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A single gene (*Eu4*) encodes the tissue-ubiquitous urease of soybean

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Abstract. We sought to determine the genetic basis of expression of the ubiquitous (metabolic) urease of soybean. This isozyme is termed the metabolic urease because its loss, in *eu4/eu4* mutants, leads to accumulation of urea, whereas loss of the embryo-specific urease isozyme does not. The *eu4* lesion eliminated the expression of the ubiquitous urease in vegetative and embryonic tissues. RFLP analysis placed urease clone LC4 near, or within, the *Eu4* locus. Sequence comparison of urease proteins (ubiquitous and embryo-specific) and clones (LC4 and LS1) indicated that LC4 and LS1 encode ubiquitous and embryo-specific ureases, respectively. That LC4 is transcribed into poly(A)$^+$ RNA in all tissues was indicated by the amplification of its transcript by an LC4-specific PCR primer. (The LS1-specific primer, on the other hand, amplified poly(A)$^+$ RNA only from developing embryos expressing the embryo-specific urease.) These observations are consistent with *Eu4* being the ubiquitous urease structural gene contained in the LC4 clone. In agreement with this notion, the mutant phenotype of *eu4/eu4* callus was partially corrected by the LC4 urease gene introduced by particle bombardment.

Key words: Ubiquitous urease – Embryo-specific urease – Urease clone – Polymerase chain reaction – *Glycine max*

Introduction

Soybean produces two urease isozymes (Holland et al. 1987). The embryo-specific urease is synthesized only in the developing embryo (Polacco and Havir 1979; Torisky and Polacco 1990), while the ubiquitous urease is found in all tissues examined, namely cultured cells, leaves, embryos, roots and seed coats (Polacco and Winkler 1984; Polacco et al. 1985). Although the ubiquitous urease is found at levels 1/1000 to 1/100 that of the embryo-specific urease, it is responsible for recycling metabolically derived urea since mutants lacking this isozyme accumulate urea and have necrotic leaf tips (Stebbins et al. 1991), most probably associated with urea burn. Leaf tip necrosis was also observed upon application of foliar urea plus a urease inhibitor (Krogmeier et al. 1989). In a urease-negative phenocopy, induced by nickel deprivation (Eskew et al. 1983), 2.5% (dry weight) of the necrotic leaf tip was urea.

The simplest genetic basis for expression of the two urease isozymes is that each is encoded by a single structural gene, a hypothesis consistent with the finding that mutations at the *Eu1* (Meyer-Bothling and Polacco 1987) and *Eu4* (Polacco et al. 1989) loci eliminate the embryo-specific or ubiquitous (leaf) ureases, respectively. To test further whether the *Eu4* locus identifies a single structural gene for the ubiquitous urease expressed in all tissues, we determined how many tissues lacked ubiquitous urease in *eu4/eu4* plants. We also sought independent genetic evidence that the *Eu4* locus contains a ubiquitous urease structural gene.

In pursuit of the first objective we found that the *eu4/eu4* genotype lacked the ubiquitous urease in roots and hypocotyls as well as leaves (Torisky and Polacco 1990). Ubiquitous urease, along with urease(s) of commensal bacteria, were the only ureases expressed in cell culture and the former was eliminated in *eu4/eu4* callus (Holland and Polacco 1992). We report here that *eu4/eu4* lacks the ubiquitous urease also in developing embryos and seed coats. Thus the *eu4* lesion appears to eliminate ubiquitous urease activity in all soybean tissues.

To establish independent genetic criteria for the notion that the *Eu4* locus is structural, we determined its linkage to an RFLP derived from a genomic urease clone (LC4), examined the deduced amino acid sequence and organ distribution of LC4 transcripts, and attempted to correct the *eu4* lesion by transformation with a subclone containing the LC4 urease.

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Materials and methods

Plant material. Unless stated otherwise, soybean (*Glycine max* L. Merr.) wild type was cultivar Williams or Williams 82. The embryo-specific urease-null line *eu1-sun/eu1-sun* (Torisky and Polacco 1990) and leaf urease-negative mutant *eu4/eu4* (Polacco et al. 1989) were in the Williams and Williams 82 backgrounds, respectively. Plant Introductions (PIs) used to screen for RFLP variants were obtained from the Northern Soybean Germplasm Collection maintained by the United States Department of Agriculture, Urbana, Ill. Callus was generated from hypocotyl/radicle sections of 2-day-old seedlings from surface-sterilized seeds (Polacco 1976).

Nucleic acid preparation. DNA was prepared from lyophilized callus (by the methods of Mettler 1987 or of Saghai-Maroof et al. 1984). Leaf DNA genomic mini-preparations for RFLP analyses were made by modifying the procedure of Dellaporta et al. (1983). Leaf tissue (0.5 g), powdered in liquid nitrogen, was incubated 15 min at 65° C in 5 ml extraction buffer and extracted directly with 5 ml phenol. Nucleic acids were precipitated from the aqueous phase by the addition of 2 vol 95% ethanol and pelleted by centrifugation at 10000 × *g*. The pellet was dissolved in 600 μl 10 mM TRIS-HCl, 1 mM EDTA, pH 8.0 (TE), and treated with 100 μg/ml DNase-free RNase (37° C for 1 h). After two phenol and one chloroform extractions, DNA was ethanol precipitated, and resuspended in 200 μl TE.

Clone isolation. *Eco*RI and *Alu*I/*Hae*III-derived soybean genomic libraries in Charon 4A were from R.B. Goldberg (UCLA). Clone LC4, which overlaps urease clone E15 (Krueger et al. 1987), was recovered by high stringency (65° C, 0.33 M Na) screening of 500000 plaques of an *Eco*RI library (cv. Forrest) with the 11 kb *Eco*RI insert of E15. LC4 was chosen because it contained the E15 insert plus 4.7 kb (in four *Eco*RI fragments) 5′ of E15. Clones extending further upstream were recovered by screening the *Alu*I/*Hae*III library with the 5′-most *Eco*RI subclone (pJG16) of LC4; AH13 extended the farthest in the 5′ direction. Urease coding and flanking sequence of LC4 was subcloned into pBluescript II KS+ (Stratagene, La Jolla, Calif.) from E15 and subclones pJG23 and pJG15 (Fig. 3), as described (Torisky 1992), to derive pUREASE (Fig. 9).

Clone LS1 was recovered by a low-stringency screen (53° C, 0.33 M Na) of 600000 plaques of the *Alu*I/*Hae*III library probed with combined LC4 subclones pJG14 and pJG15 (Fig. 3). Of 140 primary isolates, 10 were selected which showed specific *Eco*RI fragments hybridizing either to pJG14 or to pJG15 but lacking the E15 restriction pattern. Five clones, including LS1, hybridized to a PCR-derived 130 bp fragment containing only the first urease exon of pJG13 (amplified with primers 5′-GAATTCTCTGCTGTAACAACC-3′ and 5′-GAAGACAAGGAATCCGAC-3′).

cDNA clone 5E5 was recovered from size-fractionated poly(A)$^+$ RNA of mid-maturation embryos from cv. Dare. The library was screened with radiolabeled cDNA made from the same RNA used to construct the library. Clones were isolated based on their ability to hybridize rapidly with the enriched probe. Positive colonies which reacted with RNAs > 2.8 kb on Northern blots were chosen and virtually all were later shown to be lipoxygenase cDNAs (Yenofsky et al. 1988). One clone, 5E5, hybridized to an RNA of the same size as lipoxygenase mRNA, but with a dramatically reduced signal. A homology search of its nucleotide sequence against the Genbank database revealed that 5E5 encoded urease.

PCR analysis of urease transcripts. Total RNA was extracted from developing embryos (approximately 30 days after pollination (DAP)) and seed coats, from hypocotyl-radicles of seedlings 3 days after germination (DAG), and from emerging trifoliates of 6-week-old plants (cv. Forrest) essentially as described by Wadsworth et al. (1988). Approximately 1–3 μg RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) and oligo-$(dT)_{12-18}$ (Gibco-BRL) as a primer. PCR reactions on cDNA template were carried out according to the specifications of a commercial kit (GeneAmp PCR Reagent kit with Amplitaq DNA polymerase; Perkin-Elmer-Cetus) in a Coy Tempcycler or a Perkin-Elmer-Cetus thermal cycler at 94° C 40 s, 54° C 60 s, 72° C 90 s for 40 cycles.

Before PCR products primed with the unique 3′ UT oligomers ESU-3′ and LC4-3′ (Fig. 6A) were used to probe soybean genomic digests, they were isolated from a 3.5% acrylamide gel (Sambrook et al. 1989) and further amplified with the common upstream primer ALS-1 (Fig. 6A) and the unique 3′ UT primers. Products (with expected sizes 400 and 300 bp, respectively, for the seed and leaf transcripts) were labeled with α-[^{32}P]dCTP by priming (kit no. 70240, US Biochemicals) with a mixture of random and PCR primers.

Particle bombardment of callus. DNA-coated tungsten microprojectiles were driven at high velocity by a gunpowder-based gun (Klein et al. 1987) (in the laboratory of Tuan-Hua David Ho and John C. Rogers, Washington University, St. Louis, Mo.) Tungsten particles (1 μm, Analytical Scientific Instruments, Alameda, Calif.) were suspended in 100% ethanol (50 mg/ml) and sonicated for 10 min using an immersed thin probe at a setting of 4–5 on a Cole-Palmer ultrasonic homogenizer, immersing in ice when the suspension boiled. This suspension was stored at −80° C and, before use, 1 ml was sonicated briefly in a 1.5-ml aliquot tube, centrifuged and resuspended in 1 ml sterile water. Plasmids pUREASE and pHYG^r (a gift from Dr. J. Finer, OARDC, Wooster, Ohio) were mixed at a molar ratio of 5:1 and diluted to 1 mg/ml in sterile water. To a 1.5-ml aliquot tube were added, in the following order: 25 μl aqueous tungsten suspension, 10 μl plasmid (1 mg/ml), 25 μl sterile 1 M $CaCl_2$, and 10 μl sterile 100 mM spermidine (free base). Each component was added to the side of the tube, which was vortexed after the addition of spermidine. After DNA-tungsten aggregation, 35 μl of supernatant was discarded.

Table 1. Partial purification of ubiquitous urease from *eu1-sun/eu1-sun, Eu4/Eu4* seedling axes (4 DAG)

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification factor (fold)	Recovery (%)
Crude extract	26176	1955	7.1	1	100
Heat 60 °C/60 min	20483	209	13.4	7.3	78
40% $(NH_4)_2SO_4$ saturation	16221	24	672	50.2	62
$CHCl_3$ extraction	15264	16.8	909	67.8	58
DEAE pooled peak fractions	5403	4.5	1200	89.6	21
Hydroxyapatite pooled peak fractions	2520	0.34	7499	560	10

Callus was placed on an uncovered sterile polystyrene petri dish and positioned at 2, 3 or 4 inches from the stop plate. Immediately prior to shooting, tungsten-DNA aggregate was dispersed by placing the tube against the sonicator tip; one-half of each aggregate (ca. 10 μl) was dispensed onto the ends of each of two macroprojectiles (Analytical Scientific Instruments, Alameda, Calif.). Approximately 5–6 callus pieces (0.3 g fresh weight) were shot at a time, at four shots/sample, with tungsten particles coated with a mixture of $pHYG^r$: pUREASE (5 μg total DNA per shot, 1 : 5 molar ratio).

Sequence analysis. Single-stranded phagemid or double-stranded plasmid templates were sequenced using T7 DNA polymerase (Sequenase, US Biochemicals), and standard 6% or 8% polyacrylamide 8 M urea gels in 1×TBE (90 mM TRIS-borate, 2 mM EDTA, pH 8). DNA sequence data were analyzed with the Wisconsin GCG package (Devereaux et al. 1984) or with EuGene programs (which can access GenBank and PIR data banks for nucleic acid and amino acid searches, respectively) on a workstation provided by the University of Missouri DNA Core Facility. Sequencing primers were based on vector cloning regions or on known urease sequences. Initial 5′ sequence data of LS1 subclone pJG30 was obtained with a primer based on sequence near the 5′ end of the first exon of LC4 (5′-GAATTCTGAGTCCAAGGGAG-3′).

Preparation of crude extracts. Individual cotyledons and seed coats (Figs. 1 and 2, respectively) were ground in a mortar with 1–2 ml extraction buffer (25 mM MES, pH 6.1, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride) and cleared by centrifugation at 10000 × *g*, 8 min. Callus (0.2 g fresh weight) was frozen at -80° C in a 1.5-ml tube, thawed, and homogenized in 300 μl extraction buffer with a microcentrifuge pestle (Kontes Scientific Glassware/Instruments). After placing on ice for 40 min, extracts were cleared by centrifugation at 10 000 × *g*, 10 min.

Urease assay. Urease activity was measured at 37° C in 0.5 ml TM7 or TM9 (0.1 M TRIS-maleate, 1 mM EDTA; pH 7.0 or pH 9.0) containing 10 mM $[^{14}C]$urea (15 μCi/mmol) as described previously (Torisky and Polacco 1990). One unit of urease catalyzes the hydrolysis of 1 nmol urea per min at 37° C.

Ubiquitous urease purification and microsequencing. Partial purification was a modification of that employed by Polacco and Havir (1979) for the embryo-specific urease and is summarized in Table 1. Protein preparations (ca. 20 and 80 μg of ubiquitous and seed urease, respectively) were resolved on a denaturing gel (Laemmli 1970) which was blotted overnight to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, Mass.) according to the manufacturer's instructions. Urease bands were identified by Western analysis (Towbin et al. 1979) of a lane using rabbit anti-seed urease polyclonal antiserum (Polacco and Havir 1979). Proteins were visualized in the remaining lanes by a 3 min Coomassie stain followed by destaining, excision of urease bands (Torisky 1992), and direct protein sequencing by automated Edman degradation (Matsudaira 1987) in the Protein Sequencing Core Facility of the University of Missouri.

Results

Embryo ureases are controlled by Eu1 *and* Eu4

Urease activity was determined in developing soybean embryos and those homozygous for mutations at *Eu4* or *Eu1* (the putative structural gene for the embryo-specific urease; Meyer-Bothling and Polacco 1987) or at both loci. The embryos represent each of the four possible phenotypes with respect to the presence or absence of the embryo-specific (seed) and ubiquitous (determined in the leaf) ureases: SL, seed urease and ubiquitous (leaf) urease-positive (*Eu1/Eu1, Eu4/Eu4*); Sl, seed urease-positive, leaf urease-negative (*Eu1/Eu1, eu4/eu4*); sL, seed urease-negative, leaf urease-positive (*eu1-sun/eu1-sun, Eu4/Eu4*); and sl, seed and leaf urease-negative (*eu1-sun/eu1-sun, eu4/eu4*).

Embryos with a functional *Eu1* gene contain the preponderant embryo-specific urease, characterized by its high activity levels and maximal activity at pH 7.0 versus pH 9.0 or pH 5.5 (SL and Sl, Fig. 1 A and 1 B). In agreement with previous results (Holland et al. 1987; Polacco and Sparks 1982), embryo-specific urease activity increased as embryos matured, indicated by increasing fresh weight (until 30 DAP, at initiation of desiccation). In contrast, the ubiquitous urease activity revealed in *eu1-sun/eu1-sun, Eu4/Eu4* embryos (sL, Fig. 1 C) de-

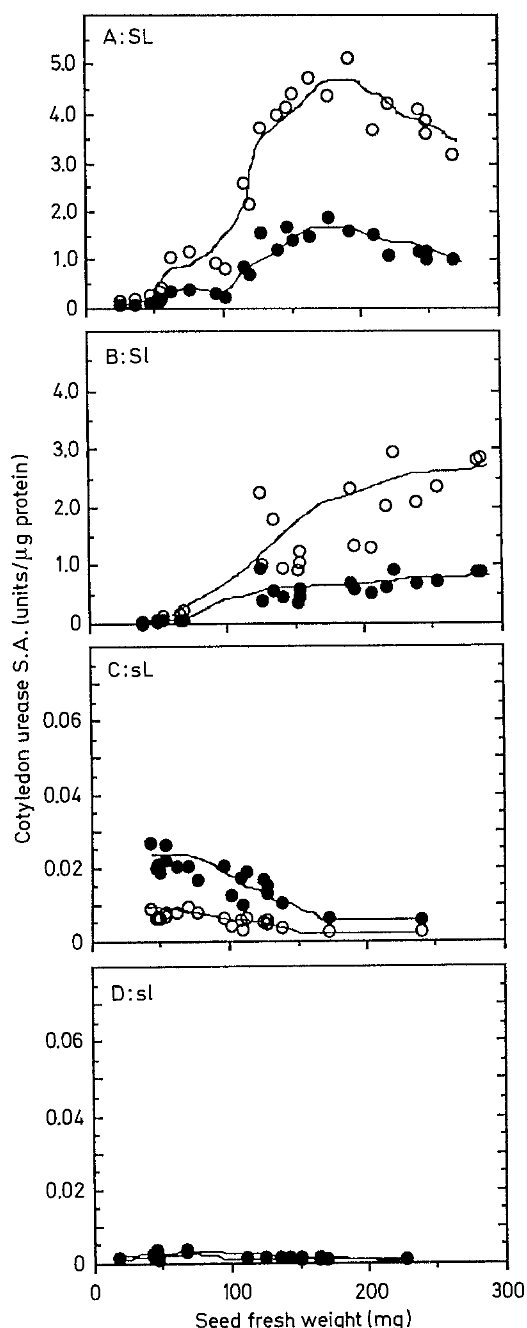

Fig. 1A–D. Urease activity of developing cotyledons. Cotyledon extracts were assayed in 10 mM [^{14}C]urea in TRIS-maleate plus MOPS (50 mM each) at pH 5.5 (not shown), pH 7.0 (*open circles*), or at pH 9.0 (*filled circles*). **A** SL, wild type (*Eu1/Eu1, Eu4/Eu4*). **B** Sl, leaf (ubiquitous) urease-negative (*Eu1/Eu1, eu4/eu4*). **C** sL, seed (embryo-specific) urease-negative (*eu1-sun/eu1-sun, Eu4/Eu4*). **D** sl, seed and leaf urease-negative (*eu1-sun/eu1-sun, eu4/eu4*). The average pH 7.0/pH 5.5 activity ratios ($\pm$SD) for the following genotypes were: SL, 1.06$\pm$0.09; Sl, 1.02$\pm$0.04; sL, 0.70$\pm$0.08

creased during embryo development. Its low pH 7.0/pH 9.0 and pH 7.0/pH 5.5 activity ratios are indicative of its biphasic pH optima at pHs 5.5 and 9.0 (Kerr et al. 1983; Torisky and Polacco 1990). Finally, double mutant *eu1-sun/eu1-sun, eu4/eu4* embryos (sl, Fig. 1D) contained little or no urease, indicating that the ubiquitous urease in *eu1-sun/eu1-sun, Eu4/Eu4* embryos is under *Eu4* control.

Seed coat urease is under Eu4 *control*

We showed earlier (Polacco et al. 1985) that the seed coat produced exclusively the ubiquitous urease; its urease had the pH profile of the ubiquitous urease with no difference in activity level between *Eu1/Eu1* and *eu1-sun/eu1-sun* genotypes. In Fig. 2 (C and D) we compared the urease activity of *Eu4/Eu4* and *eu4/eu4* developing seed coats (both in the *eu1-sun/eu1-sun* background). It is obvious that the seed coat (ubiquitous) urease is under *Eu4* control. Fig. 2C shows also that the ubiquitous urease of the seed coat did not decrease in specific activity during embryo development, unlike its pattern in the embryo (Fig. 1C).

Seed coats surrounding early *Eu1/Eu1* embryos also contained an activity with the pH preference of the ubiquitous urease (Fig. 2A), which was eliminated by the *eu4* lesion (Fig. 2B). (Late embryos were not included because it was difficult to harvest their seed coats consistently free of contaminating cotyledon tissue.)

A urease-derived RFLP cosegregates with the eu4 *allele*

It *Eu4* encodes the ubiquitous urease, alleles at *Eu4* ought to cosegregate with DNA polymorphisms detected by ubiquitous urease genomic DNA. We developed an RFLP probe from a region upstream of urease genomic clone E15 (Krueger et al. 1987), not heretofore assigned to an isozyme. Subclones of E15 and two 5′ overlapping clones, LC4 and AH13, were used to screen for RFLP variants among 58 soybean genotypes representing diverse accessions from the USDA germplasm collection (Griffin 1986). Only one subclone (pJG17, approximately 2 kb upstream of the N-terminus of LC4, Fig. 3A) detected an RFLP: either a simple 4.3 kb *Eco*RI fragment (RFLP type S) or, in some genotypes, two hybridizing *Eco*RI fragments (3.5 and 2.0 kb, RFLP type F).

Crosses were made between genotypes with RFLP F and cv. Williams (RFLP S) containing distinct alleles at one of the four urease loci: *Eu1, Eu2, Eu3* or *Eu4*. Random segregation of the LC4 S and F alleles and alleles of the *Eu1, Eu2* and *Eu3* loci occurred among F_2 progeny. However, there was complete cosegregation of LC4 RFLP S with the *eu4* allele, consistent with the LC4 urease ORF being near or within the *Eu4* locus (Table 2).

Eu1, the putative structural gene for the embryo-specific urease, is not linked to *Eu2* or to *Eu3* (Meyer-Bothling et al. 1987) and appears also to be unlinked to *Eu4*. *Eu4* cosegregated with an LC4-associated RFLP whereas *Eu1* segregated randomly (Table 2). We crossed *eu4/eu4* with *eu1-sun/eu1-sun* and confirmed lack of linkage in the F_2 (Table 3).

Urease clones LC4 and LS1 align with amino acid sequences of the ubiquitous and embryo-specific ureases, respectively

Our aim was to recover clones representing each of the two urease isozymes and to make the assignments based

Fig. 2A–D. Urease activity of developing seed coats. Seed coat extracts were assayed in 10 mM [^{14}C]urea in TRIS-maleate plus MOPS (50 mM each) at pH 5.5 (not shown), pH 7.0 (*open squares*), or at pH 9.0 (*closed squares*). **A** SL, wild type (*Eu1/Eu1, Eu4/Eu4*). **C** sL, seed (embryo-specific) urease-negative (*eu1-sun/eu1-sun, Eu4/Eu4*). **B** Sl, leaf (ubiquitous) urease-negative (*Eu1/Eu1, eu4/eu4*). **D** sl, seed and leaf urease-negative (*eu1-sun/eu1-sun, eu4/eu4*). Seed-coat urease values for larger (≥150 mg) seeds of *Eu1/Eu1* genotypes (**A** and **B**) could not be obtained due to their variable contamination by embryo-specific urease, which accumulated to high levels (Fig. 1) in the developing cotyledons of these seeds. The average pH 7.0/pH 5.5 activity ratios (±SD) for the following genotypes were: SL, 0.70±0.06; Sl, 1.25±0.35; sL, 0.68±0.05. The high standard deviation for the pH 7.0/pH 5.5 activity ratio of Sl seed coats is likely to be due to variable contamination of seed coat with cotyledon tissue bearing the preponderant embryo-specific urease

Fig. 3A, B. Urease genomic clones. **A** The LC4 "family" is three overlapping clones including the original clone E15 (Krueger et al. 1987). *Arrows* labeled N and C indicate the approximate location of the N- and C-terminal codons of the urease ORF within clone LC4. Pertinent restriction map and subclones are indicated. **B** Pertinent subclones of genomic clone LS1 which was recovered by a low stringency screen of an *Eco*RI genomic library with LC4 subclones. The C-terminal *Eco*RI fragment (common to subclones pJG28 and pJG30) hybridized to embryo cDNA clone 5E5 while the N-terminal *Eco*RI fragment of pJG30 hybridized to the PCR-amplified first exon of LC4 subclone pJG13

LC4	MKLSPREIEKLDLHNAGYLAQKRLARGL
	****************************
Ubiquitous urease protein	MKLSPREIEKLDLHNAGYLAQKRLARGL
LS1	MKLSPREVEKLGLHNAGYLAQKRLARGL
	****************************
Embryo urease protein	MKLSPREVEKLGLHNAGYLAQKRLARGL

Fig. 4. Alignment of the N-terminal 28 amino acids of the ubiquitous (Table 3) and embryo-specific ureases with deduced sequences of clones LS1 and LC4 (Fig. 3). *Asterisks* indicate identity between LS1 and embryo urease, and between LC4 and ubiquitous urease. *Arrows* indicate amino acids of LS1 (and the embryo urease) that differ from LC4 (and the ubiquitous urease)

on alignment of deduced and determined N-terminal amino acid sequences. Since the clones of Fig. 3A represent a single urease locus, and hence a single isozyme, we recovered a second urease clone, LS1 (Fig. 3B, Materials and methods).

Partial DNA sequence analysis of LC4 subclone pJG13 revealed a 30 residue ORF, which was 93% identical to the chemically determined N-terminal sequence of jack bean urease (Takishima et al. 1988). The

Table 2. F_2 segregation of LC4 urease RFLP and alleles at the *Eu1*, *Eu2*, *Eu3* and *Eu4* loci

Locus	Allele[a]	RFLP	Expected[b]	Observed
Eu1	*eu1-sun*	S	14	2
	eu1-sun	H	0	8
	eu1-sun	F	0	4
Eu1[c]	*Eu1-a/a*	S	0	0
	Eu1-a/a	H	0	1
	Eu1-a/a	F	4	3
	Eu1-a/b	S	0	4
	Eu1-a/b	H	8	1
	Eu1-a/b	F	0	3
	Eu1-b/b	S	4	1
	Eu1-b/b	H	0	1
	Eu1-b/b	F	0	2
Eu2	*eu2*	S	16	3
	eu2	H	0	6
	eu2	F	0	7
Eu3	*eu3-e1*	S	14	4
	eu3-e1	H	0	7
	eu3-e1	F	0	3
Eu4	*eu4*	S	17	17
	eu4	H	0	0
	eu4	F	0	0

The RFLP probe was subclone pJG17 (Fig. 3) which detected either a 4.3 kb *Eco*RI fragment (type S) or two fragments (2 and 3.5 kb, type F). Genotype PI 189938 (type F) was crossed with cv Williams 82 (RFLP S) containing distinct alleles at the *Eu1*, *Eu2*, *Eu3* and *Eu4* loci

[a] S and F indicate the 4.3 and 2/3.5 kb hybridizing *Eco*RI fragments, respectively; heterozygotes (H) have all three fragments

[b] Expected values assuming identity between a genetic locus and that represented by urease genomic clone pJG17

[c] Electrophoretic alleles a and b at the *Eu1* locus (Buttery and Buzzell 1971; Kloth and Hymowitz 1985)

Table 3. *Eu1* × *Eu4* F_2 segregation patterns

Cross	Phenotypic class[a]				n	χ^2	P^b
	SL	sL	Sl	sl			
eu1-sun/eu1-sun × *eu4/eu4*	23	3	4	0	30	5.79	0.122
eu4/eu4 × *eu1-sun/eu1-sun*	15	9	1	2	27	6.38	0.095
$\sum$	38	12	5	2	57	4.97	0.174
Linkage						0.22	0.823

[a] S, s and L, l refer to the presence or absence of seed or leaf urease activity, respectively (as in Figs. 1 and 2). Individuals with the sl phenotype breed true through at least the F_5 generation

[b] Probabilities are calculated based on a 9:3:3:1 theoretical ratio and a χ^2 test with 3 degrees of freedom for the segregation data, and a linkage χ^2 test with 1 degree of freedom

corresponding region of LS1 was sequenced from the homologous N-terminal subclone (the large *Eco*RI fragment of subclone pJG30; Fig. 3B). In both LS1 and LC4 the initiator methionine, two codons downstream from a stop (UGA) codon, is in the plant context (AAAA*ATG*) for translation initiation (Lütcke et al. 1987). The two ORFs differed in 8 of 90 nucleotides. More significantly, they differed in 2 of 30 amino acids (Fig. 4), differences also revealed in chemically determined amino acid sequences of each isozyme.

Ubiquitous urease was purified 500-fold from etiolated *eu1-sun/eu1-sun* seedling axes (Table 1) and its sequence was compared to that of the embryo-specific urease determined in parallel. The 28 N-terminal residues of the ubiquitous and embryo-specific ureases differed

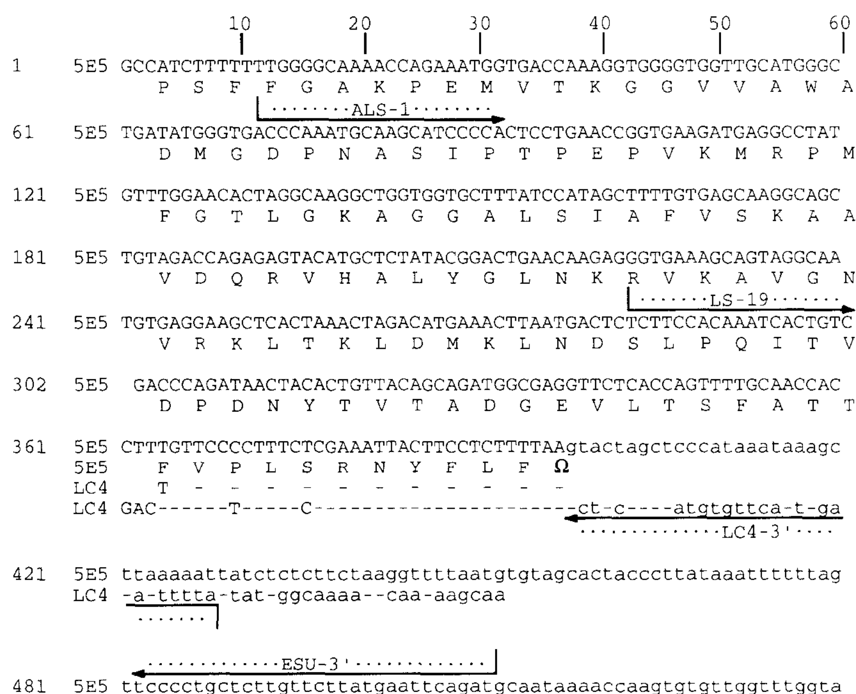

Fig. 5. Alignment of 5E5 with ubiquitous urease genomic clone LC4. ALS-1 and LS-19 are "right-pointing" (→) PCR primers while LC4-3′ and ESU-3′ are "left-pointing" (←) PCR primers

in two positions (Fig. 4). Importantly, each urease aligned perfectly with sequences predicted from clones LC4 and LS1. Thus LS1 encodes the (an) embryo-specific urease and LC4 the (a) ubiquitous urease.

Clone-specific PCR primers amplify urease poly(A)$^+$ RNA in the expected organ pattern

Gene-specific PCR primers were based on the 3′ untranslated (UT) region of each urease clone. An LC4 primer (LC4-3′) was based on sequence immediately downstream from its stop codon. The LS1 primer (ESU-3′) was chosen indirectly. It was based on the 3′ UT sequence of urease cDNA clone 5E5 derived from developing embryo RNA (Fig. 5). These primers were used in conjunction with a common upstream primer (21-1 or a longer version 21-1 LE, Fig. 6) which was based on the ORF sequence for amino acids 648–654 in clone LC4 (Krueger et al. 1987). We found an identical nucleotide sequence in clone LS1 (not shown). Under the amplification conditions employed (Materials and methods) LC4-3′ and ESU-3′, each in conjunction with primer 21-1LE, showed clone specificity in amplifying only the expected products from clones LC4 and LS1, respectively.

The same conditions were employed to amplify single-stranded cDNA from various soybean organs. Primer pair LC4-3′/21-1LE amplified a single, approximately 600 bp species (Fig. 6B) from cDNA of all tissues of both *Eu1/Eu1* and *eu1-sun/eu1-sun* genotypes: *Eu1/Eu1* (cv. Forrest) leaf, seed coat, developing embryo, hypocotyl/radicle, and *eu1-sun/eu1-sun* (cv. Itachi) developing seed and hypocotyl/radicle. In contrast, primer pair ESU-3′/21-1LE amplified a single, approximately 700 bp species only from developing embryos of *Eu1/Eu1*. It did not amplify cDNA from *eu1-sun/eu1-sun* developing embryos (which has a drastically reduced urease transcript; Meyer-Bothling and Polacco 1987) or from any other tissue (Fig. 6B). The ESU-3′/21-1LE amplification pattern agrees with conclusions that embryo-specific urease is expressed exclusively in the developing embryo and that its presence in seedling roots is not due to de novo synthesis upon germination (Torisky and Polacco 1990).

The tissue amplification pattern of LC4-3′/21-1LE indicates that LC4 (or a gene resembling LC4) is expressed in all soybean tissues known to express the ubiquitous urease. To confirm that the PCR products were urease-derived and related to the proper isozyme (embryo-specific urease from LS1 and ubiquitous urease from LC4) they were probed with LC4 (C-terminal-containing subclone pJG7, Fig. 3A) and with LS1 (C-terminal subclone pJG28, Fig. 3B). In all cases the predicted ubiquitous urease 600 bp PCR products and the single embryo-specific urease 700 bp product gave stronger hybridization

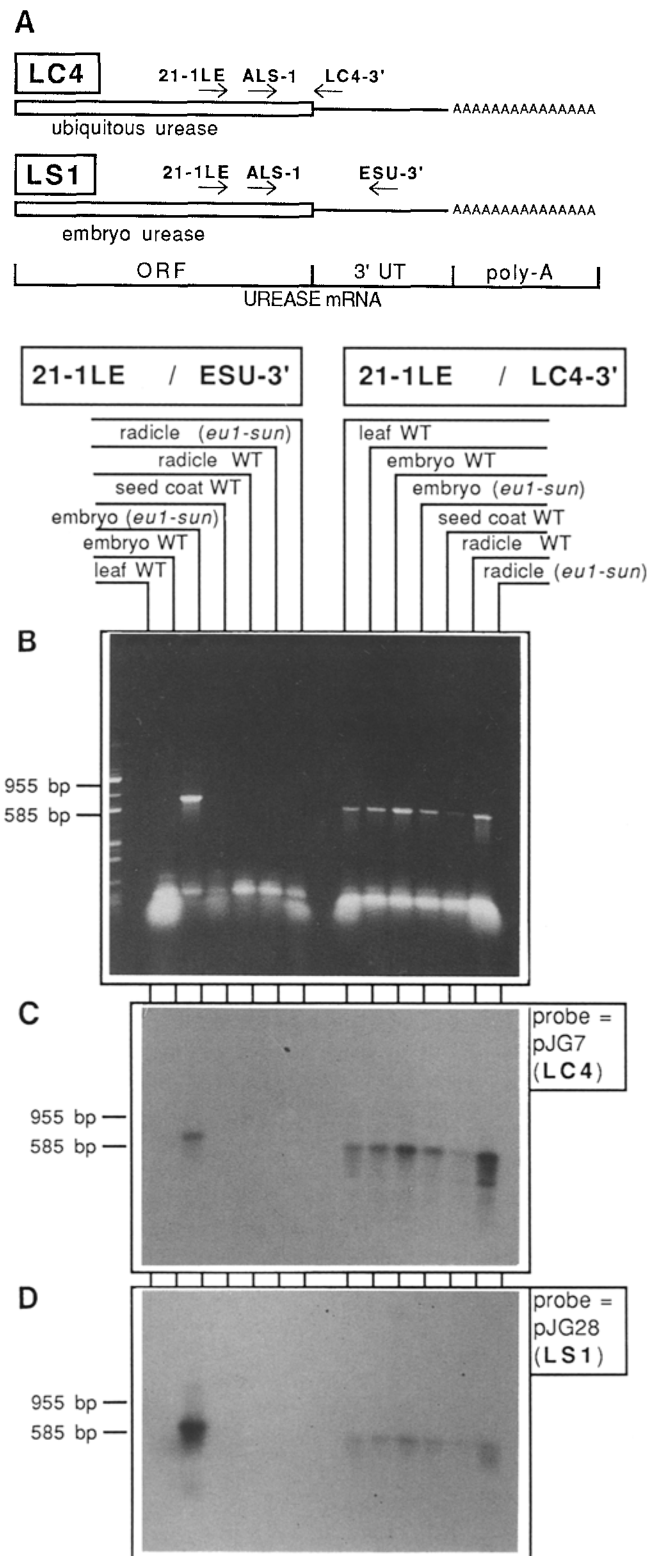

Fig. 6A–D. Clone-specific PCR amplification of urease poly(A)$^+$-derived cDNA from soybean organs. **A** PCR primer sequences and positions relative to urease message (the ORF is much larger than indicated). ESU-3′ and LC4-3′ differed both in sequence and in position relative to the terminator codon so that both size **B** and hybridization affinity (**C** and **D**) were criteria in identifying "proper" PCR products. **B** PCR products resolved on a 1% agarose gel. Primer pairs 21-1LE/ESU-3′ or 21-1LE/LC4-3′ were used to amplify oligo(dT)-primed single-stranded cDNA from leaves, developing embryos, developing seed coats and 3-day-old seedling radicles. **C** Hybridization of PCR products with LC4 C-terminal subclone pJG7. **D** Hybridization of PCR products with LS1 C-terminal subclone pJG28

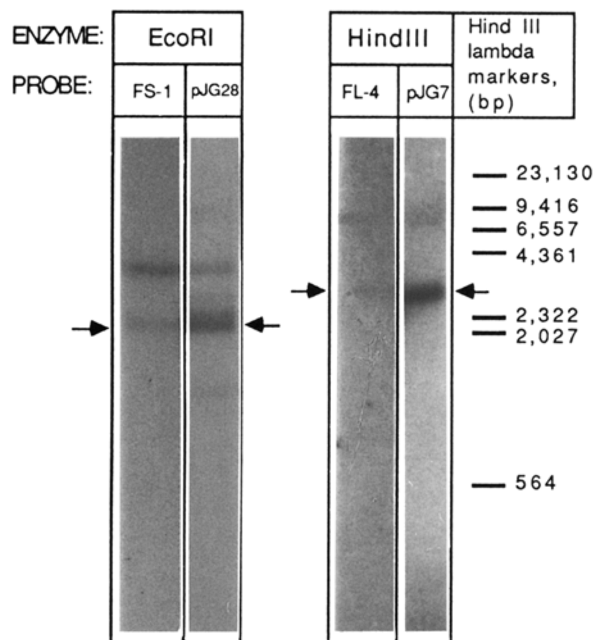

Fig. 7. Hybridization of amplified urease transcripts to genomic DNA. Genomic DNA (40 μg/lane, cv. Forrest, the source of genomic clone LC4) was hybridized either with PCR product FS-1, PCR product FL-4, LS1 C-terminal subclone pJG28 (vector plus insert; Fig. 3) or LC4 C-terminal subclone pJG7 (the *Hin*dIII insert; Fig. 3). FS-1 is a PCR subfragment of 21-1LE/ESU-3′-amplified Forrest embryo cDNA produced with primers ALS-1 (Fig. 6A) and ESU-3′. FL-4 is a PCR subfragment of 21-1LE/LCA-3′-amplified Forrest leaf cDNA produced with primers ALS-1 (Fig. 6A) and LC4-3′. The *arrows* on each *Hin*dIII lane indicate the position of the 2.9 kb *Hin*dIII insert of pJG7 on the same gel. The *arrows* on the *Eco*RI lanes indicate the position of the larger (C-terminus) 2.3 kb *Eco*RI insert of pJG28 on the same gel

signals with the LC4 and LS1 subclones, respectively (Figs. 6C and 6D).

cDNA amplified by LC4-3′ (subfragment FL-4, Fig. 7 legend) was used as a genomic probe to confirm that it was derived from LC4 transcript. FL-4 recognized a *Hin*dIII genomic fragment identical in gel migration to the (2.9 kb) *Hin*dIII insert of LC4 C-terminal clone pJG7 (Fig. 7). In addition, FL-4 recognized an approximately 7 kb *Hin*dIII genomic fragment, which may be the homologous fragment of the LS1 urease. The pJG7 *Hin*dIII insert recognized genomic *Hin*dIII fragments of similar size, although hybridizing more weakly to the 7 kb fragment. These hybridization patterns are consistent with the ubiquitous urease LC4-3′ PCR product being transcribed from LC4 genomic sequence.

Figure 7 also shows that a subfragment (FS-1) of ESU-3′-primed PCR product from developing embryo hybridized to two genomic bands: an approximately 2.3 kb *Eco*RI band similar in size to the homologous *Eco*RI insert of C-terminal LS1 subclone pJG28 and an approximately 4 kb band. pJG28 recognized two similarly sized fragments and, in addition, a weakly hybridizing 10 kb *Eco*RI fragment which is likely the E15 *Eco*RI fragment of LC4 (Fig. 3). (Clone pJG28 contains a second 1.8 kb *Eco*RI insert, Fig. 3, and thus hybridized to an approximately 1.8 kb genomic fragment, Fig. 7.) These results further validate the generation of an LS1-specific 3′ UT PCR primer (ESU-3′) from embryo urease cDNA clone 5E5. Importantly, the pattern of genomic fragment hybridization is that which would be expected if the LS1 urease were the in vivo template for the embryo-specific urease transcript.

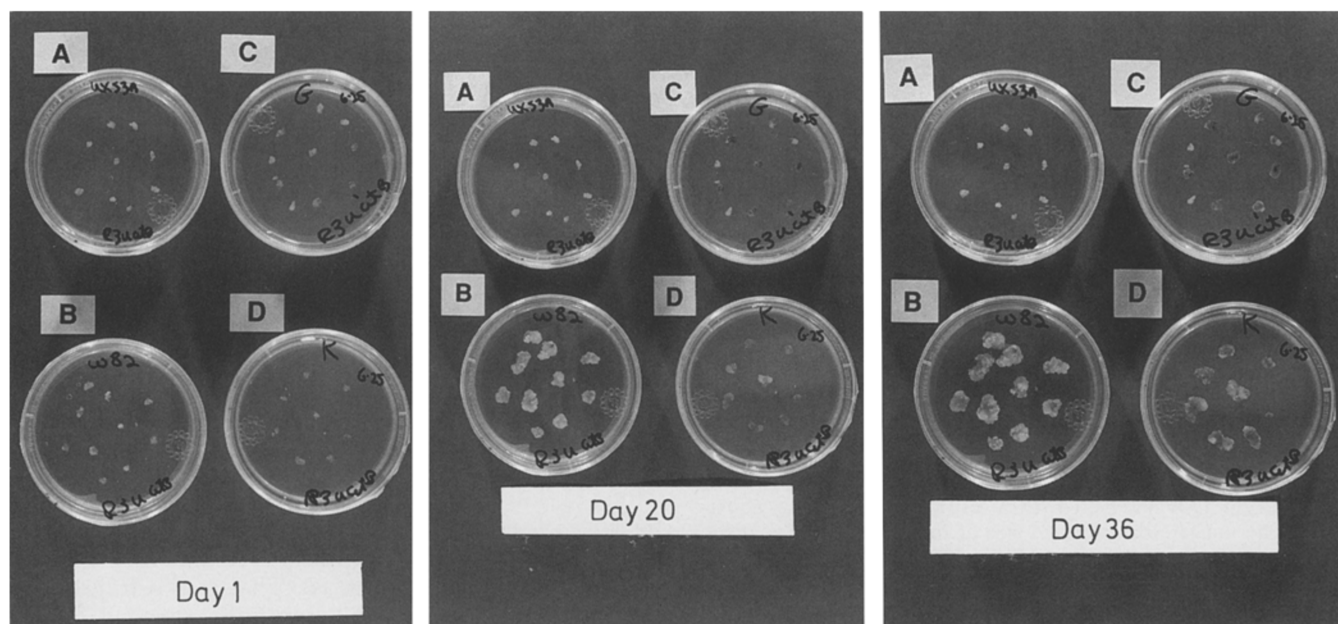

Fig. 8A–D. Urea utilization in hygromycin-resistant callus lines. **A** Host callus (*eu1-sun/eu1-sun, eu4/eu4*) used for transformation. **B** Progenitor *Eu1/Eu1, Eu4/Eu4*. **C, D** Hygromycin-resistant callus lines G and K, respectively, (containing the HPT gene encoding hygromycin phosphotransferase) maintained on medium containing 5 mM urea as sole nitrogen source. Callus is shown at 1, 20 and 36 days after transfer

Introduction of the LC4 urease gene into eu4/eu4 callus

We attempted to confirm that both LC4 and *Eu4* encode ubiquitous urease by correcting the *eu4* lesion with the LC4 urease subcloned in pBluescript II KS+ (Materials and methods; Fig. 9). The resulting pUREASE contained about 7.5 kb urease ORF, 5 kb of intron and 2.4 kb of upstream region to ensure the inclusion of a functional promoter.

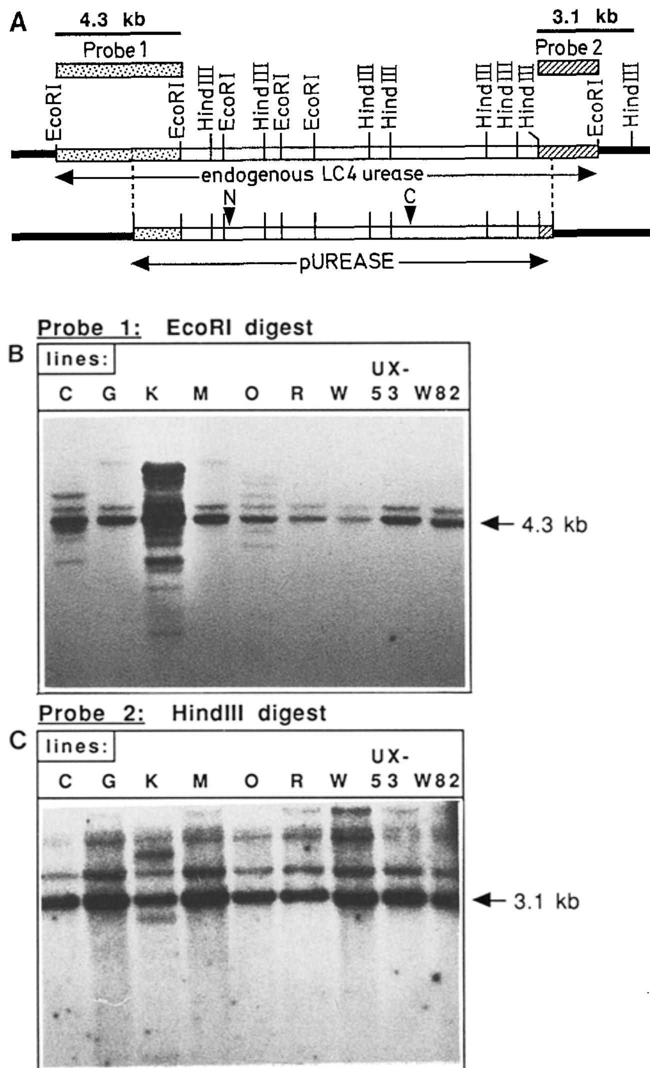

Fig. 9A–C. Genomic analysis for pUREASE integration. *Eco*RI and *Hin*dIII digests of callus DNA were blotted for analysis of 5′ and 3′ border fragments, respectively, of pUREASE. **A** Comparative restriction maps of genomic clone LC4 and its pUREASE subclone. The *solid black lines* indicate flanking genomic and vector sequences for LC4 and pUREASE, respectively. The *lines* above each probe indicate the expected size of the hybridizing fragment from the endogenous LC4 urease gene. **B** Hybridization of Southern blot of *Eco*RI-digested callus DNA (hygromycin-resistant lines C, G, K, M, O, R, W and untransformed *eu4/eu4, eu1-sun/eu1-sun* [UX53] and wild-type [W82] progenitor lines) with probe 1, the 5′ *Eco*RI border fragment subcloned in pJG17. **C** Hybridization of *Hin*dIII-digested callus DNA with probe 2, from genomic subclone pJG10 (Fig. 3), which overlaps 500 bp of the 3′ border of pUREASE

pUREASE was co-introduced into *eu1-sun/eu1-sun, eu4/eu4* callus with pHYG^r (containing the CaMV 35S promoter-hygromycin phosphotransferase (HPT) ORF-NOS (nopaline synthetase) terminator; Finer et al. 1992) by tungsten particle bombardment. We included the *eu1-sun* lesion in host callus to minimize ectopic expression of the embryo-specific urease induced by mutagenic action of introduced DNA. After a 5-day recovery/expression period, callus was transferred to medium containing 50 μg/ml hygromycin and nine independent lines, exhibiting persistent hygromycin resistance and HPT-homologous genomic sequences (not shown), were analyzed for urease expression.

Three lines (G, R and W) showed low, variable urease activity (0–10% of wild type) and urea-supported growth, always determined on cells never previously challenged with urea as sole nitrogen source. A fourth line, K, consistently grew the best in replicate experiments (e.g. Fig. 8), and had the highest urease levels determined in whole cells or in cell-free extracts (ca. 20% of wild type).

To confirm that the pUREASE construct was incorporated into the genome of K, and possibly other lines, callus DNA was probed with genomic fragments overlapping the 5′ and 3′ borders of pUREASE. The single copy 5′ fragment (the 4.3 kb *Eco*RI RFLP probe; Fig. 3A, Table 2) revealed that five of seven hygromycin-resistant lines contained extra border fragments consistent with 5′ pUREASE insertion events. Significantly, there were several-fold more 5′ integration events associated with line K than with other lines (Fig. 9B). (That at least half of the new bands were smaller than the 4.3 kb genomic fragment indicates that they did not arise spuriously from incomplete DNA digestion.) Similarly, the 3′ probe, covering only about 500 bp of the pUREASE terminus, indicated at least three separate integration events in line K for this region (Fig. 9C) and none for any other line. (A nested probe covering more of the 3′ end of pUREASE revealed several more border fragments in line K and border fragments for lines C, G, M and O, in agreement with the hybridization pattern of the 5′ probe.)

Discussion

We sought to determine the genetic basis of expression of the ubiquitous (metabolic) urease of soybean. We previously showed that the *eu4* lesion eliminates the expression of the ubiquitous urease in all tissues (Polacco et al. 1989; Torisky and Polacco 1990; Polacco and Holland 1993; this work). RFLP analysis placed urease clone LC4 near, or within, the *Eu4* locus. Sequence comparison of urease proteins (ubiquitous and embryo-specific) and clones (LC4 and LS1) indicated that LC4 encodes a (the) ubiquitous urease. That LC4 is transcribed into poly(A)$^+$ RNA in all tissues is indicated by the amplification of its transcript by an LC4-specific PCR primer. (The LS1-specific primer, on the other hand, amplifies poly(A)$^+$ RNA only from developing embryos expressing the embryo-specific urease.) These observa-

tions are consistent with two models. The first is that *Eu4* is the structural gene for the ubiquitous urease whose primary sequence is on clone LC4. The second is that *Eu4* is linked to LC4, but that it is a separate gene which controls a post-translational activation step specific for the ubiquitous urease.

Consistent with the structural gene model is the correction, albeit partial, of the *eu4* lesion with LC4 urease. We have not observed reversion of the *eu4* lesion in cell culture, but we cannot discount mutagenic effects of DNA introduced by particle bombardment causing either partial reversion or urease gene conversion during the early clonal growth of at least four hygromycin-resistant lines. However, the frequency of correction tends to militate against reversion. The second model, invoking an *Eu4* activation function unique to the ubiquitous urease, appears less convincing since the ureases appear to share common post-translational activation processes. Mutations at two loci unlinked to *Eu4*, namely *Eu2* and *Eu3*, result in inactive proteins for both the ubiquitous and embryo-specific ureases (Meyer-Bothling et al. 1987). *Eu2* and *Eu3* probably control nickel activation of apoureases (Holland and Polacco 1992).

An explanation for the poor expression of the multiple copies of LC4 urease in line K is that they are methylated; this is consistent with the inability to digest LC4 sequences in line K with methylation-sensitive *Pst*I (results not shown). Another is that one or more partial copies resulted in partial peptides acting as "negative dominants" (Herskovitz 1987) in the urease multimer.

The embryo-specific and ubiquitous ureases vary 100- to 1000-fold in protein levels (Polacco et al. 1982; Holland et al. 1987). Our PCR-reverse transcriptase approach does not provide a quantitative assessment of transcript level but an all or nothing approach for detecting specific urease message in different tissues. As such, PCR indicates that the embryo-specific urease is aptly named, i.e. its transcripts appear only in the developing embryo. The lack of amplification of a product from seedling radicles confirms our earlier conclusions that the embryo-specific urease in roots of young plants is a remnant of protein laid down during embryo development and is not made de novo after dormancy is broken (Torisky and Polacco 1990). Lack of amplification of embryo-specific transcript in *eu1-sun/eu1-sun* seed urease-null embryos indicates little or no expression of *Eu1* in this mutant.

In summary, we conclude that *Eu4* encodes the ubiquitous urease in all soybean tissues because its mutation causes pleiotropic loss of this activity and because the *Eu4* locus is near, or identical with, a ubiquitous urease-coding locus transcribed in all tissues. Correction of the *eu4* lesion with a ubiquitous urease clone is consistent with *Eu4* being the ubiquitous urease structural gene.

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