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Effects of High-Temperature Stress on Soybean Isoflavone Concentration and Expression of Key Genes Involved in Isoflavone Synthesis

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ABSTRACT: Isoflavones have been reported to have putative health-beneficial properties, which has led to increased interest and demand for soybeans and soy-based products. This study was conducted to determine the effects of high-temperature stress on isoflavone concentration and expression of four key genes involved in isoflavone synthesis (i.e., *CHS*7, *CHS*8, *IFS*1, and *IFS*2) in both soybean pods and seeds during their late reproductive stage (i.e., R5−R8). Isoflavone concentrations were quantified using high-performance liquid chromatography (HPLC), and gene expression was studied using quantitative real-time (qRT)-PCR. High-temperature stress [33/25 °C (day/night temperatures)] imposed at the late reproductive stage (R5−R8) reduced total isoflavone concentration by 46−86 and 20−73% in seeds and pods, respectively, the reduction depending on the stage of maturity. At stage R5, the reduction in total isoflavone concentration was greater in seeds than in pods, whereas at subsequent stages, the reverse was observed. High-temperature stress had a large impact on the expression of *CHS*7, *CHS*8, *IFS*1, and *IFS*2 in both seeds and pods. In seeds, temperature stress reduced the expression of one gene at the R5 stage (*CHS*8), two genes at the R6 stage (*CHS*7 and *IFS*1), and all four genes at the R7 stage, the reduction ranging between 35 and 97%. In pods, high-temperature stress affected the expression of two genes at the R6 stage (*CHS*7 and *IFS*2) and all four genes at the R7 stage. Unlike in seeds, at the R6 stage, high temperature increased the expression of *CHS*7 and *IFS*2 by 72 and 736%, respectively, whereas at R7 stage the expression of all genes was reduced by an average of 97%. The present study reveals that high-temperature stress initiated at the R5 stage and maintained until maturation (i.e., R8 stage) has a rapid and sustained negative effect on isoflavone concentration in both seeds and pods. High temperature also affects gene expression; however, there was no clear correlation between isoflavone concentration and gene expression.

KEYWORDS: *soybean, health-beneficial compounds, isoflavones, stress*

INTRODUCTION

Soybean (*Glycine max* L.) contains high concentrations of the isoflavones daidzein, genistein, and glycitein, along with their three corresponding glycosides, acetyl and malonyl ester glycoside derivatives.[1] Isoflavones have been reported to have putative health-beneficial properties.[2,3] These properties have contributed to increased interest and demand for soybeans and soy-based products and, consequently, led to an increased usage of soybean by the nutraceutical and functional food industry.[3,4]

Isoflavones are synthesized via a branch of the phenylpropanoid pathway (Figure 1). In the initial step of the pathway, the amine group of the amino acid L-phenylalanine is removed by the enzyme phenylalanine ammonia-lyase (*PAL*) to produce cinnamic acid. Cinnamic acid is subsequently converted to *p*-coumaryol CoA by cinnamate 4-hydroxylase (*C4H*) and 4-coumarate CoA ligase (*4CL*). Coumaroyl-CoA is then transformed to naringenin chalcone or isoliquiritigenin by the enzymes chalcone synthase (*CHS*) and chalcone reductase (*CHR*). Chalcone synthase (*CHS*) is encoded by a single gene in *Arabidopsis thaliana*, but represented by a large multigene family in other species including soybean.[5,6] The *CHS* gene family in soybean consists of nine members (*CHS*1−*CHS*9). Gene expression levels in developing embryos of different soybean cultivars suggest a predominant role of two specific homologues: *CHS*7 and *CHS*8.[7] Chalcone isomerase (*CHI*) then converts chalcones to flavanones. Finally, isoflavone synthase (*IFS*) is involved in the conversion of naringenin and liquiritigenin to genistein and daidzein, respectively. There are two homologues reported in soybean (*IFS*1 and *IFS*2), the expression of which varies; *IFS*2, unlike *IFS*1, is reported to be induced by stress .[8−10]

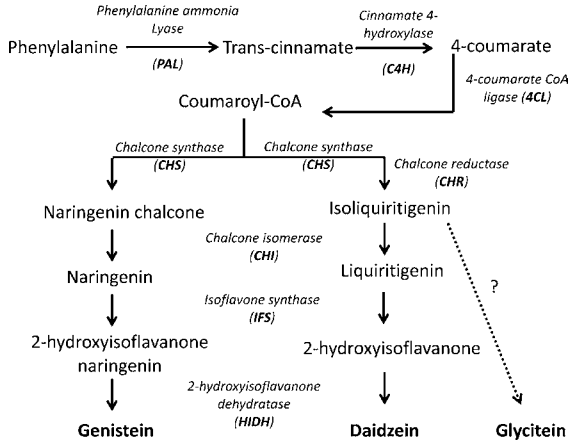

Figure 1. Simplified diagram of biosynthetic pathway of isoflavones.

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Isoflavone concentration in soybean is determined by genotypic and environmental effects, as well as environment × genotype interactions. Several studies have reported that environmental and genotype × environment effects are responsible for most of the variation observed in isoflavone concentration.[11−13] Consequently, isoflavone concentration in seeds of specific soybean cultivars often fluctuates considerably across environments. It is well documented that biotic and abiotic factors have a large impact on isoflavone concentration in soybean seeds.[1] It is therefore important to determine how specific factors mediate their effects on soybean isoflavone synthesis and ultimately accumulation. However, to date, only a few studies have investigated mechanisms by which biotic and abiotic factors may affect isoflavone concentration in soybean seeds. Gutierrez-Gonzalez et al.[10] reported that the reduction in seed isoflavone concentration observed during a period of water stress was correlated with the expression of three key genes involved in isoflavone synthesis (i.e., *CHS*7, *CHS*8, and *IFS*2). Such findings demonstrate that soybean response to water deficit stress is in part mediated through a down-regulation of the expression of a few key genes in the developing seeds. A correlation between isoflavone concentration and gene expression levels was also reported in plants exposed to biotic stresses; challenges with pathogens increased the expression of key isoflavone-related genes as well as isoflavone accumulation.[8,9,14]

Among all biotic and abiotic factors evaluated to date for their impact on soybean isoflavones, temperature has consistently been one of the most determinant factors.[15−18] High-temperature stress can affect soybean isoflavone concentration throughout plant development, but stress during seed development (i.e., R5−R8 stages)[19] has the most impact.[18,20] High-temperature stress during that specific stage can reduce isoflavone concentration in seeds by as much as 85%. The mechanisms by which high-temperature stress mediate its effects have, however, not yet been studied. The objective of the present study was thus to determine the effects of high-temperature stress during seed development on soybean isoflavone accumulation in seeds, as well as the expression of four key genes involved in isoflavone synthesis (i.e., *IFS*1, *IFS*2, *CHS*7, and *CHS*8). The impact of temperature was also studied in pods to determine if the response to high-temperature stress differs among tissues.

MATERIALS AND METHODS

Experiment and Treatment Description. Plants of the cultivar 'AC Proteina' were grown in controlled environment growth chambers (model E15, Conviron, Winnipeg, MB, Canada). Six seeds were sown in a mixture of pasteurized soil, sand, peat, and perlite in the ratio of 2:1:1:1 and thinned to two plants per pot two weeks after seeding. Seeds were inoculated with a peat-based commercial rhizobial inoculant (*Bradyrhizobium japonicum*) (Nitragin, EMD Crop Bioscience, Milwaukee, WI, USA) to ensure nitrogen fixation. Watering of plants was done when needed throughout their development. Similar levels of relative humidity were maintained in all growth chambers.

Plants were grown with a 16 h photoperiod, which was achieved by using Philips 65 W fluorescence tubes (Philips Electronics, London, ON, Canada) and 60 W incandescent bulbs (Tungsram, Oakville, ON, Canada) with a light intensity of 260 μmol m^{-2} s^{-1}. Growth chambers were set at contrasting temperatures to provide stress [i.e., 33/25 °C (day/night temperature)] and control conditions (i.e., 23/15 °C) as needed. Two treatments were thus evaluated: (i) plants grown at 23/15 °C during all stages of development and (ii) plants grown at 23/15 °C for all stages except R5−R8 (i.e., seed formation to maturity), during which they were grown at 33/25 °C (i.e., stress conditions). Exposure of plants to stress conditions was restricted to the late reproductive stages (R5−R8), as they were previously demonstrated to be the stages most responsive to high-temperature stress.[18] Each treatment was replicated four times, and each replicate included four pots of each treatment, as sampling was done at four stages of development (i.e., R5, R6, R7, and R8).

Sampling. Pods and seeds from each treatment and replicate were harvested at different developmental stages (i.e., R5, R6, R7, and R8) to determine isoflavone concentration and the expression of key genes involved in isoflavone synthesis (i.e., *CHS*7, *CHS*8, *IFS*1, and *IFS*2). Sampling for the R5 stage was done at the end of the stage, whereas the stress treatment was initiated at its onset, on average 11 days earlier. Gene expression was not determined at the R8 stage, as extraction of RNA at that particular stage was problematic. Pods and seeds of one plant from each pot were flash-frozen in liquid nitrogen and stored at −80 °C. These samples were used for gene expression analyses. The pods and seeds of the other plant from each pot were harvested for the determination of isoflavone concentration. These plants were flash-frozen in liquid nitrogen, freeze-dried, and stored at −20 °C until isoflavone extraction.

Isolation of RNA and Reverse Transcription. Samples for gene expression analyses were ground into a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated from 100 mg of powder using the RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada) and treated with RNase-free DNase I (Qiagen) according to the manufacturer's recommendations. The RNA concentration was determined at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), whereas the purity of total RNA was determined according to the A_{260}/A_{280} and A_{260}/A_{230} ratios. The quality of RNA was checked on 1.2% formaldehyde agarose gel. A total of 1 μg of RNA sample was reverse transcribed to cDNA with a Quantitect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. All cDNA samples were diluted to 100 ng μL^{-1}.

Gene Expression Analysis by Real-Time Quantitative RT-PCR. QRT-PCR was performed in 96-well plates using the Stratagene Mx3005P (Stratagene, Cedar Creek, CA, USA) targeting four key genes involved in isoflavone synthesis (i.e., *CHS*7, *CHS*8, *IFS*1, and *IFS*2) along with two reference genes (F-box protein family and α-elongation factor). Primers and PCR conditions were based on protocols of Gutierrez-Gonzalez et al.[10] and Yi et al.[21] Four biological replicates were included for each template, and one negative control was included in each run. SYBR Green Brilliant II master mix (Stratagene) was used for real-time DNA quantification. Amplification was performed in a 25 μL reaction mixture containing 160 nmol of each primer, 2× SYBR Green Brilliant II master mix, 15 μM reference dye ROX, and 2 μL of cDNA template (100 ng μL^{-1}). The amplification conditions were 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 30 s for denaturing along with different annealing temperatures (60 °C for *IFS*1, *IFS*2, F-box, and α-EF and 55 °C for *CHS*7 and *CHS*8) for 30 s and then an extension at 72 °C for 30 s. The fluorescence reading was done at 72 °C at the end of the elongation cycles. Gene expression data were estimated using Stratagene analysis software.

Quantification of Data. Data from two technical replicates were averaged before normalization. The relative expression of the target genes versus reference genes was calculated with eq 1, which was developed by Zhao and Fernald,[22] based on crossing point (CP) and efficiency obtained for each sample amplified with the reference genes (α-elongation factor and F-box protein family) and the different target genes.

$$R_0 = 1/(1 + E)^{CT} \quad (1)$$

R_0 is the initial template concentration, E is the efficiency in the exponential phase, and CT is the cycle number at threshold.

Isoflavone Extraction and Quantification. Isoflavone extraction was conducted on freeze-dried samples using a modified version of the protocol of the AOAC official method.[23] First, 4.6 mL of 70% aqueous methanol (with 50 μg mL^{-1} of apigenin as internal standard) was added to 100 mg of finely ground soybean and supersonicated for 20 min at room temperature. Further extraction was carried out by shaking for 60 min at 25 °C with orbital shaking at a speed of 200 rpm after the addition of 300 μL of 2 M sodium hydroxide. One hundred microliters of glacial acetic acid was added to achieve neutralization, which was

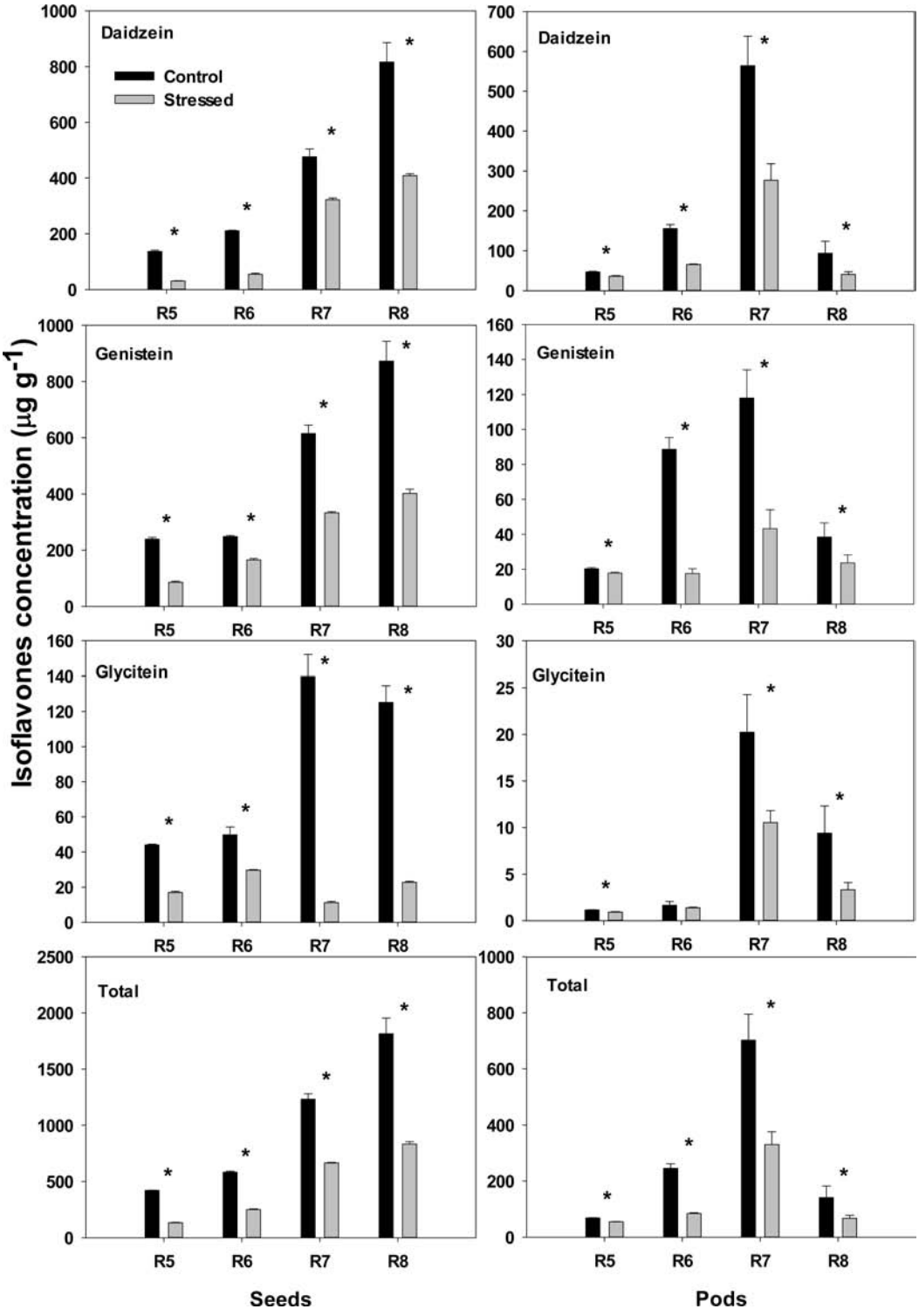

Figure 2. Isoflavone concentration in pods and seeds of soybean cultivar 'AC Proteina' at R5, R6, R7, and R8 stages. Two treatments were evaluated: plants grown at 23/15 °C (day/night temperature) during all development stages (i.e., control) and plants grown at 23/15 °C for all stages except R5−8 (seed formation to maturity), during which they were grown at 33/25 °C (i.e., stressed). Vertical bars represent SE. ∗ indicates differences between treatments for a given development stage ($P < 0.05$).

followed by centrifugation for 10 min at 10000*g*; 1.0 mL of the supernatant was then transferred to HPLC vials.

Isoflavone separation was carried out using a Varian system (Walnut Creek, CA, USA) equipped with a Prostar 210 solvent delivery system, a model 410 autosampler, and a Prostar 330 photodiode array detector (PDA). Twenty microliters of the extract was used for analyses. Separation was performed on a C18 reversed-phase column (Luna, 5 μm, 4.6 × 250 mm; Phenomenex, Torrance, CA, USA) with a flow rate of 0.65 mL min^{-1}, with the column temperature maintained constant at 40 °C. Isoflavones were detected at 260 nm. Mobile phase solvents 0.05% phosphoric acid (mobile phase A) and HPLC grade acetonitrile (mobile phase B) were used. Isoflavone elution was carried out using a linear gradient system from 10% solvent B, with no hold time after injection, to 30% solvent B over the course of 60 min, followed by a

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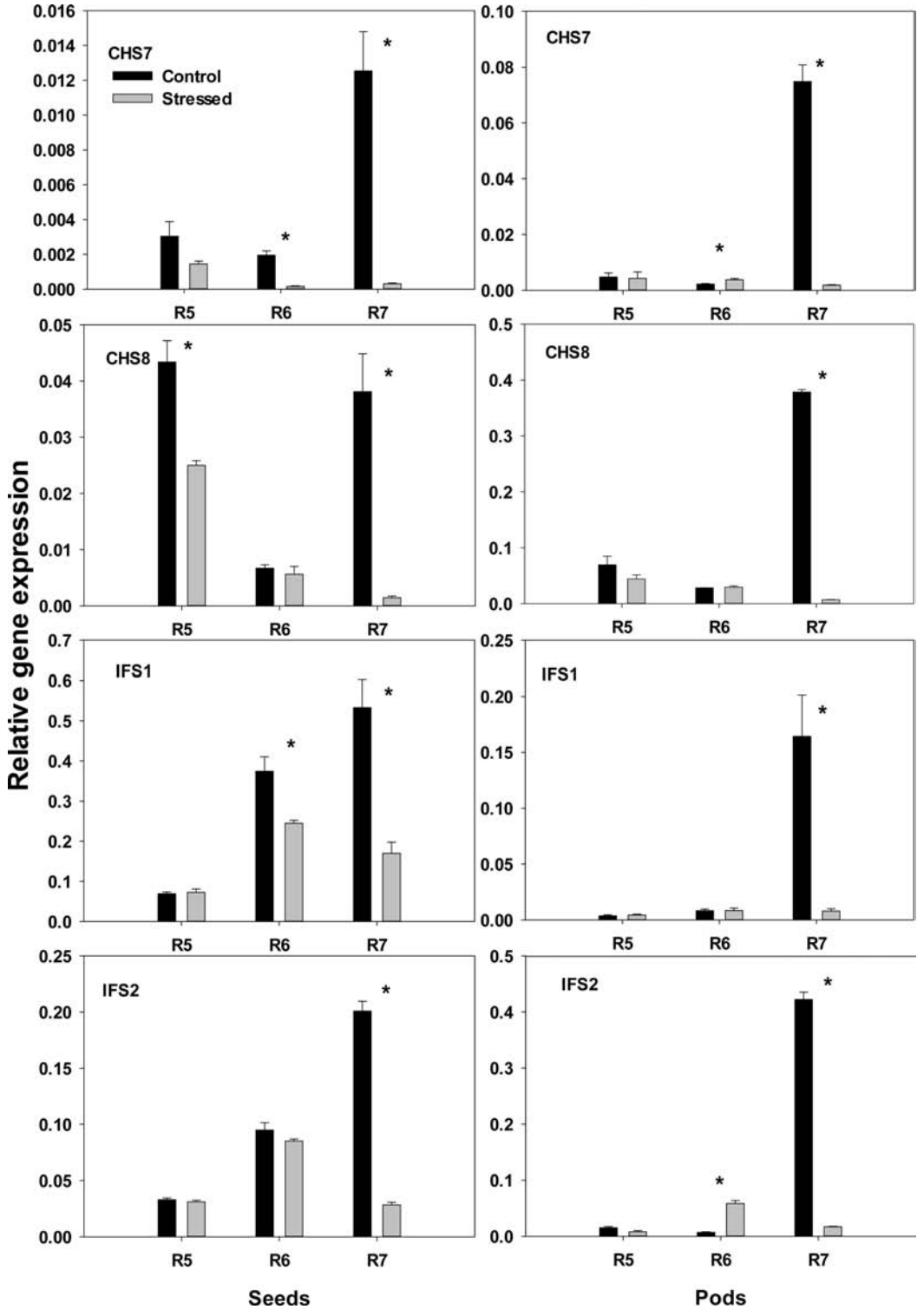

Figure 3. Relative expression of target genes *IFS*1, *IFS*2, *CHS*7, and *CHS*8 in pods and seeds of soybean cultivar 'AC Proteina' at R5, R6, and R7 stages. Two treatments were evaluated: plants grown at 23/15 °C during all development stages (i.e., control) and plants grown at 23/15 °C (day/night temperature) for all stages except R5−R8 (seed formation to maturity), during which they were grown at 33/25 °C (i.e., stressed). Vertical bars represent SE. Relative gene expression represents the number of target gene copies relative to geometric mean of two reference genes (i.e., α-elongation factor and *F*-box protein family). ∗ indicates differences between treatments for a given development stage ($P < 0.05$).

3 min wash with 90% solvent B and 10 min for equilibration with 10% of solvent B.

Purified isoflavones [daidzein, glycitein, genistein, daidzin, glycitin, and genistin (Indofine, Hillsborough, NJ, USA)] were used in the preparation of calibration curves. Concentrations of all the isoflavones detected were expressed on a dry matter (DM) basis.

Isoflavone concentrations were expressed as aglycone equivalent (i.e., daidzein, glycitein, and genistein). Total isoflavone concentration was obtained by summing the concentrations of individual isoflavones.

Statistical Analyses. The experiment design consisted of a completely randomized design (CRD) with four replicates. The relative expression of each target gene and isoflavone concentration were subjected

to an analysis of variance (ANOVA) using the general linear model (GLM) in the SAS statistical software to identify significant model and treatment effects.[24] Comparisons between means were made using LSDs at a 0.05 probability level when ANOVA indicated model and treatment significances. Throughout the text, only significant effects ($P < 0.05$) are discussed.

RESULTS

Isoflavone Concentration. Concentrations of all isoflavones were reduced by high-temperature stress in seeds and pods at all stages sampled (i.e., R5, R6, R7, and R8), except for glycitein in pods at the R6 stage (Figure 2). Depending on the stage of development, the reduction in total isoflavone concentration due to high temperature ranged between 46 and 68% and between 20 and 73% in seeds and pods, respectively. At stage R5 the reduction in total isoflavone concentration was greater in seeds than in pods (i.e., 68 vs 20%), whereas at subsequent stages (i.e., R6−R8) the reverse was observed with concentrations 8−19% greater in pods than in seeds.

In seeds, the patterns of change across stages of development for daidzein, genistein, and total isoflavones were similar. Each had a slow increase in concentration between stages R5 and R6, followed by a sharp increase between stages R6 and R8. The overall total isoflavone increase in seeds between stages R6 and R8 was of 211% for control plants and 234% for stressed plants. However, the rate of increase between stages R7 and R8 was smaller for stressed than control plants (i.e., 25 vs 47%). Changes for glycitein were different, concentrations being maximized at the R7 stage in control plants and at the R6 stage in stressed plants. In pods, the concentration of all isoflavones was maximal at the R7 stage in both control and stressed plants.

Overall, across treatments and stages of development, the concentration of all isoflavones was consistently greater in seeds than in pods. The exception was in stressed plants for which daidzein concentrations were lower in seeds than in pods at stages R5 and R6, reflecting a greater reduction caused by high temperature in seeds than in pods.

Gene Expression. High-temperature stress imposed during the late reproductive stages (i.e., R5−R8) overall greatly affected, in both seeds and pods, the expression of *CHS*7, *CHS*8, *IFS*1, and *IFS*2, four key genes involved in isoflavone synthesis (Figure 3). In seeds, high-temperature stress reduced the expression of one gene at the R5 stage, two genes at the R6 stage, and all four genes at the R7 stage. Specifically, at the R5 stage, the expression of *CHS*8 was reduced by 45%. At the R6 stage, expression of *CHS*7 and *IFS*1 was reduced by 92 and 35%, respectively. Finally, at the R7 stage, the expression of *CHS*7, *CHS*8, *IFS*1, and *IFS*2 was reduced by 97, 96, 68, and 86%, respectively. In pods, high-temperature stress affected the expression of two genes at the R6 stage and all four genes at the R7 stage. Unlike in seeds, at the R6 stage, high temperature increased the expression of *CHS*7 and *IFS*2 by 72 and 736%, respectively. However, at the R7 stage, as in seeds, the expression of all genes was reduced by an average of 97%.

In nonstressed seeds, the expression level of genes increased linearly between stages R5 and R7 for *IFS*1 and *IFS*2, levels increasing only between R6 and R7 for *CHS*7, whereas levels fluctuated for *CHS*8 (Figure 3). For all four genes, the maximum expression level was observed at the R7 stage. In contrast, in seeds of stressed plants, gene expression levels decreased between stages R6 and R7. The highest expression level in stressed seeds was observed at the R5 stage for *CHS*7 and *CHS*8 and at the R6 stage for *IFS*1 and *IFS*2. In pods of control plants an increase ranging between 12- and 59-fold of all genes was observed between stages R6 and R7. The trend differed significantly in stressed pods, gene expression levels decreasing or remaining constant between stages R6 and R7. Finally, overall, across treatments and stages of development, expression levels of *CHS*7 and *CHS*8 were lower in seeds than in pods, the reverse being observed for *IFS*1 and *IFS*2.

DISCUSSION

The objective of the present study was to provide insights on how high-temperature stress affects isoflavone concentration in soybean seeds. The effects of high temperature have consistently been reported, the late reproductive stages being the most susceptible, with isoflavone concentration being significantly reduced.[15−18] The present study revealed that high-temperature stress initiated at the R5 stage and maintained until maturation (i.e., R8 stage) has a rapid and sustained negative effect on isoflavone concentration in both seeds and pods (Figure 2). The percentage reduction in isoflavone concentration caused by high-temperature stress fluctuates across stages of development and does not increase as plants mature, averaging 55% in seeds across stages. This contrast with the response to a water deficit, by which isoflavone concentration has been reported to be reduced only when the stress is sustained.[10] Although gene expression in seeds was also reduced by high-temperature stress, the reduction was much more pronounced at the R7 stage (Figure 3). High-temperature stress reduced the expression of one gene at the R5 stage, two genes at the R6 stage, and all four genes at the R7 stage, the percentage reduction also being greater as plants matured. Our results again contrast with those reported for plants submitted to a sustained water deficit. Indeed, Gutierrez-Gonzalez et al.[10] reported that in the early phases of water stress, the expression of *CHS*7, *CHS*8, and *IFS*1 was actually increased. This response, however, changed as stress was sustained, the expression of these three genes, in addition to *IFS*2, being greatly reduced at R7 (the stage at which a reduction in isoflavone concentration was observed). It thus appears that responses to an abiotic stress (i.e., high temperature or water deficit) differ in the early stages of seed formation, the responses being comparable in the later stages. In our study, in the case of pods, whereas isoflavone concentration was reduced by high-temperature stress at all stages, gene expression was unaffected at the R5 stage, increased for two genes at the R6 stage (by as much as 736% for *IFS*2), and reduced for all four genes studied at the R7 stage. To our knowledge this is the first report of the impact of an abiotic stress on gene expression in pods at different development stages.

In both seeds and pods, in the early stages of high-temperature stress imposition, there was thus no direct correlation between isoflavone concentrations and expression of four key genes involved in isoflavone synthesis (i.e., *CHS*7, *CHS*8, *IFS*1, and *IFS*2). This suggests that in the early stages of the stress response other genes may be implicated. Indeed, *CHS*7, *CHS*8, *IFS*1, and *IFS*2 were the focus of the present study as they were previously consistently reported to be, in both control and stress conditions, determinant genes involved in isoflavone synthesis.[7−10] However, there are numerous other genes and gene homologues involved in isoflavone synthesis (Figure 1), and some could be specifically affected by high-temperature stress. Further studies looking at the impact of high-temperature stress on all genes and homologues of the phenylpropanoid pathway are thus warranted. Another hypothesis that could explain the greater initial response of isoflavones compared to that of genes may be that high temperature could promote the use of some precursor

metabolites or isoflavones for the synthesis of other secondary metabolites such as flavonols, anthocyanins, tannins, or glyceollins, many which have been implicated in stress response.[25−27] Initially, the plant response would not be to reduce gene expression but to synthesize secondary metabolites associated with stress response other than isoflavones. The pattern of the down-regulation of gene expression we observed in seeds does not provide clues about which specific secondary metabolite could be affected. Metabolomics profiling should be conducted to verify this hypothesis. Finally, a last hypothesis could be that other mechanisms such as post-translational modifications could be involved; for example, high temperature has been reported to induce phosphorylation, which down-regulated the activity of an enzyme (i.e., FAD2) involved in the conversion of oleic acid precursors to linoleic acid precursors in soybean.[28]

Gene expression and isoflavone concentration data also suggest that pods could be used as a source of isoflavones or metabolic precursors to alleviate the impact of high-temperature stress in developing seeds. Indeed, although the reduction in total isoflavone concentration caused by the high-temperature stress was greater in seeds than in pods at the R5 stage, the reverse was observed at subsequent stages (i.e., R6−R8) (Figure 2). In addition, the high-temperature stress caused an increase in the relative gene expression of *CHS*7 and *IFS*2 in pods but only at the R6 stage (Figure 3). Such data thus suggest that stressed plants upon exposure to high-temperature stress could respond by increasing the synthesis of isoflavones or key metabolic precursors in pods that would be translocated to seeds. This could then explain the greater reduction in isoflavone concentration observed in pods caused by high temperature due to their translocation into seeds. Furthermore, our data suggest that this coping mechanism would not be sustained when the stress is maintained, as was the case in the present experiment.

The possible translocation of isoflavones and/or metabolic precursors is also overall indirectly supported by the fact that across treatments and stages of development, expression levels of *CHS*7 and *CHS*8 were lower in seeds than in pods, the reverse being observed for *IFS*1 and *IFS*2 (Figure 3). Chalcone synthase (CHS) is located upstream in the phenylpropanoid pathway relative to IFS. Such differences in relative gene expression levels between seeds and pods could suggest that translocation of precursor compounds, such as chalcones, might occur from pods into developing seeds. Alternatively, it is possible that the greater expression of *CHS*7 and *CHS*8 in pods reflects a greater local synthesis of other secondary metabolites produced from the phenylpropanoid pathway. Translocation of isoflavones from maternal tissues into seeds has previously been reported experimentally.[8]

In conclusion, the present study confirms that high-temperature stress during seed development decreases isoflavone concentration in seeds.[15−18,20] It also demonstrate for the first time that high-temperature stress also reduces isoflavone concentration in pods and affects the expression of key genes involved in isoflavone synthesis in both seeds and pods. There is, however, no direct correlation between gene expression and isoflavone concentration data, suggesting that genes other than those studied could also be affected. The difference we observed between the gene expression and isoflavone response of soybean to high-temperature stress compared to that to another abiotic stress previously studied (i.e., water deficit)[10] suggests that, in terms of isoflavones, the physiological response differs greatly depending on the nature of the stress. The data also suggested a role for the translocation of isoflavones and/or metabolic precursors from pods into developing seeds in the soybean response to high-temperature stress. However, further gene expression and metabolomics studies that investigate a larger number of genes and metabolites of the phenylpropanoid pathway are required to unveil the mechanisms by which abiotic factors affect soybean isoflavone concentration.

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Notes

The authors declare no competing financial interest.

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