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Full Length Research Paper

Pathogenesis-related gene expressions in different maize genotypes under drought stressed conditions

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Aflatoxins are carcinogenic compounds produced by *Aspergillus flavus* during infection of crops including maize (*Zea mays* L.). Contamination of maize with aflatoxin is exacerbated by late season drought stress. Previous studies have implicated numerous resistance-associated proteins (RAPs) that may be responsible for resistance to *A. flavus* colonization and aflatoxin accumulation. This study examined the transcript levels of three genes encoding RAPs utilizing quantitative real-time PCR, ZmPR-10 (PR-10), glyoxalase I (GLX-I), and a 14-kDa trypsin inhibitor (TI-14), in different maize lines under drought stressed and irrigated conditions to determine their potential utility as molecular markers for germplasm. Results suggested that drought stress during kernel development affected gene expression differently in different genotypes. Results showed that physiological stress induced by drought conditions was insufficient to stimulate significant changes in the expression of genes coding for the pathogen-specific, pathogenesis-related proteins PR-10 and GLX-I. However, ti-14 transcript levels were found to be elevated significantly indicating its possible use as a selection marker.

Key words: *Zea mays*, anti-fungal protein, aflatoxin, drought stress, qPCR.

INTRODUCTION

The infection and colonization of maize (*Zea mays* L.) by the fungus *Aspergillus flavus* may result in the accumulation of several carcinogenic mycotoxins collectively referred to as aflatoxins (Davis et al., 1966; Guo et al., 2009). The production of aflatoxin is exacerbated by exposure of *A. flavus* to elevated temperatures and low relative humidity (Widstrom et al., 2003). Drought can reduce the ability of maize to resist the growth of *A. flavus* because it negatively affects the expression of genes encoding resistance-associated proteins (RAPs) (Payne et al., 1986, 1998; Guo et al., 1995; Wang et al., 2008; Scully et al., 2009). This leads to increased pre- and post-harvest aflatoxin production and accumulation.

In recent years, research practices have shifted from field based evaluations and bio-assays to an approach centered more on molecular functionality and gene expression in hopes of explaining maize resistance mechanisms within various biochemical pathways (Cleveland et al., 2003, 2004). Past experiments have identified numerous RAPs associated with anti-fungal activities in maize kernels including globulin-2, late embryogenesis abundant proteins (LEA3 and LEA14), stress-related peroxiredoxin antioxidants (PER1), heat shock proteins (HSP17.2), cold-regulated proteins (COR),

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aldose reductase, ribosome inactivating proteins (RIPs), and hydrolases (chitinase and β-1,3-glucanases) (Walsh et al., 1991; Huynh et al., 1992; Huang et al., 1997; Guo et al., 1997, 1998, 1999; Moore et al., 2004; Chen et al., 2007). From among the potential stress-related RAPs, this study focused on the expression of the three genes coding for ZmPR-10 (PR-10), 14-kDa trypsin-inhibitor (TI), and glyoxalase I (GLX-I). These genes were selected based on recent studies in which developing maize kernel tissue was found to display diminished resistance to *A. flavus* colonization when these genes were silenced using RNA interference (RNAi) techniques (Chen et al., 2008, 2009).

The RAP ZmPR-10 is a member of the pathogenesis-related (PR) protein family that functions in plant defense. Plants expressing the pr-10 gene exhibited increased ribonuclease (RNase) activity as well as anti-fungal activity against *A. flavus* infection, suggesting that ZmPR-10 could be involved in host defense against *A. flavus* infection (Van Loon et al., 1999; Chen et al., 2006; Liu et al., 2006). The 14-kDa trypsin-inhibitor (TI) inhibits extracellular α-amylase, an enzyme known to function in the metabolism of complex carbohydrates in fungi (Woloshuk et al., 1997), resulting in reduced availability of simple sugars needed for fungal growth (Chen et al., 1998, 1999a, 1999b, 2002). In the presence of glutathione and glyoxalase II, glyoxalase I (GLX-I) functions as an aflatoxin production inhibitor by converting methylglyoxal, a cytotoxic aflatoxin inducer, into non-toxic D-lactate (Chen et al., 2004).

Maize inbred lines, commonly used in breeding synthetic populations with enhanced host-defenses against detrimental conditions such as fungal colonization, were selected for use in this study. Individuals exhibiting increased resistance were expected to express defense-related genes to a higher level than individuals more susceptible to fungal colonization. This statement was based on the assumption that protein levels correlate with transcript in the inbred lines examined in this study (Guo et al., 2008).

In addition to controlling template abundance for the synthesis of RAPs in mature maize kernels and potentially the phenotypic traits of drought tolerance or resistance to *A. flavus* colonization and aflatoxin production, variation in expression may provide utility in marker-assisted germplasm evaluation (Luo et al., 2008, 2010). In this study, therefore, the expressions of three RAP-coding genes, pr-10, ti-14, and glx-I, were compared between different maize lines to determine their transcriptional responses to drought stress conditions induced at various points during kernel development from 7 days after pollination (DAP) to 21 DAP. Transcript levels were evaluated using the quantitative real-time RT-PCR (qPCR) method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference for data normalization (Schmittgen and Zakrajsek, 2000; Luo et al., 2008, 2010).

MATERIALS AND METHODS

Plant materials and drought stress induction

In 2007, inbred maize lines B73, Mo17, Lo1016, Lo964, Tex6, and CLQ-G2507 were planted in the field under six rain-out shelters with managed irrigation. Three shelters served as fully-watered controls while the remainder functioned as water-stressed treatments. Drought stress was initiated at 20 days after pollination (DAP) in treatment plots while control plots were irrigated normally without drought stress. In 2008, in order to evaluate additional lines along with selected reference lines from the previous year, two additional inbred lines, A638 and CML-454, were included and lines Mo17, Lo1016, and Tex6 were excluded due to space limitations. Inbred lines B73, A638, Lo964, CLQ-G2507, and CML-454 were planted in the field under rain-out shelters in the same configuration described for the 2007 season, and drought stress was initiated at 7 DAP for A638 and 14 DAP and 21 DAP for other lines as early and late drought stress induction in treatment samples. This was done to examine the effects of drought stress induction at different points of kernel development. All control samples on both 2007 and 2008 were irrigated normally without drought stress. All inbreds were self pollinated and dated individually for harvest timing purposes. In addition, soil moisture readings were taken periodically to measure the drought stress status in treatment shelters. All self-pollinated ear samples in both years were harvested at 30 DAP, frozen in liquid N_2, and then stored at -80°C until use.

Total RNA extraction

Total RNA was extracted from kernels of a single ear of each line selected from harvested samples of each drought stress treatment using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and performed according to the manufacturer's instructions. Isolated total RNA was then treated with DNase (Qiagen, Valencia, CA, USA) and purified using an RNeasy Cleanup Kit (Qiagen). Purified total RNA was then checked for quality and quantity using a Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Analysis of transcript levels with real-time quantitative qRT-PCR

Primer sequences were designed, based on the published sequences (GenBank accession No.: ZmPR-10, AY953127; 14-kDa trypsin-inhibitor, X54064; glyoxalase I, AY241545) (Chen et al 2007) as follows: 5'-CACCTCAGTCATGCCGTTCA-3' (PR-10-F), 5'-CCCTCGATGAGCGTGTTCTT-3' (PR-10-R), 5'-GGCGTCGC CGAATGC-3' (TI-F), and 5'-GCTCATTCCTCATGCACTATGC-3' (TI-R), and 5'-GATGCTCCATGCGGTTTACC-3' (GLX-I-F), 5'-TCAGCAGTTTCATCCCAAAGC-3' (GLX-I-R). The effects of drought stress on the transcript levels of the selected defense-related genes in these maize lines were evaluated utilizing real-time qPCR. One-step real-time qPCR was performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) according to manufacturer's instructions. A total reaction volume of 25 µl consisting of SYBR

green RT-PCR master mix, QuantiTect RT mix, and 0.5 µM of each primer.

Real-time qPCR was performed with a DNA Engine Opticon (MJ Research, Waltham, MA, USA) and cycling parameters suggested by the QuantiTect SYBR green RT-PCR kit: reverse transcription was performed at 50°C for 30 min then terminated at 95°C for 15 min; PCR was then performed with 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Primer dimerization was examined for using melting curves generated at the end of the amplification cycles. Three technical replicates were performed for each sample. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal reference for calculating relative transcript abundance for this experiment with sequences of 5'-ACTGTTCATGCCATCACTGC-3' for the forward primer and 5'-GAGGACAGGAAGCACTTTGC-3' for the reverse primer (Luo et al., 2008, 2010).

Real-time quantitative RT-PCR result verification with semiquantitative RT-PCR

Results of the real-time qPCR were then confirmed using semiquantitative RT-PCR (Marone et al., 2001). Semiquantitative RT-PCR was performed using a SuperScript III First-Strand Synthesis kit (Invitrogen) and PTC-200 DNA Engine Gradient Thermal Cycler (MJ Research) according to the manufacturer's instructions. Cycling parameters for semiquantitative RT-PCR were as follows: samples were incubated at 94°C for 10 min to halt the reverse transcription reaction; The number of cycles was chosen to be in the exponential portion of the PCR reaction with 20 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final extension of 72°C for 10 min at the end of the cycle. Generated PCR products were then subjected to MetaPhor Agarose (Cambrex, Rockland, ME, USA) gel electrophoresis. Results of the electrophoresis were then compared to the real-time qPCR data for confirmation (unpublished data).

Data analysis

Amplification curves were generated from the real-time qPCR data and the cycle threshold (C_T) was calculated based on a fluorescence threshold of 0.01, where C_T was defined as the threshold cycle of PCR at which an amplified product was first detected. Subsequently the ΔC_T for each sample was determined using the equation $\Delta C_T = C_T$ target gene – C_T reference gene to calculate the relative expression of each gene to the internal reference control. This was accomplished via a modification of the original equation to relative expression = $1 / (2^{\Delta CT})$ for both the control and treatment samples (Livak and Schmittigen, 2001; Schmittigen and Livak, 2008).

RESULTS AND DISCUSSION

The expression of three genes (pr-10, glx-I, and ti-14) encoding proteins reported to be resistance-associated (Chen et al., 1998, 2004, 2006) was analyzed in the developing kernels in different maize lines in response to drought stress. Gene expression was evaluated in 2007 (Figure 1) and 2008 (Figure 2) in field-grown plants utilizing rain-out shelters. Soil moisture readings confirmed the presence of significant drought stress one week after stopping irrigation. Soils in irrigated plots were found to possess an average volumetric water concentration of 10.34% while drought stressed plots possessed an average volumetric water concentration of 4.49% (Scully and Guo, unpublished data).

Expression of pr-10

In different maize lines, pr-10 transcript levels in maize kernels at various stages of development and in response to drought stress were assessed using real-time qPCR in 2007 (Figure 1a). The expression of pr-10 in inbred lines B73, Lo964 and CLQ-G2507 was not statistically different ($P > 0.05$) between drought stressed kernels and the irrigated control (Figure 1a, lanes 1 and 2; lanes 7 and 8, lanes 11 and 12). In contrast, Mo17 and Tex6 displayed a significant increase (Figure 1a, lanes 3 and 4, and 9 and 10) and Lo1016 displayed a significant decrease (Figure 1a, lanes 5 and 6) in pr-10 transcript levels in response to drought stress. In contrast, real-time qPCR using RNA samples collected in 2008 indicated that pr-10 RNA levels were higher in CLQ-G2507 kernels (Figures 2a, lanes 9 to 11) but generally lower in all other lines tested. However, a down-regulation of pr-10 was observed early during drought induction in Lo964, B73 and CML-454 (Figure 2a, lanes 4, 7, and 13), but RNA levels returned to those observed in well-watered samples late in drought induction. No differences in pr-10 RNA levels were observed in line A638 in response to drought stress.

It was found that pr-10 RNA levels were overall lower than those of glx-I or ti-14 in both the 2007 and 2008 treatment regiments regardless of genotypes. This trend may be due to an insufficient level of stress on the plants as pr-10 typically activates only in response to pathological or highly stringent stress-related conditions (Lo et al., 1999; Bantignies et al., 2000). The low expression may also be due to maize kernels being the source of sampled tissues. Recent studies reported that pr-10 exhibits differential expressions and is highly expressed in root tissues instead of aerial vegetative or reproductive tissues (Xie et al., 2009a, 2009b).

Expression of glx-I

Relative transcript levels of the glyoxalase I (GLX-I) coding gene were also analyzed in different maize lines under drought stress versus well-watered conditions in 2007 and 2008. In 2007, real-time qPCR revealed that glx-I transcript levels increased significantly in lines Mo17 and Tex6 in response to drought stress (Figures 1b, lanes 3 and 4, and 9 and 10), however, this was not the case for lines B73, Lo964, and CLQ-G2507 (Figure 1b,

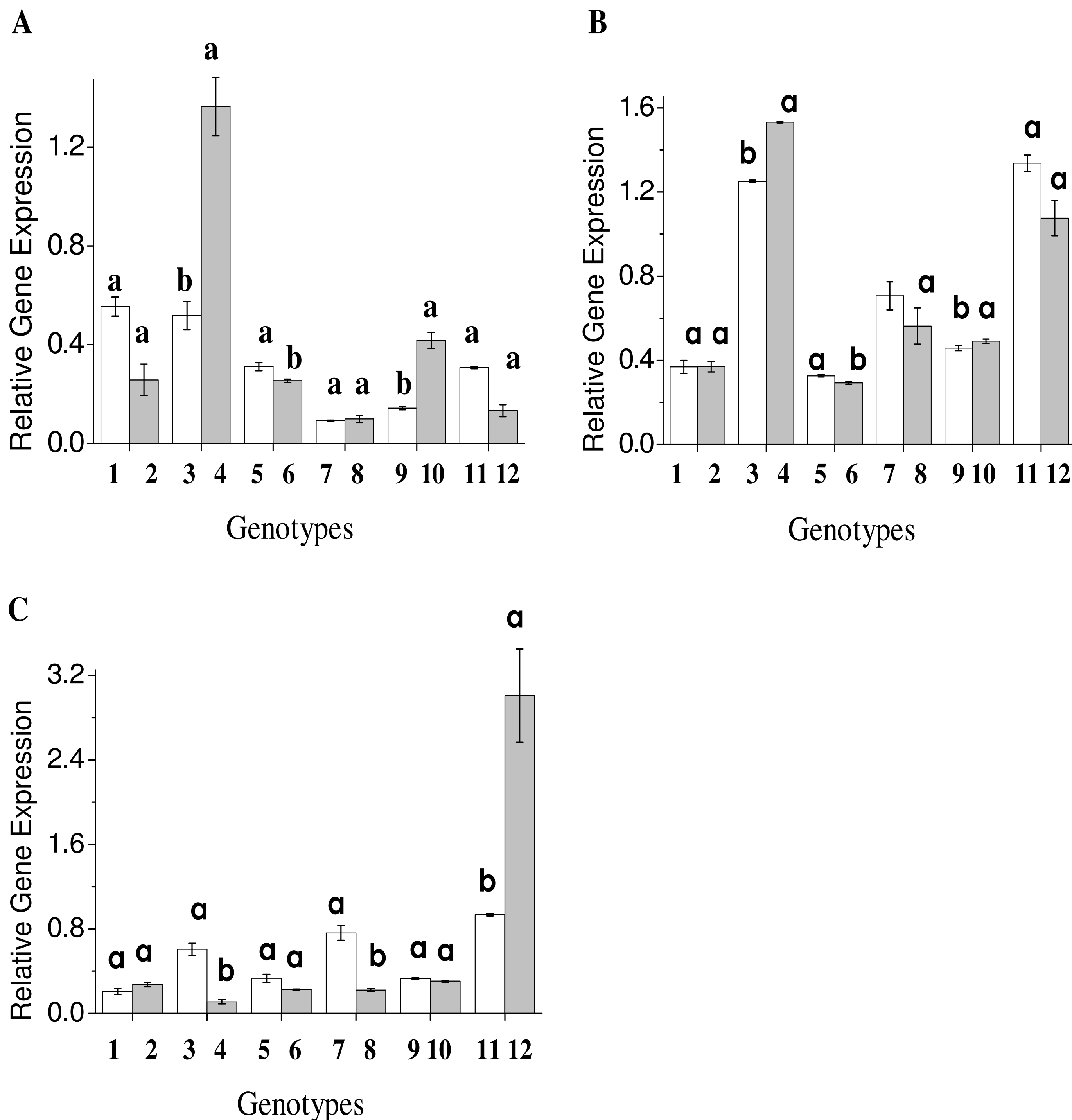

Figure 1. Expression analysis of genes encoding resistance-associated proteins (RAPs) PR-10 (A), glyoxalase I (GLX-I) (B), and 14-kDa trypsin inhibitor (TI) (C) using real-time qPCR in developing kernels in 2007. Transcript levels were obtained from the real-time qPCR analysis using the 2-ΔC_T method and expressed as relative expression of the gene of interest and the reference gene. Maize genotypes used in 2007 were B73 (lanes 1 and 2), Mo17 (lanes 3 and 4), Lo1016 (lanes 5 and 6), Lo964 (lanes 7 and 8), Tex6 (lanes 9 and 10), and CLQ-G2507 (lanes 11 and 12). Odd numbered lanes are irrigated control samples for each genotype while even numbered lanes are drought stressed treatments for each genotype. Values are means of three technical replicates (n = 3). Error bars indicate 95% confidence intervals. Values with the same letters within the same genotype are not significantly different at 95% confidence (Student's t-test).

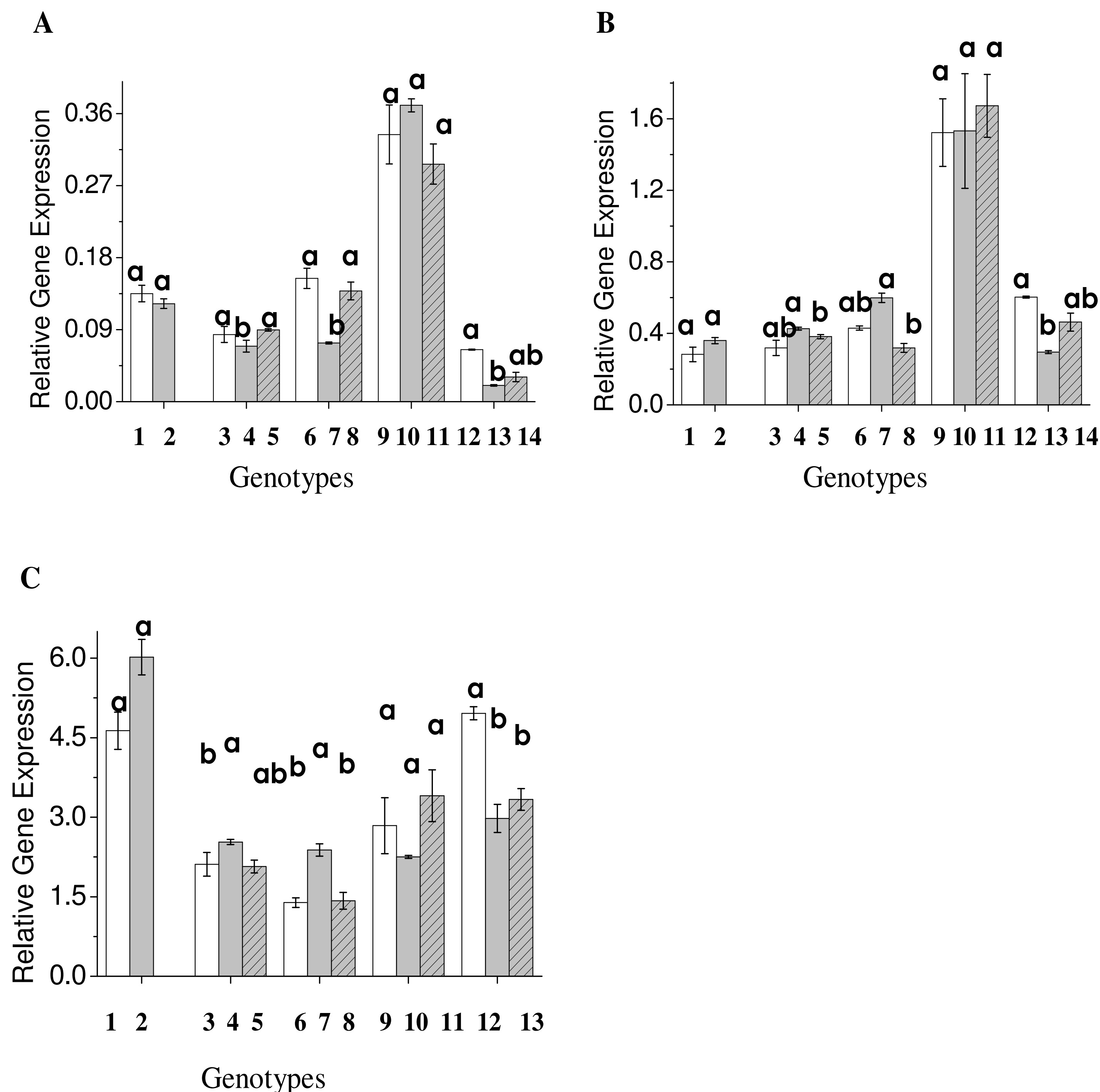

Figure 2. Expression analysis of genes encoding resistance-associated proteins (RAPs) PR-10 (A), glyoxalase I (GLX-I) (B), and 14-kDa trypsin inhibitor (TI) (C) using real-time qPCR in developing kernels in 2008. Transcript levels were obtained from the real-time qPCR analysis using the 2-ΔC_T method and expressed as relative expression of the gene of interest and the reference gene. Total RNA was extracted from developing maize kernels with drought stress or no stress. Maize genotypes used in 2008 were A638 (lanes 1 and 2), Lo964 (lanes 3, 4, and 5), B73 (lanes 6, 7, and 8), CLQ-G2507 (lanes 9, 10, and 11), and CML-454 (lanes 12, 13, and 14). The first lane of each genotype is an irrigated control sample, the second lane is an early-induced drought stress sample, and the third lane is a late-induced drought stress sample. Drought stress treatments were introduced as follows: 7 DAP for A638, 14 DAP and 21 DAP for the other genotypes. Values are means of three technical replicates (n = 3). Error bars indicate 95% confidence intervals. Values with the same letters within the same genotype are not significantly different at 95% confidence (Student's t-test).

lanes 3 and 4, and 9 and 10). Furthermore, glx-I transcript levels displayed a significant reduction in line Lo1016 in response to drought stress (Figure 1b, lanes 5 and 6). In 2008, the selected maize lines were drought stressed at varying points as previously described. Real-time qPCR revealed that the line A638 displayed no significant change in glx-I expression at 7 DAP drought stress (Figures 2b, lanes 1 and 2). CLQ-G2507 possessed the highest transcript levels of glx-I in comparison to the other lines in all treatments, however, no significant change was found to occur within the line in response to drought stress (Figures 2b, lanes 9 to 11). Lines B73 and Lo964 displayed slight increases in glx-I transcript levels during early drought stress induction, but no change during late drought stress induction relative to the well-watered control (Figures 2b, lanes 3 to 8). The line CML-454 was found to have a significant reduction in transcript levels of glx-I in response to early drought stress which increased to a level intermediate between that of the control and early drought stress treatment in later induced drought stress samples (Figures 2b, lanes 12 to 14).

Generally, there was found to be little variation in glx-I transcript levels in both 2007 and 2008 in all of the examined lines and drought stress induction schedules. Chen et al. (2004) reported that GLX-I, along with several other proteins (Chen et al., 2001), was found to be present at significantly higher levels in maize lines resistant to aflatoxin contamination in comparison to susceptible lines following inoculation with *A. flavus*. Fungal infection was found to significantly increase levels of methylglyoxal (MG), a reported inducer of aflatoxin pathway genes, in two of three examined susceptible maize lines. It was suggested that GLX-I may play an important role in controlling MG levels inside maize kernels, thereby contributing to the lower levels of aflatoxin in the resistant maize lines (Chen et al., 2004). In this study, treatment samples were not inoculated with *A. flavus*, rather they were subjected only to drought stress conditions and the expression of glx-I was found not to vary significantly among the treatments and examined lines. These observations support the findings of Chen et al. (2004) in that the expression of genes coding for GLX-I were not highly affected due to the absence of sufficient pathogenic stress as well as the absence of the enzyme complex substrate, MG, a substance produced during infection by aflatoxin producing fungi.

14-kDa trypsin inhibitor encoding gene expression

Relative transcript levels of the 14-kDa trypsin inhibitor (TI-14) coding gene (ti-14) were analyzed in different maize lines under drought versus well-watered conditions in 2007 and 2008 as described earlier. The overall expression of ti-14 was generally higher in 2007 and 2008 compared to that of pr-10 or glx-I in most of the lines examined in this study (Figures 1c and 2c). In 2007, real-time qPCR revealed that B73, Lo1016, and Tex6 possessed no significant differences in relative transcript levels in response to drought stress, but Mo17 and Lo964 showed significant reduction in response to drought stress (Figure 1c, lanes 1 to 10). In contrast, CLQ-G2507 displayed a significant up-regulation in transcript levels in response to drought stress (Figure 1c, lanes 11 and 12).

Lines examined in 2008 were drought stressed at different stages in kernel development as previously described. Real-time qPCR revealed that the maize lines A638 and CLQ-G2507 possessed elevated transcript levels of ti-14, however little variation was observed in response to drought stress in comparison to the well watered controls (Figure 2c, lanes 1 and 2, and 9 to 11). Lo964 and B73 had an up-regulation of ti-14 levels during early drought stress induction which then reduced to the control level during late drought stress induction (Figure 2c, lanes 3 to 5, and 6 to 8). However, CML-454 displayed a down-regulation of ti-14 transcript levels to a similar degree in both the early and late drought treatments (Figure 2c, lanes 12 to 14). Given the elevated transcript levels of the genes coding for TI-14, this protein may accumulate to a higher constitutive concentration in mature maize kernels than other pathogenesis-related proteins such as ZmPR-10 and GLX-I. This may result in improved post-harvest resistance to fungal colonization and subsequent mycotoxin production. This hypothesis may be confirmed in future studies using field-based and laboratory-based bioassays (Chen et al., 1998). In addition, the observed elevated transcript levels of ti-14 in the absence of pathogenic stress indicates that this gene may possess potential for utilization as a selection marker, although additional study is required to definitively confirm this observation.

SUMMARY AND CONCLUSION

In order to investigate the possibility of a relationship between the expressions of pr-10, glx-I, and ti-14 in immature maize kernels and drought stressed environmental conditions, several maize inbred lines with different phenotypic traits such as drought tolerance and fungal colonization resistance were selected from our breeding program, and planted in 2007 and 2008. Drought stress conditions were then induced at different growth stages during kernel development and relative transcript levels of pr-10, glx-I, and ti-14 were evaluated using real-time qPCR. Results suggested that drought stress during kernel development affected transcript

levels differently in different lines. Given the known putative functions of the genes examined in this study (Chen et al., 1999b, 2004, 2006), the data suggest that other biotic and/or abiotic factors also may influence phenotypic traits in developing maize kernels.

Recent genetic studies have shown that resistance to *A. flavus* infection and aflatoxin production in maize is a polygenic trait (or a quantitative trait) (Walker and White, 2001). Given the polygenic source of the overall resistant phenotype of maize as well as the apparent requirement of elevated levels of pathogenic stress for the activation of genes coding for pathogen-specific defense proteins such as PR-10 and GLX-I, this study was unable to conclusively determine whether these genes could be used as selective breeding markers in the absence of pathogen infection. However, the observed elevated transcript levels of ti-14 in the absence of pathogenic stress indicates that this gene may have potential for use as a selection marker, and may contribute more to post-harvest, host-defense phenotypes due to a possibly heightened constitutive concentration of the TI-14 protein in mature maize kernels. Additional study is required to definitively confirm these observations.

The transcript patterns and interactions observed in this study indicate that the control of certain phenotypic traits, such as low fungal colonization or aflatoxin accumulation, is a complex and interconnected process involving many gene products and transcriptional regulators as well as various host interactions with environmental factors. Future studies should focus on in-depth investigation on the progression of gene expression variation over time for the genes featured in the present study and their potential correlation with the expression of other RAP-coding genes via genomic expression analytical methods (Luo et al., 2008, 2010). These studies should also seek to examine the expression of these genes as well as other RAP-coding genes in the presence of pathogenic stress induced by fungal inoculation. Finally, variations in the expression of the genes coding for these RAPs should also be further examined in mature kernels as well as various other maize tissues, such as roots and leaf tissues, in response to biotic and abiotic factors via proteomics and gene expression analyses.

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