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Increase of peroxidase activity in tropical maize after recurrent selection to storage pest resistance

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ABSTRACT

Farmers worldwide experience substantial postharvest grain losses of maize (*Zea mays* L.) caused by the maize weevil, *Sitophilus zeamais*. Resistance to this pest has recently been demonstrated in Population 84 (P84) maize kernels. This resistance has been correlated with the presence of cell-wall–bound phenolic compounds and endosperm peroxidase (POD) activity. However, the specific role of peroxidases in insect resistance remains unknown. The aim of this study was to expand the knowledge of this role by evaluating the association between POD activity and resistance to *S. zeamais* during four cycles of recurrent selection of P84. This evaluation involved the use of kernel–insect interaction assays combined with proteomic, biochemical and histological methods. Histological staining confirmed POD activity in the germ, pedicle and aleurone layer. Endosperm POD activity was increased over three cycles of recurrent selection, mainly in advanced red kernels, but this increased activity was not associated with the thickness or number of aleurone layers in the endosperm. A significant negative correlation ($P < 0.05$) was found between endosperm POD activity and grain weight loss (GWL) in whole kernels and kernels without pericarp, adult progeny (AP) in kernels without pericarp, and number of damaged kernels (DK) in single endosperms, produced by *S. zeamais* infestation. Our findings provide strong evidence of a specific relationship of peroxidases in the biophysical and biochemical resistance mechanism against *S. zeamais*, supporting the possible application of peroxidases as a breeding trait for the development of maize varieties resistant to storage pests.

1. Introduction

Maize (*Zea mays* L.) is the most massively produced staple crop, with an estimated global production of 1026 million of tons (Cerquiglini et al., 2016). In developing countries, this cereal forms a food security base and constitutes the subsistence of small farmers. However, these countries often encounter massive grain losses (more than 30% of total production), caused mainly by deficient storage conditions that allow attacks by fungi, rodents and insect pests like the maize weevil, *Sitophilus zeamais* (Pingali and Pandley, 2001; Bergvinson and García-Lara, 2004). The consequences of inadequate storage practices include declines in grain quality and by-products and/or health problems due to the presence of toxins (Tefera et al., 2011; García-Lara et al., 2013a,b). Integrated postharvest practices, including hermetic storage structures and chemical protection, are effective strategies for reducing these losses (Tefera et al., 2011; García-Lara et al., 2013a,b). However, farmers with small cultivated areas and little income cannot afford the costs associated with these strategies (Boyer et al., 2012; Midega et al., 2016). Consequently, international maize breeding programs have directed their efforts towards the development and characterization of new varieties that are resistant to postharvest pests (Pingali and Pandley, 2001; Bergvinson and García-Lara, 2004). In the new improved varieties, this resistance is imparted by elevated levels of cell wall cross-linking components in the pericarp of the maize kernel.

The principal cell wall components associated with resistance are soluble and cell wall phenolic acids, heteroxylans, hydroxyproline-rich glycoproteins and functional proteins, such as peroxidases (Arnason et al., 1993; Sen et al., 1994; García-Lara et al., 2004, 2007; Santiago et al., 2013; Ayala-Soto et al., 2014).

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Peroxidases (POD) have attracted particular attention due their involvement in several biological processes, including defense against pathogens and insects (Passardi et al., 2005; Dowd et al., 2010; Francoz et al., 2014). These monomeric, glycosylated hemoproteins have oxidoreductase activity and use hydrogen peroxide as an oxygen acceptor to catalyze the oxidation of diverse substrates, such as phenolic compounds, amines and certain inorganic ions (Hiraga et al., 2001; Duroux and Welinder, 2003; Bunzel, 2010; Santiago and Malvar, 2010). The peroxidases in maize are involved in diverse resistance mechanisms; for example, expression of the PER1 peroxidase protein has been associated with kernel resistance to *Aspergillus flavus* infection (Chen et al., 2007).

Studies in maize kernels have suggested a correlation between endosperm POD activity and resistance to a *S. zeamais,* the major postharvest insect pest in tropical climates. Interestingly, POD activity has been detected in the germ and endosperm of insect resistant kernels, but not in the pericarp (García-Lara et al., 2007). However, examination of P84 maize population, which shows resistance to the tropical insects, and the use of a new methodology developed to detect proteins with peroxidase activity in maize grain tissues recently confirmed the presence of POD activity in kernels with resistance to *S. zeamais* (Winkler and García-Lara, 2010). This finding implies a positive correlation between endosperm POD activity and resistance parameters, such as grain weight loss and grain damage (Winkler and García-Lara, 2010). Furthermore, recent QTL mapping studies have identified the peroxidase gene *px1* in genomic areas associated with maize weevil resistance (Castro-Álvarez et al., 2015).

The maize population 84 (P84) has been developed by recurrent selection under artificial infestation with *P. truncatus* and *S. zeamais* to obtain multiple storage pest resistance varieties after four cycles of selection (García-Lara et al., 2004). This improvement in maize pest resistance was associated with an increase in the content of major cell wall phenolic acids in the kernel pericarp (García-Lara and Bergvinson, 2014). In addition to the primary POD detected in P84 (García-Lara et al., 2007; Winkler and García-Lara, 2010), another more active POD, the class III peroxidase B6T173 (ZmPrx35), has recently been identified in P84 kernels (López-Castillo et al., 2015). However, the mechanisms by which peroxidases function in insect resistance remain unknown.

The aim of the present work was therefore to extend the knowledge of the role of peroxidases in the development of resistance to postharvest pests in maize. Our approach was to use cycles of recurrent selection of P84 to evaluate the association between POD activity at the grain tissue level and the increased resistance to *S. zeamais*.

2. Material and methods

2.1. Maize genotypes

Population 84 (P84) was developed at CIMMYT from twenty Caribbean accessions that exhibited natural resistance to the maize weevil *(S. zeamais)*. Recurrent selection was conducted during four cycles using the intrapopulation improvement method. Details of the breeding methods were fully described previously (García-Lara and Bergvinson, 2014). Cycles of selection of P84 were termed C0 red (C0R), C0 yellow (C0Y), C1 red (C1R), C1 yellow (C1Y), C2 red (C2R), C2 yellow (C2Y), C3 red (C3R), C3 white (C3W) and C3 yellow (C3Y). All cycles were increased during 2014 at the CIMMYT experimental station at Agua Fria, Puebla Mexico (19° N, 60 masl). After harvest, the grains were dried and stored at 13% grain moisture and 4 °C until used in biochemical and insect assays.

2.2. Insect bioassays

Insect–kernel interactions were assessed by infesting three replicates, containing 10 g of 1) whole kernels, or 2) kernels without pericarp or 3) hand-dissected endosperms, with 10 unsexed adult weevils no older than 2 weeks of age. The adults were removed after 10 days of interaction. The infested kernels and tissues were stored under controlled conditions (27 °C ± 1 °C, 70 ± 5% relative humidity). The grain weight loss percentage (GWL), number of damaged kernels/structures (DK) and adult progeny (AP) were recorded 60 days after infestation. The level of resistance was determined using the Dobie Index method (Dobie, 1974).

2.3. Enriched kernel tissue fractions

For all genotypes, grain tissue samples were prepared from three separate replicates generated from 10 g of kernel samples. Studies were conducted using enriched tissue fractions (germ and endosperm) dissected by hand as described by García-Lara et al. (2007). The pedicle was first removed from the intact seed by a transversal cut to the kernel cap area. Following pedicle cleavage, the seed was imbibed in distilled water for 10 min at 4 °C and then each tissue was removed by hand dissection. The resulting samples, identified as 1) endosperm and 2) germ, were then dried and ground using a mixer ball mill (MM 400; Retsch/Verder Scientific, Col. Germany), sifted through a 60-mesh sieve and stored at −20 °C until further analysis.

2.4. Determination of peroxidase activity

Protein was extracted using a modification of the method reported by López-Castillo et al. (2015). Endosperm flour was homogenized in 50 mM sodium phosphate (pH 6.8) at a 3:1 ratio (tissue:buffer), whereas germ flour was homogenized at a 10:1 ratio. The protein was then extracted by incubating the homogenates at 25 °C for 30 min with continuous shaking at 3000 rpm on a vortex mixer. After incubation, the samples were centrifuged at 10 000 g for 30 min at 4 °C and the supernatant was recovered. Protein was quantified using the Bradford method (Bradford, 1976) and a Coomassie dye binding protein assay kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. POD activity was quantified using the protocol reported by García-Lara et al. (2007). Activity was measured at 470 nm after a 30 min incubation at 25 °C in a solution of 20 mM guaiacol in 50 mM phosphate buffer (pH 6.8) and 0.3% H_2O_2. Peroxidase activity was expressed in units per mg (U mg^{-1}) of protein. One activity unit was defined as the oxidation of 1 μmole of guaiacol per minute.

2.5. Electrophoretic profile of peroxidases

Peroxidase activity was detected using the activity-directed methodology for peroxidases in maize grain tissues reported by Winkler and García-Lara (2010). Active protein extracts from kernel tissues were separated in a non-reducing SDS-PAGE gel (10% acrylamide). The POD activity was then detected after incubation with a solution of 20 mM guaiacol in 50 mM phosphate buffer (pH 6.8) and 0.03% H_2O_2 for 30 min at room temperature. POD activity was observed as brown bands. As a subsequent staining step, the protein in the gel was fixed in 40% (v/v) ethanol and 10% (v/v) acetic acid for 1 h and stained with 0.1% Brilliant Blue R250 solution (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Peroxidase localization

The peroxidase activity in the maize kernels was detected and localized by histological staining with guaiacol-H_2O_2 stain,