Gene Expression and Isoflavone Concentrations in Soybeans Treated with Chitosan

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Abstract

Isoflavones in soybean seeds have putative health-beneficial effects. Previous studies demonstrated that foliar application of chitosan could increase soybean isoflavone concentrations. In this study we determined the impact of different chitosan solutions on gene expression and isoflavone concentrations of two soybean cultivars [AC Orford (low isoflavone cultivar) and AC Proteina (high isoflavone cultivar)]. QRT-PCR was used to quantify genes encoding enzymes at key points of the phenylpropanoid pathway, including phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*) and chalcone reductase (*CHR*), and at branch point enzymes in isoflavone biosynthesis [isoflavone synthase (*IFS*)]. Foliar applications of chitosan solutions [low (75-150 kDa) and high (>100 kDa) molecular weight] were done at early podding; untreated plants served as control. Plants were harvested 1 and 7 days after treatment and separated into leaf, stem, root, flower, pod, and seed parts; other plants were grown to maturity to sample mature seeds. Gene expression 1 day after treatment and isoflavone concentrations 7 days after treatment were not affected by chitosan in any plant part for both cultivars. Expression of genes was however consistently greater in AC Proteina than AC Orford, and was generally higher in roots than other plant parts. Differences in isoflavone concentrations between cultivars were observed in leaves only. Concentrations were greatest in roots and negligible in pods and seeds. Isoflavone concentrations in mature seeds were unaffected by treatments, but differed between cultivars being greater in AC Proteina.

Media Summary

Foliar applications of chitosan did not alter the expression of genes involved in isoflavone synthesis nor did it increase seed isoflavone concentrations.

Key Words

Elicitors, isoflavones, QRT-PCR, phenylpropanoid pathway, soybean, gene expression

Introduction

Interest in soybean and soybean products has increased in recent years, in part due to their beneficial effects on human health. Soybean contains isoflavones that have been reported to reduce menopausal symptoms and the incidence of osteoporosis, cardiovascular diseases, and certain cancers (Seguin et al., 2007). Although partially genetically determined, isoflavone concentrations in soybeans are affected by a range of biotic and abiotic factors (Seguin et al., 2007). Chitosan, a natural biopolymer derived by deacetylation of chitin, has been reported in some cases to enhance crop growth and increase resistance to diseases and stress (e.g., Bittelli et al., 2001). In addition, it has also been reported to increase isoflavone concentrations in soybean seeds. Seed and foliar treatments with chitosan caused a marked increase in isoflavone concentrations (by as much as 96%) of mature seeds when compared to untreated control plants (Al-Tawaha et al., 2005; Al-Tawaha et al., 2006). It was hypothesized that the observed response to chitosan was the result of an induction or promotion of the phenylpropanoid pathway leading to increases in the synthesis of isoflavones in soybeans. The present study was conducted to verify this hypothesis. The effect of chitosan was studied by measuring transcript levels of key-encoding genes in the phenylpropanoid pathway using real-time QRT-PCR, and by measuring isoflavone concentrations using HPLC.

Methods

Growth conditions and chitosan preparation: Two soybean cultivars (AC Orford and AC Proteina, low and high isoflavone seed content cultivars, respectively) were treated with one of two types of chitosan [low molecular weight (LMW) of 75-150 kDa and degree of *N*-acetylation (DA) of 80-90%, and high MW (>1000 kDa; HMW) and DA of 75-85% (Marinard Biotech, QC, Canada)]. Chitosan was dissolved in a 0.5% acetic acid solution at a concentration of 1.75g/L with a pH of 6.5. Control plants received the same solution but without chitosan. All solutions were prepared on the day of use. Plants were treated before sowing (seeds soaked in solutions for 30 min) and at the R4-5 growing stage (foliar treatment; Al-Tawaha et al. 2005). Fifteen replicate pots for each treatment were arranged in a randomized complete block design in a greenhouse. Five replicates were harvested 1-day after foliar treatment for gene expression quantification, five after 7-days as well as at seed maturity (R8) for isoflavones quantification. Plants were grown with a 14 h photoperiod and air temperature held constant at 25ºC. At harvest, 1- and 7-days after foliar treatment, plants were separated into leaf, stem, root, flower, pod, and seed parts.

Isoflavones extraction and quantification: Isoflavones were extracted from each part using the method of Seguin et al. (2004). Isoflavones were separated by HPLC and were detected at 254 nm. Purified isoflavones (daidzein, genistein, glycitein; Sigma-Aldrich, ON, Canada) were used as standards and concentrations were expressed on a DM basis. Concentrations of aglycones were summed to obtain total isoflavone concentration.

Primer design: Based on soybean protein sequences or corresponding mRNA sequences published in Genbank primer pairs for *PAL*, *CHS*7,8, *CHR*, *IFS*1,2 genes were designed using online software Primer 3 in biology workbench (http://workbench.sdsc.edu). The primers were evaluated by alignments with the original contigs from EST soybean bank and online standard nucleotide-nucleotide BLAST search (blastn in http://www.ncbi.nlm.nih.gov/BLAST/). For normalization purposes of quantification of target genes expression, the housekeeping genes (HKG) encoding actin was used. All amplifiable products were sequenced to ensure that the right sequence of the gene is amplified.

Isolation of RNA and reverse transcription and quantitative PCR: Total RNA was isolated using the RNeasy Plant Mini Kit, treated with RNase-free DNase I (Qiagen) and reverse transcribed to cDNA using QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's recommendations.

Quantitative reverse transcription (QRT)-PCR: The reactions (20 µL) were performed for each of the target gene and for the HKG, actin using Mx3000P (Stratagene) and SYBR Green QPCR master mix. Each run included a negative control and a set of standard solutions prepared from the RT-PCR product.

Data analysis and statistics: Expression of the target genes was normalized against actin and relative transcript level ratios were calculated using equation 1 from Roche Applied Science: Ratio = $(E_{ref})^{CT\ ref}/(E_{target})^{CT\ target}$ where E_{ref} and E_{target} represent the PCR amplification efficiencies for the HKG and target gene, respectively, and which were calculated using equation 3 from Liu and Saint (2002): $E=(R_{n,A}/R_{n,B})^{1/(Ct,A-Ct,B)}$. Rn,B and Rn,A are two fluorescence levels taken during the exponential phase at two crossing points (Cts), Ct,B and Ct,A. Data were analyzed using PROC GLM in SAS. Comparisons between means were made using least significant differences (LSD) at a 0.05 probability level when ANOVA indicated model and treatment significances.

Results

Gene expression: Gene expression 1 day after treatment was not affected by chitosan in any plant part or cultivar ($P>0.05$). Expression of all 4 genes or gene clusters was however consistently greater in AC Proteina than AC Orford ($P<0.01$), thus paralleling the well established difference in isoflavone concentrations in seeds of these two cultivars (Seguin et al., 2004; Al-Tawaha et al., 2005). Plant part and cultivar by plant part effects ($P<0.0001$) indicate that differences between plant parts depended on the cultivar; the response varying for each gene or gene cluster (Figure 1). In AC Orford, gene expression levels were often higher in roots and leaves and

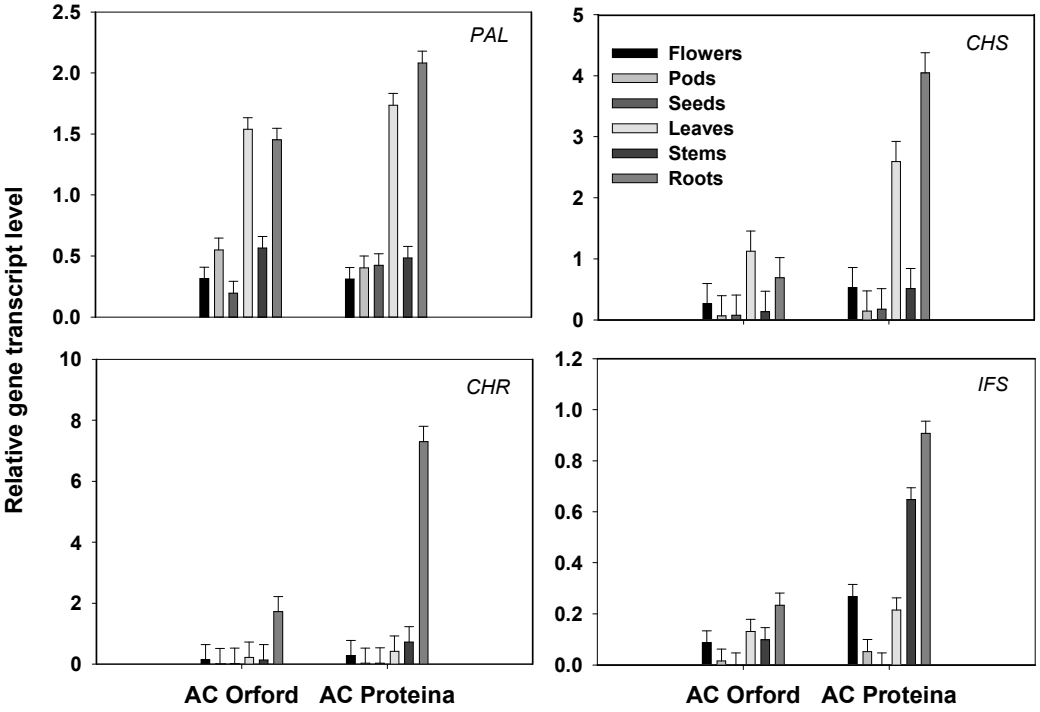

Figure 1. Relative transcript levels ratios of *PAL*, *CHS* (7 and 8), *CHR*, and *IFS* (1 and 2) in different plant parts of soybean cultivars at the R4-5 stage. Relative gene abundance represents number of target genes relative to actin (n=15). Vertical bars indicate SE.

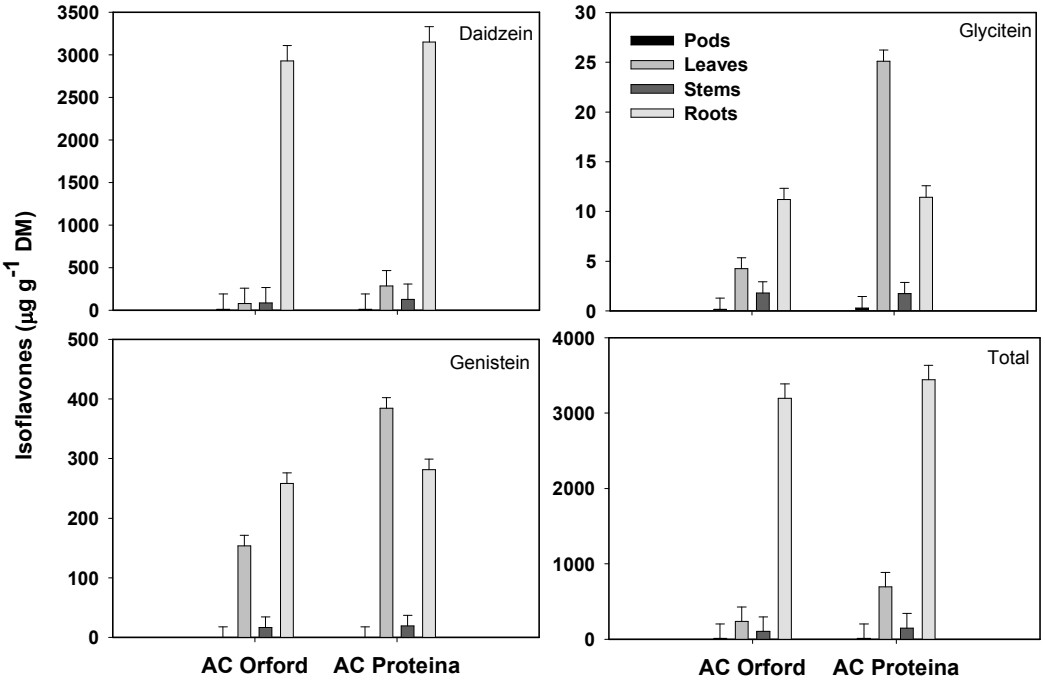

Figure 2. Isoflavone concentrations in different plant parts of soybean cultivars at the R4-5 stage (n=15). Vertical bars indicate SE.

lowest in developing pods and seeds. In the case of AC Proteina, gene expression levels were consistently higher in roots compared to other parts.

Isoflavone concentrations: Chitosan did not affect isoflavone concentrations 7 days after treatment in any plant part for both cultivars. Differences in isoflavone concentrations between cultivars were observed for glycitein and genistein ($P<0.05$). Plant part by cultivar interactions ($P<0.0001$) reflect that differences between cultivars existed only in leaves. Concentrations differed between plant parts ($P<0.0001$) and were often greatest in roots and negligible in pods (which included seeds). Isoflavone concentrations in mature seeds were unaffected by treatments, but differed between cultivars being greater in AC Proteina ($P<0.001$).

Conclusions

Foliar applications of chitosan did not alter the expression of genes involved in isoflavones synthesis nor did it increase seed isoflavone concentrations. Differences in soybean response to chitosan between the current study and those of Al-Tawaha et al. (2005, 2006) might be attributable to differences in the characteristics of chitosan used. Indeed the present study used highly purified and well characterized chitosan sources (Marinard Biotech, QC, Canada) while Al-Tawaha et al. (2005, 2006) used a crude chitosan source of unknown molecular weight and degree of *N*-acetylation (Sigma-Aldrich, ON, Canada). Our study however revealed large differences in relative gene expression levels between plant parts and cultivars. This information will be useful in the development of cultivars with specific isoflavone concentrations and in efforts to further the understanding of factors affecting isoflavone concentrations in soybean.

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