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Genetic Tests of the Roles of the Embryonic Ureases of Soybean[1]

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ABSTRACT

We assayed the *in vivo* activity of the ureases of soybean (*Glycine max*) embryos by genetically eliminating the abundant embryo-specific urease, the ubiquitous urease, or a background urease. Mutant embryos accumulated urea (250-fold over progenitor) only when lacking all three ureases and only when developed on plants lacking the ubiquitous urease. Thus, embryo urea is generated in maternal tissue where its accumulation is not mitigated by the background urease. However, the background urease can hydrolyze virtually all urea delivered to the developing embryo. Radicles of 2-day-old germinants accumulated urea in the presence or absence of the embryo-specific urease (2 micromoles per gram dry weight radicle). However, mutants lacking the ubiquitous urease exhibited increased accumulation of urea (to 4–5 micromoles urea per gram dry weight radicle). Thus, the ubiquitous and not the embryo-specific urease hydrolyzes urea generated during germination. In the absence of both of these ureases, the background urease activity (4% of ubiquitous urease) may hydrolyze most of the urea generated. A pleiotropic mutant lacking all urease accumulated 34 micromoles urea per gram dry weight radicle (increasing 2.5-fold at 3 days after germination). Urea (20 millimolar) was toxic to *in vitro*-cultured cotyledons which contained active embryo-specific urease. Cotyledons lacking the embryo-specific urease accumulated more protein when grown with urea than with no nitrogen source. Among cotyledons lacking the embryo-specific urease, fresh weight increases were virtually unchanged whether grown on urea or on no nitrogen and whether in the presence or absence of the ubiquitous urease. However, elimination of the ubiquitous urease reduced protein deposition on urea-N, and elimination of both the ubiquitous and background ureases further reduced urea-derived protein. The evidence is consistent with the lack of a role in urea hydrolysis for the embryo-specific urease in developing embryos or germinating seeds. Because the embryo-specific urease is deleterious to cotyledons cultured *in vitro* on urea-N, its role may be to hydrolyze urea in wounded or infected embryos, creating a hostile environment for pest or pathogen. While the ubiquitous urease is operative in leaves and in seedlings, all or most of its function can be assumed by the background urease in embryos and in seedlings.

Urease is a common enzyme in the plant and bacterial kingdoms. It probably aids in recycling nitrogen by converting "waste" urea from animal and plant sources to utilizable ammonia. In plants, urea is presumed to be a metabolite, although its metabolic origin(s) has not been definitively identified. We show here that viable soybean mutants, which lack all urease activities (8), accumulate considerable urea in leaves and seeds. These observations indicate that urea is indeed a plant metabolite, although not an essential intermediate in soybean nitrogen metabolism.

Micallef and Shelp (10) suggested that during soybean embryo development urease hydrolyzes urea released by arginase acting on free arginine. Studies of developing peas, however, indicate that arginase and urease do not break down arginine, which accumulates to levels representing two thirds of all free amino acid nitrogen (1). The authors presented evidence, nevertheless, that during pea germination arginase and urease actively evolved CO_2 from the guanido group of arginine.

In this communication, we describe a biochemical genetic approach to examine three aspects of urea metabolism in the developing and germinating soybean embryo: (a) the origin (maternal *versus* embryonic) of the urea accumulating in the embryos of urease-negative plants, (b) the roles of each of the urease isozymes in hydrolyzing urea accumulating in the pre- and postdormancy embryo, and (c) the effectiveness of urea as a nitrogen source for *in vitro*-cultured cotyledons lacking one or more ureases.

The biochemical genetic approach relates urea accumulation to the genetic elimination of the activity of one or more urease isozymes. This selective inactivation provides more specificity than urease inhibitors and can be extended to independent manipulation of maternal and embryonic genotypes which, in turn, allows an assessment of maternal and embryonic contributions to urea accumulated in developing embryos.

Soybean produces two urease isozymes (3): ubiquitous urease and embryo-specific urease. Ubiquitous urease is a constitutive enzyme synthesized in all organs (8, 12, 13, 16), and embryo-specific urease is synthesized exclusively in the developing embryo, although roots of young soybean plants retain considerable embryo-specific urease derived from the embryonic axis (14, 18). It accumulates at 0.2 (11) to 0.6% (14, 19) of total seed protein and has a specific activity 100-fold that of the ubiquitous urease (16, 18).

In addition to these two ureases is a third activity that we have designated as "background." This activity is revealed in

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a mutant lacking ubiquitous urease; in this communication we assign values of 2% (in expanded leaves [12]), 10% (in developing embryos [R. S. Torisky and J. C. Polacco, unpublished results], and 4% (in seedling roots [18]) for this activity relative to that of the ubiquitous urease. In some mutant tissues, however, notably callus (4, 12) and expanding unifoliates (J. C. Polacco and M. A. Holland, unpublished results), the background urease activity approaches 40% of wild type. Although it is possible that some of this activity is due to "leaky" ubiquitous urease expression by the mutant, we have attributed much of this urease activity to microbial commensals in soybean tissues: heat treatment of seeds reduces the endogenous bacterial population and reduces background urease activity to the same extent (4). In addition, bacteria living on soybean mutants that lack the background activity (8, 12) are impaired in the ability to produce fully active urease and hydrogenase, two enzymes containing active site nickel. This impairment persists in short-term culture of bacteria separated from the host plant (5; M. A. Holland and J. C. Polacco, manuscript in preparation).

We exploited urease-negative soybean mutants with lesions at the *Eu1*, *Eu3*, or *Eu4* loci to help clarify the role of urease in nitrogen metabolism. The *Eu1* locus has been postulated to contain the structural gene for embryo-specific urease because its various alleles produce either reduced levels of embryo-specific urease mRNA or altered protein (9). The *eu1-sun/eu1-sun* mutant used here lacks both transcript (7, 9) and protein (9) for the embryo-specific urease. We purified approximately 60-fold the residual urease of developing *eu1-sun/eu1-sun* embryos, and, by several criteria, it appeared to be exclusively ubiquitous urease (13). Because even 1% of the normal level of the embryo-specific urease in *eu1-sun/eu1-sun* would result in its contributing half of the total embryo urease activity, we conclude that the *eu1-sun* lesion eliminates virtually 100% of embryo-specific urease activity.

Homozygous *eu4* plants have 2% wild-type urease activity in expanded leaves (12), and similarly reduced activity in roots and hypocotyls (18). Recently, we have extended the observations of *eu4*-induced loss of ubiquitous urease activity to seed coats and to *eu1-sun/eu1-sun* embryos. Allelism (or tight linkage) between *Eu4* and a urease restriction fragment length polymorphism indicates that *Eu4* is likely the structural gene for the ubiquitous urease (J. D. Griffin and J. C. Polacco, unpublished results). The 2 to 10% activity level in *eu4/eu4* maternal tissues and *eu4/eu4,eu1-sun/eu1-sun* embryos represents the background urease described above.

The *eu3-e1/eu3-e1* mutant lacks the activities of all ureases (embryo-specific, ubiquitous, and background; its whole leaf activity is <10% that of *eu4/eu4* [12]). We suspect that this pleiotropic mutation affects a nickel insertion function common to the plant (8) and commensal bacterial (5) ureases.

Both *eu4/eu4* and *eu3-e1/eu3-e1* plants exhibit necrotic leaf tips (Fig. 1) which are likely due to accumulated urea because low-urease plants induced by nickel deprivation produce leaves that accumulate urea and have necrotic tips (2). Mutations at *Eu1*, *Eu3*, and *Eu4* are all recessive and unlinked (8, 12; N. Stebbins, Mark A. Holland, S. R. Cianzio, and J. C. Polacco, unpublished observations).

MATERIALS AND METHODS

Plant Material

The urease mutants used in this study have been previously characterized. The *eu3-1/eu3-1* (8) and *eu4/eu4* (12) genotypes, recovered from an ethylmethane sulfonate-derived M_2 population of soybean (*Glycine max*) cv 'Williams,' were first-backcross material. The *eu1-sun/eu1-sun* genotype (seed urease-null phenotype) was originally identified in PI 229.324 (6, 15) and was introgressed into the Williams genetic background by five backcrosses by Dr. R. Bernard (U. S. Department of Agriculture, Urbana, IL).

Seeds were germinated in rolled germination paper, at 28°C, and planted in a 1:2:1 mix of Pro-mix:high-clay soil:perlite. Plants were greenhouse-grown under natural light conditions. Crosses were made in April, May, and September 1990.

Preliminary identification of seed urease phenotypes was made by a seed chip assay (9). A seed chip, opposite the hilum, was removed with a razor blade and placed in 1.0 mL of test solution (0.1 M urea, 0.01 M KPO_4 [pH 7.0], 1 mM EDTA, 500 mg/L cresol red, and 0.2% NaN_3). The time for the pH-dependent color change from yellow to purple was characteristic of each phenotype (Williams and *eu4/eu4*, 15–30 min; *eu1-sun/eu1-sun*, 3–5 d; *eu3-e1/eu3-e1* and the *eu4/eu4,eu1-sun/eu1-sun* double mutant, no color change after 5 d [8; N. Stebbins, Mark A. Holland, S. R. Cianzio, and J. C. Polacco, unpublished observations]).

Tissue Extraction for Urea Analysis

Leaves (fully expanded from either flowering or pod-bearing plants) were frozen in liquid nitrogen and lyophilized. Before determinations were made, samples were stored at −20°C in a desiccator over P_2O_5. Weighed portions were ground in a mortar, extracted with approximately 40 volumes of ice cold 3.0% (w/v) $HClO_4$, and centrifuged in a clinical centrifuge (approximately 2 min). The supernatant was saved, the pellet was reextracted with 10 volumes of 3.0% $HClO_4$, and the supernatants were combined. Seeds and germinants were extracted in a similar fashion except that ground material was extracted three times in approximately 10 volumes (total) of 3.0% $HClO_4$. Perchlorate was removed from pooled supernatants by the addition of 1.25 volumes of 2.5 M K_2CO_3 and 1 mM EDTA. The potassium perchlorate precipitate was removed by centrifugation, and the volume of the neutralized supernatant was measured.

Urea Analysis

Urea was determined primarily by the Berthelot determination of ammonia following urease treatment (9). Extracts (5–30 μL) were analyzed directly by the addition of 25 μL of 0.4 M $KH_2/NaHPO_4$ buffer (pH 7.0), 1.9 units of Sigma type IX jack bean urease (5 μL, 380 units/mL; 1 unit releases 1 μmol of NH_3/min from urea at 25°C), and H_2O to 200 μL. Control samples were prepared the same way except that they were treated with urease inactivated by phenyl phosphorodiamidate (ICN Biochemicals, 0.06 nmol PPD/unit urease). Samples and controls were prepared in triplicate and incubated 10 min at 37°C, followed by the addition of 1.8 mL of H_2O and 0.2 mL each of alkaline hypochlorite and phenol nitroprusside solutions (Sigma). After the samples were heated at 65°C, for 10 min, they were cooled in a room temperature water bath, and A_{625} was determined. Urea was calculated from a urea standard that was mock extracted and assayed in

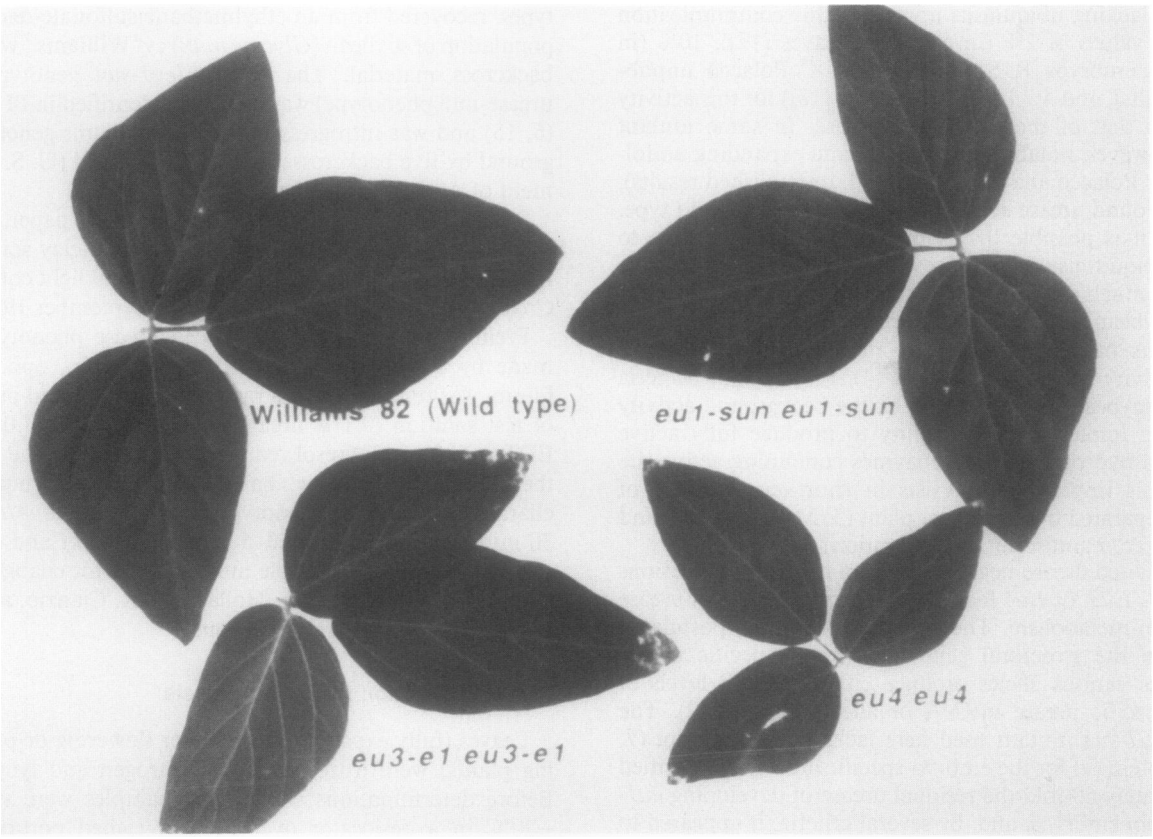

Figure 1. Leaves of genotypes used in this study. Only those genotypes that lack the ubiquitous urease, *i.e. eu4/eu4* and *eu3-e1/eu3-e1*, exhibit necrotic leaf tips. The *eu1-sun/eu1-sun,eu4/eu4* double mutant (not shown) had the leaf phenotype of the *eu4/eu4* single mutant. All fully expanded leaves were harvested from pod-bearing plants.

an identical fashion. Routinely, 1 nmol of urea gave an A_{625} of 0.018. Compounds in tissue extract did not affect reaction sensitivity.

Some of the earlier determinations of leaf urea were by the blood urea nitrogen (diacetyl monoxime) assay (as modified in ref. 13). These were discontinued because of unacceptably frequent sample turbidity. Leaf extracts (3 mL) were applied to a Dowex AG-1-X10 (formate form) anion exchange column (13) which was then rinsed twice with a total volume of 3 mL of water. Urea was determined in 0.5-mL aliquots of the pooled effluents (appropriately diluted with K_2CO_3/$HClO_4$-treated mock extracts). To control aliquots were added 12 units of jack bean urease. All samples were incubated in triplicate, for 10 min at 37°C, before the blood urea nitrogen determination (13). Control (urease-treated) values were subtracted from those of untreated samples. Urea was determined from a standard curve generated by known quantities of urea treated identically to the assay samples.

Cotyledon Culture

Developing cotyledons were cultured as described by Thompson *et al.* (17), except that vitamins were at half-strength, glycine was omitted, and nitrogen sources, urea or glutamine, were added at 40 meq N/L. Cotyledon protein was determined by boiling 25 mg of powdered, lyophilized cotyledon for 10 min in 2 mL of 10% TCA, collecting the insolubles by precipitation, boiling the resuspended pellet for 10 min in 2 mL of 0.1 N NaOH, and removing remaining insolubles by filtration (0.45 mm Metricel GA-6 filter, Gelman Sciences, Inc.). Protein in the filtrate was determined in the Missouri Agricultural Experiment Station Chemistry Laboratory from the total amino acid profile (less values for Cys and Trp) of acid hydrolysates.

RESULTS AND DISCUSSION

Maternal/Embryo Interactions Affecting Embryo Urea Accumulation

Seeds of the urease-negative mutant, *eu3-e1/eu3-e1*, which exhibits a pleiotropic loss of all activities (8, 12), were found to accumulate approximately 250 times as much urea (51 μmol/g dry weight, Table IB, line 2) as the Williams progenitor. To investigate the origin of this urea, we compared *eu3-e1/eu3-e1* seeds derived from selfed *eu3-e1/eu3-e1* plants with those from selfed *Eu3/eu3-e1* heterozygous plants. Although both seeds are urease negative, only those from urease-negative plants accumulated urea (Table I). That maternal tissue of *Eu3/eu3-e1* plants contains urease (8) and does not accumulate urea suggests that the urea in seeds from selfed *eu3-e1/eu3-e1* plants derives from the maternal tissue during seed development. A urease-positive embryo is able to hydrolyze

Table I. *Maternal/Embryo Interactions Affecting Embryo Urea Accumulation*

A. Urease Genotypes

Maternal (P)	Embryo (F_1)	How F_1 derived?
1. Williams 82 (*Eu3/Eu3*)	Williams 82 (*Eu3/Eu3*)	Self
2. *eu3-e1/eu3-e1*	*eu3-e1/eu3-e1*	Self
3. *Eu3/eu3-e1*	*eu3-e1/eu3-e1*	Self
4. *eu3-e1/eu3-e1*	*Eu3/eu3-e1*	Maternal × Williams 82
5. *Eu3/eu3-e1*	*Eu3/( )*	Self

B. Urease Phenotypes

Maternal (P)		Embryo (F_1)		
Ubiquitous + background urease	Leaf urea[a]	Ubiquitous + background urease	Embryo-specific urease	Seed urea
%	*μmol/g dry wt*	%	%	*μmol/g dry wt*
1. 100	0.07 (*n* = 2)	100	100	0.2 ± 0.2 (*n* = 3)
2. 0	96 ± 68 (*n* = 4)	0	0	51 ± 16 (*n* = 4)
3. 100	[b]	0	0	0.14 ± 0.24 (*n* = 3)
4. 0	96 ± 68 (*n* = 4)	100	100	0.4 ± 0.3 (*n* = 3)
5. 100	[b]	100	100	0.8 ± 0.2 (*n* = 3)

[a] Much of variability is stage specific; flowering plants usually exhibited less leaf urea than those bearing developing pods. Both plants were pooled (*n* = number of samples). Results are means ± SD. [b] Not determined. However, *Eu3/eu3-e1* plants exhibit wild-type levels of leaf urease (8).

this urea because *Eu3/eu3-e1* embryos developed on *eu3-e1/eu3-e1* plants did not accumulate urea (0.4 μmol/g dry weight, Table IB, line 4).

Test of Seed Urease Function by Genetic Ablation of Specific Isozymes

We set up crosses whereby maternal plants (*eu3-e1/eu3-e1*) lacked all urease activity and their heterozygous embryos lacked either the embryo-specific or the ubiquitous ureases and which retained the background activity in each case: homozygous double mutants at *Eu3* and at either *Eu1* or *Eu4* (*i.e. eu3-e1/eu3-e1,eu1-sun/eu1-sun* or *eu3-e1/eu3-e1,eu4/eu4*) were crossed with homozygous single mutants (either *eu1-sun/eu1-sun* or *eu4/eu4*, respectively) (Table IIA, lines 4 and 3). Because *eu3-e1* is recessive (8) F_1 heterozygous embryos are phenotypically *Eu3* but contain homozygous lesions at either *Eu1* or at *Eu4*, the putative structural genes for the embryo-specific and ubiquitous ureases, respectively. There was no urea accumulation in mature embryos that contained either the embryo-specific (Table IIB, line 3) or the ubiquitous (Table IIB, line 4) isozyme in addition to background urease activity.

The results presented so far are consistent with hydrolysis of delivered urea by any of the three enzymes in the developing embryo: embryo-specific, ubiquitous, or background urease (relative specific activities are 100, 1, and 0.1, respectively). To focus on the urea hydrolysis role of the background urease, we used the double mutant plant, *eu1-sun/eu1-sun,eu4/eu4* (Table II, A and B, line 2). Its maternal and embryonic tissues lack the ubiquitous urease (12, 18). Maternal *eu4/eu4* tissue accumulated considerable urea (185 ± 305 [8–800] μmol/g dry weight, mean ± SD, Table IIB, line 1), but the *eu1-sun/eu1-sun,eu4/eu4* embryo accumulated little, if any. Thus, any urea that may have been delivered to the embryo was hydrolyzed by its background urease.

To determine the quantity of urea delivered to the embryo by *eu4/eu4* maternal tissue, we examined completely urease-negative *eu3-e1/eu3-e1,eu4/eu4* embryos developed on selfed *Eu3/eu3-e1,eu4/eu4* plants (whose maternal tissues are effectively *eu4/eu4* and produce only the background urease). These "indicator" embryos accumulated a variable amount of urea, 35 ± 58 μmol urea/g dry weight (0.5–157), indicating that maternal background urease (in *eu4/eu4*, in *eu4/eu4,eu1-sun/eu1-sun*, or in *eu4/eu4,Eu3/eu3-e1* plants) did not completely suppress production of transportable urea; whereas in developing *eu4/eu4,eu1-sun/eu1-sun* embryos, the background urease quantitively hydrolyzed delivered urea.

Urea Accumulation in 2-d-Old Urease Mutant Seedlings

We reported that close to 100% of the urease activity of seedling radicles is actually a remnant of the embryo-specific urease laid down during embryogenesis (18). Thus, *eu1-sun/eu1-sun* seedlings provide a test of the functionality of this predominant embryo-specific urease in seedling radicles as

Table II. *Test of the Functions of the Seed Ureases by Genetic Ablation of Specific Isozymes*

A. Urease Genotypes

Maternal (P)	Embryo (F_1)	How F_1 derived?
1. *eu4/eu4*	*eu4/eu4*	Self
2. *eu4/eu4,eu1-sun/eu1-sun*	*eu4/eu4,eu1-sun/eu1-sun*	Self
3. *eu3-e1/eu3-e1,eu4/eu4*	*Eu3/eu3-e1,eu4/eu4*	Maternal × *eu4/eu4*
4. *eu3-e1/eu3-e1,eu1-sun/eu1-sun*	*Eu3/eu3-e1,eu1-sun/eu1-sun*	Maternal × *eu1-sun/eu1-sun*
5. *eu3-e1/eu3-e1*	*eu3-e1/eu3-e1*	Self

B. Urease Phenotypes

Maternal (P)		Embryo (F_1)		
Ubiquitous + background urease	Leaf urea[a]	Ubiquitous + background urease	Embryo-specific urease	Seed urea
%	*μmol/g dry wt*	%	%	*μmol/g dry wt*
1. 2	185 ± 305 (*n* = 6)	10	100	0 (*n* = 3)
2. 2	[b]	10	0	0.6 ± 0.2 (*n* = 10)
3. 0	56	10	100	0.4 ± 0.4 (*n* = 10)
4. 0	[b]	100	0	0 ± 0.1 (*n* = 3)
5. 0	96 ± 68 (*n* = 4)	0	0	51 ± 16 (*n* = 4)

[a] Much of variability is stage specific; flowering plants usually exhibited less leaf urea than those bearing developing pods. Both plants were pooled (*n* = number of samples). Results are means ± SD. [b] Although leaf urea levels were not determined for genotypes 2 and 4, it is expected that their urea levels would not differ from those of genotypes 1 and 5, respectively, (*i.e. eu4/eu4* and *eu3-e1/eu3-e1*), the only pertinent genotypes affecting leaf urease activity (8, 12).

well as in cotyledons. When compared with Williams 82 seedlings, *eu1-sun/eu1-sun* shows no enhanced accumulation of urea in either the radicle or the cotyledon (approximately 2 μmol/g dry weight in radicles of both genotypes; first two entries, Table III), indicating that the embryo-specific urease does not hydrolyze urea generated in germinating seeds. In contrast, elimination of the ubiquitous urease in *eu4/eu4* seedlings doubles the accumulated urea to about 4 μmol urea/g dry weight radicle (Table III).

The *eu1-sun/eu1-sun,eu4/eu4* double mutant seedling accumulated no more urea than the *eu4/eu4* single mutant, confirming the lack of a role in urea hydrolysis for the embryo-specific urease. Dormant *eu1-sun/eu1-sun,eu4/eu4* double mutant seeds accumulated no significant urea (0.6 μmol/g dry weight; Table II, line 2), indicating that the urea in double mutant seedlings was generated during germination.

To assess the maximal amount of urea generated from seed nitrogen stores during germination, we determined urea in *eu3-e1/eu3-e1* seedlings generated from urea-free seeds (*i.e.* from selfed heterozygous plants; line 3, Table IA). These seedlings lack, in addition to the embryo-specific and ubiquitous ureases, the background urease of the *eu1-sun/eu1-sun,eu4/eu4* double mutant. As can be seen in Table III, 2-d *eu3-e1/eu3-e1* seedlings accumulated even higher levels of radicle urea (34 μmol/g dry weight) than the double mutant. Urea levels increase 2.5-fold in 3-d seedlings. If 10% of the dry weight of *eu3-e1/eu3-e1* radicle tissue is protein (6.25% N), then urea (48% N) not utilized at day 3 (83 μmol/g dry weight) represents 37% of the nitrogen incorporated into protein.

Growth Response of Cotyledon Cultures of Soybean Urease Mutants on Urea and Glutamine Nitrogen Sources

Developing cotyledons were cultured *in vitro* (17) with urea or glutamine nitrogen source (40 meq N/l; Table IV). We observed, similarly to Thompson *et al.* (17), that standard (Williams 82) cotyledons gained in fresh weight even in the absence of added nitrogen. Also in agreement with their observation was our finding that urea was not only a poor nitrogen source but that it actually reduced fresh weight increase relative to that on either glutamine or on medium lacking a nitrogen source. This is not surprising in light of the increased alkalinity (pH 8.3 *versus* pH 5.5 or 5.6 for glutamine or no-N cultured cotyledons) and obviously high ammonia content of the medium of urea-cultured Williams 82 cotyledons (Table IV). This urea toxicity was dependent on an active embryo-specific urease because the *eu1-sun/eu1-sun* genotypes lacking this activity were not growth restricted by urea nor did they induce sharp increases in the alkalinity of urea-containing medium.

We expected that the ubiquitous urease would support *in*

Table III. *Urea Accumulation in 2-d-Old Seedlings*

All seedlings were from seeds lacking urea. The *eu3-e1/eu3-e1* seedling was the progeny of a selfed heterozygous plant, and its seed had little or no urea at the start of imbibition (Table I, line 3). All entries are the averages of at least two separate experiments.

Genotype	Urease Level		Urea Level	
	Ubiquitous + background	Embryo-specific	Radicle	Cotyledon
	%	%	*μmol/g dry wt*	
Williams 82 (*Eu1/Eu1, Eu3/Eu3,Eu4/Eu4*)	100	100	2.1	0.1
eu1-sun/eu1-sun	100	0	1.8	0.2
eu4/eu4	4[a]	100	4.1	0.3
eu1-sun/eu1-sun,eu4/eu4	4[a]	0	4.9	0.8
eu3-e1/eu3-e1[b]	0	0	33.2[b]	11.7[b]

[a] From values reported for 4-d seedlings (18). [b] At 3 d the radicle and cotyledon urea levels were 83 and 16 μmol/g dry wt, respectively.

vitro cotyledon development because its activity level, being much lower than that of the embryo-specific urease, might facilitate a more measured conversion of urea to ammonia. Although the ubiquitous urease in cotyledons lacking the embryo-specific urease (compare *eu1-sun/eu1-sun* and *eu1-sun/eu1-sun,eu4/eu4*) had little bearing on urea-supported increases in fresh weight, its presence resulted in greater protein deposition. At least some of the protein deposited in *eu1-sun/eu1-sun,eu4/eu4* cotyledons is due to the action of the background urease because protein levels were further reduced in *eu3-e1/eu3-e1* cotyledons that lack all three activities (Table IV). Fresh weight increases and protein deposition supported by glutamine appeared to be in the same range for all genotypes.

SUMMARY

Selective genetic ablation of urease activities shows: (a) embryo-specific urease, produced as an abundant protein during seed development and present in the cotyledons and radicles of germinating seed (18), does not affect the ability of the plant to metabolize urea except in the unnatural situation in which cotyledons have been wounded by excision from the seed and placed in culture; (b) ubiquitous urease, produced at relatively low levels in all tissues, completely prevents the

Table IV. *Urease Mutant Cotyledon Development in Vitro with Glutamine and Urea Nitrogen Sources*

Genotype	Urease Level[a]		Growth[b]		Protein[c]		Final pH	
	Ubiquitous (+ background[d])	Embryo-specific	no-N	Urea	no-N	Urea		
			(no-N/Urea)					
	%		%		%			
Williams 82	100	100	90	32	59	25	Urea	8.3
							Gln	5.5
			(2.8)				no-N	5.6
eu1-sun/eu1-sun	100	0	64	62	39	70	Urea	6.7
							Gln	6.0
			(1.0)				no-N	5.7
eu4/eu4	10	100	80	38	43	28	Urea	7.6
							Gln	5.8
			(2.1)				no-N	5.9
eu1-sun/eu1-sun,eu4/eu4	10	0	59	69	41	48	Urea	6.3
			(0.9)				Gln	5.9
							no-N	5.5
eu3-e1/eu3-e1	0	0	64	74	32	38	Urea	5.5
			(0.9)				Gln	5.8
							no-N	5.4

[a] Urease specific activity compared with Williams 82. [b] Fold fresh weight increases during 6-d culture on no-N or on urea-N relative to fold increase of paired cotyledon on 20 mM glutamine. At least two embryos were measured for each entry. Average fold fresh weight increase on glutamine-N for all genotypes was approximately 3.5. [c] Expressed as total cotyledon protein of 6-d culture relative to protein content of paired cotyledon grown with glutamine. Average final protein content (mg/cotyledon) on glutamine for each genotype was, in order, 3.8, 7.3, 4.45, 7.35, and 5.4. Two to four embryos were assayed for protein within each genotype:N source category. [d] Background urease activity in *eu4/eu4* developing cotyledons is from data reported in ref. 18.

accumulation of urea in maternal tissues (leaves), and at least partly prevents its accumulation in developing embryos, and in germinating seed. In cotyledon culture, the low-level activity of this enzyme supports protein synthesis when urea is sole nitrogen source; (c) the background urease, which we suggest represents the activity of bacteria naturally associated with the plant (4, 5), does not prevent maternal tissues from accumulating urea, but it is sufficient to prevent accumulation of high levels of urea in developing embryos and in germinating seeds. In cultured cotyledons, the background urease activity is sufficient to support protein synthesis on a urea nitrogen source, although to an extent less than that in the presence of the ubiquitous urease. We point out that, whether the background urease is of plant or bacterial origin, its striking feature is its ability to hydrolyze all or most urea in embryos and germinants lacking the ubiquitous urease.

We conclude that the ubiquitous and background ureases are the activities normally responsible for urea hydrolysis in wild-type soybean. The embryo-specific urease does not play a physiologically meaningful role in urea metabolism. Results from cotyledon culture suggest that its primary function may be that of a toxic defense protein.

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