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Analysis of Herbivore Stress- and Phytohormone-Mediated Urease Expression in Soybean (*Glycine max*)

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Abstract Ureases catalyze the hydrolysis of urea into ammonia and carbon dioxide and, thus, are involved in the metabolism and bioavailability of nitrogen. Ureases occur in plants, fungi, and bacteria. In plants, besides their enzymatic activity, ureases as proteins play a role in defense against insect and phytopathogenic fungi. Little is known about the regulation of urease in plants under stress and whether or not phytohormones may be involved. In this study, we addressed the regulation of ubiquitous urease (*ubSBU*) gene expression after phytohormone applications, insect herbivory, and mechanical damage in soybean (*Glycine max* cv. Williams 82). Stress-related phytohormones were applied. In addition, *Spodoptera littoralis* feeding and mechanical damage by MecWorm were performed. Ureolytic activity and transcripts for *ubSBU* and *UreG* were quantified. Roots and leaves showed the highest levels of *ubSBU* transcripts. The results show a significant increase of *ubSBU* transcripts upon jasmonic acid application and after herbivory, but downregulation after MecWorm treatment. *UreG* transcripts were downregulated after MecWorm, *S. littoralis*, and application of gibberellic acid, but upregulated by jasmonic acid. However, the ureolytic activities in leaves were influenced neither by phytohormones nor by herbivory and MecWorm. We conclude that the enzymatic activity of ureases is constitutive and basal levels of the enzyme are sufficient to perform the ureolytic activities in defense against insects and fungi. The defense role of ureases, which does not require the ureolytic activity, may underlie their differential regulation in response to different stress stimuli.

Keywords Soybean · Urease · Phytohormones · Herbivory · Gene expression

Introduction

Soybean (*Glycine max* (L.) Merril) is considered one of the oldest cultivated plants, with records of its cultivation in Asia dating back more than 5000 years (Costa 1996). Diseases caused by fungi, bacteria, nematodes, and viruses are the most common challenges faced by farmers, negatively affecting production and encouraging the use of pesticides that are hazardous to the environment (Rigotto and others 2014). Soybeans have a high nutritional value: 40% of the total seed mass is protein, 21% oil, and 34% carbohydrates. Similarly to other leguminous plants, soybeans are considered a valuable source of important bioactive proteins such as lectins, trypsin inhibitors, and ureases (Becker-Ritt and others 2004). These proteins are considered crucial in the defense of plants against various pathogens and pests.

Angela Menegassi and Roberta Da Silva e Silva have contributed equally.

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Urease is a soybean defense protein (EC 3.5.1.5; urea amidohydrolase), a nickel-dependent enzyme (Dixon and others 1975) that catalyzes the hydrolysis of urea into two molecules of ammonia and one molecule of carbon dioxide. This enzyme is widely distributed in plants, fungi, and bacteria, but not in animals (Krajewska 2009). Eukaryotic ureases consist of identical 90-kDa subunits assembled to homo-oligomeric trimers or hexamers, whilst most bacterial ureases are composed of three distinct subunits (α–β–γ; for example, *Klebsiella aerogenes)*. All ureases in plants and microbes need two nickel atoms in their active site for enzymatic activity (Witte 2011; Real-Guerra and others 2012), except canatoxin (CNTX), extracted from *Canavalia ensiformis*, which contains one atom of nickel and one atom of zinc (Follmer and others 2001). A urease accessory protein UreG is necessary for enzyme activation. UreG is involved in GTP hydrolysis, which generates the energy to form the urease activation complex and the delivery of Ni^{2+}, which is essential for urease activity (Moncrief and Hausinger 1997; Witte 2011). Three urease isoforms (overall 87% amino acid sequence identity) can be found in soybean (Wiebke-Strohm and others 2016). The so-called ubiquitous urease (*ub*SBU) is encoded by the *Eu4* gene and present in all plant tissues. Embryo-specific urease (*e*SBU) is encoded by the *Eu1* gene. The *e*SBU protein is mainly present in the embryo and its activity is much higher in mature seeds than the *ub*SBU activity in all other plant tissues (Polacco and Winkler 1984). Some lower but significant *e*SBU levels can also be found in young roots and in the cotyledon of mature seeds (Torisky and Polacco 1990). A third, enzymatically inactive urease isoform (SBU-III; *Eu5* gene) has recently been discovered to be expressed in germinating seeds, young roots, and embryos (Wiebke-Strohm and others 2016).

Although ureases extracted from *C. ensiformis* seeds were the first enzymes to be isolated in crystalline form (Sumner 1926), the biological role of ureases in plants is not yet entirely understood. These enzymes are considered essential for recycling of nitrogen from urea that originates from arginine degradation. Additionally, *e*SBU and *ub*SBU, and even the catalytically inactive SBU-III, are considered to be involved in the biochemical defense of soybean (Carlini and Ligabue-Braun 2016; Wiebke-Strohm and others 2016; Martinelli and others 2017). Fungicide and insecticide effects exhibited by ureases are independent of their ureolytic activity, that is, they are still observed after blockage of the active site of ureases by ρ-(hydroxymercury) benzoate, which is a thiol-oxidizing agent that irreversibly inhibits the enzyme (Becker-Ritt and others 2007).

Plants are challenged by biotic stressors, such as pathogens, herbivores, parasites, and allelopathy, and by abiotic stressors, such as drought, extreme temperature, UV-B radiation, salinity, and heavy metals (Redondo-Gómez 2013). Plants contain several constitutive as well as inducible defense mechanisms against stress (Mithöfer and Boland 2012). Upon the experience of (a)biotic stress, phytohormone-mediated processes leading to a reprogramming of the genetic machinery that finally increases plant tolerance and minimizes biological damage caused by stressors take place (Rejeb and others 2014). For example, the transcriptional and post-transcriptional regulation of RNA is drastically altered and alternative splicing can occur as a consequence of stress (Nakaminami and others 2012). Clearly, elevated levels of phytohormones such as jasmonic acid (JA), salicylic acid (SA), gibberellic acid (GA), and abscisic acid (ABA) are linked to plant responses towards (a)biotic stresses. Also, urease gene expression seems to be regulated by phytohormones. Pires-Alves and others (2003) have shown that urease genes were induced in *C. ensiformis* by ABA. Considering that ureases are involved in plant defense and that phytohormones are crucial for several defensive pathways, here we analyzed the gene expression levels of tissue-ubiquitous urease, *ub*SBU, and *UreG* during the development of soybean plants upon treatment with different phytohormones (ABA, SA, JA, and GA) and upon herbivory-related stresses. The transcript levels were quantified using Real-Time qPCR (RT qPCR) and compared with urease enzyme activity.

Materials and Methods

Experimental Design

Soybean (*Glycine max*) seeds (cultivar Williams 82) were germinated, planted in pots with soil, and kept inside plant growth chambers (16-h light 24 °C /8-h dark 22 °C; 65% relative humidity) until the V3 stage. At this stage, the plants show a fully developed trifoliate second leaf which was used if not otherwise indicated. Cotyledons were used only when they were green, above the ground, and growing straight from the hypocotyl (VE, stage-6). Experiments were performed using a completely randomized design in a unifactorial scheme; kinetics and tissue expression assays were carried out in triplicate; phytohormone assays in quadruplicate; and herbivory and MecWorm tests in quintuplicate. For both protein and RNA extraction, all excised parts of the plants were immediately frozen in liquid nitrogen; samples were ground using a GenoGrinder (1100 strokes/min for 50 s) and stored at −80 °C if not used immediately. Soybean seeds used for tissue expression assay were imbibed for 2 days in wet cotton wool and ground using liquid nitrogen, mortar, and pestle.

Phytohormone Treatments

V3 leaves of soybean plants were treated with aqueous solutions of ABA, JA, SA, or GA (100 µM). When necessary, phytohormones were initially dissolved in ethanol and then brought to the desired final concentration through the addition of ultrapure water. Plants used as controls were subjected to foliar sprinkling with solvent only. In all treatment experiments, the first trifoliate leaf of each plant was harvested in liquid nitrogen and stored at −80 °C for further analyses.

Herbivory and Robotic Mechanical Wounding (MecWorm) Assay

Spodoptera littoralis larvae were reared as described previously (Mithöfer and others 2005). Larvae of the 3rd instar were placed for 2 h on the central leaflet of the V3 stage trifoliate leaf of soybean plants. Control and treated plants were kept under identical conditions. Similarly, V3 stage trifoliate leaves were treated for 2 h with MecWorm, that is, a robotic worm that pierces the leaf at regular intervals with a sharp metal pin, mimicking the feeding of larvae (Mithöfer and others 2005). Control and treated plants were in contact with MecWorm, but the equipment was operational only on the treated leaves.

Protein Extraction and Urease Enzyme Analysis

Protein extraction was performed under agitation (4 °C, 3 h) using 20 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM sodium azide, and 0.02% β-mercaptoethanol. Trifoliate leaves were ground and buffer was added in a 1:4 ratio (w:v). Subsequently, plant extracts were centrifuged (5 min, 8000×*g*) and filtered using a PVDF membrane (45 µm). Protein quantification was carried out according to the Bradford method (1976) using the Coomassie (Bradford) Protein Assay kit (Thermo Scientific). Urease activity was measured using the phenol–nitroprusside method (Weatherburn 1967) in 96-well plates containing 10 µL of phosphate buffered saline (PBS) and 80 µL of trifoliate leaves' protein extracts obtained after different treatments. To each sample, 10 µL of urea (200 mM) was added, whereas 10 µL of distilled water was added to the blank samples. Samples were incubated for 16 h at 37 °C and subsequently 80 µL of phenol–nitroprusside and 80 µL of sodium hypochlorite were added. The plates were left in the dark for 30 min and absorption at 570 nm was measured. A standard curve of $(NH_4)_2SO_4$ was used as a reference and *C. ensiformis* urease (C3 type, Sigma) was used as a positive control. One unit of urease activity was defined as the amount of enzyme that releases 1 µmol NH_3 min^{-1} under the conditions described.

Real-Time qPCR

The isolation of RNA was carried out using TRIzol (Life Technologies). TRIzol (1 mL) was added to a 1.5-mL Eppendorf tube containing 200 mg of ground leaf matter. The resulting mixture was homogenized and incubated (20 min, RT). Chloroform (300 µL) was added, the tube was placed on ice for 20 min, centrifuged (30 min, 4 °C, 16,000×*g*), and the supernatant was transferred to a new 1.5-mL Eppendorf centrifuge tube with isopropanol (600 µL) and incubated 16 h at −20 °C. Thereafter, the mixture was centrifuged again (30 min, 4 °C, 16,000×*g*), the supernatant was discarded and the pellet was washed with 80% ethanol (800 µL), followed by centrifugation (10 min, 4 °C, 16,000×*g*). This washing step was repeated twice. After drying the samples in vacuo, the solid residue was dissolved in nuclease-free water (80 µL, previously heated to 60 °C). Following RNA extraction, a DNase treatment was carried out using a Turbo DNA-free kit (Applied Biosystems) according to the manufacturer's instructions. The RNA was quantified using a NanoDrop (ND1000) spectrophotometer. The OmniScript Reverse Transcription kit (QIAGEN) was used for synthesis of cDNA, following the manufacturer's instructions. The cDNA obtained was incubated (37 °C, 60 min) and stored at −20 °C. Prior to use, the cDNA was diluted 1:4 (v/v). The primers employed for RT qPCR are summarized in Table 1. RT qPCR was performed in 96-well plates using a BioRad CFX96 thermocycler. The reaction media consisted of cDNA (4 µL), nuclease-free water (4.5 µL), forward primer (2 µL), reverse primer (2 µL), and Brilliant II SYBR Green (12.5 µL), according to the manufacturer's instructions. The housekeeping *β-actin* gene was used as the internal control for normalizing the amount of mRNA present in each sample. The efficiency of each pair of primers used in this study was evaluated by the construction of a standard curve with different amounts of the specific product for each pair (Table 2). The relative expression of the genes of interest was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001; Pfaffl 2001).

Table 1 Sequences of primers for qPCR analysis

Primer	Sequence	Amplicon (nt)
*Gm*Actin Fw	GAGCTATGAATTGCCTGATGG	121
*Gm*Actin Rev	GTCGTTTCATGAATTCCAGTAGC	
ubSBU Fw	AACACAGATTTTGGAATTTGT TCG	138
ubSBU Rev	TGGCTTCAACCTGAACACTTTC	
*Gm*UreG Fw	CTGATTTGGCAGTCATGCAGCG	115
*Gm*UreG Rev	GCTTATTCCCTGTGGCTGCTTCC	

Table 2 Efficiency of primers

Primer pair	Efficiency (%)
Actin	95.3
Ubiquitous urease (*ubSBU*)	95.1
UreG	89.0

Efficiency was calculated according to Livak and Schmittgen (2001) and Pfaffl (2001)

Statistical Analyses

The obtained data were analyzed for normality using the Shapiro–Wilk test and for homoscedasticity using the Hartley test, whereas the independence of residuals was probed using graphic analysis. Afterwards, an analysis of the variance (ANOVA) was carried out using the F test ($p \leq 0.05$). When statistical significance was found for dependent variables (for example, urease activity and relative gene expression), the effects of phytohormone treatment in relation to controls were compared using the Dunnett's test ($p \leq 0.05$), whereas the comparison of phytohormones was performed using the Waller–Duncan test ($p \leq 0.05$). The Tukey's test was used for the data obtained from the kinetics assay and a Student's t test ($p \leq 0.05$) was applied to the data obtained from the herbivory and MecWorm assays.

Results and Discussion

Although urease was the first enzyme crystallized (Sumner 1926), its regulation at the genetic level in plants under stress conditions has still not been elucidated. We initiated an investigation to address this issue in soybean, an agriculturally important crop plant, focusing on herbivory-related stresses. In *Arabidopsis* leaves, jasmonates control the expression of approximately 67–85% of genes influenced by wounds and insects (Fraire-Velázquez and others 2011). JA accumulates rapidly after herbivory or mechanical damage and is involved in the activation of defense genes, inducing the production of low- and high-molecular mass compounds (for example, protease inhibitors and phytoalexins) that act in plant defense against herbivorous insects (Mithöfer and Boland 2012).

Urease Expression in Soybean Tissue

To identify the tissue that exhibited a satisfactory expression of the investigated genes, we compared seeds, cotyledons, stem, leaves, and radicles. Due to its expression within the whole plant (Wiebke-Strohm and others 2016), we chose to study the ubiquitous urease (*ubSBU*) gene. Using imbibed seeds as a reference, the highest transcription levels of the *ubSBU* gene were found in the leaves and radicle (Table 3), the latter showing levels twofold higher than the former. Due to technical reasons, experiments with phytohormones, herbivory, and MecWorm treatments were performed with young leaves.

Table 3 Relative expression of ubiquitous urease gene (*ubSBU*) in different soybean tissues, using actin as a housekeeping gene

Tissue	Relative expression of *ubSBU*
Seed	1.00
Radicle	8.95 ± 1.79 a
Primary leaf	4.19 ± 0.56 b
Young leaf	3.94 ± 0.26 b
Stem	1.41 ± 0.13 c
Cotyledon	0.68 ± 0.21 c

Average of $n = 3 \pm$ SE. Different letters indicate significant differences, by the Waller–Duncan test ($p \leq 0.05$)

Stem and young leaves were of stage V3; as young leaves we named the third trifoliate leaf when leaflet edges did not touch each other anymore. Primary leaves were of stage VC. Cotyledons and radicles were of stage VE. Seeds were imbibed for 2 days

Expression of *ubSBU* and *UreG* Upon Various Treatments

To determine the optimal exposure time for phytohormone treatments, we performed a kinetic analysis of the relative transcription of *ubSBU*. Time points of $t = 0$, 15, 30, 60, and 180 min after treatment with JA (100 µM) were analyzed. The results showed a transient, weak, albeit significant increase of *ubSBU* gene transcription after $t = 30$ and 60 min in comparison to $t = 0$ (Fig. 1). The expression of the *UreG* gene was not altered by JA treatment. Considering the aforementioned results, we decided that the exposure time would be $t = 30$ min during the other tests with phytohormones. After foliar treatment, a significant difference of *ubSBU* gene transcription was seen not only for JA, but also for SA treatment (Table 4); however, a downregulation was found. According to Tamaoki and others (2013), SA and JA are antagonists, even though both coordinate plant defense responses. The function of SA in regulating *ubSBU* gene transcription remains obscure because ureases are described to act not only against fungi, but also insects. It is known that SA is less important in the responses to damage by chewing herbivores. However, sucking herbivores such as white flies, aphids, and spider mites, which engage in intimate and long-lasting interaction with plant cells, can also activate

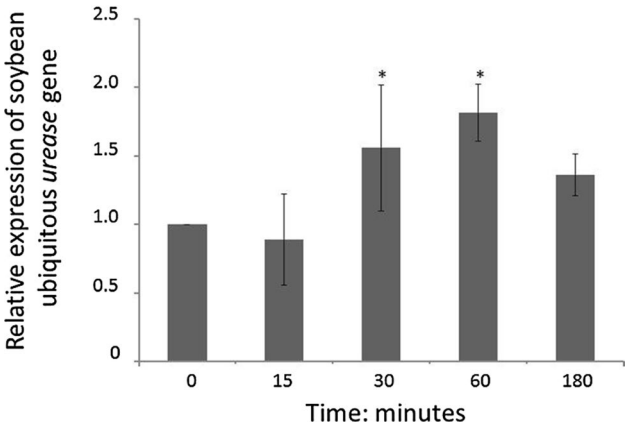

Fig. 1 Relative expression of the ubiquitous urease gene after foliar spraying with 100 µM JA. The β-actin gene was taken as reference. For each time point, different plants were used. Significant differences between different time points and control were analyzed by *t* test, $*p \leq 0.01$; $n = 3$

Table 4 Relative expression of ubiquitous urease (*ubSBU*) and *UreG* genes in soybean leaves treated with JA, SA, ABA, and GA, using actin as a housekeeping gene

Treatment	*ubSBU*	*UreG*
Control	1.00	1.00
JA	1.26 ± 0.32^{NS}	1.14 ± 0.10^{NS}
SA	0.32 ± 0.05*	0.99 ± 0.19^{NS}
ABA	1.06 ± 0.34^{NS}	0.80 ± 0.07^{NS}
GA	0.56 ± 0.07^{NS}	0.64 ± 0.10^{NS}

Average of $n = 4 \pm$ SE. Differences analyzed by Dunnett's test ($p \leq 0.05$)

*Significant

^{NS}Not significant

SA-dependent defense responses in the same way as pathogens (Leitner and others 2005; War and others 2012).

Because GA accumulates upon exposure of plants to both biotic and abiotic stress (Javid and others 2011), we analyzed the effect of GA on *ubSBU* gene transcription. We found that upon GA treatment there was a tendency for the transcription levels of *ubSBU* and *UreG* to drop by nearly a half and one-third, respectively (Table 4). None of the tested phytohormones significantly affected the transcription levels of the *UreG* gene (Table 4). These results differ somewhat from those previously reported by our group (Pires-Alves and others 2003), the only publication available on this topic. In comparison to controls, it was found that in ABA-treated detached *C. ensiformis* leaves the transcript levels of JBU urease (equivalent to *eSBU* in soybean) increased about 17-fold. Apart from using a different species and focusing on the embryo-specific urease, this difference could also be attributed to the experimental conditions. In their study, the petiole of *C. ensiformis* leaves was dipped in phytohormone solution, while in this study leaves were sprayed with the phytohormone solutions without being excised from the plant.

In plant defense against insect attacks and some microbial pathogens, JA is a crucial component. Because JA can weakly, but significantly, upregulate the *ubSBU* gene, we also tested insect feeding and mechanical wounding. In comparison to control plants, the relative transcription level of the *ubSBU* gene increased significantly in soybean leaves subjected to *S. littoralis* larvae feeding, whereas *UreG* gene transcription was significantly downregulated (Table 5). In contrast, when submitted to mechanical damage by MecWorm, soybean leaves exhibited a significant decrease in relative transcription of both the *ubSBU* and *UreG* genes (Table 5). UreG proteins are involved in the hydrolysis of GTP, which provides energy for nickel insertion and the activation of ureases. The hydrolysis of GTP occurs concomitantly with the carbamylation of a lysine residue in the active site of urease (Witte 2011). As urease activity is not required for the protein to exert insecticidal and antifungal effects, the fact that the *UreG* gene is repressed in herbivory and mechanical damage treatments can be interpreted in terms of plant economy, as it eliminates the necessity of the metallocenter assembly of the enzyme.

Interestingly, *ubSBU* gene repression was caused only by MecWorm and not by insect feeding. For insect herbivores, the presence of elicitors in their oral secretions may contribute to the plants' responses, in this case, the increase in *ubSBU* gene transcription. The collection of oral secretions from herbivores has led to the isolation of various compounds with elicitor capacity (Mithöfer and Boland 2008). Mechanical damage alone often does not initiate the same response as seen upon herbivorous attack, for example, volatile emissions in *Phaseolus lunatus* or induction of a calmodulin-like protein gene, *CML42*, in *Arabidopsis* (Bricchi and others 2010; Vadassery and others 2012).

Arabidopsis plants grown on different *N* sources show several genes, including *UreG*, that are differentially

Table 5 Relative expression of ubiquitous urease (*ubSBU*) and *UreG* genes in soybean leaves after mechanical and *Spodoptera littoralis* wounding, using actin as a housekeeping gene

Treatment	*ubSBU*	*UreG*
Control	1.00	1.00
S. littoralis	1.54 ± 0.15*	0.69 ± 0.07*
Control	1.00	1.00
MecWorm	0.70 ± 0.07*	0.71 ± 0.09*

Average of $n = 5 \pm$ SE

*Significant differences analyzed by Student's *t* test ($p \leq 0.05$)

regulated (Mérigout and others 2008). The plants used in this study were grown in soil without the addition of nitrogen as a fertilizer, and this can explain why no differences in *UreG* transcripts were observed, not even after phytohormone treatment. Tomato leaves treated with JA or mechanical wounding showed high arginase activity (Chen and others 2004). Besides the involvement of arginase in nitrogen metabolism, the enzyme may also be involved in the control of transcription levels when cells are in an environment with limited access to nitrogen (Hall and others 2004; Chen and others 2004). This might result in reduced or only weakly increased quantities of *ubSBU* and *UreG* transcripts, as seen in this study (Tables 4, 5). In *Arabidopsis* plants, only *UreG* transcripts varied according to *N* levels, but neither *UreD* nor *UreF* transcript levels were affected (Mérigout and others 2008). Further experiments with soybean plants growing on varying nitrogen concentrations, in particular high nitrogen levels, could provide more insight into whether or not the N level has an impact on the transcription of urease-related genes. In *Arabidopsis*, it was found that only *UreG*, but neither *UreD* nor *UreF*, transcript levels were affected by different nitrogen concentrations (Mérigout and others 2008). Such a study might also provide evidence on the putative composition of UreD, UreF, and UreG activation complex.

Urease Activity

We next investigated whether the qPCR results corresponded to urease enzyme activity. The differences between the catalytic urease activities in treated and control trifoliate leaves were not significant (ANOVA), neither upon phytohormone treatments nor after herbivory or MecWorm damage (Table 6). Of course, it is possible that after 30 min of phytohormone treatment and 2 h of herbivory and MecWorm, respectively, the time for the activation of *ub*SBU transcription or synthesis of the protein was not enough. Expression of *ubSBU* in soybean transgenic plants was upregulated 1 h after inoculation with *P. pachyrhizi* and a strong downregulation after 24 h of infection was reported (Wiebke-Strohm and others 2016). However, in *Arabidopsis*, both the transcript accumulation and enzymatic activity of JAR1, the JA, and isoleucine conjugating enzyme increased upon feeding within one hour (Scholz and others 2014).

Table 6 Ureolytic activities of soybean leaves after different treatments

Sample	Urease activity (U/min/µg protein)
Control	1.63 ± 0.13
JA	1.81 ± 0.27^{NS}
SA	1.75 ± 0.09^{NS}
ABA	1.50 ± 0.03^{NS}
Control	3.31 ± 0.30
S. littoralis	3.32 ± 0.31^{NS}
Control	4.52 ± 1.01
MecWorm	4.38 ± 0.79^{NS}

Average of $n = 5 \pm$ SE

^{NS}Not significant

Conclusions

From the present work, we concluded that the treatment with neither phytohormones nor herbivory and MecWorm had any influence on ureolytic activity of soybean plants, indicating a sufficient constitutive basal expression of urease in its active form. From our data, it is tempting to speculate that phytohormones may present antagonistic responses in the regulation of *ubSBU* gene expression. Herbivory, and consequently JA, but not mechanical damage with MecWorm, increased the transcription level of the *ubSBU* gene, and both treatments repressed the *UreG* gene. After treatment with phytohormones, no change in *UreG* expression was observed. Independent of the enzyme activation machinery, urease may have its expression regulated in response to different sources of stress.

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