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Transgenic expression of a maize geranyl geranyl transferase gene sequence in maize callus increases resistance to ear rot pathogens

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ABSTRACT

Determining the genes responsible for pest resistance in maize can allow breeders to develop varieties with lower losses and less contamination with undesirable toxins. A gene sequence coding for a geranyl geranyl transferase-like protein located in a fungal ear rot resistance quantitative trait locus was cloned from an inbred with reported resistance to *Fusarium proliferatum* and *Fusarium verticillioides* ear rot. Transgenic expression of the gene in maize callus reduced colonization by these two *Fusarium* species and also *Fusarium graminearum* relative to a β-glucuronidase (GUS) transformant control. Some transformants were also more insect resistant. The more fungal resistant transformant lines produced higher levels of headspace ethanol which were significantly associated with antifungal activity, especially for *F. verticillioides*. Maize pyruvate decarboxylase appears to have a moiety capable of interacting with the geranyl geranyl transferase, suggesting ethanol production is enhanced due to more efficient transfer of pyruvate through the mitochondrial membrane. Other undetermined mechanisms may also be enhancing resistance of the transformants to the *Fusarium* fungus, however. This is the first report of the involvement of a geranyl geranyl transferase-like sequence in fungal resistance in plants, and represents a novel mechanism for producing higher yielding and better quality maize.

1. Introduction

Economic losses due to maize insects and diseases are considerable worldwide (Oerke, 2006). Additionally ear rot pathogens reduce grain quality and can also contaminate the grain with toxins (mycotoxins) that are hazardous to humans and animals and are strictly regulated worldwide (Van Egmond and Jonker, 2005). Many different strategies have been explored to reduce the problems with maize pests. One of the most effective is the use of host plant resistance (Fritsche-Neto and Borem, 2012). Problems with maize foliar pathogens (Carson, 1999), stalk rots (White, 1999) and insect pests (Smith, 2005) have been greatly reduced through breeding efforts. Incorporation of bacterial genes into maize has greatly reduced damage due to insects, although many markets find transgenic materials objectionable (Gilbert, 2013), and several pests have developed resistance to the transgene product (Fatoretto et al., 2017). There is still a considerable need to identify genes involved in maize insect and disease resistance in order to promote incorporation by breeding or new technologies such as CRISPR/Cas.

Many studies on ear rot pathogens that are mycotoxin producers have identified regions of chromosomes, called quantitative trait loci (QTL) that are associated with resistance trait inheritance. A number of these studies were subjected to meta-analysis, and identified many chromosomal regions that are associated with inherited resistance through the comparison of progeny of crosses of resistant and susceptible inbreds (Xiang et al., 2010). However, these QTLs involve thousands of genes, requiring additional efforts to determine which genes are most relevant to pursue in breeding for increased resistance. QTL mapping, coupled with the sequencing of the maize genome, has made it possible to identify candidate genes potentially involved in insect and fungal resistance. A meta QTL associated with resistance to ear rot and mycotoxin producing fungi *Aspergillus flavus*, *Fusarium proliferatum*, *F.*

Abbreviations: AMU, atomic mass unit; *BAR*, bialaphos resistance; BMS, black Mexican sweet; DMSO, dimethyl sulfoxide; GGT, geranyl geranyl transferase; GUS, β-glucuronidase; HPLC, high pressure liquid chromatography; MALDI, mixed assisted laser desorption ionization; MS, mass spectrometry; *NOS*, nopaline synthase; NPGE, native polyacrylamide gel electrophoresis; OD, optical density; PAGE, polyacrylamide electrophoresis; PCR, polymerase chain reaction; PDA, photo diode array; QTL, quantitative trait locus; sCLIPS, self-calibrating line-shape isotope profile; SPME, solid phase micro extraction; TOF, time of flight; *Ubi1*, ubiquitin 1

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verticillioides and *F. graminearum* spanned portions of chromosome 3 (Xiang et al., 2010).

As part of this process, we identified a geranyl geranyl transferase-like gene (previously annotated in Gramene as a terpene cyclase) located in a resistance QTL on chromosome 3. Geranyl geranyl transferases (GGTs) are responsible for adding a long chain hydrocarbon to a protein to assist in membrane interactions and protein-protein interactions (Running, 2014). In several instances, proteins derivatized by geranyl groups have been associated with plant pathogen resistance in different plant species (Crowell and Huizinga, 2009). Because of a potential role in plant disease resistance, we decided to investigate this role through transgenic expression in maize callus along with evaluation of potential increases in resistance to representative maize ear insect pests and mycotoxin-producing ear rot pathogens. The gene was cloned and expressed in maize callus, which was evaluated for resistance to different species of maize pest insects and ear rot fungi. The relationship between resistance seen and potential gene product influences was determined through chemical analysis.

2. Materials and methods

2.1. Insects

Corn earworms (*Helicoverpa zea*) and fall armyworms (*Spodoptera frugiperda*) were reared on pinto bean based diet as described previously (Dowd, 1988). First instars were used for bioassays.

2.2. Fungi

Original stocks of *F. graminearum* (strain III-B, David Schisler, from Illinois), *F. proliferatum* (NRRL 13569, NRRL Culture Collection, originally from California) and *F. verticillioides* (AMRF-4, Robert Proctor, from Illinois) were collected from maize.

2.3. Transgenic callus production

The candidate gene was located as part of a selection process involving inspection of chromosome regions (QTLs) associated with ear rot resistance and/or mycotoxin production of multiple species of fungi (Xiang et al., 2010). The candidate gene was located on chromosome 3, 17,442,021–17,443,896 and had Gramene designation GRMZM2G451327 when originally accessed. Primers were designed for insertion into a biolistic construct as described previously (Dowd and Johnson, 2016) and were forward GCC-CGG-GAC-ATG-CTA-GAT-GAC-TGG-ACA-GGA-ATG-GAC-AAG, and CCC-GAG-CTC-CTA-TAA-TGC-AGC-AGC-GGA-GAG-GAT-CCC-GAG reverse. Thermal cycling conditions were the same as those described previously (Dowd and Johnson, 2016), except that the annealing temperature was 69.6 °C. RNA was isolated from a *Fusarium proliferatum/F. verticillioides* resistant inbred GE440 (Robertson et al., 2006) milk stage kernels using previously published methods (Wang et al., 2012), and cDNA was produced (Dowd and Johnson, 2016). Cloning, construction and bombardment of Black Mexican Sweet (BMS) maize cells were performed as described previously (Dowd and Johnson, 2016). In addition to the GGT gene, the construct also contained a *Bar* selectable marker gene, and both genes had a maize *Ubi-1* promoter and *Agrobacterium tumefaciens Nos* terminator. Clones and constructs were sequenced using cloning primers and PCR Mastermix (Roche, Indianapolis, Indiana) according to manufacturer's instructions. The transgene was detected using a primer located in the promotor region (CTG-CAG-GTC-GAC-TCT-AGA-GGA-TTC-CCC) and the reverse primer CTA-TAA-TGC-AGC-AGC-GGA-GAG-GAT located in the GGT gene.

2.4. Determination of potential GGT substrate proteins

Because of the potential role of the transgenically introduced GGT in ethanol production (see below) sequences of enzymes involved in ethanol production (alcohol dehydrogenase and pyruvate decarboxylase) that were potentially targets of GGT were obtained from Genbank and examined for sequence previously reported to be a target; C-C (Yalovsky et al., 1996) and CaaX, where a is an aliphatic amino acid and X is often leucine (Caldelari et al., 2001; Running, 2014). Those possessing the appropriate amino acid sequence if not localized at the C-terminus were further analyzed three dimensionally to determine if the sequence occurred on the surface of the enzyme and thus would be accessible to GGT. The predictive program SWISS MODEL (www.expansy.org, Biasini et al., 2014) was used to make this determination.

2.5. Bioassays

Insect and fungal bioassays were performed as described previously (Dowd and Johnson, 2016). Briefly, callus was placed on a Teflon® disk in a Petri dish with a tight fitting lid containing 3% agar. Ten larvae were added to each plate, and run in duplicate for each transformant type for 2–3 days. The larvae were evaluated for growth stage, frozen, and weighed after two days. Fungal assays were set up similarly, except there were six disks in each dish, and assays were rated after 40–46 h, depending on the species and inoculum load (target of 10–20 spores per callus clump). Experiments were repeated at least 3 times for both insect and fungal assays. Fungal growth was evaluated on a whole number zero to ten scale, with zero being no growth visible, and ten being callus covered by fungal growth; evaluations were performed using a dissecting microscope. The GUS callus served as a transformation control. The remaining callus was frozen at − 80 °C for use in subsequent analysis.

2.6. Secondary metabolite determination

Frozen callus samples were freeze-dried overnight and then ground to a fine powder. Weighed samples were placed in a vial and 5 mL methanol was added. The vials were capped and wrapped with sealing tape and sonicated for 30 min and allowed to stand at room temperature overnight. The extracts were recovered from the marc, evaporated to dryness, then resuspended in 200 μL of a methanol:DMSO (1:1) solvent. The extract was filtered through a 0.45 μM nylon 66 filter for HPLC analysis.

HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector, running under Shimadzu LCSolutions version 1.22 chromatography software, Columbia, MD, USA). The column was an Inertsil ODS-3 reverse phase C-18 column (5 μM, 250 × 4.6 mm, from GL Sciences). For phenolic analysis, the initial conditions were from 2% to 20% methanol and 0.025% trifluroacetic acid (TFA) in water, at a flow rate of 1 mL per minute. The effluent was monitored at 285 nm, 340 nm, or 520 nm on the variable wavelength detector depending on the nature of the compounds to be examined. After injection (typically 25 μL), the column was held at the initial conditions for 2 min, then developed to 100% methanol and 0.025% TFA in a linear gradient over 52 to 72 min. The GGT transformant samples showing obvious differences in metabolite composition were subjected to LC-MS analysis.

Samples were run on an Thermo Electron LTQ Orbitrap Discovery Mass Spectrometer – a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with a high energy collision (HCD) cell – with an Ion Max electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system (ACCELA 1250 UHPLC pump, ACCELA1 HTC cool stack autoinjector, and a ACCELA 80 Hz PDA detector) all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software. The MS was typically calibrated at least weekly with a standard calibration mixture recommended by Thermo Scientific and the signal detection optimized by running the autotune software feature as needed. The MS was run with the ESI probe in the