

Characterization and Expression of a Novel Member (JBURE-II) of the Urease Gene Family from Jackbean [*Canavalia ensiformis* (L.) DC]

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Canavalia ensiformis (jackbean) seeds contain the proteins urease and canatoxin, a variant form of the jackbean urease. Here we have cloned a cDNA encoding another isoform of urease, called JBURE-II. This cDNA was obtained by RT-PCR using as template total RNA extracted from *C. ensiformis* tissues. Nucleotide sequence analysis showed that JBURE-II clones share 86% similarity with known jackbean urease. The presence in *C. ensiformis* of a family of urease-related genes with at least three members was demonstrated by Southern blot analysis. In order to understand the pattern of expression of the JBURE-II gene, we collected tissue samples from different stages of flower and embryo development. The results of RT-PCR show that JBURE-II is expressed from flower buds throughout seed maturation. Semi-quantitative RT-PCR indicates that expression of urease and JBURE-II genes is induced in seedlings and in leaves treated with abscisic acid, a phytohormone involved in seed maturation and wound response. This work constitutes the first report on the presence of a family of urease genes in jackbean, and provides characterization of a cDNA encoding a new member of this gene family.

Keywords: Abscisic acid — *Canavalia ensiformis* — Gene — Jackbean — Seed maturation — Urease (EC 3.5.1.5).

Abbreviations: ABA, abscisic acid; CNTX, canatoxin; DPA, days-post-anthesis; MS, Murashige and Skoog medium.

The nucleotide sequence reported in this paper has been submitted to the GenBank under accession no. AF468788.

Introduction

The seeds of many members of the Leguminosae are rich sources of urease, lectins and toxic proteins. Jackbean (*Canavalia ensiformis*) seeds contain urease (EC 3.5.1.5), the first protein ever crystallized (Sumner 1926), and concanavalin A, a glucose-mannose-specific lectin (Sumner and Howell 1936).

Takashima et al. (1988) determined the primary structure of the 840-amino acid subunit of the jackbean urease and Riddles et al. (1991) reported the nucleotide sequence of its cDNA. In 1981, Carlini and Guimarães isolated canatoxin (CNTX), a potent neurotoxic protein (a dimer of 95 kDa subunits) that is lethal to rats and mice when injected intraperitoneally (LD₅₀ 2.0 mg per kg body weight) (Carlini and Guimarães 1981). CNTX also displays insecticidal activity when fed to some insects, being cleaved by their digestive cathepsins to give entomotoxic peptide(s) (Carlini et al. 1997, Ferreira-Da Silva et al. 2000, Carlini et al. 2000).

The primary role of ureases is to allow the organisms to use external or internally generated urea as a nitrogen source (Mobley and Hausinger 1989, Mobley et al. 1995). In leguminous plants, the widespread presence of urease and CNTX-related proteins (Carlini et al. 1988) as well as their accumulation pattern during seed maturation (Sehgal and Naylor 1966, Polacco and Sparks 1982, Barcellos et al. 1993) can be taken to indicate an important physiological role for these proteins, possibly related to plant defense (Polacco and Holland 1993).

The maturation phase of seed formation includes the accumulation of storage products, the suppression of precocious germination, the acquisition of desiccation tolerance, water loss, and often, the induction of dormancy (Harada 1997). The endogenous content of abscisic acid (ABA) increases during the last two-thirds of seed development, returning to low levels in the dry seed (Rock and Quatrano 1995). ABA is thus thought to regulate several essential processes in the developmental stages that follow the formation of the embryo, such as induction of seed dormancy, tolerance to desiccation, and accumulation of reserves in the seed (Leung and Giraudat 1998).

Recently, CNTX was shown to be a variant form of urease with lower ureolytic activity and distinct physicochemical and biological properties (Follmer et al. 2001). Taking these observations into consideration, in this study we have cloned a new cDNA by RT-PCR based on the similarity of these proteins. This cDNA was used to study the pattern of mRNA expression and the effect of ABA during development of

⁵ In memoriam.

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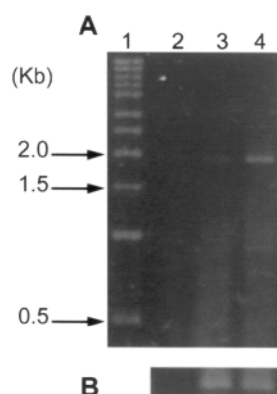


Fig. 1 RT-PCR amplification of RNA isolated from 8-day-old seedlings treated with water or 100 μ M ABA. (A) Amplification with URE-3 and URE-4 primers: lane 1, DNA ladder; lane 2, negative control (without cDNA); lane 3, seedlings treated with water; lane 4, seedlings treated with ABA. (B) Amplification with actin primers. Sizes (in kb) of markers are indicated by arrows at left.

C. ensiformis seeds and seedlings. Our data also demonstrate for the first time the presence of a family of urease-related genes in jackbean.

Results

Accumulation of urease mRNA in seedlings and genomic hybridization

The accumulation of putative urease transcripts was analyzed by RT-PCR using primers based on the sequence of urease cDNA (Riddles et al. 1991) and total RNA isolated from 8-day-old jackbean seedlings treated with 100 μ M ABA. Primers (URE-3 and URE-4; see Materials and Methods) were designed according to conserved regions of the deduced amino-acid sequence of urease and partial internal amino-acid sequences of the urease isoform, CNTX (Follmer et al. 2001). As illustrated in Fig. 1, DNA fragments of approximately 900 and 1,800 bp were amplified from RNA isolated from seedlings treated with ABA. Accumulation of actin transcripts was analyzed in parallel as a positive control for RNA quality.

Genomic hybridization analysis was carried out to confirm the RT-PCR result, using the 1,800 bp product as a probe (Fig. 2). DNA digested with *Hind*III and *Pst*I showed three bands with medium to high intensities, suggesting that the urease gene family in *C. ensiformis* has at least three members. Genomic DNA digested with *Eco*RI produced four DNA fragments identified by the probe.

Cloning of JBURE-II by RT-PCR

Following amplification, the two RT-PCR products derived from ABA-treated seedlings were purified, ligated into pGEM-T Easy Vector (Promega) and sequenced. Nucleotide sequence analysis indicated that the 900 bp product corresponded to the previously known urease gene (accession no.

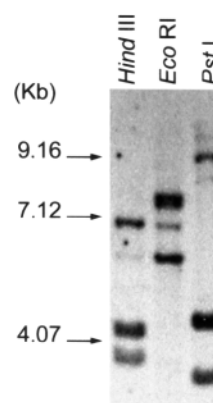


Fig. 2 Southern blot analysis of the urease gene family from *C. ensiformis*. Total DNA isolated from *C. ensiformis* (16 μ g) was digested to completion with each of the following restriction enzymes: *Hind*III, *Eco*RI or *Pst*I. The fragments were separated by gel electrophoresis followed by transfer to Hybond-N+ (Amersham Pharmacia Biotech). The blot was probed with the partial cDNA of 1,800 bp (JBURE-II). Lane 1, *Hind*III digests; lane 2, *Eco*RI digests; lane 3, *Pst*I digests. Positions and sizes (in kb) of markers are indicated on the left.

M65260; Riddles et al. 1991). However, the 1,800 bp product exhibited 86% similarity with the urease cDNA, and was designated JBURE-II. The sequencing data indicated that primer URE-3 annealed in to another internal site of the urease mRNA, resulting in the 900 bp fragment and not the expected ~1,800 bp fragment.

Based on the 1,800 bp fragment (JBURE-II) we designed primers (indicated in Fig. 3A) for 5' RACE (URE-9 and URE-14) and 3' RACE (URE-7). The complete cDNA sequence of JBURE-II was obtained by overlapping 3' and 5' RACE products. These products were purified, ligated into pGEM-T Easy Vector and sequenced. The complete cDNA sequence (2,618 bp) is shown in Fig. 3A. The putative amino-acid sequence encoded by JBURE-II was deduced by comparison with the primary sequence of urease (Takashima et al. 1988, Riddles et al. 1991). The putative protein encoded by JBURE-II contains 725 amino acids and has a molecular mass of 78 kDa and a predicted isoelectric point at pH 5.36. Fig. 3B illustrates the similarity of 77% observed between the amino-acid sequences of this protein and urease.

Accumulation of the JBURE-II Transcripts in Seeds and Leaves Treated with ABA

The presence of JBURE-II transcripts during seed development was analyzed by RT-PCR on total RNA isolated from flower buds, flowers and seeds collected at different days-post-anthesis (DPA). JBURE-II transcripts with the expected size of 1,800 bp were detected even in the flower buds, and were present in flowers and seeds collected at 7 DPA (Fig. 4A) as well as in seeds at 70 DPA (data not shown). Accumulation of actin transcripts was used as an RNA control (Fig. 4B). Both JBURE-II and urease transcripts were detected in all tissues

AATGATGATTTGGAACTAGAAAGAGCTGAATGAACCATTTTAAAGAGGACAGGCTGCTTCCAAGAGTCCACATCTGTGAACATTTATCAGGTTGAGGCTACCTTACCTAATGGGACA 120
 MNHFNRNRQVLPVPHLLNIQVEATITGCTTK
 AGTTAGTGACAGTTTCATGATCCGATTCGCAATGAGATGGAGATCTAGAAGAAGCATATATGGTTCTTTCTCCAGTTCTCTCGCTTGACAAGTTTGTCTGAGAGTAAAGAGGCATA 240
 LVTVHDPIDANENGNDLEALYGSFLLPVPSLDKFAESKEEHK
 AAATTCCTGAGAAATAATATGTGCAGATGGAAGACTAATCTTAACCTTGAAGGAAAGCAGTATTTCTCAAAGTTGTCAACCATGGAGCAGGCGCAATCCAGGTAGGGACCATATAT 360
 IPGEIICADGRLTLNPNRKAVFLKVVNVNHGDRPIQVGSHEYH
 CATTTTATITGAAGTAAATCCATACCTGACTTTTGTATCGAAGAAAGCTTATGGCATGCGCTGAATATAGCTGCAGGGAGTTCTGTTCCGTTTGAGCTCGGGGATCAATAAATCTTTAAT 480
 FIEVNPYLTFTFDRRKAYGMRLLNIAAGDSVRFEPFGDHKTVNL
 CTTGTAAAGCATGGAGGTAACAAATCATTAGAGGAGTAAATGCCATTGCTGATGGTCCAGTAAATGAAGCCAAATGTAAAGCAGCATGGAGATTTGTGCAGAAAGGAAATTTGGACAT 600
 VSGISGKNIIRGGNAIADGPVNEANCKAAMEIVCRCREFGHK
 AAGGAAGAAGGAAGCAAGTGGGTTTCAACAGGAGACCTGATTGCTCTTCAACAAAGCATTCCTCGTGAAGAATATGCTAAACAGTATGGTCCGACTATTTGGTGACAAATC 720
 EEEASESEGVTTTGDDPDCTTKAIPREYANKYPTIGDKIR
 CGTCTTGGTGACACTGATTGATTGCTGAAATGAAAAGGATTTGCCCTTATGGTGATGAAAGTTTITGGAGGTGGAAAGTTATAAGAGATGGAATGGGTCATCAAGTGGGCAT 840
 LGDTDLDLIAEIEKDFALYGDSESVFGGGKVIDRDMGQSSGHP
 CCACCTGCTATGCTCTTGGATATCTGTATCAACAGTGCAGTGATTAATGACTATATCGAATCATCAAGCAGATATAGGTATCAAGATGGCTATTGCTCTTATGGAAAGACAGGA 960
 PAMSLDTVITSAVIDYTGIIKAGDGLIASIGKAGN
 AATCCAGACATCATGAATGGTGATTTCCTAATATGATCATTTGGGTAAATCATGAATATATGCTGGGAGAGGGTTGATCGTAACCTGCTGGGGGTATAGACTGTCACTGTTATATATA 1080
 PDIMNGVFPNMIIGVNVTEVICGEGGLIVTAGGIDCHVHYIC
 TGCCTCATCATCTTGATGAGGCGCATATCAAGCGGCATCACAACTAGTATAGGAGGTGAACACAGCAACCGGATGGAAGTCGTGCCACCATGTACACCGGCACCAACTCAGATGAAG 1200
 PQSLDEAIESSGITTTVVGGGTTGTDTGSRATTTCTPTAPTQMKL
 CTAATGCTGCAATCAACAGATGACATCCCGTAAACTTTGGTTTCACTGGCAAGGAGTGGTTCACATCTGTGAATCAATGAATAATCAAGGCTGGAGCATGGGACTGAAGCTG 1320
 MLQSTTDIPLNFVFGTCTGKSGSGSHPEDELHEIIKAGAMGLKLH
 CATGAGGACTGGGATGCAGCGCAGCTGCAATAGATAATTGTTGGCTGTGCTGAACACATGATATCCAGGTAAATATACACACTGACACCGCTAAATGAATCTGGATTCGTGGAAAC 1440
 EDWGGCTTGAADIANCLVAEQLHDIIQVNIHTDTVNESGFVEHT
 ACTATGCTGCATTTAATGGAGAACTATTCATCTTACCACTGAGGCTGAAGGTGGTGATGCTCCAGATATCATCAAGTATGTAGTATGAATAATGCTTACCTTCATCAACC 1560
 IAAFNGRTHIYHSEAGGAGGHAPDIIKVCSCMNVLPSSTN
 AACCCACACGCGCTTTAACTATTAATACCGTAGATGAGCATCTTGACATGCTGATGGTCTGCCATAGTTGAATAGGGAATTCAGAAAGATTAGCTTTTGCAAGTTCCAGGGTABA 1680
 TTRPLTSTNTVDHLDMLMVCHKLNRREIPEDLAFASRRVRE
 GBAACAACATTCGTGCTGAAGATTTTGCACCATTTGGGGGATATGACATATATCTTGATGCACAGGCTGTGGTGCAATTTGAGAGGATGATTAAGCTGCATTTGGCAACATGCT 1800
 CTIAAEDILHHIGGISIISSDQAVRGIVGEVISTCTWQTAD
 GATAAGATGAAGCGAAGAGGAGCACTTCAGCGCTGATGGTTGAGCAATGATAACTTTGCAATCAACGATATATTTGCAAAATACACTATTAATCCAGGCATAGTAAATGGCATCTCA 1920
 KMKAAERGPLQPDGSDNDNFRIKRYIAKYTINPAIVNGISQ
 CAATATGTTGGTTTCAGTTGAGGTGGGAAAGTTAGCTGCTTGTGATTTTGAAGGCCATTTTTCGGGGCAAAACAGACATTTGTGATCAAAAGGTGGTTCAATTGCAATGGGCTGACAT 2040
 YVSGSEVVGKGLADLVITWKPSFPGAKPDDIVIKGGSIAWADMG
 GGTGACCCAAATGGCAGCATACCTACACCTGAACCGGTGCTGATGAGGCGTATGTATGGAACACTTGGCAAGGCTGGTATGCTTTTATCCATAGCTTTTGTGAGCAAGCGGCTTTAGAC 2160
 DPNPGSITPTPEAVGLMRFMYGTLGKAGSALSIAFVSKAALDL
 CTTGGAGTGAAGTTTATATGGACTGAACAAAGGCTGAAACCCGCTGAGCAATGTGAGAAAGCTCACCAAACTAGACTTGAACATCAATCACTCTCTTCCAGAAATCACTGTGTGCCAG 2280
 GVKVLYGLNKGNF
 AAACATTCACAGTTACAGTATGATGGCAGGCTCTCTCAAGCAGGAGCAGTCACCACTTTGCCAATTTCTCAAAATTACTTTCATCTTTTAAGTACTCCTACAAATTAAGGTTAATTATTGTT 2400
 AAACATTCACCTACAATGACTGATTTGATGGAAGTTGTTTAAATACCTGATTAAAGTCAAAGTGTGTTTCTGATGTTTGGAGGCTTTGTTTATCCTATAATGATGATTGATTAACTAT 2520
 TGTCTCTAATTTGTATGAGTTTGTATTATGATGCAAGTGTATTTTTTATTAATATAATTTGATGTGCTACAAAAAATAAAAAA 2640

analyzed and had the same band pattern (900 bp and 1,800 bp) as that observed in the ABA-treated seedlings.

23: with ABA, the amounts were at least 1.5-fold greater with JBURE primers and 17-fold greater with URE primers. These results indicate that urease and JBURE-II transcript levels are increased by ABA induction.

Discussion

We reported here a partial cDNA sequence from JBURE-II, an urease-like gene present in *C. ensiformis* (jackbean). To our knowledge, this is the first evidence for a family of urease genes in the jackbean. We have also analyzed by RT-PCR the expression pattern for the JBURE-II and urease cDNA clones, after ABA treatment and during seed development.

In our experimental approach, we used information based on the cDNA of urease (Riddles et al. 1991) and the partial internal amino-acid sequences of the urease isoform, CNTX (Follmer et al. 2001) to design primers. CNTX and urease accumulate with a similar time course in maturing seeds

JBURE-II	-----MHHFNRRQVLPAVPHLLNIQVEATLPNGTKLVTVHDFIARENGDLEALYGSFLFVPS	59
UREASE	MKLSPREVEKLGLHNAGYLAQKRLARGVRLMYTEAVALIASQIMEYARDGEKTVQAIGLCGQHLLRRQVLPAVPHLLNAVQVEATEPPDGTKLVTVHDFISRENGELQALPGSLLPVPS	120
JBURE-II	LDKFAESKEEHKIPGEILCADGRLLPMPKAVFLKVVNHGDRPIQVGSHYHFEVNPYLTFDRRKAYGMRLNTAAAGDSVRFEPGDHKTVNLVSIGGNKIIRGGNAIADGPNVEANKKAA	119
UREASE	LDKFAETKEDNRIPGEILCEDCLTNIGKAVILKVTSGKDRPIQVGSHYHFEVNPYLTFDRRKAYGMRLNTAAAGTAVRFEPGDCKSVTLVSTEGVKVIRGGNAIADGPNVETLKEAA	240
JBURE-II	MEIVCRREFGHKEEHEEASGEVTTGDPDPCFTKALPREEYANKYGPITGDKIRLGGTDLIAEIEKDFALYGDSESVGGGKIVIRDMGQSGGHPFAMS LDTVITSAVI IDYTGIIKADIGIK	299
UREASE	MHAVRSKGFGEHEESKDSAGFTKEDPNCPFFTFIHRKEYANKYGPITGDKIRLGGTDLNLLAEIEKDVALYGDSEVGGGKIVIRDMGQSGGHPAIS LDTVITNAVI IDYTGIIKADIGIK	360
JBURE-II	DGLIASIGKAGNPDIMNMGVFFNMIIIGVNTVEIVCGEGLVITAGGIDC-----IINICPQSLDEAISSGITT VGGGTGPTDGRATTCTFPAPTMKMLQSTDDIPLNFGFTKGSGSGHPDELHE	419
UREASE	DGLIASIGKAGNPMNMGVFSNMIIIGANTVEIAGEGLVITAGAIDC-----IINICPQLVYVAISSGITT VGGGTGPAAGTRATTTCPSPQMRMLQSTDYPLNFGFTKGSGSSKDELHE	480
JBURE-II	IIKAGAMGLLHEDMGCTPAAIDNCLAVAEQHDQVNI-----IDTVNSESGFVERTIAAFNGRTIHTVISEGAGCGHAPDI IKVCSMKQNLPSSTNTRPLTSNTIDEHLMLMVCHKLNRFP	539
UREASE	IIKAGAMGLLHEDMGSTPAAIDNCLTIAEHHDQVNI-----IDTVLNEAGFVESHIAAFKGRRTIHTVISEGAGCGHAPDI IKVCGIKNVLPSSTNTRPLTSNTIDEHLMLMVCHHLDRFP	600
JBURE-II	EDLAFASSRVRQETIAAEDILHHIGGISIISS-----IQAVGRIGVEISRTWQTADMKMAERGLPQDGSNDNFRIRRYIAKYTINPAIVNGISQYVGSVEVGKLADLVINWKPSEFFGAKPDIV	659
UREASE	EDLAFASHRIRKKTIAAEDVLNDIGAISIISS-----IQAMGRVGEVISRWTQADPMKQAQTGLPKDCSSNDNFRIRRYIAKYTINPAIANGFSQYVGSVEVGKLADLVINWKPSEFFGKTFEMV	720
JBURE-II	IKGGSIAWADMGDPNGSIPTPEVIMRMFYGTGLKAGSALSIAFVSKAALDLGVVLYGLNKGWNP-----	725
UREASE	IKGGHVAWADIGDNGASIPTEPEVIMRMFYGTGLKAGSALSIAFVSKAALDQVNVVLYGLNKRVEAVSNVRKLTLMKLNDALPEITVDFESYTVKADGKLLCVSEATTVPLSRNYFLF	840

(Sehgal and Naylor 1966, Barcellos et al. 1993) and both proteins are localized in the cytosol of storage parenchyma cells in the mature seed (unpublished results; Faye et al. 1986), prompting us to test whether ABA, a plant hormone involved in this process, was able to induce their expression. As expected, RT-PCR carried out on preparations from jackbean seedlings treated with ABA and amplified with these primers successfully resulted in two products (Fig. 1) that were cloned and sequenced. The sequence data revealed the smaller product to be a fragment of the urease cDNA (M65260). The 1,800 bp product was a partial cDNA of the JBURE-II gene that exhibited 86% similarity with that of urease. This sequence was submitted to GenBank under accession no. AF468788. The high degree of similarity between these cDNAs allows them to be classified as members of a urease gene family.

Semi-quantitative RT-PCR demonstrated an increase in relative amounts of transcripts with ABA treatment, with JBURE primers and URE primers. Although comparisons of mRNA levels by this technique could be misleading, as slight differences in band intensity against cycle number may have considerable effect on the amount of transcript estimated (Raeymaekers 1999), such differences were not seen for RT-PCR products of actin primers.

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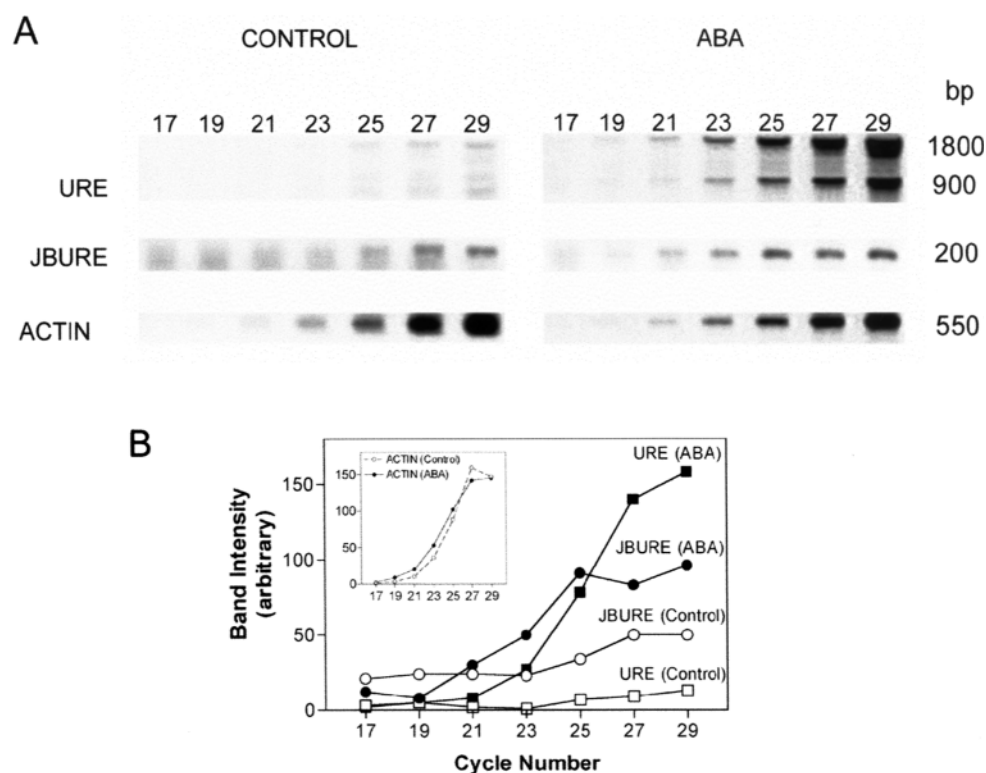


Fig. 5 Semi-quantitative RT-PCR analysis. (A) Agarose gel of RT-PCR products from cycles 17–29 using as the template RNA extracted from leaves treated with MS medium (CONTROL) or ABA in MS medium (ABA). URE, JBURE and ACTIN indicates the primers used in the reactions. (B) Plot of band intensities against cycle number for the bands seen in (A), as quantified by GraphPad Prism software. The inset shows the same analysis for actin transcripts.

each other by a two-step chromatographic procedure using immobilized metal affinity matrices (C. Follmer and C.R. Carlini, unpublished results). One of these isoforms is CNTX, a dimer of non-covalently linked 95-kDa polypeptide chains with an isoelectric point at pH 4.7 (Follmer et al. 2001). However, the putative protein encoded by JBURE-II does not correspond to CNTX, as it would have a shorter polypeptide chain, a more basic isoelectric point and little similarity with the available partial sequence of CNTX (Follmer et al. 2001). From the analysis of the deduced amino-acid sequence, the JBURE-II-encoded protein is expected to display ureolytic activity, since all of the residues implicated in nickel binding and catalytic properties are present (Jabri et al. 1995). Thus, JBURE-II may encode a third urease isoenzyme, in agreement with the content of urease isoforms detectable in *C. ensiformis* seeds (Follmer et al. 2001; unpublished results).

Two urease isoenzymes are known to exist in soybean plants, one of them a highly active, hexameric enzyme present only in the embryo, and the other an ubiquitous, trimeric isoenzyme of lower specific activity. No deleterious effect was seen in mutant plants that lacked the embryo-specific urease, implying that its high ureolytic activity is not relevant for its physiological role, which is not known (Polacco and Havir 1979, Polacco and Winkler 1984, Polacco and Holland 1993). The

complete primary sequences of these ureases have not yet been determined. Recently, the nucleotide sequence of a soybean urease isoenzyme was deposited in the GenBank (accession no. AJ276866).

In summary, in this report we have identified a second member of a family of urease-related genes in *C. ensiformis* and demonstrated that ABA induces the expression of some members of this gene family. The pattern of JBURE-II mRNA accumulation during seed development suggests increased levels at early stages of seed maturation. At present, we are investigating the precise number of members of the urease gene family. These data, together with the expression of JBURE-II as a transgene, will improve our understanding of the physiological role of JBURE-II and other related urease proteins in *C. ensiformis*.

Materials and Methods

Plant material

C. ensiformis plants were grown under greenhouse conditions. Flower buds, flowers and seeds were collected at different developmental stages (days post-anthesis; DPA). *C. ensiformis* seedlings were grown on vermiculite in a growth chamber under controlled conditions ($24 \pm 2^\circ\text{C}$, 40% relative humidity, fluorescent illumination supplemented with GroLux® lamp) on a 12-h light–dark cycle.

ABA treatment

ABA (Life Technologies) was dissolved in ethanol (100 mM stock solution) and subsequently diluted in Murashige and Skoog medium (MS) to 100 μ M. At day 8, seedlings were removed from vermiculite and the roots were placed in 40 ml MS (control) or in MS containing 100 μ M ABA. After 24 h in the dark, RNA was isolated. Alternatively, leaves from 15-day-old seedlings were cut at the petiole, which was then immersed in MS (control) or in MS containing 100 μ M ABA for 18 h in the dark, before RNA isolation.

Plant nucleic acid isolation

Total RNA was isolated from tissue ground in the presence of liquid nitrogen, followed by phenol/chloroform extraction and precipitation with LiCl to a final concentration of 2 M (Bugos et al. 1995). Genomic DNA was extracted from freeze-dried young leaves of *C. ensiformis* according to a modified CTAB procedure (Murray and Thompson 1980).

RT-PCR analysis

Total RNA isolated from different tissues was used as a template in RT-PCR (Murphy and Taiz 1995) with primers based on the sequence of urease cDNA (Riddles et al. 1991), URE-3 (5'-CCATTTGCTATGGCTGGATT-3') and URE-4 (5'-AGTCACAGTTCATGATCGATT-3') or with primers for actin – ACT-REV (5'-AGGAAGCTCTGAGCTCTTCTC-3') and ACT-FOR (5'-GATCTGGCATCACACCTTCTAC-3'). The reverse transcription reaction was carried out with 0.34 mM of each dNTP, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 6.8 mM DTT, 1.36 μ M URE-3 primer and 68 U of M-MLV reverse transcriptase (Life Technologies). After 1 h at 37°C, 5.0 μ l of this reaction mixture was used as template for PCR in a MiniCycler Thermal Cycler (MJ Research). Amplification was performed in a volume of 50 μ l containing the cDNA template, 200 μ M of each dNTP, 0.4 μ M of each primer, 50 mM KCl, 2 mM MgCl₂, 20 mM Tris-HCl pH 8.4 and 2.5 U of *Taq* (DNA) polymerase (Life Technologies). The PCR program was 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by incubation at 72°C for 5 min. Ten microliters of each PCR product was analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide (0.5 μ g ml⁻¹).

cDNA cloning

RT-PCR products from seedlings treated with ABA were gel-purified using a Gene-Clean kit (Bio 101) according to supplier's recommendations. These products were ligated directly into plasmid pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. *E. coli* XL-1 Blue cells were transformed with the ligation products. After mini-preparation from recombinant colonies, plasmids were sequenced on an ABI 377 automated sequence analyzer (Applied Biosystems-Perkin Elmer) using the M13 Universal Sequence Primer or M13 Reverse Sequence Primers (Amersham Pharmacia Biotech).

Full-length cDNA was obtained by 3' and 5' RACE. For the 3' end, reverse transcription reaction was performed with an oligo(dT)-adapter primer (5'-GCGGCCGCT(dT)₁₈-3'). The synthesized cDNAs were used as templates in 3' RACE. The PCR product was amplified using primer URE-7 (5'-AAGTTCAAGGGTAAGAGAAC-3') and the adapter primer. Two anti-sense primers for 5' RACE were designed. First-strand cDNAs synthesized by reverse transcription reaction with URE-9 primer (5'-ATCTCCATAGCTGCTTTTAC-3') were purified on a Glassmax column (Life Technologies); then homopolymeric dA tails were added to their 3' ends by terminal deoxynucleotidyl transferase.

The dA-tailed cDNAs were first amplified with primer URE-9 and oligo(dT)-adapter primer complementary to the dA tails. In order to obtain a higher yield of the specific cDNAs, 5 μ l of the first PCR

product was used as template for a second PCR amplification with the adjacent primer URE-14 (5'-GATTGGCCTGCTCCATGGTTGAC-3') and the oligo(dT)-adapter primer. The 3' RACE and 5' RACE products were cloned and sequenced.

Semi-quantitative RT-PCR

Reverse transcription was performed as indicated in the RT-PCR analysis. Primers used were URE (URE-3 and URE-4), ACTIN (ACT-F and ACT-R) and JBURE (JBURE-1 – 5'-AAGGTTAATTATTGTTA-AACATGCACC-3' and JBURE-2 – 5'-TCACACTTTGACTCAATATAAACA-3'). PCR samples were taken after each two cycles from cycle 17 on and separated on an agarose gel. The gel images were analyzed for peak intensity by QuantiScan Version 1.25 software. Band intensities were plotted against cycle number using the GraphPad Prism software. Regression analysis was performed for data in cycle 23 using peak intensities of actin bands as an external control.

Genomic hybridization

DNA samples (16 μ g) were digested to completion with *Hind*III, *Eco*RI or *Pst*I (Amersham Pharmacia Biotech) and separated by electrophoresis on a 0.7% agarose gel, denatured, soaked in 20 \times SSC (0.3 M sodium citrate and 3 M NaCl) and blotted onto a nylon membrane (Hybond-N⁺; Amersham Pharmacia Biotech) by capillary transfer for at least 16 h. DNA was covalently cross-linked to the membrane at high temperature. An insert of approximately 1,800 bp released from the pGEM-T (easy) (Promega) by *Eco*RI and containing the JBURE-II partial cDNA was used as a probe. Hybridization was performed at 60°C overnight according to supplier's recommendations (Gene Images, Amersham Pharmacia Biotech).

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References

- Barcellos, G.B.S., Almeida, L.M., Moreira, R.A., Cavada, B.S., Oliveira, J.T.A. and Carlini, C.R. (1993) Canatoxin-, concanavalin A- and canavalin-cross-reactive materials during maturation of *Canavalia brasiliensis* (Mart.) seeds. *Planta* 189: 397–402.
- Bugos, R.C., Chiang, V.I., Zhang, X.H., Campbell, E.R., Podila, G.K. and Campbell, W.H. (1995) RNA isolation from plant tissues recalcitrant to extraction in guanidine. *Biotechniques* 19: 734–737.
- Carlini, C.R., Barcellos, G.B.S., Baeta-Neves, A.D.V. and Guimarães, J.A. (1988) Immunoreactivity for canatoxin and concanavalin A among proteins of legume seeds. *Phytochemistry* 27(1): 25–30.
- Carlini, C.R., Ferreira-Da Silva, C.T. and Gombarovits, M.E.C. (2000) Peptídeo Entomotóxico da Canatoxina: Processo de Produção. Patent No. 0003334-0, Instituto Nacional de Propriedade Industrial, Rio de Janeiro, Brazil.
- Carlini, C.R. and Guimarães, J.A. (1981) Isolation and characterization of a toxic protein from *Canavalia ensiformis* (jackbean) seeds, distinct from concanavalin A. *Toxicon* 19: 667–676.
- Carlini, C.R., Oliveira, A.E., Azambuja, P., Xavier-Filho, J. and Wells, M.A. (1997) Biological effects of canatoxin in different insect models: evidence for

- a proteolytic activation of the toxin by insect cathepsinlike enzymes. *J. Econ. Entomol.* 90: 340–348.
- Faye, L., Greenwood, J.S. and Chrispeels, M.J. (1986) Urease in Jack-bean (*Canavalia ensiformis* (L.) DC) seeds is a cytosolic protein. *Planta* 168: 579–585.
- Ferreira-Da Silva, C.T., Gombarovits, M.E.C., Masuda, H., Oliveira, C.M. and Carlini, C.R. (2000) Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. *Arch. Insect Biochem. Physiol.* 44: 162–171.
- Follmer, C., Barcellos, G.B., Zingali, R.B., Machado, O.L., Alves, E.W., Barja-Fidalgo, C., Guimarães, J.A. and Carlini, C.R. (2001) Canatoxin, a toxic protein from jackbeans (*Canavalia ensiformis*), is a variant form of urease (EC 3.5.1.5): biological effects of urease independent of its ureolytic activity. *Biochem. J.* 15: 360: 217–24.
- Harada, J.J. (1997) Seed maturation and control of germination. In *Cellular and Molecular Biology of Plant Seed Development*. Edited by Larkins, B.A. and Vasil, I.K. pp. 545–592. Kluwer Academic Publishers, Dordrecht.
- Jabri, E., Carr, M.B., Hausinger, R.P. and Karplus, P.A. (1995) The crystal structure of urease from *Klebsiella aerogenes*. *Science* 268: 998–1004.
- Leung, J. and Giraudat, J. (1998) Absciscic acid signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49: 199–222.
- Mobley, H.L. and Hausinger, R.P. (1989) Microbial ureases: significance, regulation, and molecular characterization. *Microbiol. Rev.* 53: 85–108.
- Mobley, H.L., Island, M.D. and Hausinger, R.P. (1995) Molecular biology of microbial ureases. *Microbiol. Rev.* 59: 451–480.
- Murphy, A. and Taiz, L. (1995) Comparison of metallothionein gene expression and nonprotein thiols in ten *Arabidopsis* ecotypes. *Plant Physiol.* 109: 945–954.
- Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.* 8: 4321–4325.
- Polacco, J.C. and Havir, E.A. (1979) Comparisons of soybean urease isolated from seed and tissue culture. *J. Biol. Chem.* 254: 1707–1715.
- Polacco, J.C. and Holland, M.A. (1993) Roles of urease in plant cells. *Int. Rev. Cytol.* 145: 65–103.
- Polacco, J.C. and Sparks, R.B. Jr. (1982) Patterns of urease synthesis in developing soybeans. *Plant Physiol.* 70: 189–194.
- Polacco, J.C. and Winkler, R.G. (1984) Soybean leaf urease: a seed enzyme? *Plant Physiol.* 74: 800–803.
- Raeymaekers, L. (1999) General principles of quantitative PCR. In *Quantitative PCR Protocols*. Edited by Kochanowski, B. and Reischl, U. pp. 31–33. Humana Press, Totowa, NJ.
- Riddles, P.W., Whan, V., Blakeley, R.L. and Zerner, B. (1991) Cloning and sequencing of a jackbean urease-encoding cDNA. *Gene* 108: 265–267.
- Rock, C.D. and Quatrano, R.S. (1995) The role of hormones during seed development. In *Plant Hormones*. Edited by Davies, P.J. pp. 671–697. Kluwer Academic Publishers, Dordrecht.
- Sehgal, P.P. and Naylor, A.W. (1966) Ontogenetic study of urease in jackbeans, *Canavalia ensiformis* (L.) DC. *Bot. Gaz.* 127: 27–34.
- Sumner, J.B. (1926) The isolation and crystallization of the enzyme urease. *J. Biol. Chem.* 69: 435–441.
- Sumner, J.B. and Howell, S.F. (1936) Identification of the hemagglutinin of jackbean with Con A. *J. Bacteriol.* 32: 227–240.
- Takashima, K., Suga, T. and Mamiya, G. (1988) The structure of jackbean urease: the complete amino acid sequence, limited proteolysis and reactive cysteine residues. *Eur. J. Biochem.* 175: 151–165.

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