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Cytochrome P_{450}, CYP93A1, as defense marker in soybean

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Abstract

Cytochrome P_{450}, CYP93A1, is involved in the synthesis of the phytoalexin glyceollin in soybean (*Glycine max* L. Merr). The gene encoding CYP93A1 has been used as defense marker in soybean cell cultures, however, little is known regarding how this gene is expressed in the intact plant. To further understand the tissue-specific role of CYP93A1 in soybean defense, we analyzed the expression of this gene in mechanically damaged leaves and stems. In leaves, *CYP93A1* was constitutively expressed; its expression did not change in response to mechanical damage. In stems, however, expression of *CYP93A1* was induced as quickly as 4 h after mechanical damage and remained upregulated for at least 48 h. The induction of *CYP93A1* was associated with the synthesis of glyceollins. In comparison to several other defense-related genes encoding cysteine protease inhibitors L1 and R1 and storage proteins vspA and vspB, *CYP93A1* was the most strongly induced by stem wounding. The induction of *CYP93A1* was observed only locally, not systemically. Similar stem expression patterns were consistently observed among three different soybean genotypes. The strong induction of *CYP93A1* in mechanically damaged stems suggests an important role in the soybean stem defense response; therefore, this study expands the use of *CYP93A1* as a defense response marker to stems, not just soybean cell cultures.

Additional key words: cysteine protease inhibitors, glyceollin, jasmonic acid, mechanical damage, storage proteins.

Introduction

Soybean (*Glycine max* L. Merr) is an agriculturally important plant species and as such, there has been an extensive effort to identify genes that function in defense against pathogens and insect herbivory. One gene of interest is cytochrome P_{450}, *CYP93A1*. This gene encodes a cytochrome P_{450} (CYP) enzyme CYP93A1 that has dihydroxy-pterocarpan 6a-hydroxylase activity and is involved in the synthesis of the phytoalexin glyceollin (Schopfer *et al.* 1998). Glyceollin has anti-pathogen activity (Lygin *et al.* 2010), and it has been associated with resistance against nematodes (Veech 1982). It also appears to function as a feeding deterrent against insects (Hart *et al.* 1983, Liu *et al.* 1992).

The gene encoding CYP93A1 was originally identified in soybean suspension-cultured cells treated with methyl jasmonate, a derivative of jasmonic acid (JA) (Suzuki *et al.* 1996). Jasmonic acid is the primary signaling hormone involved in inducible insect defense (Howe and Jander 2008, Erb *et al.* 2012) and defense against necrotrophic pathogens (Glazebrook 2005). *CYP93A1* is also induced in soybean suspension-cultured cells treated with fungal extracts (Schopfer and Ebel 1998, Schopfer *et al.* 1998) and the soybean peptide defense signals GmSubPep, GmPep914, and GmPep890 (Pearce *et al.* 2010a,b, Yamaguchi *et al.* 2011). In elicitor treated soybean cell cultures, an increase in glyceollin

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Abbreviations: CystPI - cysteine proteinase inhibitor; CYP - cytochrome P_{450}; ELF1B - translational elongation factor 1 subunit beta; JA - jasmonic acid; qPCR - quantitative PCR; UKN1 - unknown 1; VSP - vegetative storage protein.
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accumulation has been positively correlated to the induction of the gene encoding CYP93A1 (Schopfer *et al*. 1998). Even though CYP93A1 appears to be a crucial component of the soybean defense response, only one study has analyzed its expression in intact plants, and this was in leaf tissue (Pearce *et al*. 2010b). The synthesis of glyceollin, however, is not limited to leaves. Glyceollin accumulates in roots in response to pathogen infection (Hahn *et al.* 1985, Boué *et al.* 2000) and nematode inoculation (Huang and Barker 1991, Kaplan *et al.* 1980a, 1980b) and is induced in hypocotyls by various chemical elicitors (Liu *et al*. 1992). It is therefore important to study the expression of *CYP93A1* in other tissues as it may be an important defense response marker in these tissues.

To gain a better understanding of the role of CYP93A1 in soybean defense responses, we analyzed the expression of *CYP93A1* and the presence of glyceollin in wounded leaves and stems. As compared to leaves, relatively little is known about the soybean stem defense response even though soybeans are susceptible to stem canker disease and infection by stem rot pathogens. They are also subjected to damage by the stem borer, *Dectes texanus*, which causes economic damage to soybean crops by tunneling through stems and then girdling the stems near the base of the plants resulting in lodging (Hatchett *et al.* 1975). The expression of *CYP93A1* was compared to other defense-related genes including the cysteine proteinase inhibitors (CystPIs) *L1* and *R1*, and the vegetative storage proteins (VSPs) *vspA* and *vspB*. The *R1*, *vspA*, and *vspB* are strongly upregulated when soybean leaves are mechanically damaged or treated with methyl jasmonate (Mason and Mullet 1990, Mason *et al.* 1992, Botella *et al.* 1996), but like *CYP93A1,* very little is known concerning other tissue-specific expression patterns. Furthermore, to determine the consistency of the observed responses, the expression of *CYP93A1* was analyzed in the stems of three different soybean genotypes.

Materials and methods

Plants and treatments: Three different genotypes (PI 186021, PI 518671, and PI 90763) of soybean (*Glycine max* L. Merr) were used in this study. PI 186021 was obtained from the *Victory Seed Company*, Molalla, OR, USA. The other two, PI 518671 and PI 90763, were obtained from the *USDA Soybean Germplasm Collection*, Urbana, IL, USA. PI 518671 is the cultivar Williams 82 that was used for the soybean genome sequencing project (Schmutz *et al.* 2010), and PI 90763 is known to be resistant to a field population of soybean cyst nematode (Anand and Gallo 1984). Soybean seeds were germinated and grown in pots containing *Baccto Professional Plant Mix* (Houston, TX, USA). Plants were maintained in growth chambers (*Environmental Growth Chambers*, Chagrin Falls, OH, USA) at a 12-h photoperiod, an irradiance of 300 µmol m^{-2} s^{-1}, day/night temperatures of 28/22 °C, and a relative humidity of 70 %. The plants were watered every other day and fertilized weekly with the 1.25 mg dm^{-3} *Peters 20-20-20* water soluble fertilizer (*JR Peters*, Allentown, PA, USA). Plants used for experiments were approximately 14-d-old and had one fully developed trifoliate leaf.

For stem gene expression, enzyme activity, and phytohormone analyses, the second internodes were mechanically damaged by inserting a dissecting needle completely through the stem three times. The wound sites were approximately 0.5 cm apart. The damaged tissue samples were collected at the indicated time points. To evaluate the systemic defense response, damaged second internodes as well as non-damaged first internodes, unifoliate leaves, and trifoliate leaves from the same plant were collected 48 h post-damage treatment. For all experiments, corresponding tissue samples were also collected from non-damaged control plants. To analyze gene expression and enzyme activity, three biological replicates were collected for each treatment, and each replicate consisted of pooled tissue from four individual plants. To analyze the phytohormone JA, eight biological replicates, each consisting of a pool of tissue from four individual plants, were collected for each treatment. For glyceollin quantification, three biological replicates were collected, and each replicate consisted of tissue pooled from six individual plants.

For leaf experiments, each leaflet of the trifoliate leaf was mechanically damaged 20 times along their edge using a hemostat, and damaged tissue samples were collected 48 h after the initial wounding. Corresponding tissue samples were also collected from non-damaged control plants. For gene expression and enzyme analyses, three biological replicates, consisting of tissue from one plant, were collected for each treatment. To analyze glyceollins I, II, and III, three biological replicates were collected from each treatment and consisted of tissue pooled from six individual plants.

Gene expression analysis: A modified *Trizol*® method (*Invitrogen*, Carlsbad, CA, USA) described in Dafoe *et al*. (2011) was used to extract RNA from soybean tissue. One µg of RNA was reverse transcribed into cDNA using an *iScript* reverse transcription supermix (*BioRad*, Hercules, CA, USA). For real-time quantitative PCR (qPCR), 10 ng of cDNA was added to a 1× *SsoAdvanced SYBR Green* supermix (*BioRad*), 300 nM each of forward and reverse primers, and nuclease-free double distilled H_2O to a final volume of 0.02 cm^3. Quantitative PCR was performed using the *CFX Connect*

real time PCR detection system (*BioRad*) following standard conditions: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 62 °C for 30 s, and then 65 - 95 °C in 0.5 °C increments, 5 s per increment. Data were analyzed using *CFX Manager™ 3.1* (*BioRad*). The reactions were run in triplicate. Melting curves were used to validate the specificity of the gene-specific oligonucleotides listed in Table 1. Quantification cycles were normalized to the housekeeping genes unknown 1 (*UKN1*, Hu *et al*. 2009) or translational elongation factor 1 subunit beta (*ELF1B*, Pearce *et al*. 2010a,b). For PI 186021, *CYP93A1*, *R1*, and *L1* were normalized to *UKN1*, whereas *vspA* and *vspB* were normalized to *ELF1B*. For the strain PI 518671, *CYP93A1* was normalized to *UKN1* and for the strain PI 90763, *CYP93A1* was normalized against *ELF1B*. Gene expressions were calculated by comparing the quantification cycle values in mechanically damaged plant tissue to the corresponding untreated control tissue. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to calculate the fold-changes of the transcripts. Student's *t*-tests were used to make comparisons between the two treatments.

Content of jasmonic acid and glyceollins: The JA content was estimated using a method described by Schmelz *et al.* (2004). Briefly, the phytohormone was extracted with 1-propanol and methylene chloride and collected by vapor-phase extraction following carboxylic acid methylation. Analysis was performed using gas chromatography-mass spectrometry (*Agilent Technologies*, Santa Clara, CA, USA) and quantification was based on a calibration curve created by an external standard of pure JA (*Sigma-Aldrich*, St. Louis, MO). Two-way *ANOVA* was performed to compare JA content in undamaged and damaged tissues at the various time points.

Content of glyceollins I and II were quantified and glyceollin III estimated using the liquid chromatography-mass spectrometry method described in Malik *et al*. (2015). Glyceollin data for 48 h damaged stem tissue was $\log_{10}$ transformed prior to statistical analysis. One-way *ANOVA* and Tukey's range test were used to compare mean values for glyceollins I, II, and III.

Cysteine protease inhibitor activity assay: As described in Zavala *et al*. (2009), the total soluble proteins were extracted from soybean tissue using a 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2 mM EDTA-Na_2. The concentration of protein in each extract was determined using the *Quick Start™* Bradford dye reagent (*BioRad*). Activity of CystPI was measured against the cysteine protease papain (*Sigma-Aldrich*), using a synthetic substrate *p*Glu-Phe-Leu *p*-nitroanilide (*Sigma-Aldrich*). Papain releases *p*-nitroaniline from the substrate and this compound can be measured at 410 nm (Zavala *et al*. 2009). For the assay, papain (a 5.6 μg cm^{-3} final assay concentration) was incubated for 10 min at 37 °C with 0, 0.0125, 0.025, and 0.05 mg cm^{-3} soybean tissue extracts before adding the substrate at a final concentration of 0.5 mM. The reaction mixture was then incubated for an additional 20 min at 37 °C and the change in absorbance at 410 nm was used to calculate the percentage of protease inhibition. The assays were run in duplicate for three biological replicates. For both assays, inhibition and protein content were normally distributed. The difference in slopes was tested using the interaction term for indicator variables for control and mechanically damaged plant tissue, and then comparing the reduced to full regression models (Neter *et al.* 1989).

Table 1. Primer sequences used to amplify defense-related genes.

Symbol	NCBI acc. No.	Forward primer	Reverse primer	Amplicon size [bp]
ELF1B	XM_003545405	GGTGATGAGACAGAGGAAGATAAG	GCTTAACATCGAGAAGGACAGA	109
UKN1	BU578186	TGGTGCTGCCGCTATTTACTG	GGTGGAAGGAACTGCTAACAATC	74
CYP93A1	D83968	ACACGTCAGCTGTAAGCATAG	TTCCAACCACCGCATCTATC	99
vspA	M76980	CGATATGTTCCCTCTCCGAATG	GAATGGTCTCAAAGCCAAAGATG	116
vspB	M76981	GAGCGTTCTTCGGAGGTAAA	GTTGGTTCAACGCACTCTTC	101
R1	BT089060	TGGAGTTTGTGAGGGTGATTAG	ATGGTTTCTCCAACACCTTAGT	126
L1	AK285650	ATGTGACAGGAAGCCAGAAC	CACTTGCTGTTTCGCAGTTAC	125

Results

The second internodes of the stems of soybean genotype PI 186021 were mechanically damaged and samples were collected at either 4 or 48 h in order to examine the expression of *CYP93A1* as well as several other known defense-related genes including the *L1, R1*, *vspA*, and *vspB*. Of these genes, only *CYP93A1* was significantly induced 4 h after stems were mechanically damaged (Fig. 1*A*). Its expression increased 24-fold above non-damaged control. The expression of *vspA* and *vspB* appeared to begin to increase at 4 h 2- and 3.6-fold,

respectively, but neither change was significant at that time point (Fig. 1*A*). *CYP93A1* remained significantly upregulated at least 48 h after the initial stem damage (Fig. 1*B*, Table 2). The expression of *CYP93A1* was 39-fold higher in damaged stem tissue than in control tissue. At 48 h, *vspA* and *vspB* were also significantly upregulated in damaged stem tissue, 3- and 6.6-fold, respectively (Fig. 1*B*, Table 2). The expression of the *CystPIs*, *L1* and *R1*, did not change in 4 or 48 h damaged tissues; both appeared to be expressed at comparable levels in the tissues tested (Fig. 1*A*,*B*, Table 2).

The induction of *CYP93A1*, *vspA*, or *vspB* in mechanically damaged stem tissue was not correlated with an increase in JA content. Mechanically damaging stems of PI 186021 did not result in a statistically significant change in JA content at 4 and 48 h post-damage as compared to control plants (Fig. 2).

To determine if *CYP93A1* is systemically induced in response to stem wounding, damaged tissue as well as non-damaged tissue were collected for gene expression analysis. Forty-eight hours after damaging the second internode of soybean stems, *CYP93A1* was strongly induced locally at the site of wounding, but the induction of this gene was limited to the damaged tissue (Fig. 3). No induction of *CYP93A1* was observed in the systemic tissues, which included the first internode, unifoliate leaves, or trifoliate leaves.

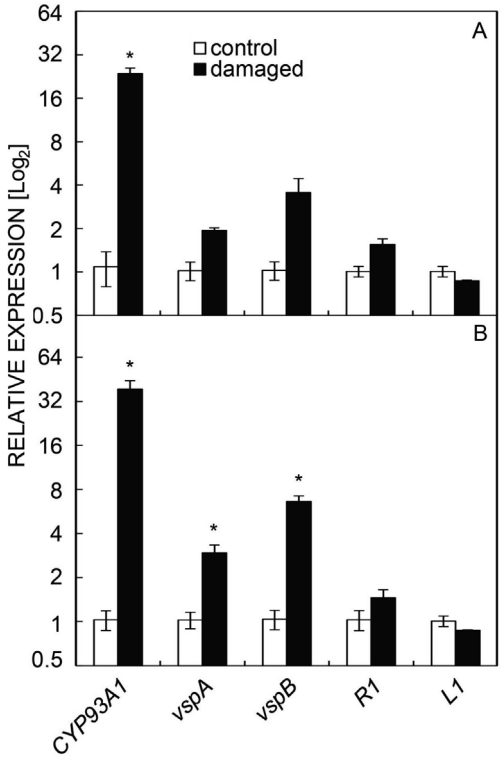

Fig. 1. Expression of defense-related genes *CYP93A1*, *vspA*, *vspB*, *R1*, and *L1* in mechanically wounded soybean stem tissue after 4 h (*A*) and 48 h (*B*). Means ± SEs, $n = 3$, significant differences indicated by *asterisks* (Student's *t*-test, $P < 0.05$).

Table 2. Quantification cycle values for *CYP93A1, vspA, vspB, R1*, and *L1* in control and 48 h damaged stem and leaf tissues. Means ± SEs, $n = 3$.

Gene	Stem control	damaged	Leaf control	damaged
CYP93A1	32.7 ± 0.2	27.6 ± 0.2	30.3 ± 1.1	31.2 ± 0.2
vspA	20.5 ± 0.2	17.8 ± 0.3	21.3 ± 0.9	19.1 ± 0.7
vspB	21.6 ± 0.2	18.4 ± 0.3	23.9 ± 1.3	21.2 ± 0.8
R1	22.5 ± 0.1	22.3 ± 0.3	21.3 ± 0.1	20.7 ± 0.5
L1	20.2 ± 0.1	20.4 ± 0.2	22.9 ± 0.2	22.3 ± 0.2

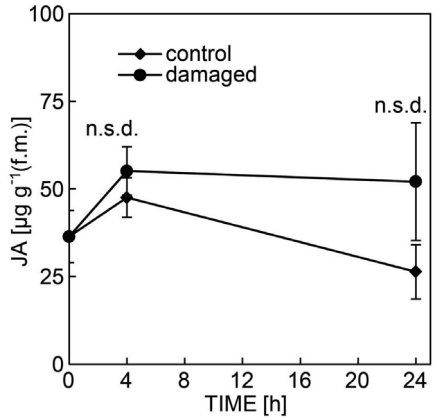

Fig. 2. Content of jasmonic acid (JA) in stems in response to mechanical damage. Means ± SEs, $n = 8$, no significant difference was detected ($P > 0.05$).

CYP93A1 as well as *vspA, vspB,* and *R1* have been previously shown to be upregulated in other soybean genotypes in response to leaf wounding (Mason and Mullet 1990, Botella *et al*. 1996, Pearce *et al*. 2010b). To determine if these genes are inducible in the leaves of the soybean genotype PI 186021, we heavily damaged the first trifoliate leaf by using a hemostat 20 times around the edge of each leaflet and analyzed samples 48 h post-damage. Even with this severe wounding, no significant change in expression was observed for *CYP93A1*, *vspA*, *vspB*, *R1*, or *L1* (Table 2). All genes appeared to be expressed at comparable levels in the tissue tested.

Given that differences may occur between soybean genotypes, we compared the expression of *CYP93A1* in mechanically damaged stems of the soybean genotypes PI 518671 and PI 90763 to that of the PI 186021. When analyzing the stems of all three genotypes 48 h post-damage, *CYP93A1* was strongly induced after stem damage (Fig. 4).

CYP93A1 is necessary for the biosynthesis of glyceollins, therefore we assessed glyceollins I, II, and III content in control and mechanically damaged stem and leaf tissues 48 h after treatments to determine if there is a relationship between *CYP93A1* induction and the

presence of glyceollins. Similar to the induction of *CYP93A1*, glyceollins were detected only in 48 h mechanically damaged stem tissue (Table 1 Suppl). The average total content of glyceollin in 48 h damaged stem tissue was 57.5 ng per 100 mgof dry mass. Of the three glyceollins, the concentration of glyceollin III was statistically higher than either glyceollin I or glyceollin II. *CystPI* expression was also directly associated with CystPI control and mechanically damaged tissues (Fig. 1 Suppl.). There was no significant difference in activity between the two treatments for either tissue.

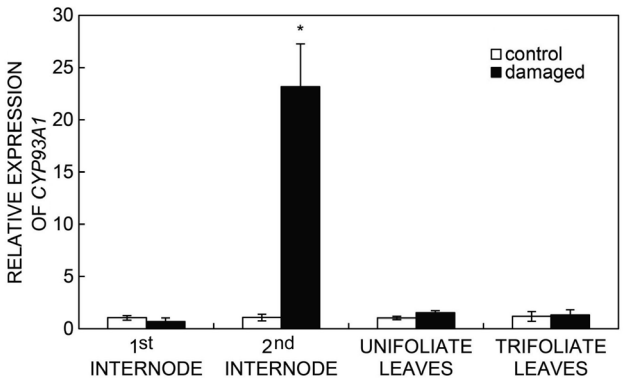

Fig. 3. Expression of *Cyp93A1* in damaged tissue (2nd internode) and non-damaged systemic tissues (1st internode, unifoliate leaves, and trifoliate leaves) after 48 h. Means ± SEs, $n = 3$, significant difference indicated by an *asterisk* (Student's *t*-test, $P < 0.05$).

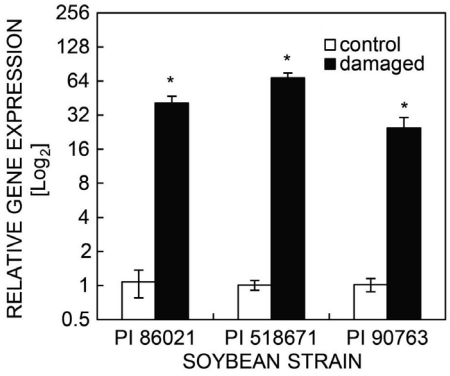

Fig. 4. Expression of *Cyp93A1* in three genotypes (PI 186021, PI 518671, and PI 90763) of soybean 48 h after mechanical damage. Means ± SEs, $n = 3$, significant differences indicated by *asterisk*s (Student's *t*-test, $P < 0.05$).

Discussion

CYP93A1 is a dihydroxypterocarpan 6a-hydroxylase that is involved in the synthesis of glyceollins, one of the most important defense in soybean (Schopfer *et al*. 1998). Despite its importance, we continue to know relatively little about the expression of *CYP93A1* in an intact plant. To better understand the role of CYP93A1 *in planta*, we analyzed the expression of *CYP93A1* in mechanically damaged leaves and stems, a method commonly used to mimic insect feeding (Howe and Jander 2008). *CYP93A1*, *vspA*, *vspB*, and *R1* have all previously been shown to be wound-inducible in leaves (Mason and Mullet 1990, Botella *et al*. 1996, Pearce *et al*. 2010b). However, in the leaves of soybean genotype PI 18601, mechanical damage did not change the expression of these genes. Extensive mechanical damage of leaves also did not elicit the synthesis of glyceollins or increase the activity of CystPIs. It is difficult to make comparisons with previous studies considering that they utilized different growing conditions, different mechanisms for imposing damage, and different soybean genotypes. These variables could account for the differences observed. It has, for example, been shown that glyceollin accumulation can vary based on the soybean species (Bhattacharyya and Ward 1988, Huang and Barker 1991), the age of the plant (Bhattacharyya and Ward 1988), and the environmental conditions in which they are grown (Graham and Graham 1996, Aisyah *et al*. 2013). Alternatively, leaves are expendable and under the optimal defense theory, the allocation of resources for leaf defense may not be as high priority for some plants under certain conditions.

Unlike leaves, stems are not as readily expendable, and *CYP93A1* was strongly induced in mechanically damaged stems. It was upregulated as early as 4 h after wounding and its expression remained induced for at least 48 h. Of the other genes tested, only *vspA* and *vspB* were also induced in response to stem damage. These genes were induced to a lesser extent than *CYP93A1*, and they were only significantly induced at 48 h. The fast, strong induction of *CYP93A1* is associated with the synthesis of glyceollins. These results suggest that *CYP93A1* has an important role in the soybean stem defense response, especially since it was consistently upregulated in damaged stems of multiple soybean genotypes.

The induction of CYP93A1 in stems was localized to the site of mechanical damage. It was not upregulated in systemic leaf tissue or neighboring, unwounded stem tissue. There is also evidence that the accumulation of glyceollin is limited to local tissue (Hahn *et al*. 1985, Graham and Graham 1991, Huang and Barker 1991). It is also possible that the inflicted damage may have not been sufficient to initiate a systemic defense response. The content of JA, a hormone known to play an important role in the leaf systemic defense response (Howe and Jander 2008), did not significantly change in response to this wounding treatment. In maize stems, it was also observed

that mechanical damage alone does not result in an increase of JA, but feeding by *Ostrinia nubilalis* does (Dafoe *et al*. 2011). This correlates with a higher expression of certain defense-related genes and a higher content of defensive compounds in tissue damaged by *Ostrinia nubilalis* (Dafoe *et al*. 2011, 2013). This is consistent with the notion that stems may also receive mechanical damage from the wind, and under such circumstances, the plant is not supposed to waste too much energy on induction of defenses in the absence of an insect herbivore or pathogen infection (Howe and Jander 2008).

Previously, *CYP93A1* has been used as a defense response marker in soybean cell cultures, but a suspension of undifferentiated cells that do not represent an intact plant that consists of interconnected tissues and organs (Pearce *et al*. 2010a,b, Yamaguchi *et al*. 2011). Cell cultures are an important tool, but it is important to study whole plants. Upon analyzing its tissue-specific regulation, it was apparent that *CYP93A1* was a good defense response marker in stems, but not in leaves. *CYP93A1* was strongly induced in mechanically damaged stems, more than *CystPIs* and *vsps*, and it was consistently induced in the stems of several soybean genotypes. It will be important to continue studying the expression of CYP93A1 in different tissues and under different stresses because the synthesis of glyceollins in response to abiotic and biotic stresses is important for the protection of plants.

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