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Research article

Structural and transcriptional characterization of a novel member of the soybean urease gene family

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ABSTRACT

In plants, ureases have been related to urea degradation, to defense against pathogenic fungi and phytophagous insects, and to the soybean–*Bradyrhizobium japonicum* symbiosis. Two urease isoforms have been described for soybean: the embryo-specific, encoded by *Eu*1 gene, and the ubiquitous urease, encoded by *Eu*4. A third urease-encoding locus exists in the completed soybean genome. The gene was designated Eu5 and the putative product of its ORF as SBU-III. Phylogenetic analysis shows that 41 plant, moss and algal ureases have diverged from a common ancestor protein, but ureases from monocots, eudicots and ancient species have evolved independently. Genomes of ancient organisms present a single urease-encoding gene and urease-encoding gene duplication has occurred independently along the evolution of some eudicot species. SBU-III has a shorter amino acid sequence, since many gaps are found when compared to other sequences. A mutation in a highly conserved amino acid residue suggests absence of ureolytic activity, but the overall protein architecture remains very similar to the other ureases. The expression profile of urease-encoding genes in different organs and developmental stages was determined by RT-qPCR. *Eu*5 transcripts were detected in seeds one day after dormancy break, roots of young plants and embryos of developing seeds. *Eu*1 and *Eu*4 transcripts were found in all analyzed organs, but *Eu*4 expression was more prominent in seeds one day after dormancy break whereas *Eu*1 predominated in developing seeds. The evidence suggests that SBU-III may not be involved in nitrogen availability to plants, but it could be involved in other biological role(s).

1. Introduction

Ureases (EC 3.5.1.5), also referred as urea amidohydrolases, are nickel dependent enzymes that catalyze the hydrolysis of urea to form ammonia and carbamate. The later compound spontaneously hydrolyzes at physiological pH to form carbonic acid and a second molecule of ammonia. This enzyme has been isolated from a variety of natural sources including plants and microorganisms. The high similarity among all ureases suggests that they share a common ancestral gene (Ligabue-Braun et al., 2013) and catalytic mechanisms (Follmer, 2008). Ureases are present virtually in all plants, but are especially abundant in many seeds of the leguminosae and the cucurbitaceae. *Arabidopsis* and soybean (*Glycine max* [L.] Merr.) ureases are the best characterized at the genetic and biochemical levels, while jackbean urease is one of the best characterized at the biochemical level (Follmer, 2008), including solving its three-dimensional structure (Balasubramanian and Ponnuraj, 2010). So far, two isozymes were described for soybean (Torisky et al. 1994;

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[3] BWS, CRC and MHBZ: wrote the manuscript.

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Goldraij et al. 2003) and three for jackbean (Sumner, 1926; Follmer et al. 2001; Mulinari et al., 2011). In soybean, the ubiquitous urease, encoded by the *Eu*4 gene (Glyma11g248700; GenBank accession AJ276866), is found in all plant tissues, in which it catalyzes urea hydrolysis and thereby allows organisms to use exogenous and internally generated urea as a nitrogen source (Torisky et al. 1994; Witte et al. 2002). The embryo-specific urease, encoded by the *Eu*1 gene (Glyma05g146000; GenBank accession NM001249869), is present in developing embryos and mature seeds (Polacco and Holland, 1993). The relevance of the catalytic function of soybean embryo-specific and jackbean ureases remain widely unclear. However, the toxicity of ureases against some insects and fungi have been demonstrated suggesting participation of these proteins in defense mechanisms of plants. The toxic properties were shown to be independent from the ureolytic activity, although these enzymes have fully active catalytic sites (Follmer et al. 2004; Becker-Ritt et al. 2007).

Soybean and jackbean belong to the family Fabaceae, subfamily Papilionoideae (Sato et al. 2010), thus they are phylogenetically related. Although a family of three urease-related genes was suggested for the jackbean, embryo-specific and ubiquitous ureases were believed as the only functional genes in soybean. This conclusion was based on experiments with *eu*1/*eu*4 double mutants that are virtually devoid of ureolytic activity (Stebbins and Polacco, 1995; Goldraij et al. 2003). The soybean whole-genome sequence was reported in 2010 and brought new insights by allowing new and more accurate studies on the urease gene family. In fact, the presence of the third urease-encoding gene or an urease-like-encoding gene in the soybean genome was previously identified (Witte, 2011; Real-Guerra et al. 2013; Polacco et al. 2013). In the present study we characterized this novel soybean urease-encoding gene.

2. Materials and methods

2.1. Bioinformatic analyses

A search to identify urease isoforms was carried out using BLAST analysis in Phytozome v.10.2 - *G. max* v1.1 (http://www.phytozome.org) and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Protein sequences encoded by urease genes were identified and downloaded from the databases. A total of 41 plant-, algae- and moss-ureases sequences were selected. The multiple sequences alignments of ureases were performed with MUSCLE software (Edgar, 2004) implemented in MEGA5 (Molecular Evolutionary analysis) software (http://www.megasoftware.net) (Tamura et al. 2007).

Phylogenetic analysis was conducted with protein sequences using Bayesian approach implemented in BEAST 1.8.1 software (Drummond and Rambaut, 2007). The best-fit model of protein evolution was determined using ProTest (Abascal et al. 2005), which selected the JTT model for protein matrix substitution. The Yule tree was selected as the tree prior for Bayesian analysis and 20.000.000 generations. The input file for BEAST was setup with BEAUti v. 1.8.1. The trees were summarized with TreeAnnotator v. 1.8.1 and visualized with FigTree.

The construction of three-dimensional (3D)-structural models of the three soybean ureases was performed using homology modeling techniques, employing MODELLER 9.14 (Sanchez et al. 2000). The template used for modeling was the jackbean urease crystal structure (PDB id 3LA4, 2.05 Å resolution) (Balasubramanian and Ponnuraj, 2010). Ten models were built for each protein. These models were stereochemically evaluated with PROCHECK (Laskowski et al. 1993) and had their one-dimensional (1D)-3D profile theoretically validated with Verify3D (Luthy et al. 1992). The best model for each urease was selected based on these assessments.

The *Glyma* codes (locus names) that correspond to ureases isoforms were used to investigate the expression pattern in RNA-seq experiments at SoyBase and the Soybean Breeder's Toolbox (http://www.soybase.org/soyseq). For the tissue-specific analyses, raw digital gene expression counts were normalized using a variation of the Reads/Kb/Million (RPKM) method. The 14 analyzed tissues were grouped into three main clades according to Severin et al. (2010), as follow: underground tissues (root and nodule), seed development (seed 10-days after flowering (DAF), seed 14-DAF, seed 21-DAF, seed 25-DAF, seed 28-DAF, seed 35-DAF and seed 42-DAF) and aerial tissues (young leaf, flower, 1 cm pod, pod shell 10-DAF and pod shell 14-DAF). A Z-score analysis was performed. The obtained values measure the number of standard deviations in gene expression level in a specific tissue in relation to the mean expression level in all tissues (Severin et al., 2010).

2.2. Plant growth conditions

The Brazilian cv. MGBR-46 Conquista was chosen for qPCR expression analyses. A first pool of seeds was placed on dishes containing wetted germination paper and maintained in the dark for one day. A second pool of seeds was sowed in vermiculite and plants were grown for two weeks in a culture room at 26 ± 1 °C with 16/8 h light/dark at a light intensity of 250 μmol $m^{-2}.s^{-1}$. A third pool of seeds was planted in pots containing organic soil and plants were grown until complete development in a growth chamber at 28 ± 1 °C with 16/8 h light/dark at a light intensity of 250 μmol m^{-2} s^{-1}. Different plant organs were collected in four phenological stages as shown in Table 1. The developmental stages of flowers was based on the association between flowers bud sizes and the corresponding microspore developmental stage as previously reported (Lauxen et al. 2003). For each organ, four biological replications were collected; each replication was represented by material from 4 different plants. All samples were quickly frozen in liquid nitrogen and stored at −80 °C.

2.3. Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, USA) and further treated with DNAse (Promega, Madison, USA) according to the manufacturer's instruction. The first-strand cDNAs were obtained by using approximately 2 μg of DNA-free RNA, M-MLV reverse transcriptase system (Invitrogen, Carlsbad, USA) and 24-polyVT primer.

RT-qPCR was performed using a StepOne Applied Biosystem real-time cycler™, based on SYBR fluorescence. Each 25 μL reaction comprised 12.5 μL cDNA (1:100 dilution), 1x PCR buffer (Invitrogen, São Paulo, Brazil), 2.4 mM $MgCl_2$, 0.024 mM dNTP, 0.1 mM each primer, 2.5 μL SYBR-Green (1:100,000, Molecular Probes Inc., Eugene, USA) and 0.03 U of Platinum Taq DNA Polymerase (5 U/μl, Invitrogen, São Paulo, Brazil). PCR-cycling conditions were implemented as follows: 5 min 94 °C, followed by 40 repetitions of 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C, by the end 2 min at 40 °C. A melting curve analysis was performed at the end of the PCR run, over the range 55–99 °C, increasing the temperature stepwise by 0.1 °C every 1 s. All PCR reactions were carried out in quadruplicate. No-template reactions were used as negative controls.

A set of four candidate reference genes was selected from previous reports (Table 2) (Libault et al. 2008). Specific primer pairs were projected for each urease-encoding genes using Primer3 (v. 0.4.0) software (Table 2). Expression data analyses were performed after comparative quantification of amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1
Plant organs in four phenological stages collected for qPCR gene expression analyses.

	Plant phenological stage	Plant organ
1	1 day after dormancy break	Seed
2	Young plants[a]	Root
		Stem
		Leaf
3	Adult plants – flowering[b]	Stem
		Leaf
		Flower before fertilization
		Flower at fertilization
		Flower after fertilization
4	Adult plants – seed development[c]	Pod with seeds
		Pod without seeds
		Seed

[a] Two week-old plants after expansion of the first trifoliolate leaf.
[b] Organs were collected at the moment the first flowers opened, about three month after sowing. An association between soybean flower bud size and the microspores developmental stage was previously reported (Lauxen et al. 2003). Flower buds with 4–5 mm in length, presenting immature anthers, were considered not fertilized. Flower buds with 6–7 mm in length, with mature anthers and pollen, were determined at fertilization. The opened flowers were considered fertilized.
[c] Organs were harvested about three weeks after flowering, at the moment the seeds achieved 3–4 mm in length.

3. Results

3.1. Bioinformatic analyses

Based on the soybean whole-genome sequence the Glyma08g103000 was identified as a new putative urease isoform. The third urease encoding-gene was designated *Eu*5 and its deduced amino acid sequence, SBU-III (soybean urease III). A comparison among the three urease-encoding genes is shown in Table 3. The *Eu*5 cDNA sequence share 79.6% identity with *Eu*4 and 91.8% identity with *Eu*1 cDNA.

Urease-encoding genes were identified in 34 different plants-, moss- and algae-species which have their genome sequence available in Phytozome plus *Canavalia ensiformis*. The 41 ureases' complete amino acid sequences available in databases were aligned to reconstruct a phylogenetic tree (Fig. 1). Ureases from *Micromonas pusilla* [Butcher] Manton & Parke (algae), *Physcomitrella patens* [Hedw.] Bruch & Schimp. (moss), *Selaginella moellendorffii* Hieron. (ancient vascular plant) were grouped together, forming a basal clade. The other clade consists of ureases from monocot and eudicot plants; however, ureases from these groups formed two independent sub-clades: the first with monocot ureases and the second with eudicot ureases. These findings suggest that all analyzed ureases have diverged from a common ancestor protein in Viridiplantae, but ureases from monocots, eudicots and ancient species have evolved independently along the time.

Leguminous plants were also shown to have a common ancient urease. It was found that SBU-III (GmaIII08g10850) is closely related to soybean embryo-specific (GmaE05g27840) and to two *C. ensiformis* ureases (CenUREI and CenUREIIB) (Fig. 1). Curiously, soybean ubiquitous urease (GmaU11g37250) was grouped with *Phaseolus vulgaris* L. (Phv001G260000) and *Medicago truncatula* Gaertn. (MtrMedtr3g085640) ureases, in a different sub-clade than that formed by the other soybean ureases.

Most analyzed species contain a single copy of urease-encoding gene into their genome, including the basal species (*M. pusilla*, *P. patens*, *S. moellendorffii*) and all monocots. However, two gene copies were found into *Brassica rapa*, *Theobroma cacao* L. and *Linum usitatissimum* L. genomes, whereas three gene copies were identified into *G. max*. The genome of *C. ensiformis* has not been sequenced so far, but it is known that this species produces three urease isoforms, at least two of which are encoded by independent genes (Sumner, 1926; Carlini and Guimarães, 1981; Mulinari et al., 2011). The DNA and amino acid sequences of two of these genes were previously determined and therefore included in our analyses (Riddles et al. 1991; Mulinari et al., 2011). It is noteworthy that species showing two or three urease isoforms are all eudicots, but were grouped in different sub-clades. These findings suggest that ancient organisms presented a single urease-encoding gene in their

Table 2
Primers set for RT-qPCR.

Target gene	Orientation	Primer sequence
*Eu*1 (embryo-specific urease)	Forward	5′-ACCAGTTTTGCAACCACCTT-3′
	Reverse	5′-AAGAACAAGAGCAGGGGAACT-3′
*Eu*4 (ubiquitous urease)	Forward	5′- TCACTGTGGACCCAGAAACA -3′
	Reverse	5′- CTTGCTTATTGTTTTTTGCCAAT -3′
*Eu*5 (urease III)	Forward	5′-GTCGAGTTGGAGAGGTCCTTTAT-3′
	Reverse	5′-GAGAAATGTCACATGCACACTG-3′
Metalloprotease	Forward	5′- ATGAATGACGGTTCCCATGTA -3′
	Reverse	5′- GGCATTAAGGCAGCTCACTCT -3′
FBox protein	Forward	5′- AGATAGGGAAATGTTGCAGGT -3′
	Reverse	5′- CTAATGGCAATTGCAGCTCTC -3′
Actin 11	Forward	5′- CGGTGGTTCTATCTTGGCATC -3′
	Reverse	5′- GTCTTTCGCTTCAATAACCCTA -3′
Cyclophilin	Forward	5′- ACGACGAAGACGGAGTGG -3′
	Reverse	5′- CGACGACGACAGGCTTGG -3′

Table 3
Urease encoding-genes characteristics according to Phytozome database.

Urease	Encoding-gene (ID)	Chromosome	Gene size (bp)	Number of introns	CDS (nt)	Peptide (aa)	Molecular mass (kDa)
Embryo-specific	*Eu*1 (Glyma05g146000)	5	8076	17	2520	839	94
Ubiquitous	*Eu*4 (Glyma11g248700)	11	7534	17	2514	837	94
SBU-III	*Eu*5 (Glyma08g103000)	8	5849	14	1932	643	73[a]

[a] Predicted molecular size of the putative protein was obtained by submitting the sequence to the ProtParam tool available at the Expasy site (http://ca.expasy.org/tools/protparam.html).

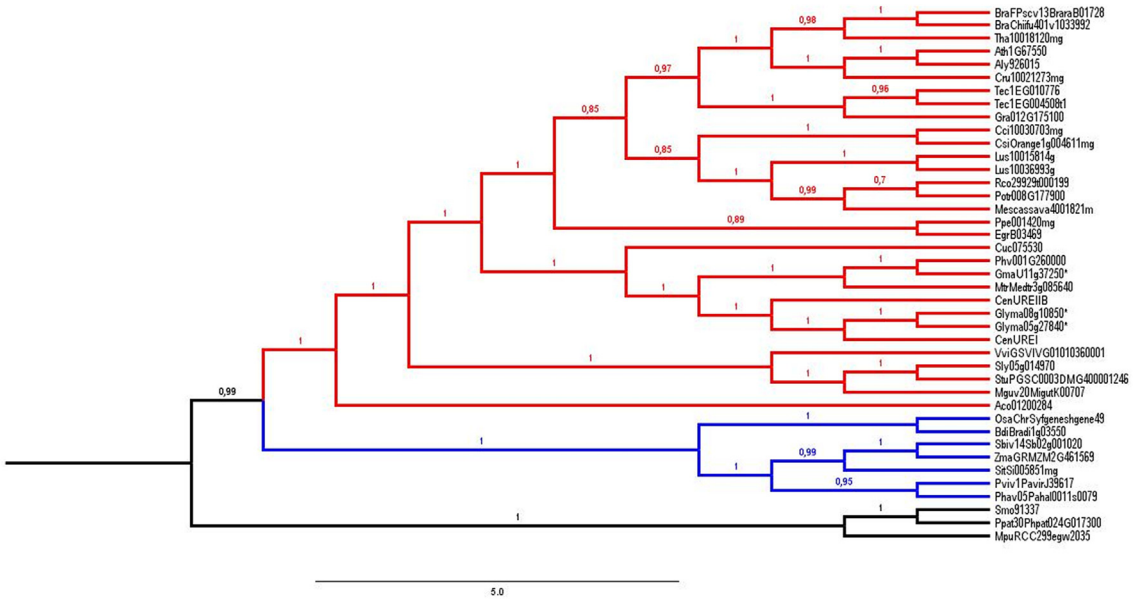


Fig. 1. Phylogenetic analysis of plant, moss and algae ureases. Phylogenetic analysis was conducted with protein sequences using Bayesian approach (20.000.000 generations). Canavalia ensiformes [L.] DC. (CenUREI, CenUREIIB), Glycine max [L.] Merr. (GmaU11g37250 = Glyma11g248700, GmaE05g27840 = Glyma05g146000, GmaIII08g10850 = Glyma08g103000), Manihot esculenta Crantz (Mescassava4001821m), Ricinus communis L. (Rco29929t000199), Linum usitatissimum L. (Lus10015814g, Lus10036993g), Populus trichocarpa Torr. & Gray (Potr008G177900), Phaseolus vulgaris L. (Phv001G260000), Cucumis sativus L. (Cuc075530), Arabidopsis thaliana [L.] Heynh. (Ath1G67550), Arabidopsis lyrata [L.] O'Kane & Al-Shehbaz (Aly926015), Capsella rubella Reut. (Cru10021273 mg), Brassica rapa L. – Chiifu-401 v1.2 (BraChiifu401v1033992), Thellungiella halophila [Mey.] Schulz (Tha10018120 mg), Gossypium raimondii Ulbr. (Gra012G175100), Theobroma cacao L. (Tec1EG010776, Tec1EG004508t1), Citrus sinensis [L.] Osbeck (CsiOrange1g004611 mg), Citrus clementina hort. ex Tanaka (Cci10030703 mg), Eucalyptus grandis Hill ex Maid. (EgrB03469), Vitis vinifera L. (VviGSVIVG01010360001), Solanum lycopersicum L. (Sly05g014970), Aquilegia coerulea James (Aco01200284), Zea mays L. (ZmaGRMZM2G461569), Oryza sativa L. (OsaChrSyfgeneshgene49), Brachypodium distachyon [L.] Beauv. (BdiBradi1g03550), Selaginella moellendorffii Hieron. (Smo91337), Micromonas pusilla [Butcher] Manton & Parke RCC299 (MpuRCC299egw2035), Brassica rapa L. FPsc v1.3 (BraFPscv13BraraB01728), Mimulus guttatus DC. v2.0 (Mguv20MigutK00707), Physcomitrella patens [Hedw.] Bruch & Schimp. v3.0 (Ppat30Phpat024G017300), Sorghum bicolor (L.) Moench v2.1 (Sbiv14Sb02g001020), Panicum hallii Vasey v0.5 (Phav05Pahal0011s0079), Panicum virgatum L. v1.1 (PviviPavirJ39617), Medicago truncatula Gaertn. (MtrMedtr3g085640), Solanum tuberosum L. (StuPGSC0003DMG400001246), Setaria italica (L.) Beauv. (SitSi005851 mg), Prunus persica (L.) Batsch (Ppe001420 mg). Clade in blue consists of ureases from monocot, in red eudicot plants and in black the basal clade. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genome and that urease-encoding gene duplication have occurred independently along the evolution of eudicot species. Exceptions are *G. max* and *C. ensiformis* that may have the same gene duplication origin. To better access this question it would be necessary to obtain the entire sequence of the missing *C. ensiformis* urease (canatoxin).

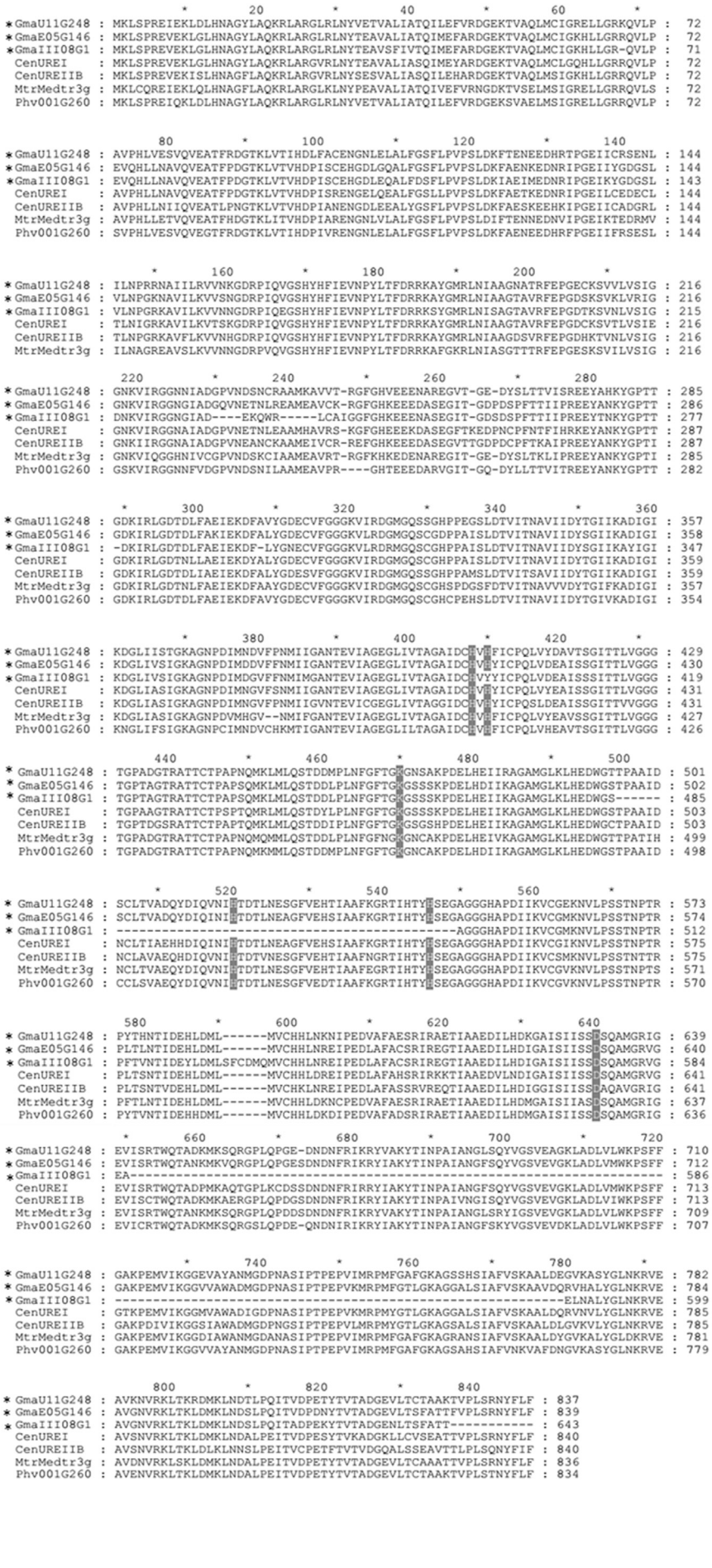
Alignment of SBU-III amino acid sequence (GmaIII08g1) with other leguminous urease sequences revealed a high similarity (Fig. 2). However, SBU-III presented a mutation in one of the highly conserved residues of the active site (Jabri et al. 1995; Balasubramanian and Ponnuraj, 2010), in which a histidine residue (His409 in CenUREI) was substituted by a tyrosine. Long gaps were also observed in SBU-III, including part of the entomotoxic peptide region (residues 245–258 in CenUREI, Mulinari et al., 2007), part of the catalytic domain (residues 644–772 in CenUREI) and a carboxy-terminal segment (residues 830–840 in CenUREI).

The 3D-structure of the three soybean ureases was predicted in order to compare SBU-III with its counterparts. The modeled structures share the hammer or T-shape, as observed so far in all urease crystal structures (Jabri et al. 1995; Balasubramanian and Ponnuraj, 2010) (Fig. 3). The ureolytic active site cavity and the mobile flap of SBU-III exhibit an overall architecture that is similar to embryo-specific and ubiquitous ureases. Nevertheless, the SBU-III structure seemed to have a more flexible conformation when compared to the other soybean ureases, especially in domain connections. The absence of part of the entomotoxic region (Mulinari et al., 2007) and of helical segments near the active site in the α domain were also observed. Apart from these major differences, some other smaller variations are present.

3.2. Urease expression in different plant organs

A set of four reference genes previously tested for other studies was selected (Table 2). The expression stability of the genes was examined by geNorm software v3.5 (Vandesompele et al. 2001) and Normfinder software (Andersen et al. 2004). Fbox, Metalloprotease and Actin 11 genes had an M value below 1.5 which is recommended by geNorm. Results indicated FBox protein and Metalloprotease genes as the most stable reference genes for expression normalization under our experimental conditions. The same results were found when data were analyzed by Normfinder software. The specificity of the primer pairs for the different ureases-encoding genes was confirmed by (1) the presence of a single peak in the melting curve, (2) a single fragment of the expected size in electrophoresis agarose gel and (3) amplicon sequencing (data not shown).

As expected, ubiquitous urease transcripts were identified in all analyzed samples (Fig. 4A), but a higher level was detected in seeds one day after the dormancy break. Surprisingly, embryo-specific urease transcripts were also present in all organs (Fig. 4A), but, as expected, the higher levels were observed in samples containing embryos, i.e., developing pods with seed and seeds. The developing seed is the developing embryo enclosed by maternal (and some endosperm) tissue comprising the seed coat. In early embryo development, the seed coat is a dynamic a metabolically active tissue. In contrast to the other ureases expression pattern, SBU-III mRNA was identified in specific organs and developmental


*GmaU11G248 : MKLSPREIEKLDLHNAGYLAQKRLARGLRLNYVETVALIATQILEFVRDGEKTVAQLMCIGRELLGRKQVLP : 72
*GmaE05G146 : MKLSPREVEKLGLHNAGYLAQKRLARGLRLNYTEAVALIATQIMEFARDGEKTVAQLMCIGKHLLGRRQVLP : 72
*GmaIII08G1 : MKLSPREVEKLGLHNAGYLAQKRLARGLRLNYTEAVSFIVTQIMEFARDGEKTVAQLMCIGKHLLGR-QVLP : 71
CenUREI : MKLSPREVEKLGLHNAGYLAQKRLARGVRLNYTEAVALIASQIMEYARDGEKTVAQLMCLGQHLLGRRQVLP : 72
CenUREIIB : MKLSPREVEKISLHNAGFLAQKRLARGVRLNYSESVALIASQILEHARDGEKTVAQLMSIGKHLLGRRQVLP : 72
MtrMedtr3g : MKLCQREIEKLQLHNAGFLAQKRLARGLKLNYPEAVALIATQIVEFVRNGDKTVSELMSIGRELLGRRQVLS : 72
Phv001G260 : MKLSPREIQKLDLHNAGYLAQKRLARGLRLNYVETVALIATQILEFVRDGEKSVAELMSIGRELLGRRQVLP : 72
*GmaU11G248 : AVPHLVESVQVEATFRDGTKLVTIHDLFACENGNLELALFGSFLPVPSLDKFTENEEDHRTPGEIICRSENL : 144
*GmaE05G146 : EVQHLLNAVQVEATFPDGTKLVTVHDPISCEHGDLGQALFGSFLPVPSLDKFAENKEDNRIPGEIIYGDGSL : 144
*GmaIII08G1 : EVQHLLNAVQVEATFPDGTKLVTVHDPISCEHGDLEQALFDSFLPVPSLDKIAEIMEDNRIPGEIKYGDGSL : 143
CenUREI : AVPHLLNAVQVEATFPDGTKLVTVHDPISRENGELQEALFGSLLPVPSLDKFAETKEDNRIPGEILCEDECL : 144
CenUREIIB : AVPHLLNIIQVEATLPNGTKLVTVHDPIANENGDLEEALYGSFLPVPSLDKFAESKEEHKIPGEIICADGRL : 144
MtrMedtr3g : AVPHLLETVQVEATFHDGTKLITVHDPIARENGNLVLALFGSFLPVPSLDIFTENNEDNVIPGEIKTEDRMV : 144
Phv001G260 : SVPHLVESVQVEGTFRDGTKLVTIHDPIVRENGNLELALFGSFLPVPSLDKFAENEEDHRFPGEIIFRSESL : 144
*GmaU11G248 : ILNPRRNAIILRVVNKGDRPIQVGSHYHFIEVNPYLTFDRRKAYGMRLNIAAGNATRFEPGECKSVVLVSIG : 216
*GmaE05G146 : VLNPGKNAVILKVVSNGDRPIQVGSHYHFIEVNPYLTFDRRKAYGMRLNIAAGTAVRFEPGDSKSVKLVRIG : 216
*GmaIII08G1 : VLNPGRKAVILKVVNNGDRPIQEGSHYHFIEVNPYLTFDRRKSYGMRLNISAGTAVRFEPGDTKSVNLVSIG : 215
CenUREI : TLNIGRKAVILKVTSKGDRPIQVGSHYHFIEVNPYLTFDRRKAYGMRLNIAAGTAVRFEPGDCKSVTLVSIE : 216
CenUREIIB : TLNPGRKAVFLKVVNHGDRPIQVGSHYHFIEVNPYLTFDRRKAYGMRLNIAAGDSVRFEPGDHKTVNLVSIG : 216
MtrMedtr3g : ILNAGREAVSLKVVNNGDRPVQVGSHYHFIEVNPYLTFDRRKAFGKRLNIASGTTTRFEPGESKSVILVSIG : 216
*GmaU11G248 : GNKVIRGGNNIADGPVNDSNCRAAMKAVVT-RGFGHVEEENAREGVT-GE-DYSLTTVISREEYAHKYGPTT : 285
*GmaE05G146 : GNKVIRGGNGIADGQVNETNLREAMEAVCK-RGFGHKEEEDASEGIT-GDPDSPFTTIIPREEYANKYGPTT : 286
*GmaIII08G1 : DNKVIRGGNGIAD----EKQWR-----LCAIGGFGHKEEENASEGIT-GDSDSPFTTIIPREEYTNKYGPTT : 277
CenUREI : GNKVIRGGNAIADGPVNETNLEAAMHAVRS-KGFGHEEEKDASEGFTKEDPNCPFNTFIHRKEYANKYGPTT : 287
CenUREIIB : GNKIIRGGNAIADGPVNEANCKAAMEIVCR-REFGHKEEEDASEGVTTGDPDCPFTKAIPREEYANKYGPTI : 287
MtrMedtr3g : GNKVIQGGHNIVCGPVNDSKCIAAMEAVRT-RGFKHKEDENAREGIT-GE-DYSLTKLIPREEYANKYGPTI : 285
Phv001G260 : GSKVIRGGNNFVDGPVNDSNILAAMEAVPR----GHTEEEDARVGIT-GQ-DYLLTTVITREEYANKYGPTT : 282
*GmaU11G248 : GDKIRLGDTDLFAEIEKDFAVYGDECVFGGGKVIRDGMGQSSGHPPEGSLDTVITNAVIIDYTGIIKADIGI : 357
*GmaE05G146 : GDKIRLGDTDLFAKIEKDFALYGDECVFGGGKVLRDGMGQSCGDPPAISLDTVITNAVIIDYSGIIKADIGI : 358
*GmaIII08G1 : -DKIRLGDTDLFAEIEKDF-LYGNECVFGGGKVLRDRMGQSCGHPPAISLDTVITNAVIIDYSGIIKAYIGI : 347
CenUREI : GDKIRLGDTNLLAEIEKDYALYGDECVFGGGKVIRDGMGQSCGHPPAISLDTVITNAVIIDYTGIIKADIGI : 359
CenUREIIB : GDKIRLGDTDLIAEIEKDFALYGDESVFGGGKVIRDGMGQSSGHPPAMSLDTVITSAVIIDYTGIIKADIGI : 359
MtrMedtr3g : GDKIRLGDTNLFAEIEKDFAAYGDECVFGGGKVIRDGMGQSCGHSPDGSFDTVITNAVVVDYTGIFKADIGI : 357
Phv001G260 : GDKIRLGDTDLFAEIEKDFAVYGDECVFGGGKVIRDGMGQSCGHCPEHSLDTVITNAVIIDYTGIVKADIGI : 354
*GmaU11G248 : KDGLIISTGKAGNPDIMNDVFPNMIIGANTEVIAGEGLIVTAGAIDCIVGFICPQLVYDAVTSGITTLVGGG : 429
*GmaE05G146 : KDGLIVSIGKAGNPDIMDDVFPNMIIGANTEVIAGEGLIVTAGAIDCIVGYICPQLVDEAISSGITTLVGGG : 430
*GmaIII08G1 : KDGLIVSIGKAGNPDIMDGVFPNMIMGANTEVIAGEGLIVTAGAIDCIVYYICPQLVDEAISSSITTLVGGG : 419
CenUREI : KDGLIASIGKAGNPDIMNGVFSNMIIGANTEVIAGEGLIVTAGAIDCIVGYICPQLVYEAISSGITTLVGGG : 431
CenUREIIB : KDGLIASIGKAGNPDIMNGVFPNMIIGVNTEVICGEGLIVTAGGIDCIVGYICPQSLDEAISSGITTVVGGG : 431
MtrMedtr3g : KDGLIASIGKAGNPDVMHGV--NMIFGANTEVIAGEGLIVTAGAIDCIVGFICPQLVYEAVSSGITTLVGGG : 427
Phv001G260 : KNGLIFSIGKAGNPCIMNDVCHKMTIGANTEVIAGEGLILTAGAIDCIVGFICPQLVHEAVTSGITTLVGGG : 426
*GmaU11G248 : TGPADGTRATTCTPAPNQMKLMLQSTDDMPLNFGFTGGGNSAKPDELHEIIRAGAMGLKLHEDWGTTPAAID : 501
*GmaE05G146 : TGPTAGTRATTCTPAPSQMKLMLQSTDDLPLNFGFTGGGSSSKPDELHDIIKAGAMGLKLHEDWGSTPAAID : 502
*GmaIII08G1 : TGPTAGTRATTCTPAPSQMKLMLQSTDDLPLNFGFTGGGSSSKPDELHEIIKAGAMGLKLHEDWGS------ : 485
CenUREI : TGPAAGTRATTCTPSPTQMRLMLQSTDYLPLNFGFTGGGSSSKPDELHEIIKAGAMGLKLHEDWGSTPAAID : 503
CenUREIIB : TGPTDGSRATTCTPAPTQMKLMLQSTDDIPLNFGFTGGGSGSHPDELHEIIKAGAMGLKLHEDWGCTPAAID : 503
MtrMedtr3g : TGPADGTRATTCTPAPNQMQMMLQSTDDLPLNFGFNGGGNCAKPDELHEIVKAGAMGLKLHEDWGTTPATIH : 499
Phv001G260 : TGPADGTRATTCTPAPNQMKMMLQSTDDMPLNFGFTGGGNCAKPDELHDIIKAGAMGLKLHEDWGSTPAAID : 498
*GmaU11G248 : SCLTVADQYDIQVNIGTDTLNESGFVEHTIAAFKGRTIHTYGSEGAGGGHAPDIIKVCGEKNVLPSSTNPTR : 573
*GmaE05G146 : SCLTVADQYDIQINIGTDTLNEAGFVEHSIAAFKGRTIHTYGSEGAGGGHAPDIIKVCGMKNVLPSSTNPTR : 574
*GmaIII08G1 : ----------AGGGHAPDIIKVCGMKNVLPSSTNPTR : 512
CenUREI : NCLTIAEHHDIQINIGTDTLNEAGFVEHSIAAFKGRTIHTYGSEGAGGGHAPDIIKVCGIKNVLPSSTNPTR : 575
CenUREIIB : NCLAVAEQHDIQVNIGTDTVNESGFVEHTIAAFNGRTIHTYGSEGAGGGHAPDIIKVCSMKNVLPSSTNTTR : 575
MtrMedtr3g : NCLTVAEQYDIQVNIGTDTLNESGFVEHTIAAFEGRTIHTYGSEGAGGGHAPDIIKVCGVKNVLPSSTNPTS : 571
Phv001G260 : CCLSVAEQYDIQVNIGTDTLNESGFVEDTIAAFKGRTIHTYGSEGAGGGHAPDIIKVCGVKNVLPSSTNPTR : 570
*GmaU11G248 : PYTHNTIDEHLDML------MVCHHLNKNIPEDVAFAESRIRAETIAAEDILHDKGAISIISSGSQAMGRIG : 639
*GmaE05G146 : PLTLNTIDEHLDML------MVCHHLNREIPEDLAFACSRIREGTIAAEDILHDIGAISIISSGSQAMGRVG : 640
*GmaIII08G1 : PFTVNTIDEYLDMLSFCDMQMVCHHLNREIPEDLAFACSRIREGTIAAEDILHDIGAISIISSGSQAMGRVG : 584
CenUREI : PLTSNTIDEHLDML------MVCHHLDREIPEDLAFAHSRIRKKTIAAEDVLNDIGAISIISSGSQAMGRVG : 641
CenUREIIB : PLTSNTVDEHLDML------MVCHKLNREIPEDLAFASSRVREQTIAAEDILHDIGGISIISSGAQAVGRIG : 641
MtrMedtr3g : PFTLNTIDEHLDML------MVCHHLDKNCPEDVAFAESRIRAETIAAEDILHDMGAISIIASGSQAMGRIG : 637
Phv001G260 : PYTVNTIDEHHDML------MVCHHLDKDIPEDVAFADSRIRAETIAAEDILHDMGAISIISSGSQAMGRIG : 636
*GmaU11G248 : EVISRTWQTADKMKSQRGPLQPGE-DNDNFRIKRYVAKYTINPAIANGLSQYVGSVEAGKLADLVLWKPSFF : 710
*GmaE05G146 : EVISRTWQTANKMKVQRGPLQPGESDNDNFRIKRYIAKYTINPAIANGFSQYVGSVEVGKLADLVMWKPSFF : 712
*GmaIII08G1 : EA---------- : 586
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CenUREIIB : EVISCTWQTADKMKAERGPLQPDGSDNDNFRIKRYIAKYTINPAIVNGISQYVGSVEVGKLADLVIWKPSFF : 713
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*GmaU11G248 : GAKPEMVIKGGEVAYANMGDPNASIPTPEPVIMRPMFGAFGKAGSSHSIAFVSKAALDEGVKASYGLNKRVE : 782
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*GmaIII08G1 : ----------ELNALYGLNKRVE : 599
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Phv001G260 : GAKPEMVIKGGVVAYANMGDPNASIPTPEPVIMRPMFGAFGKAGSAHSIAFVNKVAFDNGVKASYGLNKRVE : 779
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*GmaE05G146 : AVGNVRKLTKLDMKLNDSLPQITVDPDNYTVTADGEVLTSFATTFVPLSRNYFLF : 839
*GmaIII08G1 : AVGNVRKLTKLDMKLNDSLPQITADPEKYTVTADGENLTSFATT---------- : 643
CenUREI : AVSNVRKLTKLDMKLNDALPEITVDPESYTVKADGKLLCVSEATTVPLSRNYFLF : 840
CenUREIIB : AVSNVRKLTKLDLKLNNSLPEITVCPETFTVTVDGQALSSEAVTTLPLSQNYFIF : 840
MtrMedtr3g : AVDNVRKLSKLDMKLNDALPEITVDPETYTVTADGEVLTCAAATTVPLSRNYFLF : 836
Phv001G260 : AVENVRKLTKLDMKLNDALPEITVDPETYTVTADGEVLTCTAAKTVPLSTNYFLF : 834

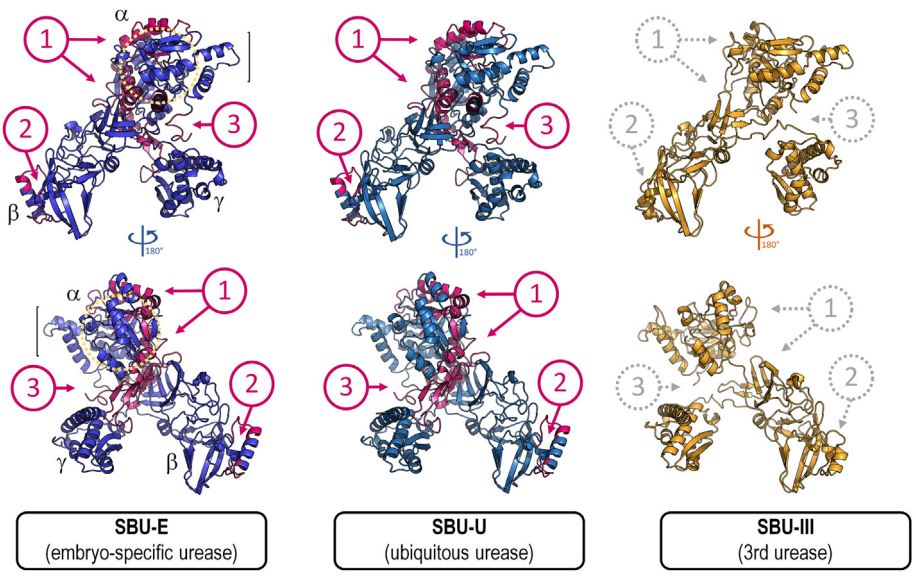

Fig. 3. Comparison of the three-dimensional (3D)-structural models of the three soybean ureases. Molecular modeling was carried out based on JBURE-I X-ray crystallographic structure (PDB3LA4). Regions that are missing in SBU-III are highlighted in purple in the ubiquitous and embryo-specific ureases isoforms. These missing regions encompass (1) long segments of the catalytic domain, (2) part of the entomotoxic peptide region, and (3) a carboxy-terminal segment. α, β and γ are the different structural domains. The region containing the ureolytic active site is indicated by a yellow dashed circle and the mobile flap is indicated by a bracket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stages: seeds one day after the dormancy break, roots of young plants, developing pods with seeds and developing seeds (Fig. 4A). Transcripts observed in developing pods with seeds and developing seeds may have derived from developing embryos or seed coat, since there was no amplification in pods without seeds. Nevertheless, when expression levels of the three ureases were compared in specific organs, the lower amount of SBU-III transcripts become evident (Fig. 4B), justifying the lack of enzyme identification by traditional techniques.

Based on RNA-Seq Atlas of *G. max* data (http://www.soybase.org/soyseq/), which provides a high-resolution gene expression in a diverse set of fourteen tissues, the expression pattern of the three urease-encoding genes was determined. After data normalization (Reads/kilobase/million normalization of the raw data) no expression of *Eu*5 (Glyma08g103000) and *Eu*1 (Glyma05g146000) was observed in young leaf, flower, 1 cm pod, pod shell (10 and 14 days after flowering - DAF), root and nodule (Fig. 5A and B). In seeds, *Eu*5 transcripts were detected 25 DAF and the highest levels were observed 42 DAF. *Eu*1 (Glyma05g146000), the embryo-urease, presented the same pattern of *Eu*5 (Glyma08g103000), but, as expected, the *Eu*1 expression was much higher (Fig. 5C). The ubiquitous urease presented transcripts in all analyzed tissues (Fig. 5). The results of qPCR (Fig. 4) and the RNA-seq (Fig. 5) data are similar and confirm the expression of SBU-III in seeds. The expression of *Eu*5 is detectable by qPCR only in young roots and probably this is the reason why no transcripts were detected in RNA-seq experiment after data normalization.

4. Discussion

In the present study, the *Eu*5 gene (Glyma08g103000) in the soybean genome was characterized. *Eu*5 gene shows high sequence similarity with the urease-encoding genes. The *Eu*5 predicted product, SBU-III, was shown to be more closely related to the soybean embryo-specific isoform than to the ubiquitous isoform (Fig. 1). Low levels of *Eu*5 transcripts were detected in specific developing organs: seeds one day after dormancy break, young roots and developing seeds (Figs. 4 and 5). It is noteworthy that SBU-III predicted molecular size is 73 kDa (Table 3) and that Torisky and Polacco (1990) identified a smaller isoform of soybean urease with approximately 80 kD in denaturing gels. This lower weight-urease was detected in an 800-fold purified protein extract derived from root tissues of young plants, hypocotyl/radicle of seedlings and embryo root axes (Torisky and Polacco, 1990). At that time, *Eu*1 and *Eu*4 were believed to be the only urease-encoding genes in soybean and the 80 kD-urease was proposed to be an additional smaller species, which may be a transient product of urease turnover. Bioinformatics predictions applied to Phytozome do not identify alternative transcripts of soybean urease-encoding genes. Altogether, the identification of *Eu*5 gene, whose transcripts were detected in the same tissues and its predicted product is 73 kDa, lead us to believe that SBU-III was previously isolated by Torisky and Polacco (1990).

The presence of a third urease-encoding gene in soybean genome was surprising, because double mutants for ubiquitous and embryo-specific urease are virtually devoid of ureolytic activity (Stebbins and Polacco, 1995; Goldraij et al., 2003). The very low urease activity detected in the double mutant was explained by the presence of urease-positive bacteria living on the plant (Holland and Polacco, 1992). It has been suggested that SBU-III may not display ureolytic activity (Witte, 2011; Real-Guerra et al. 2013; Polacco et al. 2013). In agreement with this previous postulation, we suggest that SBU-III may be enzymatically inactive due to a mutation and two deletions in important residues of the active site (Fig. 2), which would probably impair the ureolytic activity. The ureases catalytic site consists of a bi-nickel center and the mutated

Fig. 2. Alignment of urease amino acid sequences of leguminous plants. The alignment was performed using MUSCLE software implemented in MEGA5 software. Residues from the ureolytic active site are shaded in black. Canavalia ensiformes (CenUREI, CenUREIIB), Glycine max (Glyma11g248700, Glyma05g146000, Glyma08g103000), Phaseolus vulgaris (Phv001G260000), Medicago truncatula (MtrMedtr3g085640).

residue is one of the six ligands of Ni atoms, binding to Ni-2 (Balasubramanian and Ponnuraj, 2010). This amino acid is highly conserved in ureases from plants as well as in microorganisms (Follmer, 2008; Balasubramanian and Ponnuraj, 2010). A mutation (H134A) affecting one of the six conserved residues was produced in *Klebsiella aerogenes* urease, rendering the protein able to bind only one (Ni-1) of the two nickel ions. This mutant urease was enzymatically incompetent, demonstrating that Ni-2 is required to produce an active urease (Park and Hausinger, 1993). Additionally, α-helical segments are absent in the α domain of SBU-III. This domain comprises the active site and is traditionally described as a distorted TIM-barrel (Jabri et al. 1995). These barrels, also known as $(\alpha\beta)_8$-barrels, are formed by alternating 8 α-helixes and 8 β-strands and the absence of constitutive helices may further hinder the SBU-III ureolytic activity. *Eu*5 has also a premature stop codon and misses the last 11 amino acid residues of the other soybean ureases. Together these data highly suggest that SBU-III might not be involved in urea degradation.

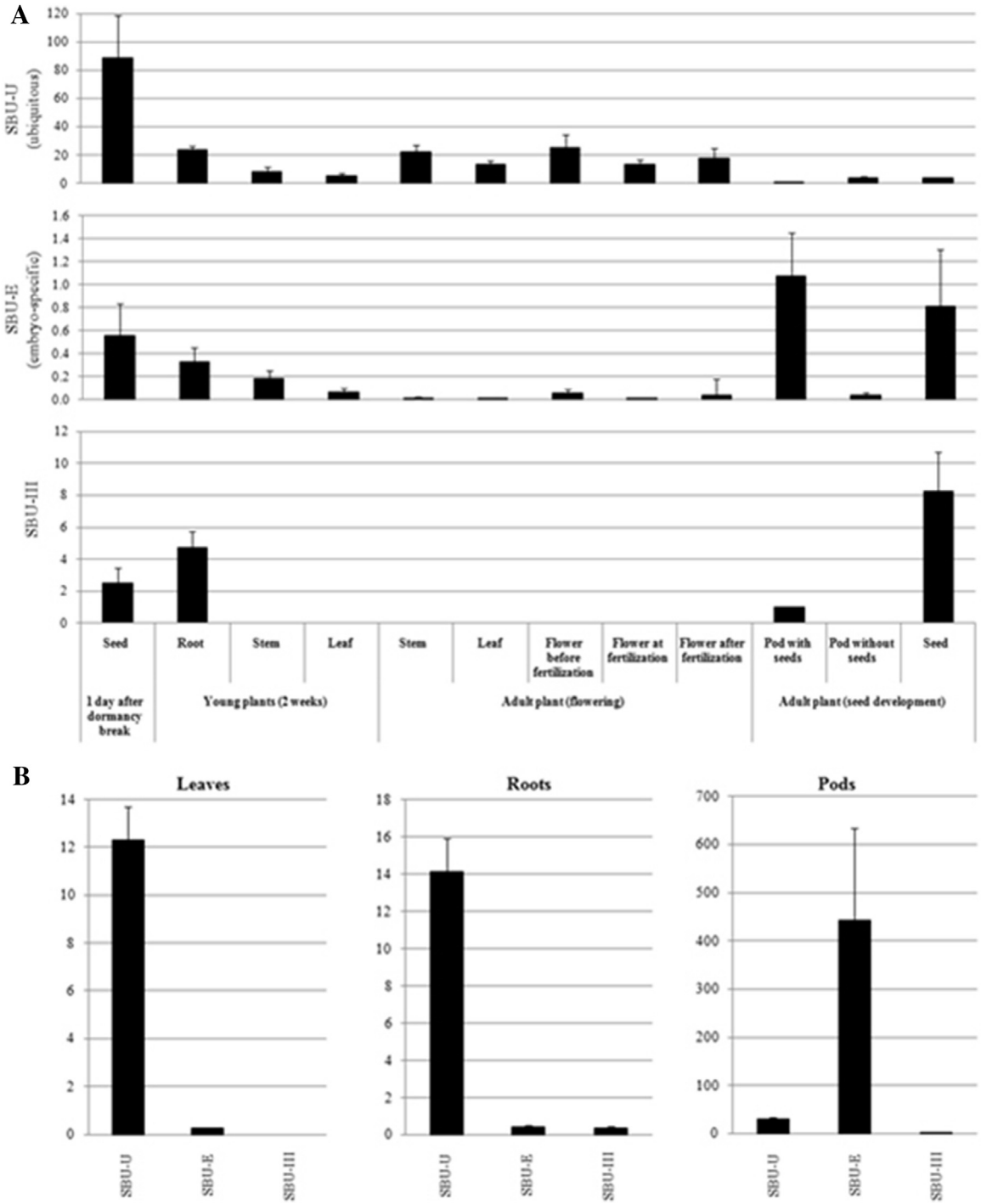

Fig. 4. Relative expression levels (RT-qPCR) of the three urease-encoding genes in different soybean organs and developmental stages. A. Expression pattern of each urease-encoding gene was determined relative to developing pods with seeds. B. Comparison of expression levels among the urease-encoding genes in three different organs: leaves and roots of young plants and developing pods with seeds. Values are means of four biological replicates and four technical replicates.

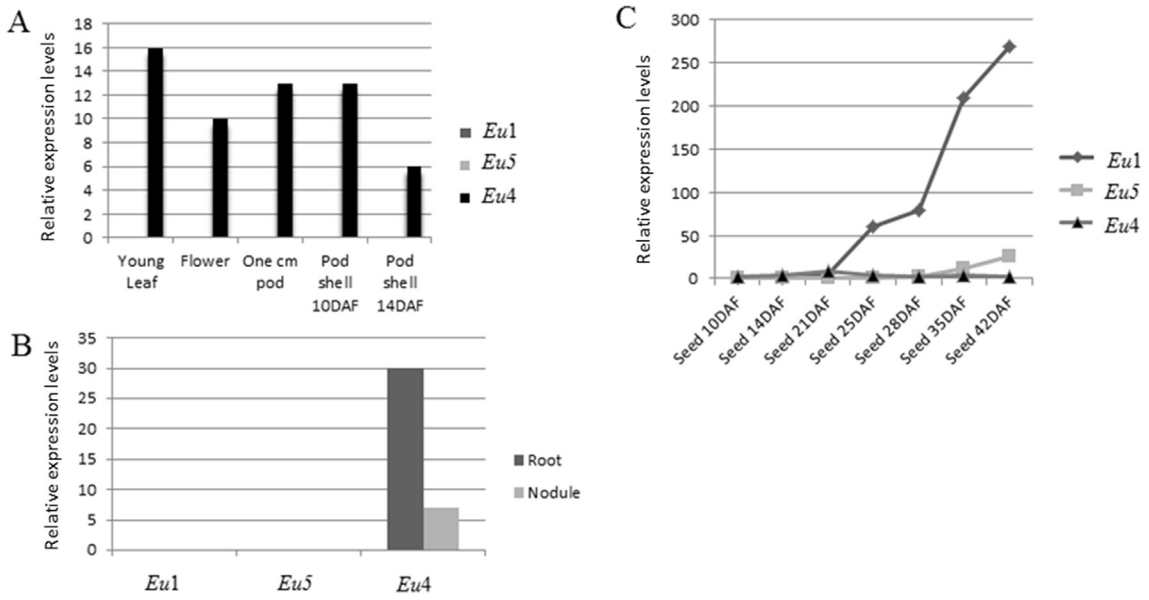

Fig. 5. Relative expression levels based on Z-score analysis. Expression patterns of specific genes of interest in A. Aerial tissues (young leaf, flower, 1 cm pod, pod shell 10-DAF and pod shell 14-DAF). B. Underground tissues (root and nodule). C. Seed development (seed 10-DAF, seed 14-DAF, seed 21-DAF, seed 25-DAF, seed 28-DAF, seed 35-DAF and seed 42-DAF).

On the other hand, it is known that bacterial and plant ureases display several biological roles that are independent of their ureolytic activity. Plant ureases have been related to antifungal and insecticidal properties (Follmer et al. 2004; Becker-Ritt et al. 2007), as well to the soybean–*Bradyrhizobium japonicum* [Kirchner] Jordan symbiosis (Medeiros-Silva et al. 2014). Witte (2011) and Real-Guerra et al. (2013) proposed that SBU-III could also be involved in other physiological roles in the plant. SBU-III was found to resemble more closely the embryo-specific urease, which displays insecticidal and antifungal effects comparable to those of the jackbean urease JBU, in both case proved to be independent of their ureolytic activities (Follmer et al., 2004; Becker-Ritt et al., 2007). At least part of the entomotoxic and fungitoxic properties reside within the region comprising amino acid 222 to 314 of the JBU molecule (the so called entomotoxic region), represented by the recombinant peptide Jaburetox (Mulinari et al., 2007). Structure-versus-activity studies performed on Jaburetox have shown that although the 41 residues of its N-terminal half (of which 8 are equivalent residues missing in SBU-III) are critical for the insecticidal effect, its C-terminal half (which is present in SBU-III) has membrane-disruptive properties that contributes to the overall entomotoxicity (Martinelli et al., 2014). Jaburetox is also deterrent to fungi, but it probably does not represent the main fungitoxic domain of JBU or of the embryo-specific soybean urease, since these proteins display at least 20 times more potent antifungal activity not related to urea hydrolysis. Thus, putative entomotoxic and fungitoxic effects could be expected properties of an enzymatically inactive SBU-III protein.

In contrast to soybean, most sequenced plant genomes possess a single urease-encoding gene. Our phylogenetic analysis showed that the genome of ancient organisms presented a single urease-encoding gene. It has been postulated that the presence of several urease paralogous genes as is the case of soybean appears to be the exception and not the rule (Witte, 2011). Our results indicate that urease-encoding gene duplication have occurred independently during the evolution of different eudicot species, with exceptions of *G. max* and *C. ensiformis* that may have the same gene duplication origin. Soybean ancestral genome was duplicated ca. 13and 59 million years ago (Schmutz et al. 2010). Maintenance of urease-encoding genes as well as urease accessory genes, after genome duplications, rearrangements, chromosome loss, etc. indicates selective pressure to maintain ureolytic activity (Polacco et al. 2013). Additionally, genome duplications will also allow the divergent roles of individual family members.

In conclusion, in the present work we characterized a new soybean urease-encoding gene and its expression pattern and putative product were compared to the already known urease isoforms. This is the first report showing *Eu*5 expression at the mRNA level and its phylogenetic relations in Viridiplantae. SBU-III is probably not involved in nitrogen availability in soybean because of accumulated mutations. However mutations do not exclude the possibility of protein endowed of ureolysis-independent defense-related biological properties which remain to be determined.

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References

Abascal, F., Zardoya, R., Posada, D., 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21, 2104–2105.
Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250.
Balasubramanian, A., Ponnuraj, K., 2010. Crystal structure of the first plant urease from jack bean: 83 years of journey from its first crystal to molecular structure. J. Mol. Biol. 400, 274–283.
Becker-Ritt, A.B., Martinelli, A.H., Mitidieri, S., Feder, V., Wassermann, G.E., Santi, L., Vainstein, M.H., Oliveira, J.T., Fiuza, L.M., Pasquali, G., Carlini, C.R., 2007. Antifungal activity of plant and bacterial ureases. Toxicon 50, 971–983.
Carlini, C.R., Guimarães, J.A., 1981. Isolation and characterization of a toxic protein from *Canavalia ensiformis* (jackbean) seeds, distinct from concanavalin A. Toxicon 19, 667–676.
Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214–222.
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Follmer, C., Barcellos, G.B., Zingali, R.B., Machado, O.L., Alves, E.W., Barja-Fidalgo, C., Guimaraes, J.A., Carlini, C.R., 2001. Canatoxin, a toxic protein from jack beans (*Canavalia ensiformis*), is a variant form of urease (EC 3.5.1.5): biological effects of urease independent of its ureolytic activity. Biochem. J. 360, 217–224.

Follmer, C., Real-Guerra, R., Wasserman, G.E., Olivera-Severo, D., Carlini, C.R., 2004. Jackbean, soybean and *Bacillus pasteurii* ureases: biological effects unrelated to ureolytic activity. Eur. J. Biochem. 271, 1357–1363.

Follmer, C., 2008. Insights into the role and structure of plant ureases. Phytochemistry 69, 18–28.

Goldraij, A., Beamer, L.J., Polacco, J.C., 2003. Interallelic complementation at the ubiquitous urease coding locus of soybean. Plant Physiol. 132, 1801–1810.

Holland, M.A., Polacco, J.C., 1992. Urease-null and hydrogenase-null phenotypes of a phylloplane bacterium reveal altered nickel metabolism in 2 soybean mutants. Plant Physiol. 98, 942–948.

Jabri, E., Carr, M.B., Hausinger, R.P., Karplus, P.A., 1995. The crystal structure of urease from Klebsiella aerogenes. Science 268, 998–1004.

Laskowski, R.A., McArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291.

Lauxen, M.S., Kaltchuk-Santos, E., Hu, C., Callegari-Jacques, S.M., Bodanese-Zanettini, M.H., 2003. Association between floral bud size and developmental stage in soybean microspores. Braz. Arch. Biol. Technol. 46, 515–520.

Libault, M., Thibivilliers, S., Bilgin, D.D., Radwan, O., Benitez, M., Clough, S.J., Stacey, G., 2008. Identification of four soybean reference genes for gene expression normalization. Plant Genome 1, 44–54.

Ligabue-Braun, R., Andreis, F.C., Verli, H., Carlini, C.R., 2013. 3-to-1: unraveling structural transitions in ureases. Naturwissenschaften 100, 459–467.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.

Luthy, R., Bowie, J.U., Eisenberg, D., 1992. Assessment of protein models with three-dimensional profiles. Nature 356, 83–85.

Martinelli, A.H.S., Kappaun, K., Ligabue-Braun, R., Defferrari, M.S., Piovesan, A.R., Stanisçuaski, F., Demartini, D.R., Verli, H., Dal Belo, C.A., Almeida, C.G., Follmer, C., Carlini Celia, R., Pasquali, G., 2014. Structure-function studies on Jaburetox, a recombinant insecticidal and antifungal peptide derived from jack bean (Canavalia ensiformis) urease. Biochim. Biophys. Acta 1840, 935–944.

Medeiros-Silva, M., Franck, W.L., Borba, M.P., Pizzato, S.B., Strodtman, K.N., Emerich, D.W., Stacey, G., Polacco, J.C., Carlini, C.R., 2014. Soybean Ureases, but not that of *Bradyrhizobium japonicum*, are involved in the process of soybean root nodulation. J. Agric. Food Chem. 62, 3517–3524.

Mulinari, F., Stanisçuaski, F., Bertholdo-Vargas, L.R., Postal, M., Oliveira Neto, O.B., Ridgen, D.J., Grossi-de-Sá, M.F., Carlini, C.R., 2007. Jaburetox-2Ec: an insecticidal peptide derived from an isoform of urease from the Canavalia ensiformis plant. Peptides 28, 2042–2050.

Mulinari, F., Becker-Ritt, A.B., Demartini, D.R., Ligabue-Braun, R., Stanisçuaski, F., Verli, H., Fragoso, R.R., Schroeder, E.K., Carlini, C.R., Grossi-de-Sá, M.F., 2011. Characterization of JBURE-IIb isoform of Canavalia ensiformis (L.) DC urease. Biochim. Biophys. Acta - Proteins Proteom. 1814, 1758–1768.

Park, I.S., Hausinger, R.P., 1993. Site-directed mutagenesis of *Klebsiella aerogenes* urease: identification of histidine residues that appear to function in nickel ligation, substrate binding, and catalysis. Protein Sci. 2, 1034–1041.

Polacco, J.C., Holland, M.A., 1993. Roles of urease in plant cells. Int. Rev. Cytol. 145, 65–103.

Polacco, J.C., Mazzafera, P., Tezotto, T., 2013. Nickel and urease in plants: still many knowledge gaps. Plant Sci. 199–200, 79–90.

Real-Guerra, R., Stanisçuaski, F., Carlini, C.R., 2013. Soybean urease: over a hundred years of knowledge, a comprehensive survey of international soybean research. InTech 317–339.

Riddles, P.W., Whan, V., Blakeley, R.L., Zerner, B., 1991. Cloning and sequencing of a jackbean urease-encoding cDNA. Gene 108, 265–267.

Sanchez, R., Pieper, U., Mirkovic, N., Bakker, P.I.W.D., Wittenstein, E., Sali, A., 2000. ModBase, a database of annotated comparative protein structure models. Nucleic Acids Res. 28, 250–253.

Sato, S., Isobe, S., Tabata, S., 2010. Structural analyses of the genomes in legumes. Curr. Opin. Plant Biol. 13, 146–152.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.C., Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., Jackson, S.A., 2010. Genome sequence of the palaeopolyploid soybean. Nature 463, 178–184.

Severin, A.J., Woody, J.L., Bolon, Y., Joseph, B., Diers, B.W., Farmer, A.D., Muehlbauer, G.J., Nelson, R.T., Grant, D., Specht, J.E., Graham, M.A., Cannon, S.B., May, G.D., Vance, C.P., Shoemaker, R.C., 2010. RNA-Seq Atlas of Glycine max: a guide to the soybean transcriptome. BMC Plant Biol. 10, 160.

Stebbins, N.E., Polacco, J.C., 1995. Urease is not essential for ureide degradation in soybean. Plant Physiol. 109, 169–175.

Sumner, J.B., 1926. The isolation and crystallization of the enzyme urease. J. Biol. Chem. 69, 435–444.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Torisky, R.S., Griffin, J.D., Yenofsky, R.L., Polacco, J.C., 1994. A single gene (*Eu4*) encodes the tissue-ubiquitous urease of soybean. Mol. General Genet. 242, 404–414.

Torisky, R.S., Polacco, J.C., 1990. Soybean roots retain the seed urease isozyme synthesized during embryo development. Plant Physiol. 94, 681–689.

Vandesompele, J., Preter, K.D., Pattyn, F., Poppe, B., Roy, N.V., Paeppe, A.D., Speleman, F., 2001. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1–12.

Witte, C.P., Tiller, S.A., Taylor, M.A., Davies, H.V., 2002. Leaf urea metabolism in potato. Urease activity profile and patterns of recovery and distribution of (15)N after foliar urea application in wild-type and urease-antisense transgenics. Plant Physiol. 128, 1129–1136.

Witte, C.P., 2011. Urea metabolism in plants. Plant Sci. 180, 431–438.