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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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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).