cinnamomi* growth and infection. If *P. cinnamomi* overcome those barriers, specific pathogen recognition may occur, by Cast_LRR-RLK. Hence, host transcription is reprogrammed via MAPK cascades and SA signaling. Cast_WRKY 31 should activate transcription of LRR-RLK. Cast_ABR1 regulate SA accumulation via ABA suppression. HR could be activated by many mechanisms: SA or calcium signaling, via Cast_Gnk2-like (actin-dependent) or by vital protein degradation (by Cast_RNF5). Cell walls not infected may be reinforced and antifungal proteins may be secreted in more abundance, inhibiting further colonization.

Resistant genotypes present a higher expression of genes in non-inoculation conditions that may be part of a constitutive defense mechanism that prepare and protect the plant in advance to *P. cinnamomi* infection by secreting antifungal proteins and having stronger cell walls even before the contact with the pathogen. If *P. cinnamomi* overcomes those chemical and physical barriers, specific pathogen recognition proteins are earlier and more expressed in the resistant genotypes when compared to the susceptible ones. Thereafter, the transcription of the host will probably be reprogrammed via signal transduction and SA signaling. HR-related cell death is probably activated and cell walls may be reinforced in non-infected tissues, preventing further colonization.

In conclusion, the first layer of defense seems to be active and decisive in the resistance of *C. crenata* to *P. cinnamomi*. A lower and delayed expression of the eight studied genes was found in *C. sativa*, which may be related with the sensitivity of this species towards the disease. One probable explanation for this difference can be the allelic variation of the genes or gene-promoters that in *C. sativa* may condition the levels of gene expression before inoculation. *C. mollissima*, also a resistant species, may share with *C. crenata* some of the allelic variants that allow an efficient level of resistance against *P. cinnamomi*. This will be the object of further research. Natural selection could have had an active role in keeping those allelic variants, since Asian species have evolved in contact with *P. cinnamomi*. This study is part of an ongoing Portuguese breeding program to introduce resistance to *P. cinnamomi* in *C. sativa*. This knowledge may contribute for the development of strategies to control ink disease in chestnut and other woody plants, which may include early selection of resistant genotypes.

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