Agri Gene 9 (2018) 16–23

Contents lists available at ScienceDirect

Agri Gene

journal homepage: www.elsevier.com/locate/aggene

Overexpression of a maize (*Zea mays*) defensin-like gene in maize callus enhances resistance to both insects and fungi

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ARTICLE INFO

Keywords:
Defensin
Fusarium
Helicoverpa
Maize
Spodoptera

ABSTRACT

Identification of genes responsible for pest resistance in maize will assist with breeding attempts to reduce crop losses and hazards due to toxins produced by mold infecting ears. A gene coding for a defensin-like gene was cloned from an inbred reported to be resistant to *Fusarium proliferatum* and *Fusarium verticillioides* ear rot, based on its location in a QTL associated with resistance to those and other species of ear rot molds that produce mycotoxins. The gene was expressed transgenically in maize callus and the construct presence confirmed in transformants by PCR analysis and by detection with antibody made to a portion of the protein. Positive transformants were more resistant to corn earworms (*Helicoverpa zea*) and fall armyworms (*Spodoptera frugiperda*) as indicated by significantly lower weights compared to control callus expressing a β–glucuronidase (GUS) gene. Positive transformants also had significantly less visible growth of *F. proliferatum* and *F. verticillioides*, but not *Fusarium graminearum*, than controls. This indicates for the first time a defensin that is active against both insects and fungi, thereby allowing for more effective breeding for resistance to both major classes of pests attacking maize.

1. Introduction

Maize (*Zea mays* L.) is one of the most important food crops in the world (Shiferaw et al., 2011). Recent uses for ethanol production have increased demands and prices (Shiferaw et al., 2011), especially in years where production is reduced due to abiotic or biotic factors. Trends toward increased temperatures have already reduced maize production due to stress in several regions of the world (Lobell and Gourdji, 2012). Insects and disease also are responsible for decreasing maize production (Oerke, 2006). Mycotoxins produced by ear mold fungi further reduce the quality and safety of maize and cause further economic hardships (Robens and Cardwell, 2005). A variety of defensive chemicals and proteins are produced by maize that are targeted to insects and plant pathogens. Identification of defensive proteins has lagged behind that of chemicals, but some proteins have been positively identified as conferring resistance to insect pests (Dowd et al., 2008). A few maize proteins have demonstrated activity against both insects and fungi when expressed transgenically in plant tissue, such as a ribosome-inactivating protein (Dowd et al., 2003; Nielsen et al., 2001) and a peroxidase (Dowd and Johnson, 2016).

One class of proteins that has been identified as antifungal are the small, cysteine-crosslinked proteins called thionins. A diversity of structural types are reported, although they fall into different classes depending on the number of disulfide bridges and charges on the amino acids (Garcia-Olmedo et al., 1992). The γ-thionins have become known as defensins (Terras et al., 1995). Similar defensins are produced by plants, insects, vertebrates and fungi (Van der Weerden and Anderson, 2013). Antifungal activity has been reported for defensins from a wide variety of plant monocots and dicots (Van der Weerden and Anderson, 2013), including maize (Balandin et al., 2005, Wang et al., 2011). There appear to be few reports of *in vivo* activity of defensins against insects, although some can inhibit α-amylase activity of insect gut homogenates (Bloch Jr. and Richardson, 1991; Franco et al., 2002; Vi et al., 2017). Wheat derived purothionin and related thionins are toxic to some insects when injected, but not when administered orally (Kramer et al., 1979). Oral toxicity of bacterial expressed mung bean defensin VrCRP has been reported to one insect species, however (Chen et al., 2002). The mode of action of thionins/defensins against different organisms is often unclear, but proposed activities include causing membrane leakage, interfering with transcription, and inhibition of digestive

Abbreviations: DTT, dithiothreitol; GUS, β-glucuronidase; NEM, N-ethyl maleimide; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; QTL, quantitative trait locus; SDS, sodium dodecyl sulfate; TCEP, tris (2-carboxyl ethyl) phosphine hydrochloride

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https://doi.org/10.1016/j.aggene.2018.07.003
Received 23 March 2018; Received in revised form 17 July 2018; Accepted 18 July 2018
Available online 19 July 2018
2352-2151/ Published by Elsevier Inc.

enzyme activity (Garcia-Olmedo et al., 1992, Lay and Anderson, 2005, Wijaya et al. (2000). Membrane leakage caused by thionins and defensins may involve oligomerization of the monomers to form pores in the membranes (Stec et al., 2004; Lay et al., 2012).

QTL mapping, coupled with the sequencing of the maize genome, has made it possible to identify potential candidate genes that may be involved in insect and fungal resistance. A meta QTL associated with resistance to ear rot and mycotoxin producing fungi *Aspergillus flavus*, *Fusarium proliferatum*, *F. verticillioides*, and *F. graminearum* spanned portions of chromosome 5 (Xiang et al., 2010). Our examination of one QTL in the Gramene database identified a gene with thionin-like homology that was a potential candidate as a source of resistance to fungi, which had not previously been examined. We cloned this gene from a *F. proliferatum/F. verticillioides* resistant maize inbred, expressed it trasngenically in maize callus, and examined the callus for increased resistance to maize ear feeding insects and pathogens.

2. Materials and methods

2.1. Cloning and construct preparation

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 5, 211,666,179-211,666,773 and had Gramene designation GRMZM2G149869. Primers were designed for insertion into a biolistic construct as described previously (Dowd and Johnson, 2016) and were CCCCCGGGACATGAAGACCGGCCTCGTGA, forward; and CCCCCGAG CTCTCACGCGATTGATTCGCTACTA, reverse. Thermal cycling conditions were the same as those described previously (Dowd and Johnson, 2016), except that the annealing temperature was 73.5 °C. RNA was isolated from a *F. proliferatum/F. verticillioides* resistant inbred NC300 (Robertson et al., 2006) milk stage kernels using previously published methods (Wang et al., 2012). Cloning, vector construction and bombardment were performed as described previously (Dowd and Johnson, 2016). Clones and constructs were sequenced using cloning primers and PCR Mastermix (Roche, Indianapolis, Indiana) according to the manufacturer's instructions. The transgene was detected using a primer located in the promoter region (CTGCAGGTCGACTCTAGAGGATTCCCC) and the reverse primer used in cloning. Sequencing reactions were performed with the ABI BigDye® 3.1 (Foster City, CA) according to manufacturer's instructions; each reaction was repeated 3 times.

2.2. Analysis of protein properties

Protein properties were analyzed using DNAstar programs Editseq, Megalign, and Protean (DNAstar, Madison, Wisconsin). Potential effects of amino acid substitutions were determined using SNIP and SNAP analysis (Hecht et al., 2013).

2.3. Insect and fungi

Assays were performed as described previously (Dowd and Johnson, 2016). Briefly, ten first instar larvae of corn earworms (*Helicoverpa zea*) or fall armyworms (*Spodoptera frugiperda*) were placed in a Petri dish with a tight fitting lid (Falcon 351,006) containing 5 ml of 3% agar, in which the callus was placed on a 1 cm diameter Teflon® disk. Assays were performed in duplicate and run for 2–3 days. Fungal assays were set up similar to the insect assays, except six Teflon® disks with callus were placed in each dish, and 3 µl of a 0.1% Triton-X-100 solution containing a target quantity of 10–20 spores was placed on each callus clump. *F. graminearum* (strain III-B, David Schisler) assays were rated after 40 h, while *F. proliferatum* (NRRL 13569, NRRL Culture Collection) and *F. verticillioides* (AMRF-4, Robert Proctor) assays were rated after 45 h. All strains were originally collected from maize. Visual ratings for fungal growth on callus are significantly correlated with fungal DNA levels (Johnson and Dowd, 2016).

2.4. Preparation of antibody

Examination of the protein sequence with Protean indicated a highly antigenic region at the C-terminus of the protein. The antibody was produced by Bethyl Laboratories (Montgomery, Texas) using a synthesized peptide CPAVSPLAKNESIA.

2.5. Electrophoretic separation of protein

Transformed callus samples used in bioassays and previously frozen at −80 °C were homogenized in 100 µl of 0.01 M, pH 7.0 Tris HCl buffer containing 1% Brij35, in approximately a 1:1 volume ratio, using a prechilled small ground glass homogenizer (Radnotti Econo-grind, Covina, California). Homogenates were centrifuged at 10,000 x g for 15 min at 4 °C. The supernatant was removed, and an approximate volume of 100 µl resulted. 100 µl of a 0.1 M solution of Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was added to the supernatant to break cysteine crosslinking, and incubated at room temperature for 5 min. Then, 100 µl of a 0.1 M solution of *N*-ethyl maleimide (NEM) was added to derivatize the reduced free sulfhydryl group to prevent recrosslinking during the electrophoresis process, and the samples were again incubated for 5 min at room temperature. Samples were separated electrophoretically, Western blotted, and the protein detected with antibody using methods similar to those reported previously (Dowd et al., 2012). Samples were loaded on a 16% tricine or urea gel, in some cases with a 10% stacking region, which was run at 30 V until molecular weight markers began to leave the wells, and then at 200 V until molecular weight markers were well separated. Proteins were transferred to the PVDF membrane at 200 mA/h in CAPS buffer. Antibody detection was performed as described previously (Dowd et al., 2012). Deglycosylation reactions with PNGase A and F (NEB) were also performed prior to preparing the material with TCEP and NEM according to manufacturer's instructions.

2.6. Statistical analysis

Significant differences in insect weights and fungal growth were determined by analysis of variance, using Proc GLM, and correlations between biological activity and detected defensin levels were determined using Proc Reg of SAS version 8.0 for Windows.

3. Results

The sequence of the cloned gene (282 bp, 94 amino acids) was similar to that reported for GRMZM2G149869 and was identical for all three clones examined. However, the C-terminal region methionine (amino acid 88) of the sequence reported in Gramene, and for Genbank entry XP_008646345.1 [(defensin-like protein 124) (*ZmDef124*)] from inbred B73, was coded to a lysine in the cloned gene. Because of the similarity of the existing entry in Genbank, we will refer to the gene we cloned as *ZmDef124* throughout the rest of the paper. The difference between a methionine and lysine would potentially cause a functional difference, as respective SIFT and SNAP values are relatively high at 0.61/0.71 and 0.67/0.79, depending on which version is the reference. Eight cysteines were present and spaced approximately the same as other defensin examples from different monocots and dicots (Van der Weerden and Anderson, 2013). A putative glycosylation site (NES) was located at the C-terminus region in positions 89–91. There were 6 strongly acidic, 9 strongly basic, 28 hydrophobic and 35 polar residues, resulting in a calculated pI of 8.13 and charge of +2.83. However, cleavage of the N-terminal endoplasmic reticulum targeting sequence has typically been reported for defensins just prior to the first cysteine (Lay and Anderson, 2005). Assuming a similar cleavage pattern at the