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Quantitative Expression Analysis of *GH3*, a Gene Induced by Plant Growth Regulator Herbicides in Soybean

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Symptoms resembling off-target plant growth regulator (PGR) herbicide injury are frequently found in soybean fields, but the causal agent is often difficult to identify. The expression of *GH3*, an auxin-regulated soybean gene, was quantified from soybean leaves injured by PGR herbicides using real-time RT-PCR. Expression of *GH3* was analyzed to ascertain its suitability for use in a diagnostic assay to determine whether PGR herbicides are the cause of injury. *GH3* was highly induced by dicamba within 3 days after treatment (DAT) and remained high at 7 DAT, but induction was much lower at 17 DAT. *GH3* was also highly induced at 7 DAT by dicamba + diflufenzopyr, and to a lesser extent by the other PGR herbicides clopyralid and 2,4-D. The non-PGR herbicides glyphosate, imazethapyr, and fomesafen did not significantly induce *GH3* expression above a low constitutive level. These results indicate that a diagnostic assay for PGR herbicide injury based on overexpression of auxin-responsive genes is feasible, and that *GH3* is a potential candidate from which a diagnostic assay could be developed. However, time course analysis of *GH3* expression indicates the assay would be effective for a limited time after exposure to the herbicide.

KEYWORDS: Auxin; dicamba; *GH3*; plant growth regulator; real-time RT-PCR

INTRODUCTION

Plant growth regulator (PGR) herbicides are widely used in corn (*Zea mays*) and wheat (*Triticum aestivum*) production, due to their effectiveness at controlling broadleaf weeds, including weed biotypes resistant to other classes of herbicides. However, corn and soybean are generally grown in rotation and are often grown in close proximity to each other. This can lead to off-target injury because soybean (*Glycine max*) is highly sensitive, and these herbicides can induce abnormal foliar development at very low doses. Among the PGR herbicides in common use today, dicamba is the most active and can induce off-target injury in dicot crops at extremely low concentrations (*1*, *2*). Foliar soybean injury symptoms include leaves that are cupped or puckered, strapped, or with parallel venation. Injury from PGR herbicides can also result in delayed maturity, decreased yield, and reduced germination of harvested seed (*1*, *3*). Yield reductions are usually accompanied by more severe symptoms such as terminal bud kill, swollen petioles, and splitting of the stem (*4*−*6*). Symptoms resembling PGR herbicide damage are commonly reported during the growing season, and the increased use of postemergence herbicides has coincided with an increase in the frequency of these injury reports (L. M. Wax, personal communication).

There are various ways in which soybean may be exposed to a PGR herbicide. The herbicide can move off-target through spray particle drift or volatilization, or residues can be dislodged from application equipment that was used for previous applications to a corn or wheat crop (*4*, *6*). The cause of reported cases of foliar injury is often difficult to determine, however. The source of PGR herbicide is often not readily determined, and injury from other stresses can be mistaken for damage by a PGR herbicide. For example, other postemergence herbicides as well as the soybean viruses bean pod mottle virus and soybean mosaic virus can cause foliar injury symptoms that may be confused with PGR herbicide injury. Currently, there is no diagnostic tool available to determine whether a reported case of injury is caused by a PGR herbicide or not, which complicates the resolution of grower complaints.

Because PGR herbicides cause injury at extremely low rates, the herbicide itself can be present in plant tissues below the detection threshold of analytical equipment and still cause injury (*7*). However, the mode of auxinic herbicide action yields biochemical markers specific to this class of herbicides. Many classes of herbicides inhibit a specific enzyme in a biochemical pathway, causing a detectable buildup of precursors and/or the depletion of end-products that are a sign of the phytotoxic action of the herbicide (*8*, *9*). PGR herbicides over-stimulate the auxin hormonal pathway to produce their phytotoxic effect, and specific genes are induced in injured plants (*10*). Several soybean genes have been identified that are induced in response to auxin application (*11*−*14*). By identifying genes that are overexpressed in response to PGR herbicides and not other plant stresses, a diagnostic assay can be developed to help identify

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or rule out PGR herbicides as the causal agent of abnormal foliar symptoms in soybean.

To develop this assay, we have examined the differential expression of the soybean gene *GH3* in response to PGR herbicides. *GH3* is considered a primary-response gene in the auxin signal pathway (*15*). Its expression has been shown to be induced within 5 min of auxin application, and little to no expression has been detected in untreated soybean leaves (*16*, *17*). Preferably, a diagnostic assay for PGR herbicide injury would be based on a gene that is normally expressed at a low level (or not at all) in soybean leaves, but is induced to a high, easily detectable level of expression only by PGR herbicides over an extended period of time.

In the current study, *GH3* expression levels in uninjured and PGR-herbicide injured soybean leaves were quantified using a PCR-based technique. Real-time RT-PCR was used for expression analysis because it allows for highly reproducible quantification (*18*, *19*). In addition, this method has a high level of specificity and is very sensitive, theoretically able to detect expression of a single transcript copy per reaction (*20*−*22*). These qualities are ideal for quantification of *GH3* transcripts under different circumstances to evaluate the feasibility of using *GH3* as an expressed marker in a diagnostic assay. The objectives of this study were to (1) verify the responsiveness of *GH3* to auxin in soybean leaves by exogenous application of indole-3-acetic acid (IAA), (2) evaluate *GH3* response to PGR herbicides, with non-PGR herbicides included as an alternative stress to the plant, and (3) evaluate *GH3* expression in response to dicamba over several time points to determine the length of time during which overexpression could be detected. Our results show that *GH3* is highly induced by auxin and dicamba and could be useful as a diagnostic marker for PGR herbicide injury.

MATERIALS AND METHODS

Plant Material. The glyphosate-resistant Pioneer soybean variety 94B01RR was planted in all experiments. Plants for treatment with auxin were grown under greenhouse conditions and treated when plants were in the V3 growth stage (25 cm tall). A solution containing 0.5% (w/v) of the sodium salt of IAA was applied with a paintbrush to the upper surfaces of all leaves until leaf surfaces were wet with the solution. Plants for all other treatments were grown under field conditions in the summer of 2003 at the Crop Science Research and Education Center in Urbana, IL. Herbicides were applied to soybean in the V3 growth stage (23 cm tall). Treatments were applied with a CO_2-pressurized backpack sprayer equipped with 8003 flat-fan nozzles that delivered 187 L/ha. Treatments included the diglycolamine salt of dicamba at two rates (5.6 g ae/ha and 0.56 g ae/ha), a commercially available premix containing the sodium salt of dicamba plus the sodium salt of diflufenzopyr (1.4 g ae/ha + 0.56 g ae/ha, respectively), clopyralid (2.1 g ae/ha), and the isooctylester formulation of 2,4-D (170 g ae/ha). These application rates were selected to induce foliar leaf symptoms without causing plant death and represent fractions of labeled use rates applied in corn or wheat. All PGR herbicides were applied with 0.25% (v/v) of a nonionic surfactant (83% alkylphenyl hydroxypolyoxyethylene, 17% natural fatty acids). Glyphosate, imazethapyr, and fomesafen were applied at the labeled soybean use rates of 840 g ae/ha, 71 g ae/ha, and 313 g ae/ha, respectively. Ammonium sulfate was applied with glyphosate at 1.9 kg/ha. Methylated seed oil and 28% urea ammonium nitrogen were both applied with imazethapyr at 1.25% (v/v) and with fomesafen at the rates 1.0 and 2.5% (v/v), respectively. These three non-PGR herbicides were selected because they have three different modes of action and are three of the most commonly used herbicides in soybean production. At different time points after symptom development, the newest trifoliate leaves were harvested and stored at −80 °C until RNA extraction.

RNA Extraction and cDNA Preparation. Total RNA was extracted from leaf tissue using the TRIzol total RNA isolation reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's recommendations. RNA was quantified at OD_{260} using a GeneQuant pro spectrophotometer (Amersham Pharmacia, Cambridge, England). DNA contamination was removed from RNA using DNA-free DNase Treatment and Removal Reagent (Ambion Inc., Austin, TX) according to manufacturer's instructions. The following manufacturer-recommended modification was used: RNA was diluted to 100 μg/mL, and the incubation time was extended to 1 h at 37 °C. Reverse transcription (RT) reactions were carried out in a 20 μL volume using 0.67 μg total RNA as template, annealed to 0.5 μg oligo$(dT)_{12-18}$ primer (Invitrogen Life Technologies) and 200 units of Superscript II RT (Invitrogen Life Technologies) at 42 °C for 50 min followed by inactivation at 70 °C for 15 min according to instructions provided by the supplier.

Oligonucleotide Primers and Probe for Real-Time RT-PCR. The sequences for *GH3* oligonucleotide primers and probe were designed using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA) to span an intron to reduce amplification of contaminating genomic DNA. The probe was labeled with the fluorescent FAM reporter dye on the 5′ end and a nonfluorescent minor groove binding dye on the 3′ end.

GH3 Primers. Forward: 5′-AGA GAT GGA CCG TCG TCA ATT AA-3′. Reverse: 5′-TTG TCC ATA TCA GTC ACG TAT TGA TT-3′. Probe: 5′-TCA GCC TTC CCA TGC C-3′.

To verify the quality of the RNA and that reverse transcriptase reactions were successful, the expression of the constitutive gene *Gmpcc16* was also examined. This gene is a member of a gene family of phosphoenolpyruvate carboxylases (PEPC) and has been shown to be expressed in soybean at similar levels in several different tissues (*23*). The oligonucleotide primers and probe for *Gmpcc16* were designed from the 3′ untranslated region to amplify the cDNA from this specific *PEPC* and not other members of the same gene family (*24*). The probe was labeled with the fluorescent FAM reporter dye on the 5′ end and the TAMRA quencher dye at the 3′ end.

Gmpcc16 Primers. Forward: 5′-TTC CTT TAT CAG AAA TAA CGA GTT TAG CT-3′. Reverse: 5′-TGT CTC ATT TTG CGG CAG C-3′. Probe: 5′-CCC TCC CCT GTA CCC ATG TTT CCA TTA TAA-3′. (*24*).

DNA Standards Used for Quantification. The DNA standard for *GH3* quantification was the full-length *GH3* cDNA in pBluescript KS + plasmid (*12*). The plasmid was used to transform *E. coli* competent cells and was then purified from liquid overnight cultures using the QIAfilter Plasmid Midi Kit (Qiagen Inc., Valencia, CA). Template copy numbers were determined from OD_{260} using a GeneQuant pro spectrophotometer (Amersham Pharmacia Biotech, Cambridge, England) and the size of the plasmid (5.6 kb). Values were multiplied by 2 because a dsDNA standard provides twice as much template as a single-stranded cDNA.

The DNA standard for *Gmpcc16* quantification was obtained from cloning a PCR fragment into the pCR2.1-TOPO plasmid using the TOPO-TA cloning kit (Invitrogen Life Technologies). The same oligonucleotide primers used for real-time RT-PCR were used to amplify the fragment used for cloning from a cDNA template. The sequence of the standard in the plasmid was verified by DNA sequencing (University of Illinois W. M. Keck Center for Comparative and Functional Genomics, Urbana, IL). The *Gmpcc16* plasmid standard was purified and its template copy number determined in the same manner as the *GH3* standard. Using the copy number, dilution series were prepared for each gene in 10-fold dilutions to develop a standard curve during real-time PCR.

Quantitative Real-Time RT-PCR. Quantitative real-time RT-PCR was performed using the 7900 HT Sequence Detection System (Perkin-Elmer Applied Biosystems) according to manufacturer's instructions, except that the reaction volume was reduced to 25 μL. The reaction mixture included (as template) 1/20 of a 20 μL RT reaction that had been diluted 5-fold with TE buffer (10 mM Tris-HCl, pH 8; 1 mM Na_2EDTA). The reaction also contained oligonucleotide primers and probe as described below, and Taqman Universal PCR Master Mix (Perkin-Elmer Applied Biosystems) including AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, and ROX dye as an internal reference.

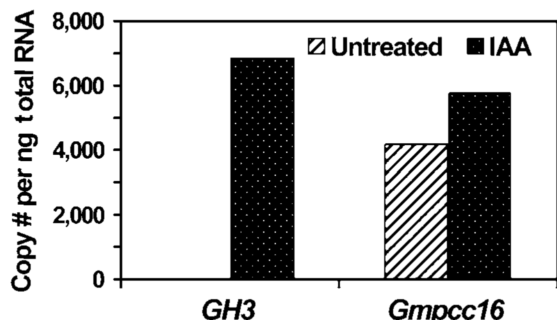

Figure 1. Quantitative expression analysis of *GH3* and *Gmpcc16* (control gene) from newly developing soybean leaves harvested 24 h after being treated with a 0.5% (w/v) solution of indole-3-acetic acid brushed onto leaves. There were 5 copies of *GH3* per ng total RNA detected from the untreated sample (not visible on the graph). Values were corrected for any genomic DNA contamination by subtracting the negative RT control values.

For *GH3* amplification, primer and probe concentrations were 900 nM and 200 nM, respectively. For amplification of *Gmpcc16*, primer and probe concentrations were 800 nM (forward primer), 400 nM (reverse primer), and 50 nM (probe). Reaction conditions included an initial temperature of 50 °C for 2 min, followed by 95 °C for 10 min. This was followed by 40 cycles of 95 °C for 15 s and then 60 °C for 60 s. RNA that had not undergone reverse transcription was also used as a template (negative RT control) to determine the presence of genomic DNA contamination. Any value detected from the negative RT control was subtracted from the positive RT corresponding reactions.

Quantification is based on a Ct value, which is the PCR cycle number when probe fluorescence exceeds a threshold value which is arbitrarily set in the exponential phase of reaction fluorescence. Copy numbers of mRNA transcript per ng total RNA were determined using absolute quantification based on a standard curve generated by plotting copy numbers of DNA standards versus Ct values (*19*). All standard curves used to quantify real-time RT-PCR assays had R^2 values that exceeded 0.97 with the majority exceeding 0.99. Apart from some negative RT reactions that were conducted in duplicate, all reactions were conducted in triplicate.

Data Analysis. All experiments involving herbicide application were conducted twice, beginning with independent RNA extractions. The natural auxin treatment (positive control) was conducted once. Data were subjected to ANOVA, and where appropriate, means were separated by Fischer's protected least significant difference test at the 0.05 probability level. Copy number data were transformed by base 10 logarithm prior to statistical analysis to stabilize variances. This is in agreement with the method of quantification since the standard curve used to quantify the data was based on a dilution series with an order of magnitude between dilutions. Untransformed data are presented with statistical interpretation based on transformed data.

RESULTS AND DISCUSSION

Natural Auxin. Soybeans were treated with IAA, an endogenous auxin that would not cause plant injury under normal circumstances. However, at the high concentration applied in this experiment, IAA treatment resulted in injury resembling symptoms caused by PGR herbicides. Symptoms caused by IAA included epinasty, stem curvature and foliar malformations. The newest trifoliate leaves were harvested 24 h after exogenous IAA application to soybean. RNA was extracted and used for real-time RT-PCR analysis to quantify the expression of *GH3* and the control gene *Gmpcc16*. Expression differences for both genes are shown in **Figure 1**. Genomic DNA contamination from negative RT controls was very low for *GH3* (0.18 copies/ng or less) and not detected for *Gmpcc16*. While *Gmpcc16* was expressed at a similar level in both treated and untreated leaves (**Figure 1**), *GH3* expression was barely detectable (5 copies per ng total RNA) in untreated leaves, but was induced to nearly 7000 copies per ng total RNA by IAA application. These results are consistent with previous reports of the auxin inducibility of *GH3* (*25*) and act as a reference for further experiments testing the effects of PGR herbicides on *GH3* expression.

Herbicide Treatments. *GH3* expression was evaluated following the application of several PGR and non-PGR herbicides to soybean. The lower of the two rates of dicamba mentioned previously (0.56 g ae/ha) was used for this experiment. Dicamba and dicamba + diflufenzopyr caused comparable injury in the field including leaf cupping and short, highly branched plants. Clopyralid also caused leaf malformations and short, branched plants, but injured leaves were more strapped with parallel veins. However, 2,4-D had very little effect on leaf morphology, though there was some epinasty. Imazethapyr temporarily stunted plant growth but resulted in a minimal reduction in height. Fomesafen caused temporary contact leaf burn but had no effect on subsequent growth.

Newly developing trifoliate leaves were harvested 7 days after treatment, and *GH3* and *Gmpcc16* expression was quantified using real-time RT-PCR. **Figure 2** shows the differences in gene expression between treatments. Genomic DNA contamination from negative RT controls was very low for *GH3* (1.1 copies/ng or less) and not detected for *Gmpcc16*. *Gmpcc16* expression was detected at high levels in all samples tested, ranging from 7024 to 14 921 copies per ng total RNA (**Figure 2B**). *GH3* was expressed at a very low level in leaves that were not injured by PGR herbicides (7.3 to 17.1 copies/ng), but was highly expressed in response to dicamba (1367 copies/ng), and dicamba + diflufenzopyr (1207 copies/ng) (**Figure 2A**). Clopyralid also induced *GH3* expression, but to a lesser degree than dicamba (403 copies/ng). *GH3* expression in response to 2,4-D was much lower (48 copies/ng) than expression in response to other PGR herbicides, but was still significantly higher ($P < 0.05$) than the untreated control. Expression results correlate well with injury levels because almost no leaf injury was caused by 2,4-D. In contrast, dicamba caused clearly visible leaf injury and also induced *GH3* expression the most (187-fold higher than untreated).

These results demonstrate the ability of PGR herbicides to induce expression of *GH3* in soybean leaves. Different PGR herbicides were shown to induce *GH3* to varying degrees. Non-PGR herbicides with three different modes of action had no effect on *GH3* expression, even though two of them caused visible injury to the plant. Because *GH3* expression was clearly induced by PGR herbicides to levels that correlated well with injury, an assay for *GH3* expression has potential to indicate whether foliar leaf injury found in soybean fields is caused by PGR herbicides. However, the length of time after herbicide exposure that *GH3* overexpression can be detected must be determined to realize the time limitations of the assay.

Dicamba Time Course. Newly developing trifoliate leaves were harvested and assayed for *GH3* expression at several timepoints following application of two rates of dicamba to determine the duration of *GH3* induction after application. Foliar leaf malformations were visible within 3 days of the highest rate of dicamba but took longer to develop from the lower rate, and the higher rate caused more severe damage. Leaves were harvested at 3, 7, 17, and 21 days following application, though no sample was collected for the lower rate of dicamba at 3 days because symptoms had not yet developed. **Figure 3** shows the differences in gene expression over time at different rates of dicamba. Genomic DNA contamination from negative RT controls was low for both *GH3* (0.39 copies/ng or less) and for

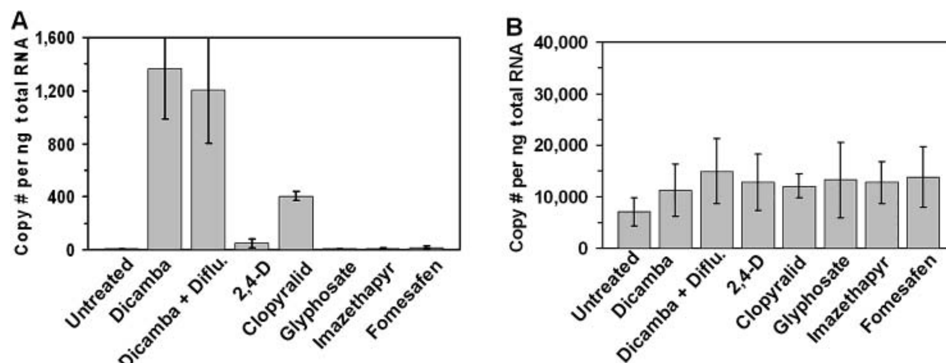

Figure 2. Quantitative real-time RT-PCR expression of *GH3* (A) and *Gmpcc16* (B) from newly developing soybean leaves harvested 7 days following treatment with plant growth regulator (PGR) and non-PGR herbicides. Data represent means between two separate experiments. Positive RT reactions were corrected for any genomic DNA contamination by subtracting the negative RT controls. Error bars represent standard errors of positive RTs.

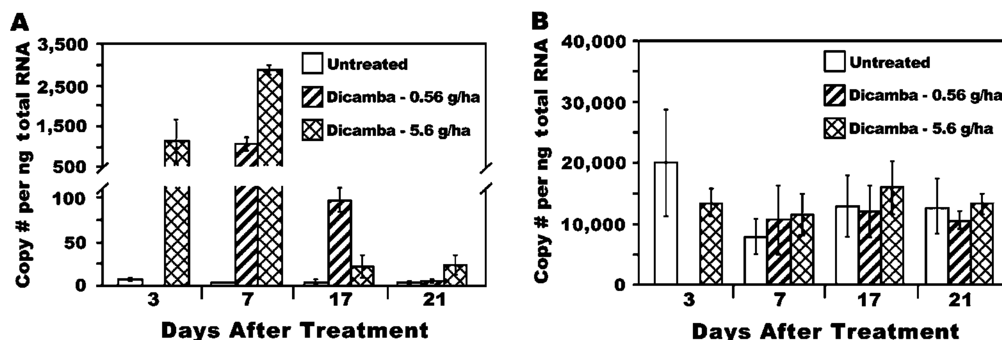

Figure 3. Quantitative real-time RT-PCR expression of *GH3* (A) and *Gmpcc16* (B) from newly developing soybean leaves harvested at different time points following application of two different rates of dicamba. Leaves were not harvested at 3 days after treatment from plants treated with the lower rate of dicamba (0.56 g/ha). Positive RT reactions were corrected for any genomic DNA contamination by subtracting the negative RT controls. Error bars represent standard errors of positive RTs between two separate experiments. The break in graph A represents values from 125 to 500 copies per ng total RNA, and the scale of the graph is different above and below the break.

Gmpcc16 (26 copies/ng or less). *Gmpcc16* expression was again detected at high levels from all samples (7925−20 021 copies/ng) (**Figure 3B**). *GH3* was highly induced by dicamba at 3 and 7 days (1160−2874 copies/ng) after application (**Figure 3A**). By 17 days, expression had dropped considerably (22−98 copies/ng), though both rates of dicamba still resulted in expression that was significantly higher ($P < 0.05$) than the untreated control. By 21 days, only the higher rate of dicamba resulted in *GH3* expression (24 copies/ng) that was significantly higher ($P < 0.05$) than that of the untreated control (4.1 copies/ng). The higher rate caused more injury than the lower rate and resulted in a more significant yield reduction (Kelley and Riechers, manuscript in preparation), demonstrating a further correlation between *GH3* expression and dicamba injury.

Induced *GH3* expression in response to dicamba peaked at 7 days after treatment, and still retained significant levels of induction 21 days after treatment with the highest rate examined (5.6 g ae/ha). At 3−7 days after treatment, real-time RT-PCR could easily distinguish soybean leaf samples injured by dicamba from uninjured leaves due to the >100-fold induction of *GH3*. This should be sufficient time for injury symptoms in the field to be recognized and leaf tissue collected by field scouts. Hence, this technique has potential as a diagnostic assay for injury from dicamba and possibly from other PGR herbicides in soybean. Variations of this technology are being developed, including improved primer and probe design and simplified experimental protocols (*18*, *19*, *21*) to make the method less expensive and labor intensive. Real-time RT-PCR or a similar RNA detection system will be a useful tool to respond to grower complaints of soybean injury symptoms resembling PGR herbicide injury.

An alternative to an RNA detection method such as real-time RT-PCR would be detection of GH3 protein by an enzyme-linked immunosorbant assay (ELISA) (*26*, *27*). However, *GH3* transcript levels may not correlate directly with protein abundance (*28*). An ELISA may not be sensitive enough to detect small differences in GH3 protein expression. However, given the high level of induction of *GH3* at the RNA level, it is highly probable that an ELISA for GH3 would be able to differentiate between soybean leaves injured by dicamba and uninjured leaves, at least up to 7 days after treatment. Additionally, the rate at which the GH3 protein accumulates and is degraded is not known, and it is possible that the difference in GH3 protein abundance between dicamba injured and uninjured leaves could be detectable for several days longer than the difference in RNA levels. The initial findings of this study give positive indications to the possibility that a field test based on an ELISA could be developed.

A field assay for PGR herbicide injury in soybean would have to be cost-effective and selective for PGR herbicides and not other sources of plant stress. The results of this study show that *GH3* expression is highly induced by PGR herbicides, remains induced for a sufficient amount of time for injury to be recognized and leaf samples to be collected and analyzed, and is not affected by herbicides with other modes of action. For an assay measuring *GH3* expression to be used as a diagnostic tool, *GH3* expression under many stresses commonly found in a soybean field must be examined to ensure that an unrelated stress is not mistaken for dicamba injury by the assay (i.e., false positive). Therefore, further expression analyses will be conducted to examine the effects of several plant stresses including

drought stress, salt stress, heat stress, and soybean virus infection on *GH3* expression. This will ensure that when the assay detects high levels of *GH3* expression from collected field samples, the overexpression can be attributed to a PGR herbicide (most likely dicamba) with relative confidence. In addition, development of the assay would require a reproducible, straightforward procedure, conducive to the collection of large numbers of field samples and may take the form of an ELISA or an RNA detection system. Future experiments will determine whether *GH3* proves to be a useful tool in developing a diagnostic assay for PGR herbicide injury. However, the results given here demonstrate that the products of gene expression can be used as a marker for PGR herbicide injury.

ABREVIATIONS USED

DAT, days after treatment; IAA, indole-3-acetic acid; PEPC, phosphoenolpyruvate carboxylase; PGR, plant growth regulator; RT-PCR, reverse-transcriptase Polymerase Chain Reaction; RT, reverse transcription.

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