Interallelic Complementation at the Ubiquitous Urease Coding Locus of Soybean

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Soybean (Glycine max [L.] Merrill) mutant aj6 carries a single recessive lesion, aj6, that eliminates ubiquitous urease activity in leaves and callus while retaining normal embryo-specific urease activity. Consistently, aj6/aj6 plants accumulated urea in leaves. In crosses of aj6/aj6 by urease mutants at the Eu1, Eu2, and Eu3 loci, F1 individuals exhibited wild-type leaf urease activity, and the F2 segregated urease-negative individuals, demonstrating that aj6 is not an allele at these loci. F2 of aj6/aj6 crossed with a null mutant lacking the Eu1-encoded embryo-specific urease showed that ubiquitous urease was also inactive in seeds of aj6/aj6. The cross of aj6/aj6 to eu4/eu4, a mutant previously assigned to the ubiquitous urease structural gene (R.S. Torisky, J.D. Griffin, R.L. Yenofsky, J.C. Polacco [1994] Mol Gen Genet 242: 404–414), yielded an F1 having 22% ± 11% of wild-type leaf urease activity. Coding sequences for ubiquitous urease were cloned by reverse transcriptase-polymerase chain reaction from wild-type, aj6/aj6, and eu4/eu4 leaf RNA. The ubiquitous urease had an 837-amino acid open reading frame (ORF), 87% identical to the embryo-specific urease. The aj6/aj6 ORF showed an R201C change that cosegregated with the lack of leaf urease activity in a cross against a urease-positive line, whereas the eu4/eu4 ORF showed a G468E change. Heteroallelic interaction in F2 progeny of aj6/aj6 × eu4/eu4 resulted in partially restored leaf urease activity. These results confirm that aj6/aj6 and eu4/eu4 are mutants affected in the ubiquitous urease structural gene. They also indicate that radical amino acid changes in distinct domains can be partially compensated in the urease heterotrimer.

The main function of urease in plants is to recycle N from urea. In soybean (Glycine max [L.] Merrill), this is particularly important during germination when storage proteins are mobilized to nourish the seedling. Most of the large endogenously generated urea pool comes from Arg (Stebbins and Polacco, 1995), which constitutes 18% of storage protein N (Micallef and Shelp, 1989) and is actively degraded to urea and Orn upon germination (Goldraij and Polacco, 1999). Urease catalyzes N reconversion from urea to ammonia, which is subsequently assimilated via Gln synthetase (Lam et al., 1996). Urease also recycles N derived from urea exogenously applied as a foliar fertilizer (Witte et al., 2002). A potential urease role in maintaining N2 fixation under water stress was posited in a model by Purcell et al. (2000) and Vadez and Sinclair (2001), whereby urea is generated directly from ureides in soybean cultivars (e.g. Maple arrow) able to fix N2 under water deficit. Ureides are converted to urea in reactions that are not affected by water deficit thus avoiding ureide buildup and its inhibition of N fixation (Serraj et al., 1999). In this model, urease is essential to N use in soybean cultivars adapted to dry areas where fixed N is the sole or major N source. Drought-sensitive cultivars, however, are purported to bypass urea production by generating ammonia directly from ureides.

All plant and bacterial ureases described so far are metalloenzymes having a nickel metallocenter (Dixon et al., 1975; Hausinger et al., 2001). In Klebsiella aerogenes, where urease structure and its activation are best understood, the enzyme is a trimer of trimers (α, β, γ)3 in which α, β, and γ are encoded by ureC, ureB, and ureA, respectively. To become functional, apoureae is activated by four known accessory proteins (UreD, UreE, UreF, and UreG) to incorporate two nickel atoms per active site (Jabri et al., 1995; Hausinger et al., 2001).

Plant ureases are multimers (Polacco et al., 1985) of a single subunit that is colinear with the three bacterial subunits (Mobley et al., 1995). In soybean, there are two distinct, nonallelic urease isoforms, differing in both structural and biochemical properties (for a summary, see Bacanamwo et al., 2002a). The embryo-specific urease is synthesized exclusively in developing embryos (Torisky and Polacco, 1990) and is encoded by the Eu1 locus (Meyer-Bothling and Polacco, 1987). Apparent electrophoretic variants are trimers or hexamers (Buttery and Buzzell, 1971), although the genetic hexamer can be converted to the trimer by treatment with heat, Suc, or glycerol (Polacco and Havir, 1979). The ubiquitous urease is a constitutive enzyme expressed in all organs and encoded by the Eu4 locus (Polacco et al., 1989). It is trimeric in all varieties that we have examined (Polacco et al., 1985). The absence of ubiquitous urease activity in soybean

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results in necrotic leaf tips due to urea accumulation (Stebbins et al., 1991).

At least two other genes, Eu2 and Eu3, encode accessory proteins necessary for urease activity (Meyer-Bothling et al., 1987). A mutation in either of them is pleiotropic, eliminating the activities of both ureases. Although Eu3 encodes a nickel-binding protein, the plant ortholog of bacterial UreG (Freyermuth et al., 2000), the identity and specific function of the Eu2 protein is still unknown. Eu2 and Eu3 proteins interact to activate urease; incubation of mixed crude extracts from developing embryos of eu2/eu2 and eu3/eu3 mutants slowly gain urease activity (Polacco et al., 1999). Also, the orthologs of bacterial urease accessory genes ureD and ureF have been recently identified in soybean, and at least ureF has been showed to be functional by complementation of the homologous urease-negative mutant of fission yeast (Schizosaccharomyces pombe; Bacanamwo et al., 2002b).

We report here the genetic and molecular characterization of a urease-negative mutant, aj6/aj6, and complete the previous characterization of the eu4 lesion, which was assigned to the ubiquitous urease structural gene (Torisky et al., 1994). We demonstrate that both mutants have lesions in different regions of the Eu4 locus. Moreover, in the heterozygous progeny derived from a cross of the two urease-negative mutants, urease activity is partially restored, indicating that the products of both alleles interact, with at least one heterotrimer regaining catalytic activity.

RESULTS

Soybean aj6 Contains a Homozygous Recessive Lesion Eliminating Ubiquitous Urease Activity

Soybean mutant aj6 was recovered from a pooled M2 seed population. It exhibited urease-negative leaves and seeds with variable activity in the M3 and M4 generations (Polacco et al., 1989). We quantified urease activity and urea content of seeds and leaves in a true breeding line of aj6 advanced for several generations (Fig. 1). Seed urease activity and total seed urea content were similar in both aj6 and in its urease-positive progenitor, soybean cv Williams. In contrast, leaf urease activity was undetectable in aj6. Consistently, urea accumulated in leaves of aj6 to levels about 65-fold over wild type. aj6 adult plants exhibited necrotic leaf tips likely due to “urea burn,” a typical trait of plants lacking leaf ubiquitous urease activity (Eskew et al., 1983; Stebbins et al., 1991).

In an aj6 × wild type cross, the F2 segregation pattern of leaf urease-positive to leaf urease-negative individuals was close to 3:1, suggesting that the aj6 trait is due to a single recessive mutation (Table I). Henceforth, the lesion is called aj6, and the mutant genotype is designated aj6/aj6.

To test the activity of ubiquitous urease in aj6/aj6 seeds it was necessary to remove the masking embryo-specific urease. The eu1-sun/eu1-sun mutant has normal levels of ubiquitous urease but lacks the embryo-specific urease whose wild-type-specific activity, being almost $8 \times 10^3$ times higher, masks the expression of ubiquitous urease in the embryo (Meyer-Bothling and Polacco, 1987). F2 progeny from an aj6/aj6 × eu1-sun/eu1-sun cross were analyzed by the seed chip assay, a qualitative assay in which the time for alkalinization of the medium, detected by cresol red, was used to distinguish the activities of urease isozymes in soybean seeds (Meyer-Bothling and Polacco, 1987). The segregation ratio in the F2 generation (Table II) was consistent with the presence of two independent lesions in different isozymes and confirmed that the ubiquitous urease isozyme is inactive in aj6/aj6 seeds. The urease phenotype of the double mutant seeds, eu1-sun/eu1-sun, aj6/aj6, resembled that of eu3-e1/eu3-e1, a pleiotropic

Figure 1. Urease phenotype in the soybean mutant aj6. Urease activity (A) and urea content (B) determined in dry seed and trifoliate leaf of wild type and aj6. Results are means ± SD of three independent experiments.
mutation in the urease accessory gene UreG that lacks all urease activities (Freyermuth et al., 2000).

Consistent with the absence of leaf urease activity, a leaf-derived callus of aj6/aj6 was unable to grow in 2.5 mm urea as sole N source (Fig. 2, A and B). Conversely, in the presence of a permissible N source, it was not affected by 50 mm urea, a concentration toxic to urease-positive callus due to the abundant ammonia generated by urease action (Polacco et al., 1989; Stebbins et al., 1991). Callus urease activity was almost undetectable in aj6/aj6 (Fig. 2C).

Thus, the pattern of urease activity and urea accumulation in aj6/aj6 was similar to that of the partially characterized eu4/eu4 mutant (Torisky et al., 1994), in which a mutation linked to or within the structural gene of the ubiquitous urease was responsible for the loss of its enzymatic activity.

Allelism Tests and Molecular Analyses of aj6/aj6

In the crosses of aj6/aj6 to mutants at the Eu1, Eu2, and Eu3 loci, all F1 individuals were urease positive, and their activities were close to that of the F1 of aj6/aj6 × wild type (Tables I and III). These data along with the patterns of segregation in the F2 generations (Table III) suggested that the aj6 mutation was not an allele of these genes. Consistent with this conclusion was the distinct phenotype of aj6/aj6, in which only the leaf urease was affected. In contrast, the levels of leaf urease activities in the F1 generation derived from the aj6/aj6 × eu4/eu4 cross were notably lower than those of the F1 of the aj6/aj6 crosses to the other mutants. Given the similarities of the phenotypic traits between aj6 and eu4, we considered the possibility that both mutations could be affecting the same locus, complementing each other to produce a partially active F1 progeny. If this were the case, both mutations would be linked, and the phenotypic segregation ratio expected in the F2 generation would be 1:1 (negative; partially positive), which was close to that experimentally obtained (Table III).

To identify the aj6 and eu4 mutations, the ubiquitous urease open reading frame (ORF) was obtained by reverse transcriptase (RT)-PCR from leaf RNA isolated from aj6/aj6, eu4/eu4, and soybean cv Williams, progenitor of both mutants. No significant differences were seen in the levels of the amplified products, suggesting that both mutants have normal levels of ubiquitous urease transcripts (data not shown). The deduced amino acid sequence of the ubiquitous urease ORFs (Fig. 3) showed that in aj6/aj6, Arg was changed to Cys at position 201 (R201C). In eu4/eu4, Gly at position 468 was replaced by Glu (G468E), corroborating at the molecular level that the previously reported mutation in the locus Eu4 (Torisky et al., 1994) is a lesion in the ubiquitous urease structural gene. The ubiquitous urease nucleotide sequence in the wild type was essentially identical (one silent nucleotide change) to the GenBank cDNA sequence of soybean urease ( accession no. AJ726866) from an unidentified cultivar and tissue.

The embryo-specific urease ORF from wild type (Meyer-Bothling et al., 1987), whose complete coding sequence has not heretofore been reported, was cloned by RT-PCR from dry seeds. A comparison of the deduced amino acid sequence between both soybean ureases and the products of ureA, ureB, and ureC genes (i.e. y, β, and α, respectively) of K. aerogenes is shown in Figure 3. We term the single subunits for soybean ureases αuu and αeu, for the ubiquitous and embryo-specific ureases, respectively. αuu and αeu share 87% identity and 92% similarity. Soybean αuu was found to share 73% and 76% amino acid identity and 82% and 86% similarity with leaf ureases of rice (Oryza sativa; accession no. BAB78715) and potato (Solanum tuberosum; accession no. CAC43859), respectively.

Table 1. F2 transmission of leaf urease trait in aj6

<table>
<thead>
<tr>
<th>Cross</th>
<th>F1 Urease Activity</th>
<th>Observed F2 Phenotypic Ratio</th>
<th>n</th>
<th>χ^2</th>
<th>P^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>aj6 (♀) × soybean cv Kenwood (♂)</td>
<td>27.8 ± 11.3(n=4)</td>
<td>+:::</td>
<td>145</td>
<td>19</td>
<td>0.017</td>
</tr>
</tbody>
</table>

a Parental activities: soybean cv Kenwood, 26.2 ± 10 (n = 9); aj6, 0 (n = 10) nmol urea h^-1 leaf disk^-1. b Urease activity was 23.55 ± 15 and 0 for urease-positive and urease-negative individuals, respectively. c Probability of random deviation from a 3:1 ratio.

Table 2. F2 segregation of seed urease phenotype determined by the seed chip assay

<table>
<thead>
<tr>
<th>Cross</th>
<th>Purple^a after 20 min (Embryo-Specific +; Ubiquitous or −)</th>
<th>Pale Pink after 48 h (Embryo-Specific −; Ubiquitous +)</th>
<th>Yellow after 5 d (Embryo-Specific −; Ubiquitous −)</th>
<th>n</th>
<th>χ^2</th>
<th>P^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>eu1-sun/eu1-sun (♀) × aj6/aj6 (♂)</td>
<td>77 (72)</td>
<td>15 (18)</td>
<td>4 (6)</td>
<td>96</td>
<td>1.52</td>
<td>&gt;0.3</td>
</tr>
</tbody>
</table>

a Color due to the pH increase produced by ammonia generated by urease action. b Probability of random deviation from the expected ratio (12:3:1) with two degrees of freedom assuming non-linkage between the eu1-sun and aj6 lesions. Ideal expectations are in parentheses. c Parental phenotypes are pale pink after 48 h (eu1-sun/eu1-sun) and purple after 20 min (aj6/aj6).
As was shown previously for jack bean (Canavalia ensiformis) urease (Mobley et al., 1995), both α_uu and α_eu align with three urease bacterial subunits (Fig. 3). K. aerogenes γ, β, and α and the corresponding regions from α_uu share 56%, 49%, and 69% identity and 71%, 61%, and 80% similarity, respectively. It is important to point out that the aj6 and eu4 mutations are allelic, but they are in different “domains,” homologous to bacterial β and α, respectively. A BLASTp using the deduced amino acid sequence of soybean ubiquitous urease as a query in the GenBank (Altschul et al., 1997) showed that all urease amino acid sequences reported conserve both the R201 altered in aj6/aj6 and the G468 altered in eu4/eu4.

The Lack of Ubiquitous Urease Activity in aj6/aj6 Cosegregates with R201C

To confirm the association between the absence of ubiquitous urease activity and the mutation at position 201 in α_uu of aj6/aj6, the cDNA sequences of five urease-negative and six urease-positive F2 individuals from the aj6/aj6 × wild type cross were analyzed (Table IV). As expected, leaf urease-positive individuals were either homozygous or heterozygous at codon 201, encoding either the wild-type Arg or both Arg and Cys, respectively. All leaf urease-negative plants, however, were homozygous at position 201, encoding Cys. The cosegregation of both traits suggests strongly that R201C in the ubiquitous urease ORF of aj6/aj6 eliminates ubiquitous urease activity.

Partial Interallelic Complementation between aj6 and eu4

The F1 of the aj6/aj6 × eu4/eu4 cross was urease positive but its activity was clearly less than either the wild-type or the F1 levels in crosses of aj6/aj6 with wild type and with the mutants eu1-sun/eu1-sun, eu2/eu2, and eu3-e1/eu3-e1 (Table III). F2 from the aj6/aj6 × eu4/eu4 cross were analyzed for leaf urease activities and the segregation of the aj6 and eu4 mutations (Table V). Only those individuals heterozygous for both the aj6 and eu4 alterations were urease positive and their activity levels ranged from 14% to 47% of wild-type activity. Thus, in the F2

**Table III. Allelism tests of aj6/aj6**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Locus</th>
<th>Leaf Urease Activity</th>
<th>F1 Urease Activity</th>
<th>Expected F2 Phenotypic Ratio</th>
<th>F2 Phenotypic Distribution</th>
<th>n</th>
<th>χ²</th>
<th>P *</th>
</tr>
</thead>
<tbody>
<tr>
<td>eu1-sun</td>
<td>Eu1</td>
<td>+</td>
<td>26.9 ± 5.4(n=4)</td>
<td>3:1</td>
<td>16:4</td>
<td>20</td>
<td>0.27</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>eu2</td>
<td>Eu2</td>
<td>−</td>
<td>21.5 ± 5.9(n=6)</td>
<td>9:7</td>
<td>20:8</td>
<td>28</td>
<td>2.61</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>eu3-e1</td>
<td>Eu3</td>
<td>−</td>
<td>25.7 ± 5.7(n=4)</td>
<td>9:7</td>
<td>20:8</td>
<td>20</td>
<td>0.62</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>eu4</td>
<td>Eu4</td>
<td>−</td>
<td>5.8 ± 3.1(n=7)</td>
<td>9:7(non-linked)</td>
<td>16:0</td>
<td>16</td>
<td>0.95</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

* aj6 plants were ? in all crosses except when crossed with eu1-sun/eu1-sun. ** Expected F2 ratios assuming that the lesion in aj6/aj6 is at a locus unlinked to Eu1, Eu2, or Eu3 and either unlinked to (9:7) or allelic with (1:1) Eu4. * Probabilities of random deviation from the expected values with 1 degree of freedom.
generation the two alleles partially complement to restore some leaf urease activity. The activity in these heterozygous individuals showed high instability after prolonged tissue storage at \(-80^\circ C\) or tissue extraction with buffer (data not shown). Despite this, urease activity was detected in native gels (Fig. 4). The identical migration of both ureases suggests strongly that urease subunits in the heterozygous progeny are also assembled into a trimer. A trimeric conformation was previously reported for soybean ubiquitous urease (Polacco et al., 1985). Urease activity in the F\(_2\) progeny also demonstrates that the lack of activity in \(aj6/aj6\) is not due to the absence of ubiquitous urease protein. Ubiquitous urease protein was reported to be present in \(eu4/eu4\) (Polacco et al., 1989). Both genotypic and phenotypic segregation ratios were experimentally close to those expected in the F\(_2\) for two allelic or tightly linked mutations (1:2:1).

**Figure 3.** Deduced amino acid sequences of soybean ureases aligned with *K. aerogenes* urease subunits. UreA (γ), UreB (β), and UreC (α) are the subunits of the urease heterotrimer in most bacteria. These subunits are roughly colinear with urease amino acid sequence of plants. Identities are indicated in black boxes, similarities in gray boxes. The arrows indicate the R201C and G468E alterations in the ubiquitous ureases of \(aj6/aj6\) and of \(eu4/eu4\), respectively. Accession numbers: soybean ubiquitous urease, AY230156; soybean embryo-specific urease, AY230157; and *K. aerogenes* urease subunits UreA, P18316; UreB, P18315; and UreC, P18314.
Table IV. F2 cosegregation of the aj6 lesion with the leaf urease-negative phenotype

<table>
<thead>
<tr>
<th>Plant</th>
<th>Urease Activity Phenotype</th>
<th>Urease Locus Genotype</th>
<th>Urease Amino Acid at Position 201</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>+</td>
<td>aj6/+</td>
<td>Arg/Cys</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>aj6/+</td>
<td>Arg</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>aj6/+</td>
<td>Arg/Cys</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>aj6/+</td>
<td>Arg/Cys</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>aj6/+</td>
<td>Arg/Cys</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>aj6/+</td>
<td>Arg</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>aj6/aj6</td>
<td>Cys</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>aj6/aj6</td>
<td>Cys</td>
</tr>
<tr>
<td>13</td>
<td>–</td>
<td>aj6/aj6</td>
<td>Cys</td>
</tr>
<tr>
<td>17</td>
<td>–</td>
<td>aj6/aj6</td>
<td>Cys</td>
</tr>
<tr>
<td>D</td>
<td>–</td>
<td>aj6/aj6</td>
<td>Cys</td>
</tr>
</tbody>
</table>

*Urease activity was 25.7 ± 19.3 and 0 nmol urea h⁻¹ leaf disk⁻¹ in urease-positive and urease-negative individuals, respectively.

**DISCUSSION**

The aj6 lesion was demonstrated to be a single recessive mutation (Table I). Like other soybean mutants lacking leaf urease activity, aj6/aj6 accumulated considerable leaf urea (Fig. 1). The analysis of F2 segregants in the cross of aj6/aj6 to eu1-sun/eu1-sun, the latter a null mutant in the Eu1 locus that encodes the embryo-specific urease (Meyer-Bothling and Polacco, 1987), confirmed that the aj6 mutation also affects the ubiquitous urease activity in seeds (Table II). Thus, the aj6 mutation effects were similar to those of the eu4 mutation, which was assigned to the ubiquitous urease structural gene (Torisky et al., 1994). Allelism tests of aj6 with eu1-sun and with the pleiotropic mutations eu2 and eu3-e1, the latter two involved in the activation of the two soybean urease isozymes (Meyer-Bothling et al., 1987; Polacco et al., 1999), demonstrated that the aj6 mutation is not in Eu1, Eu2, or Eu3 (Table III). The aj6/aj6 × eu4/eu4 F1 was urease positive but its activity was clearly lower than wild-type activity, unlike the full F1 activity levels in crosses of aj6/aj6 with mutants in the Eu1, Eu2, and Eu3 loci (Table III). These results indicate that aj6 and eu4 are different but related mutations.

The leaf urease ORFs of wild type, aj6/aj6, and eu4/eu4 suggested that the lack of ubiquitous urease activity in each mutant was due to a single nucleotide missense alteration. In aj6/aj6, the change was Arg to Cys at position 201 whereas in eu4/eu4, a Gly was replaced by Glu at position 468. Both changes are radical and all bacterial, fungal, and plant urease sequences producing significant alignments with soybean ubiquitous urease in the GenBank retain wild-type Arg (201) and Gly (468) at these positions. Thus both amino acids are likely important for maintaining urease structure and/or function. eu4/eu4 callus was partially corrected with a 15-kD genomic DNA fragment containing the wild-type ubiquitous urease coding sequence (Torisky et al., 1994). The G468E change reported in this work confirms that eu4 is a lesion in the ubiquitous urease structural gene.

**Table V. Complementation of aj6 and eu4 alleles in the F2 of aj6/aj6 × eu4/eu4**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Leaf Urease Activity</th>
<th>Genotype at aj6 Site (Residue 201)</th>
<th>Genotype at eu4 Site (Residue 468)</th>
<th>Wild-Type Activity (n = 3) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>aj6/+</td>
<td>eu4/+</td>
<td>21 ± 10</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>aj6/+</td>
<td>eu4/+</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>aj6/aj6</td>
<td>+/+</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>aj6/aj6</td>
<td>eu4/eu4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>aj6/aj6</td>
<td>eu4/eu4</td>
<td>39 ± 11</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>aj6/+</td>
<td>eu4/eu4</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>aj6/aj6</td>
<td>+/+</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>aj6/aj6</td>
<td>+/+</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>aj6/aj6</td>
<td>eu4/eu4</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>aj6/aj6</td>
<td>+/+</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>aj6/+</td>
<td>eu4/+</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>aj6/aj6</td>
<td>eu4/eu4</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>aj6/aj6</td>
<td>eu4/eu4</td>
<td>20 ± 20</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>aj6/aj6</td>
<td>eu4/eu4</td>
<td>27 ± 13</td>
</tr>
<tr>
<td>15</td>
<td>–</td>
<td>aj6/aj6</td>
<td>+/+</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>–</td>
<td>aj6/aj6</td>
<td>+/+</td>
<td>0</td>
</tr>
</tbody>
</table>

*Wild-type activity was 25 ± 8 nmol urea h⁻¹ leaf disk⁻¹ (n = 9).
genomic clone containing the ubiquitous urease structural gene (Torisky et al., 1994). This, coupled with phenotypic and genotypic segregation patterns in the eu4/eu4 × aj6/aj6 cross, shows convincingly that aj6 is a second mutant allele in the Eu4 locus.

The simplest explanation for the interallelic complementation between the aj6 and eu4 alleles is the functional interaction of polypeptides mutated in different sites. In the cross of aj6/aj6 to eu4/eu4, both mutations are linked, consistent with an experimental phenotypic ratio of leaf urease positive to leaf urease negative in the F2 close to 1:1 (Tables III and V). The heterozygous progeny produce two types of polypeptides each having a mutation in the aj6 or eu4 sites. Association of these polypeptides forms two types of heterotrimers viz. \( \alpha_{uu}(R201C)_2\alpha_{uu}(G468E) \) and \( \alpha_{uu}(R201C)\alpha_{uu}(G468E)_2 \), at least one of which has activity. Considering the clear sequence colinearity of \( \alpha_{uu} \) with the three (or two) subunits of bacterial ureases (Mobley et al., 1995), it is reasonable to assign R201C and G468E to different domains. Therefore, eu4/aj6 heterozygotes have at least one intact copy of each domain per heteromeric urease molecule.

Interallelic complementation has been extensively reported in a wide variety of organisms. A familiar example is the \( \alpha \)-complementation in \( \beta \) galactosidase of \( E. \) coli, which is the basis of identifying bacterial colonies carrying recombinant DNA (Ullman et al., 1967). Early reports in plants include interallelic complementation in maize at the alcohol dehydrogenase (Schwartz, 1975) and Suc synthase (Chourey and Nelson, 1979) loci. More recently, interallelic complementation has been reported in a protein required for coordination of cell polarity in Arabidopsis (Grebe et al., 2000). Examples are found in other organisms such as fungi (Sanz et al., 2002), fruitfly (Drosophila melanogaster; Ponomareff et al., 2001), and mouse (Steingrimsson et al., 2003).

Given the similarities between \( \alpha_{uu} \) and the urease of \( K. \) aerogenes (Fig. 3), we sought to localize the plant mutations on the three-dimensional structure of the bacterial enzyme. In the nonameric (\( \alpha, \beta, \gamma \)) structure of the \( K. \) aerogenes urease protein (Jabri et al., 1995), the homologous positions for the ubiquitous urease R201 (\( \beta R70 \)) and G468 (\( \gamma G197 \)) residues are shown in Figure 5. Neither residue is involved in the active site of the enzyme. \( \beta R70 \) is very close to the interface of two trimers, equivalent to the \( \alpha_{uu}:\alpha_{uu} \) interaction. Although not directly involved in contacting the neighboring trimer, the side chain of \( \beta R70 \) forms multiple hydrogen bonds, including one to \( \beta Q35 \), which in turn interacts with the neighbor-
of their ureases can be deduced. Eu3 is the plant UreG ortholog, but unlike the bacterial protein, it contains a His-rich N terminus and binds nickel (Freyermuth et al., 2000). Two eu2/eu2 mutants produce wild-type UreD and UreF proteins (A. Goldraij and J.C. Polacco, unpublished data), so Eu2 may be unique in plants. Likewise, the different structural and biochemical properties of embryo-specific and ubiquitous ureases (i.e. embryo-specific is 1,000 times more abundant, whereas its $K_m$ is 20 to 600 times higher than that of ubiquitous [Polacco and Holland, 1994]) raise questions about the activation of the soybean urease isozymes. Are they activated by the same pool of accessory proteins? Are these accessory proteins similarly regulated for activating each isozyme? Focusing on these topics will contribute to further elucidation of the complex urease reaction mechanism in plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Unless stated otherwise, wild-type soybean (Glycine max [L.] Merrill) was cv Williams 82. In some experiments, wild-type soybean was cv Kenwood. Both cultivars showed the same deduced amino acid sequence and similar levels of activity for the ubiquitous urease. Soybean mutants aj6/aj6, eu4/ eu4 (Polacco et al., 1989), eu2/eu2, eu3/eu3/eu3-e1 (Meyer-Bothling et al., 1987), and eu1-sun/eu1-sun (Meyer-Bothling and Polacco, 1987) were in the soybean cv Williams/Williams 82 backgrounds. Seeds were germinated in the dark at 27°C in rolls of germination paper (Anchor Paper, St. Paul) moistened with deionized water. Plants were grown in pots on regular soil in a controlled-environment greenhouse at 25°C with a 16-h/8-h light/dark regime.

F2 seed phenotypes of the eu1-sun/eu1-sun × aj6/aj6 cross were distinguished by the seed-chip assay (Meyer-Bothling and Polacco, 1987). Seeds with low (embryo-specific negative, ubiquitous positive) versus null (embryo-specific negative, ubiquitous negative) urease levels were distinguished by incubating seed chips 5 d at 37°C in capped tubes.

Callus was induced and cultured on R3 medium as reported previously (Polacco et al., 1989). In the growth experiment of Figure 2B, callus was induced on medium identical to R3 except that hormones were 4.86 mg L$^{-1}$ naphthalene acetic acid and 1.8 mg L$^{-1}$ kinetin. Callus was maintained on S3 medium in which hormones were 3.5 × 10$^{-1}$ mg L$^{-1}$ each of benzylaminopurine and 2,4-dichlorophenoxyacetic acid.

Plant Crosses and Allelism Tests

aj6/aj6 plants were crossed to wild type and to individuals homozygous for recessive urease-negative alleles at the Eu1, Eu2, and Eu3 loci at the Iowa State Soybean Breeding Nursery (Isabela Substation, University of Puerto Rico, Isabela, Puerto Rico). The eu1-sun/eu1-sun × aj6/aj6 cross was performed at Bradford Farm (University of Missouri). F1 seeds were tested non-destructively by the seed chip urease assay. Leaf urease activity was measured in leaves of F1 plants that were selfed to obtain F2 seeds. Segregation patterns of F2 progeny were statistically analyzed by $t$ tests ($P > 0.05$).

Urea Content and Ureaase Activity Analyses

Urea content was quantified by the phenol-hypochlorite method (Weatherburn, 1967), determining the ammonia generated following urease treatment. Seed and leaf (10th trifoliate) tissue extraction and urea determination were performed as described by Goldraij and Polacco (1999) and Stebbins et al. (1991), respectively.

To determine seed urease activity, the seed coat was removed, and a seed sliver was taken from the side opposite the hilum. The tissue was homog-
enzized with a plastic pestle in 300 to 400 mL of 0.1 M Tris-maleate and 1 mM EDTA, pH 7, in a microcentrifuge tube at 4°C. After centrifugation, the supernatant was mixed with 55 mm urea in a total volume of 250 mL and was incubated at 37°C for 10 min. Aliquots of 0.1 mL were taken, and the reaction was stopped by the addition of 40 mL of 0.64 N H2SO4. After dilution with 2 mL of water, 0.1 mL of Nessler’s reagent (Fisher Scientific, Fair Lawn, NJ) was added, and the absorbance read at 425 nm. Routinely, 1 reaction was stopped by the addition of 40 mL of 1.4 N H2SO4.

Leaf urease activity was determined in discs cut from mid-sections of fully expanded eighth to 10th trifoliate leaves with a number 8 cork borer. Leaf powder (0.1 g) was extracted with 0.5 mL of 50 mM NaCl, 1 mM EDTA, and 50% (v/v) glycerol. Immediately before extraction, dithiothreitol and phenylmethylsulfonyl fluoride were added to 20 mM and 2 mL of water, 0.1 mL of Nessler’s reagent (Fisher Scientific, Fair Lawn, NJ) was added, and the absorbance read at 425 nm. Routinely, 1 reaction was stopped by the addition of 40 mL of 1.4 N H2SO4. Acid-released 14CO2 was trapped and quantified as described by Meyer-Bothling and Polacco (1987). A similar procedure was followed to determine urease activity in lyophilized callus cultured on R3 medium (Polacco et al., 1989).

To determine urease activity in native gels, leaf tissue was frozen in liquid N and ground in a mortar to a fine powder. Leaf powder (0.1 g) was extracted with 0.5 mL of 50 mM phosphate buffer, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, and 50% (v/v) glycerol. Immediately before extraction, dithiothreitol and phenylmethylsulfonyl fluoride were added to 20 mM and 0.5 mM, respectively. Samples were homogenized in a 1.5 polystyrene tube with a plastic pestle, vortexed for 30 s, and centrifuged at 12,000 rpm for 10 min at 4°C. Supernatants were transferred to new tubes and centrifuged again for 20 min under the same conditions. Aliquots of clarified crude extracts were subjected to electrophoresis under non-denaturing conditions as indicated in Witte and Medina-Escobar (2001) except that the gels were run in an ice bucket. After electrophoresis, the gel was washed five times with 50 mL of 5 mM sodium acetate, pH 6, and once with 50 mL of water at 4°C. Urease activity was detected either by staining (Witte and Medina-Escobar, 2001) or by incubating gel slices (0.58 mm) with 10 mM [14C]urea following the same procedure stated above for the leaf disc urease activity assay, except that samples were incubated for 20 h.

Cloning and Sequencing of Soybean Ureases

Leaf ubiquitous urease ORFs were cloned from two soybean urease-positive wild-type cvs Williams and Kenwood and from the aj6/aj6 and eu4/eu4 mutants, which were induced in soybean cv Williams. The soybean embryo-specific urease ORF was cloned from dry soybean cv Williams 82 seeds. Total RNA (2–4 μg) isolated according to Murfett et al. (1994) was used as template for cDNA synthesis by the RT-PCR in the lmProm-II Reverse Transcription System (Promega, Madison, WI), according to the manufacturer’s instructions. Subsequent amplification of the ORF used primers designed from the chemically determined N-terminal amino acid sequences and from genomic DNA sequences containing the C-terminal regions of both soybean ureases (Toriisky et al., 1994). For ubiquitous urease, oligonucleotide primers (Integrated DNA Technologies Inc, Coralville, IA) were 5′-ATG AACGTGACCCAGGAGATG-3′ and 5′- TAAAATATCATGGACACCATGCTG-3′. For embryo-specific urease, they were 5′-ATGAAACTGAGTCCAAGGGAG-3′ and 5′-GCCTTATTTA- TGGGAGTCTAGA-3′. PCR was performed using 2 μL of RT reaction, using Takara Ex Taq (Takara Biomedicals, Kyoto) at 59°C annealing temperature for 35 cycles. All amplification products obtained had the expected size and were cloned into pGEM-Teasy (Promega). For all reactions, both strands of cDNA from the PCR products were sequenced using oligonucleotides based on internal sequences. Point mutations in aj6/aj6 and eu4/eu4 were consistently detected in five clones from different sequencing reactions. Sequencing was done in the DNA Core of the University of Missouri (Columbia). Identity of sequenced products was confirmed through BLASTn and BLASTp searches (Altschul et al., 1997).

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