Constitutive expression of pathogenesis-related proteins and antioxydant enzyme activities triggers maize resistance towards *Fusarium verticillioides*

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ABSTRACT

Fusarium verticillioides is a fungal pathogen of maize that causes ear rot and contaminates the grains with fumonisin mycotoxins. Breeding for resistance to *Fusarium* emerged as the most economic and environmentally safe strategy; therefore the discovery of resistant sources and effective molecular markers are a priority. Ears of resistant (CO441 and CO433) and susceptible (CO354 and CO389) maize lines were inoculated with *F. verticillioides* and the expression of pathogenesis-related (*PR*) genes (*PR1*, *PR5*, *PRm3*, *PRm6*) and genes that protect from oxidative stress (*peroxidase*, *catalase*, *superoxide dismutase* and *ascorbate peroxidase*) were evaluated in the kernels at 72 h post inoculation. In addition, the oxidation level and the enzymatic activity of ascorbate-glutathione cycle, catalase, superoxide dismutase and cytosolic and wall peroxidases were investigated. The uninoculated kernels of the resistant lines showed higher gene expression and enzymatic activities, highlighting the key role of constitutive resistance in limiting pathogen attack. In contrast, the susceptible lines activated defensive genes only after pathogen inoculation, resulting in increased levels of H_2O_2 and lipid peroxidation, as well as lower enzymatic activities. The constitutive defenses observed in this study from seed could be profitably exploited to develop markers to speed up conventional breeding programs in the selection of resistant genotypes.

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1. Introduction

Ear rots and mycotoxin contamination of the kernels frequently reduce the quality and yield of an economically important and widely grown crop as maize (*Zea mays* L.). *Fusarium verticillioides* (Sacc.) Nirenberg is one of the most common fungal species associated with maize in temperate regions and produces fumonisins, considered carcinogenic mycotoxins (Santiago et al., 2015). Fumonisins are able to disrupt the metabolism of sphingolipids, important signaling molecules in animal and plants (Williams et al., 2007). *F. verticillioides* enters the ear through silks or through wounds due to insect injury and mechanical damage and causes ear rot in the tip or in scattered kernels (Logrieco et al., 2002; Munkvold, 2003a). The complexity of this pathosystem lies partially in the lifestyle of pathogen, which is both parasite and saprophyte and it can be transmitted from seed to plant as a symptomless intercellular endophyte (Bacon et al., 2008; Munkvold, 2003a). The process of *F. verticillioides* infection and mycotoxin accumulation is influenced by environmental conditions, but also by host resistance and biochemical composition of kernel, including moisture, development stage and lipid composition (Battilani et al., 2008; Maschietto et al., 2015; Sagaram et al., 2006; Woloshuk and Shim, 2013).

Agronomic practices for fumonisin content reduction are often ineffective and breeding for resistance to *Fusarium* species (particularly *F. verticillioides*) emerged as the most economic and environmentally safe strategy (Munkvold, 2003b). Quantitative Trait Locus (QTL) mapping studies in maize indicated that *Fusarium* resistance and fumonisin contamination are quantitative traits determined by small effect polygenes with moderate to high heritability (Ding et al., 2008; Robertson-Hoyt et al., 2006; Zila et al., 2013, 2014). Genetic resistance to *Fusarium* ear rot and fumonisin accumulation has been identified in maize lines and hybrids (Clements et al., 2004; Henry et al., 2009; Kleinschmidt et al., 2005; Löffler et al., 2010; Lanubile et al., 2011; Pascale et al., 2002; Santiago et al., 2013), but the complexity of these polygenic traits

have so far hampered the development of resistant maize genotypes with high agronomic performance (Bush et al., 2004; Eller et al., 2008; Zila et al., 2013). Breeding for resistance will benefit from the discovery of efficient markers for phenotyping and QTLs consistent across populations, but also by a more extensive comprehension of the genetic basis underlying maize-*F. verticillioides* interaction.

Plant resistance to pathogen attack is polygenic, involving a hierarchy of genes that produce proteins and metabolites, either constitutive or induced post infection, including the synthesis and accumulation of reactive oxygen species (ROS), phytoalexins and pathogenesis-related (PR) proteins (Almagro et al., 2009; Torres, 2010). The success of infection depends on the number and abundance of these defense related products and the speed with which defense responses are mounted.

The oxidative burst is one of the main event associated to biotic stimuli. The accumulation of ROS, in particular hydrogen peroxide, suppresses pathogen entrance or induces host cell death or hypersensitive response to contain the pathogen (Baxter et al., 2014; Torres, 2010; Wirthmueller et al., 2013). The amount of ROS depends on the type and amount of enzymatic and non-enzymatic scavenging molecules that include: superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD) and the antioxidants ascorbate (ASC) and glutathione (GSH) (Mittler et al., 2004). ASC levels and ASC-GSH cycle components are tightly linked to plant tolerance to biotic and abiotic stresses (Locato et al., 2013; Paciolla et al., 2004, 2008).

In comparison to vegetative tissues, deeper and continuing efforts are still needed to unravel the mechanisms of qualitative and quantitative resistance in maize kernels against *F. verticillioides* infection. Candidate genes for host resistance towards this pathogen were searched in the last years thanks to the employment of maize mutants (Christensen et al., 2013, 2014; Gao et al., 2007) and genotypes showing contrasting level of resistance (Lanubile et al., 2010, 2012a,b, 2014; Maschietto et al., 2015). Both constitutive and induced resistance were proven to be involved in maize kernel defense against *F. verticillioides* infection, including the expression of PR proteins, lipoxygenases, ribosome-inactivating proteins (RIPs), WRKY and other transcription factors, jasmonate and ethylene signaling-related genes, genes related to primary and secondary metabolism, antioxidant enzyme activities and proteins involved in protein synthesis, folding and stabilization (Bravo et al., 2003; Campos-Bermudez et al., 2013; Christensen et al., 2013, 2014; Gao et al., 2007; Guo et al., 1997; Lanubile et al., 2014; Murillo et al., 1999; Zila et al., 2013, 2014). Host resistance was associated to a relatively high constitutive gene expression of defense-related genes in the resistant (CO441) kernels, whereas these genes were induced by pathogen attack in the susceptible (CO354) genotype starting from 48 h post inoculation (hpi) (Lanubile et al., 2010, 2012a,b, 2014). Moreover the resistant (CO433) maize genotype showed an earlier and enhanced expression of genes of the LOX pathway in comparison to the susceptible (CO354) line after *F. verticillioides* inoculation (Maschietto et al., 2015). Although hundreds of genes were detected in the studies reported above, the defense bases are still unclear, probably due to the complex nature of the pathosystem.

In addition to the previous findings reported above, this work extended the evaluation of the molecular and biochemical responses against *F. verticillioides* infection to other two maize genotypes, showing elevated levels of resistance (CO433) and susceptibility (CO389) to the pathogen. The study assessed the expression profile of selected defense-related genes (*PR* genes and genes involved in protection from oxidative stress) and the activity of the ASC-GSH cycle enzymes and the cell oxidative status with the purpose of correlating molecular and biochemical data. This study validated the results previously observed, confirming

the hypothesis that the resistant genotypes have constitutive high levels of biochemical barriers before inoculation, providing a basal defense system to the pathogen. The candidate genes and biomarkers validated in this study could be applied to speed up conventional breeding programs for *Fusarium* resistance.

2. Materials and methods

2.1. Plant material and F. verticillioides inoculation assay

Four maize genotypes with contrasting phenotypes for resistance to *Fusarium* ear rot were used in this study: the resistant lines CO441 and CO433 and the susceptible lines CO354 and CO389, as previously reported (Lanubile et al., 2010; Maschietto et al., 2015; Reid et al., 2009). All lines were developed by the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada (Ottawa, Canada) and were maintained by sibling at the Department of Sustainable Crop Production in Piacenza, Italy. Seeds from each line were planted in pots (40 cm diameter, 35 cm height) and 10 plants were grown up. Before inoculation, pots were transferred to an environmentally controlled greenhouse with day-time and night-time conditions of 28 °C and 20 °C temperatures, respectively, and a light regime of 16 h using lamps, as described in Lanubile et al., 2015. *F. verticillioides* inoculation was performed using the isolate ITEM 1744 (Institute of Sciences of Food Production, National Research Council, Bari, Italy), a high fumonisin producer strain, cultured as previously described by Lanubile et al. (2010, 2012a). Maize ears were inoculated at 15 days after hand-pollination (DAP) using a side-needle inoculator, as reported by Lanubile et al. (2015). For the detection of *F. verticillioides*, real-time RT-PCR expression analysis, and enzymatic assays, seeds adjacent to the inoculated kernels were collected at 72 hpi, in the area around the point of inoculation, to evaluate fungal growth and colonization and to avoid mechanical damage due to needle-prick (Lanubile et al., 2013, 2014). Control seeds were sampled at the same inoculation time listed above and considered as uninoculated. Three pools of kernels for the 72 hpi time-point were prepared, where each pool derived from the mixing of kernels coming from three different maize ears.

2.2. RNA isolation and real-time RT-PCR expression analysis

Maize kernels of CO441, CO433, CO354 and CO389 genotypes were ground in liquid nitrogen with mortar and pestle and total RNA was extracted from 2.5 g of seeds using the TRIzol protocol (Invitrogen, Carlsbad, CA, USA) and purified with the RNA Clean-up protocol (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The amount and the quality of the total RNA were estimated by fluorometric assay (Qubit, Invitrogen) as well as by agarose gel electrophoresis.

Real-time RT-PCR experiments were performed on kernels collected at 72 hpi using the 2 x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the CFX-96 device (Bio-Rad). cDNA synthesis and relative quantitative analysis by real-time RT-PCR followed the previous method described by Lanubile et al. (2015). Briefly, 20 ng of single strand cDNA were used for real-time RT-PCR at the following conditions: 95 °C for 3 min and 40 cycles at 95 °C 10 s, 60 °C for 25 s (Lanubile et al., 2015). A melting curve analysis was performed and three technical replicates of each biological replicate were employed (Lanubile et al., 2015). The gene-specific primers for *PR1*, *PR5*, *PRm3*, *PRm6*, *POD*, *CAT*, *SOD* and *APX* are reported in Supplementary Table S1. Primers were designed possibly within consecutive exons, separated by an intron, using Primer3 software. Relative quantification of maize genes was normalized to the housekeeping gene *β-actin* (Supplementary Table S1) and FC val-