Aspartate Kinase 2. A Candidate Gene of a Quantitative Trait Locus Influencing Free Amino Acid Content in Maize Endosperm

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The maize (Zea mays) Oh545o2 inbred accumulates an exceptionally high level of free amino acids, especially lysine (Lys), threonine (Thr), methionine, and iso-leucine. In a cross between Oh545o2 and Oh51Ao2, we identified several quantitative trait loci linked with this phenotype. One of these is on the long arm of chromosome 2 and is linked with loci encoding aspartate (Asp) kinase 2 and Asp kinase (AK)-homoserine dehydrogenase (HSDH) 2. To investigate whether these enzymes can contribute to the high levels of Asp family amino acids, we measured their specific activity and feedback inhibition properties, as well as activities of several other key enzymes involved in Lys metabolism. We did not find a significant difference in total activity of dihydrodipicolinate synthase, HSDH, and Lys ketoglutarate reductase between these inbreds, and the feedback inhibition properties of HSDH and dihyrodipicolinate synthase by Lys and/or Thr were similar. The most significant difference we found between Oh545o2 and Oh51Ao2 is feedback inhibition of AK by Lys but not Thr. AK activity in Oh545o2 is less sensitive to Lys inhibition than that in Oh51Ao2, with a Lys IC50 twice that of Oh51Ao2. AK activity in Oh545o2 endosperm is also higher than in Oh51Ao2 at 15 d after pollination, but not 20 d after pollination. The results indicate that the Lys-sensitive AK kinase 2, rather than the Thr-sensitive AK-HSDH2, is the best candidate gene for the quantitative trait locus affecting free amino acid content in Oh545o2.

The opaque-2 (o2) mutation in maize (Zea mays) improves the nutritional quality of the grain by enhancing endosperm Lys content (Mertz et al., 1964). The higher level of Lys in o2 endosperm is primarily a consequence of increased synthesis of Lys-containing proteins (Moro et al., 1996; Sun et al., 1997), but these mutants also have higher than normal levels of free Lys. Part of the explanation for the increase in free Lys is the loss of Lys ketoglutarate reductase (LKR) activity (EC 1.5.1.9), an enzyme that degrades Lys as the endosperm matures (Arruda et al., 2000). However, there is also evidence for increased Lys synthesis and/or accumulation of other amino acids (Sodek and Wilson, 1970; Misra et al., 1975; Sodek, 1976). In characterizing several wild-type and o2 maize inbred lines, we found evidence for high levels of amino acids derived from the Asp pathway (Lys, Thr, Met, and iso-Leu), as well as Ala and Ser, in o2 mutants (Wang and Larkins, 2001). By analyzing the progeny of a cross between Oh545o2 and Oh51Ao2, we identified four quantitative trait loci (QTLs) that account for about 50% of the variability in the high free amino acid (FAA) trait. A QTL on the long arm of chromosome 2 that is responsible for 11% of the phenotypic variability occurs in proxim-ity with genes encoding a monofunctional Asp kinase 2 (Ask2) and a bifunctional Asp kinase-homo-Ser dehydrogenase-2 (AK-HSDH2). As a consequence, these genes are good candidates to explain the increased synthesis of Asp-derived amino acids in this mutant.

The Asp pathway directs Lys synthesis and is feedback regulated by its end products (Gengenbach et al., 1978; Bryan, 1990; Galili, 1995; Azevedo et al., 1997; Fig. 1). AK (EC 2.7.2.4), the first enzyme in this pathway, catalyzes the conversion of Asp to β-aspartyl phosphate. In maize, there are at least five genes encoding two or more isoforms of this enzyme, based on their feedback inhibition properties (Dotson et al., 1989; Azevedo et al., 1992a; Muehlbauer et al., 1994a, 1994b). Two genes, Ask1 and Ask2, encode monofunctional AKs that have been mapped to the short arm of chromosome 7 and the long arm of chromosome 2, respectively (Azevedo et al., 1990; Muehlbauer et al., 1994a). The AK in Ask1 and Ask2 mutants is less sensitive to Lys inhibition and results in overproduction of Lys, Thr, Met, and iso-Leu (Dotson et al., 1990a; Muehlbauer et al., 1994a). Ask1 appears to be regulated by O2, because in double mutants of Ask1 and o2, AK is less sensitive to Lys inhibition than in Ask1 mutants alone (Azevedo et al., 1990; Brennecke et al., 1996). There are at least three bifunctional AK-HSDH genes in maize, and they appear to encode Thr-sensitive isoforms of AK (Azevedo et al., 1992b; Muehlbauer et al., 1994b). Two AK-HSDH genes were mapped to the long arm of chromosome 2 and the short arm of chromosome 4 (Muehlbauer et al., 1994b).

HSDH (EC 1.1.1.3), another enzyme of the Asp pathway, uses NADPH to convert Asp semialdehyde (ASA) to homo-Ser (Fig. 1). In maize, there are two
different isoforms of HSDH, one Thr sensitive and one Thr insensitive (Walter et al., 1979). Depending on the tissue and developmental stage, the relative level of the two isoforms is variable (Matthews et al., 1989). Carrot (Daucus carota) HSDH can be changed in vitro between a Thr-sensitive trimeric form and a Thr-insensitive dimeric form (Matthews et al., 1989; Turano et al., 1990). The Thr-sensitive trimeric form requires Thr, whereas the Thr- insensitive dimeric form requires potassium (Turano et al., 1990). Several genes could encode HSDH in maize. It was predicted that AK-regulated HSDH in maize. It was predicted that AK-1990). The Thr-sensitive trimeric form requires Thr, whereas the Thr-insensitive dimeric form requires potassium (Turano et al., 1990). Several genes could encode HSDH in maize. It was predicted that AK-HSDH genes encode the Thr-sensitive form, due to HSDH being feedback regulated by Thr alone, HSDH is feedback regulated by Thr, and Lys can activate LKR activity. 

DHDP$\$ (EC 4.2.1.52), a key regulatory enzyme in Lys biosynthesis, catalyzes the formation of dihydrodipicolinate acid by condensing pyruvate and ASA (Fig. 1). DHDP$\$ is highly sensitive to Lys feedback regulation; when expressed in Escherichia coli, 50% of the maize DHDDS activity is inhibited (I$_{50}$) by 7$\mu$M Lys (Vauterin et al., 2000). Plants with a mutant DHDP$\$ are less sensitive to Lys feedback inhibition and overproduce the amino acid (Ghislain et al., 1995). Because bacterial DHDP$\$ is less sensitive than plant DHDP$\$, genes encoding bacterial DHDP$\$ have been used to genetically engineer plants that overproduce Lys (Falco et al., 1995).

As previously noted, Lys degradation is another important factor influencing Lys content in maize endosperm (Arruda et al., 2000). LKR is the initial enzyme involved in Lys degradation, and its activity is dramatically reduced in o2 mutants (Brochetto-Braga et al., 1992; Kemper et al., 1999). Therefore, it is thought that the reduction in LKR activity is primarily responsible for the increased Lys content in o2 endosperm.

Here we report the analysis of key enzymes involved in Lys biosynthesis and degradation in Oh545o2 and Oh51Ao2. The specific activity of AK in Oh545o2 is higher than in Oh51Ao2 at 15 d after pollination (DAP), but not at 20 DAP. The most significant difference we found between AK activities in the endosperm of these mutants is feedback inhibition by Lys, but not by Thr. The AK in Oh545o2 has an I$_{50}$ for Lys that is twice that of the AK in Oh51Ao2, indicating that it is less sensitive to Lys inhibition. We did not find a difference in level or specific activity of HSDH and DHDP$\$ in Oh545o2 and Oh51Ao2, and the feedback inhibition properties by Lys and/or Thr are similar. These results suggest that Ask2, rather than AK-HSDH2, is the best candidate gene for the QTL on the long arm of chromosome 2 that influences FAA content.

**RESULTS**

The Effects of the QTL on the Long Arm of Chromosome 2 on the FAA Content and Composition

To evaluate the effect of the QTL on the long arm of chromosome 2 on endosperm amino acid composition, we used a flanking marker, bmc1329, to separate the F$_{2:3}$ progeny of the Oh545o2 × Oh51Ao2 cross into three genotypes: 25 homozygous-like Oh545o2, 25 homozygous-like Oh51Ao2, and 55 heterozygous. Twenty micrograms of endosperm flour from each individual was used to create the three pooled samples, and the FAA compositions were determined. The data in Table I show that the pool with the bmc1329 genotype of Oh545o2 had more than twice the FAA content of the heterozygous and the Oh51Ao2 genotype pool. In the Oh545o2 genotype pool, the concentration of amino acids from the Asp pathway is nearly double that of the other two genotypes. The relative content of most other amino acids is not significantly different between the pools, although the levels of Asp and Asn in the Oh545o2-related pool are reduced from 19% to 14% and 18% to 16%, respectively, compared with the Oh51Ao2-related pool. As a consequence, it appears that the allele in Oh545o2 for this QTL has a major effect on amino acid products of the Asp pathway.

Specific Activity and Feedback Inhibition Properties of DHDP$\$ in Oh545o2 and Oh51Ao2 Endosperm

The striking difference in Asp pathway amino acids in these F$_{2:3}$ progeny led us to investigate the properties of AK in Oh545o2 and Oh51Ao2 endosperm. We measured the specific activity and feedback inhibition properties of partially purified AK from these inbreds at 15 and 20 DAP. The results in Figure 2A show that the specific activity of AK in Oh545o2 is nearly twice that of Oh51Ao2 at 15 DAP,
but the values are nearly identical by 20 DAP. In both inbred lines, the specific activity of AK at 15 DAP is higher than at 20 DAP. Assays for AK feedback inhibition by 10 mM Lys and/or 10 mM Thr showed similar degrees of sensitivity to Thr, with only 10% inhibition at 15 and 20 DAP (Table II). However, AK sensitivity to Lys is noticeably different between the inbreds. The AK in Oh54502 is between 8% and 10% less sensitive to 10 mM Lys at both developmental stages (Table II). When 10 mM Thr and 10 mM Lys were included in the assay, Oh54502 AK had 30% of control activity, whereas Oh51A02 had about 23% of control activity.

For a more detailed comparison of the Lys feedback inhibition of these enzymes, assays were conducted with varying concentrations of Lys in the reaction. Figure 2B shows the AK in Oh54502 is significantly less sensitive to Lys than that in Oh51A02 at 20 DAP. The Lys I50 of the AK in Oh54502 is more than 500 μM, whereas that in Oh51A02 is around 250 μM. Similar results were obtained whether the enzyme from 15 or 20 DAP endosperm was assayed.

HSDH Activity in Developing Endosperm of Oh54502 and Oh51A02

Because AK-HSDH2 is also a candidate gene for the QTL on the long arm of chromosome 2, we characterized HSDH activity to obtain evidence for whether or not variation in the HSDH domain of the bifunctional AK-HSDH is involved in the regulation of the high FAA level in Oh54502. Table III shows the specific activity of HSDH in Oh54502 is lower than that in Oh51A02 at 15 DAP, but at 20 DAP the difference is not significant. The specific activity of both enzyme preparations at 20 DAP is lower than at 15 DAP, as was true for AK. We tested for feedback inhibition using high concentrations of Thr (5, 10, and 20 mM). With enzyme from 15 DAP endosperm, there was 70% to 80% of control activity in 20 mM Thr for the Oh54502 and Oh51A02 enzymes, respectively (Table IV). It is interesting that HSDH from Oh54502 is more sensitive to Thr than the enzyme from Oh51A02 at 20 DAP.

### Table I. FAA composition of pooled F2 individuals with different flanking marker (bmc1329) genotypes

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mean</th>
<th>SE</th>
<th>Mean</th>
<th>SE</th>
<th>Mean</th>
<th>SE</th>
<th>Mean</th>
<th>SE</th>
</tr>
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<td></td>
<td>A</td>
<td>H</td>
<td>B</td>
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<td></td>
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<tr>
<td>Asp</td>
<td>4.81</td>
<td>0.04</td>
<td>4.45</td>
<td>0.54</td>
<td>7.32</td>
<td>0.26</td>
<td>19.31</td>
<td>17.46</td>
</tr>
<tr>
<td>Asn</td>
<td>4.50</td>
<td>0.08</td>
<td>4.58</td>
<td>0.45</td>
<td>8.12</td>
<td>0.36</td>
<td>18.07</td>
<td>17.97</td>
</tr>
<tr>
<td>Glu</td>
<td>3.94</td>
<td>0.05</td>
<td>4.41</td>
<td>0.00</td>
<td>9.75</td>
<td>0.47</td>
<td>15.84</td>
<td>17.29</td>
</tr>
<tr>
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<td>0.01</td>
<td>0.89</td>
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<td>Ala</td>
<td>2.86</td>
<td>0.04</td>
<td>3.05</td>
<td>0.06</td>
<td>6.96</td>
<td>0.37</td>
<td>11.47</td>
<td>11.97</td>
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<tr>
<td>Ser</td>
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<td>0.01</td>
<td>0.76</td>
<td>0.07</td>
<td>1.38</td>
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<tr>
<td>Lys</td>
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<td>0.01</td>
<td>0.44</td>
<td>0.01</td>
<td>1.01</td>
<td>0.05</td>
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<td>1.73</td>
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<tr>
<td>Thr</td>
<td>0.45</td>
<td>0.00</td>
<td>0.46</td>
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<td>0.95</td>
<td>0.05</td>
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<td>Met</td>
<td>0.09</td>
<td>0.00</td>
<td>0.07</td>
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<td>0.01</td>
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<td>Ile</td>
<td>0.12</td>
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<td>0.12</td>
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<td>0.01</td>
<td>0.50</td>
<td>0.46</td>
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<td>Leu</td>
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<td>0.00</td>
<td>0.17</td>
<td>0.00</td>
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<td>0.02</td>
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<td>Gly</td>
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<td>0.01</td>
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<td>1.77</td>
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<td>0.00</td>
<td>0.45</td>
<td>0.01</td>
<td>0.95</td>
<td>0.05</td>
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<td>1.75</td>
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<td>Tyr</td>
<td>0.31</td>
<td>0.00</td>
<td>0.29</td>
<td>0.01</td>
<td>0.59</td>
<td>0.03</td>
<td>1.23</td>
<td>1.13</td>
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<tr>
<td>Phe</td>
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<td>0.00</td>
<td>0.12</td>
<td>0.01</td>
<td>0.25</td>
<td>0.01</td>
<td>0.60</td>
<td>0.49</td>
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<td>Gaba</td>
<td>1.02</td>
<td>0.02</td>
<td>1.14</td>
<td>0.04</td>
<td>2.53</td>
<td>0.12</td>
<td>4.08</td>
<td>4.48</td>
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<td>His</td>
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<td>0.00</td>
<td>0.24</td>
<td>0.02</td>
<td>0.46</td>
<td>0.02</td>
<td>1.09</td>
<td>0.94</td>
</tr>
<tr>
<td>Arg</td>
<td>0.37</td>
<td>0.01</td>
<td>0.36</td>
<td>0.02</td>
<td>0.73</td>
<td>0.03</td>
<td>1.47</td>
<td>1.40</td>
</tr>
<tr>
<td>Pro</td>
<td>1.62</td>
<td>0.01</td>
<td>1.78</td>
<td>0.07</td>
<td>3.87</td>
<td>0.20</td>
<td>6.51</td>
<td>6.97</td>
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<tr>
<td>Others</td>
<td>1.34</td>
<td>0.01</td>
<td>1.30</td>
<td>0.03</td>
<td>2.48</td>
<td>0.12</td>
<td>5.39</td>
<td>5.10</td>
</tr>
<tr>
<td>Total</td>
<td>24.90</td>
<td>0.21</td>
<td>25.50</td>
<td>1.19</td>
<td>51.43</td>
<td>2.39</td>
<td>100.00</td>
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</tr>
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</table>

Specific Activity and Feedback Inhibition Properties of DHDPS in Oh54502 and Oh51A02 Endosperm

The genes encoding maize DHDPS have not been genetically mapped, so there is no information whether or not one of the four QTLs influencing FAA composition (Wang and Larkins, 2001) is associated with this enzyme. DHDPS is a key regulatory enzyme for Lys synthesis, and Oh54502 has a much higher level of free Lys than Oh51A02. Therefore, it was of interest to investigate the activity of DHDPS in developing endosperm of these two inbreds. We used the same endosperm extracts to measure DHDPS activity as were used for HSDH assays. The DHDPS activity at 15 DAP is higher than at 20 DAP, with no difference between the two genotypes at 20 DAP (Table V). At 15 DAP, the specific activity of the
enzyme from Oh51A is higher than that from Oh545. However, the sensitivity of the two enzymes to feedback inhibition by Lys is almost identical, with an I50 of 20 to 30 mM at both developmental stages. More than 90% of the DHDPS activity in crude extracts is inhibited by 100 mM Lys (Fig. 3, A and B).

**LKR Activity in Developing Endosperm of Oh545o2 and Oh51A02**

The Lys catabolic pathway is a major factor determining the final Lys content of maize endosperm (Arruda et al., 2000). LKR is the first enzyme involved in Lys degradation, and it is highly expressed in endosperm tissue. To test whether there is a difference in LKR activity in developing endosperm of these two inbreds, we measured LKR activity at several developmental stages. At 15 DAP, we did not detect any LKR activity. At 20 DAP, the LKR activity in Oh545o2 is much lower than that in Oh51A02; however, both activities are very low (less than 0.3 units mg⁻¹ protein). The LKR activity at 25 DAP for both genotypes is higher than at earlier stages, and it is slightly greater in Oh545o2 than Oh51A02 (Fig. 4). However, total activity of LKR for both genotypes is extremely low compared with their wild-type counterparts (5 units mg⁻¹ protein; data not shown). Therefore, the slight difference in LKR activity in Oh545o2 and Oh51A02 does not appear to contribute significantly to the difference in free Lys levels.

**DISCUSSION**

The FAA analysis of pooled F2:3 flour samples, based on the flanking marker genotype of the QTL on the long arm of chromosome 2, demonstrated that this locus has a large effect on the endosperm FAA content. It appears the allele from Oh545o2 has a recessive genetic effect, and relative to the allele from Oh51A02, it effectively doubles the FAA content of the endosperm (Table I). The increased levels of Thr, Lys, Met, and iso-Leu in Oh545o2 are consistent with the hypothesis that this locus affects the Asp pathway. The reduced percentage (not absolute content) of Asp and Asn in Oh545o2 suggest that relatively more Asp enters this pathway. These data support our suggestion that AK is a good candidate gene to partially explain the high level of FAA in Oh545o2 (Wang and Larkins, 2001).

As appears to be true of other maize tissues, the Lys-sensitive AK seems to be the major form of the enzyme in endosperm (Dotson et al., 1989, 1990b; Azevedo et al., 1992a). At first, we partially purified AK by ammonium sulfate precipitation, but its specific activity was too low to assay accurately. Therefore, we further purified the enzyme by phenyl sepharose chromatography, and this led to a several-

### Table II. Inhibition of AK from developing endosperm of Oh545o2 and Oh51A02 by 10 mM Lys and/or 10 mM Thr

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>10 mM Lys (Oh545o2)</th>
<th>10 mM Lys (Oh51A02)</th>
<th>10 mM Thr (Oh545o2)</th>
<th>10 mM Thr (Oh51A02)</th>
<th>10 mM Lys + 10 mM Thr (Oh545o2)</th>
<th>10 mM Lys + 10 mM Thr (Oh51A02)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 DAP</td>
<td>34.32 ± 0.05</td>
<td>26.16 ± 0.33</td>
<td>89.98 ± 0.65</td>
<td>92.09 ± 4.59</td>
<td>30.12 ± 0.35</td>
<td>23.71 ± 0.79</td>
</tr>
<tr>
<td>20 DAP</td>
<td>43.79 ± 0.57</td>
<td>33.68 ± 1.70</td>
<td>92.32 ± 1.74</td>
<td>88.47 ± 2.47</td>
<td>35.38 ± 1.07</td>
<td>23.35 ± 2.03</td>
</tr>
</tbody>
</table>
Table III. Specific activity of HSDH from developing endosperm of Oh545o2 and Oh51Ao2

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Mean ± SE</th>
<th>OH545O2</th>
<th>OH51AO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 DAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 DAP</td>
<td></td>
<td></td>
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</table>

Values (units per milligram protein) are means of at least two independent extractions. One unit is defined as the amount of enzyme required for the oxidation of 1 mmole of NADPH per min at RT.

Table IV. Inhibition of HSDH from developing endosperm of Oh545o2 and Oh51Ao2 by Thr

<table>
<thead>
<tr>
<th>Amino Acid Concentration</th>
<th>Mean ± SE</th>
<th>OH545O2</th>
<th>OH51AO2</th>
<th>OH545O2</th>
<th>OH51AO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM Thr</td>
<td>73.84 ± 1.53</td>
<td>80.34 ± 4.66</td>
<td>61.22 ± 1.57</td>
<td>72.69 ± 1.55</td>
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</tr>
<tr>
<td>10 mM Thr</td>
<td>73.16 ± 0.69</td>
<td>78.12 ± 0.26</td>
<td>59.77 ± 1.63</td>
<td>73.66 ± 0.58</td>
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</tr>
<tr>
<td>20 mM Thr</td>
<td>70.98 ± 2.86</td>
<td>71.92 ± 1.65</td>
<td>48.98 ± 0.14</td>
<td>67.95 ± 1.28</td>
<td></td>
</tr>
</tbody>
</table>

Values are percentage of control activity (without inhibitors) averaged from two independent extractions.

The HSDH in maize endosperm is very active, with the Thr-insensitive isoform predominating. Even in the presence of 20 mM Thr, there was still 50% to 70% of the HSDH activity in our enzyme preparations. As is true of AK and DHDPS, HSDH had a higher specific activity at 15 DAP than at 20 DAP (Table III). This implies a higher activity of the Asp pathway at early stages of endosperm development. A change in HSDH sensitivity to Thr during development was also observed in maize leaves and shoots (Matthews et al., 1975). In contrast to the results of Matthews et al. (1975), we found HSDH to be more sensitive to Thr at later stages of endosperm development; there is no obvious explanation for the discrepancy between the two sets of experimental results.

The DHDPS in Oh545o2 and Oh51A02 endosperms is similarly sensitive to Lys feedback inhibition, although the specific activity of DHDPS in Oh545o2 is slightly lower than that in Oh51A02. The Lys I50 of the crude DHDPS we prepared from both genotypes is between 20 and 30 μM, similar to previous reports for DHDPS feedback inhibition (25 μM) in maize and tobacco (Negrutiu et al., 1984; Frisch et al., 1990). This suggests that DHDPS is not related to the high level of free Lys in Oh545o2. AK and DHDPS play important roles in Lys metabolism, but DHDPS primarily regulates the level of free Lys and does not influence the level of other amino acids (Negrutiu et al., 1984; Ghislain et al., 1995).

LKR does not appear to account for the difference in the FAA level in Oh545o2 compared with Oh51A02. The activity of this enzyme is very low in both genotypes, with slightly more activity at 25 DAP compared with earlier stages of development. LKR activity is somewhat lower in OH545o2 than in OH51A02 at 20 DAP (Fig. 4). The activity of this enzyme in both genotypes is substantially less (under 0.3 units mg⁻¹ protein) than in their wild-type counterparts. This difference is typical for LKR activity in wild-type and o2 mutants (Brochetto-Braga et al., 1992; Gaziola et al., 1997; Kemper et al., 1999). There

The DHDPS in Oh545o2 and Oh51A02 endosperms is similarly sensitive to Lys feedback inhibition, although the specific activity of DHDPS in Oh545o2 is slightly lower than that in Oh51A02. The Lys I50 of the crude DHDPS we prepared from both genotypes is between 20 and 30 μM, similar to previous reports for DHDPS feedback inhibition (25 μM) in maize and tobacco (Negrutiu et al., 1984; Frisch et al., 1990). This suggests that DHDPS is not related to the high level of free Lys in Oh545o2. AK and DHDPS play important roles in Lys metabolism, but DHDPS primarily regulates the level of free Lys and does not influence the level of other amino acids (Negrutiu et al., 1984; Ghislain et al., 1995).

LKR does not appear to account for the difference in the FAA level in Oh545o2 compared with Oh51A02. The activity of this enzyme is very low in both genotypes, with slightly more activity at 25 DAP compared with earlier stages of development. LKR activity is somewhat lower in OH545o2 than in OH51A02 at 20 DAP (Fig. 4). The activity of this enzyme in both genotypes is substantially less (under 0.3 units mg⁻¹ protein) than in their wild-type counterparts. This difference is typical for LKR activity in wild-type and o2 mutants (Brochetto-Braga et al., 1992; Gaziola et al., 1997; Kemper et al., 1999). There

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is no doubt that the low activity of LKR in Oh545o2 is important for maintaining the high concentration of Lys as the endosperm matures, but it appears likely that the high level of Lys in this inbred is primarily a result of high levels of biosynthetic activity.

Overall, the results of our studies indicate that Ask2 rather than AK-HSDH2 is the best candidate gene for the QTL on the long arm of chromosome 2 influencing FAA content. The similarity of HSDH activity in Oh545o2 and Oh51Ao2 and its sensitivity to Thr feedback inhibition indicate that HSDH is unlikely to be responsible for overproducing FAAs in Oh545o2. The difference in AK inhibition by Lys, but not by Thr, and the lower sensitivity of AK from Oh545o2 to Lys, suggest the monofunctional rather than the bifunctional AK is responsible for overproducing Asp pathway amino acids. In other plant species, mutants with Lys-insensitive AK overproduce Thr as well as other amino acids (Frankard et al., 1992; Shaul and Galili, 1992), so perhaps it is not coincidental that the FAA composition of Oh545o2 endosperm reflects that of the maize Ask2 mutant (Muehlbauer et al., 1994a). Therefore, we hypothesize that the Ask2 allele from Oh545o2 encodes an AK that is less sensitive to Lys. We cannot dismiss the possibility that high levels of AK expression in Oh545o2 also contribute to the high-FAA phenotype. If high levels of AK influence the FAA phenotype, allelic variation in the promoter region of this gene could also be responsible for this QTL.

There is another observation that indirectly fails to support bifunctional AK-HSDH2 as the candidate gene for this QTL. Because the promoter region of the Arabidopsis AK-HSDH contains a putative GCN4-like element (Zhu-Shimoni and Galili, 1998), the same could be true of the maize gene. Because the O2 protein can substitute for GCN4 in transformed yeast cells (Mauri et al., 1993), one would predict that in an o2 mutant this enzyme would have a lower level of expression, and consequently, a lower level of amino acids would be synthesized. Thus, it would be surprising if the QTL encodes AK-HSDH2, based on what we know about the high-FAA phenotype of Oh545o2.

The FAA analysis of pooled F2:3 samples (Table I) suggest the high FAA level regulated by this QTL is recessive. However, our mapping data suggest it is not completely recessive, i.e. it could be semidominant (Wang and Larkins, 2001). Analysis of the pooled F2:3 individuals, based on flanking-marker genotype, may not accurately reflect the genotype of the QTL. As a consequence, the FAA composition of these samples may inaccurately represent the phenotype of the alleles of this QTL. We identified several QTLs that influence endosperm FAA content, and if the size of the population is not large enough to
analyze AK activity and FAA composition in indi-
prepare developing endosperm that can be used to
Oh51A
cross, and these materials will allow us to
inbred lines from the progeny of the Oh545
o2
these genes. We have also developed recombinant
inhibition.

In the heterozygous condition, a single altered sub-
by
MATERIALS AND METHODS

Plant Materials
Oh545o2 and Oh51o2 were grown in a greenhouse at the
Campus Agricultural Center (University of Arizona). De-
veloping kernels were harvested at 15, 20, and 25 DAP,
frozen with liquid nitrogen and stored at −80°C. The ker-
nels were degemerized before use in enzyme assays.

Endosperm flour was prepared from F2 progeny of a
cross between Oh51o2 and Oh545o2, based on the geno-
type (25 homozygous like each parent and 55 heterozy-
gous) of the simple sequence repeat marker, bmc1329,
which flanks the QTL influencing FAA content on the long
arm of chromosome 2 (Wang and Larkins, 2001).

Sample Preparation and FAA Analysis of
Mature Endosperm

Extraction and analysis of mature endosperm FAs was
performed as described by Wang and Larkins (2000).
Twenty milligrams of flour was defatted for 1 h in a 1.5-mL
centrifuge tube with 1 mL of petroleum ether. The ether
was removed by centrifugation at 14,000 rpm for 10 min
and another 1 mL of ether was added for 10 min. Following
centrifugation, the ether was removed by aspiration, and
the defatted samples were resuspended in 1 mL of sterile
double-distilled water by shaking vigorously for 20 min at
room temperature. The supernatant was saved and filtered
through a C18 reverse phase minicartridge (Vydac, Hespe-
dia, CA) to remove soluble proteins. Five hundred micro-
liters of the supernatant was dried with a speed vacuum
drier (Southwest Instruments Biomedical Instrumentation,
Tucson, AZ), and the pellet was resuspended in 50 μL of
sterile double-distilled water for amino acid analysis.

Amino Acid Analysis
Amino acid analysis was performed by the Laboratory
for Protein Sequencing and Analysis (University of Ari-
izona) using a post column Amino Acid Analyzer (Beckman
7300, Beckman Instruments Inc., Fullerton, CA; ninhydrin
method). Amino acids were separated by ion-exchange
chromatography using citrate buffer of increasing ionic
strength and pH at varying temperatures. Amino acids
were detected by mixing with ninhydrin, and the reaction
was monitored by a colorimeter at 570 nm for primary
amino acids and 440 nm for secondary amino acids.

AK Extraction
All procedures for enzyme extraction and analysis were
carried out at 4°C. Five to 10 g of immature endosperm was
ground with a Kinematica GmbH Polytron (Brinkman In-
struments, Wesbury, NY) at a speed setting of 7 in 5:1
(v/w) buffer A (50 mM Tris-HCl [pH 7.4], 50 mM KCl, 2 mM
Lys, 2 mM Thr, 3 mM DTT [ dithiothreitol], 0.1 mM phenyl
methylsulfonyl fluoride [PMSF], 1 mM EDTA, 15% [v/v]
glycerol, and 5% [w/v] insoluble polyvinylpoly-pyrrolidone
[PVP]). The extract was centrifuged at 12,000g for 30 min,
and the particulate was filtered through four layers of Mir-
cloth (Calbiochem, La Jolla, CA). Finely ground (NH4)2SO4
was gradually added with stirring to the supernatant until
10% saturation. The solution was stirred for 30 min and
centrifuged at 12,000g for 30 min. The supernatant was
loaded onto a Phenyl Sepharose-CL-4B column (Pharmacia
Biotech, Uppsala) pre-equilibrated with buffer B [50 mM
Tris-HCl [pH 7.4], 50 mM KCl, 1 mM EDTA, 3 mM DTT, and
10% (NH4)2SO4]. The column was washed with buffer C
[50 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM EDTA, 3 mM DTT,
and 7.5% (NH4)2SO4]. Proteins bound to the column
were eluted with buffer D (50 mM Tris-HCl [pH 7.4], 50 mM
KCl, 1 mM EDTA, 3 mM DTT, and 50% [v/v] ethylene
glycol) until no significant amount remained. Proteins
were precipitated by adding 1.5 volumes of 100% saturated
(NH4)2SO4, stirring for 30 min and centrifuging at 20,000g
for 40 min. The pellet was dissolved in resuspension buffer
(50 mM Tris-HCl [pH 7.4], 50 mM KCl, 3 mM EDTA, and
15% [v/v] glycerol) and stored on ice until use.

AK Assay
The hydroxamate assay method was modified from a
procedure described by Brennecke et al. (1996). The assays
were performed in a 500-μL volume containing the follow-
ging: 50 mM Asp (sodium salt), 20 mM Tris-HCl (pH 7.4), 1
mM DTT, 3% [v/v] glycerol, 8 mM MgSO4, 20 mM ATP (pH
7.4), and 480 mM hydroxylamine (neutralized with 4.8 x
NaOH just before use). The assay was started by the addi-
tion of 100 μL of enzyme. After incubating at 37°C for 40 to
60 min, the reaction was terminated by addition of 500 μL
of stop solution (0.67 M FeCl3, containing 0.5 M HCl and
EDTA, 5 mM with a Polytron homogenizer in 5:1 (v/w) buffer E (100 mM cations. Developing kernels were degermed and ground into 2-mL aliquots, and stored at 4°C until use. The method described by Bryan and Lochner (1981) and Walter et al. (1979) was used for HSDH extraction, with minor modifications. Developing kernels were degemmed and ground with a Polytron homogenizer in 5:1 (v/w) buffer E (100 mM potassium phosphate buffer [pH 7.5], 1 mM EDTA, 5 mM L-Thr, 1.4 mM β-mercaptoethanol, 20% [v/v] glycerol, and 1 mM PMSF). The homogenate was centrifuged for 35 min at 20,000g and the supernatant was collected. Finely ground (NH₄)₂SO₄ was added to the supernatant until 70% saturation. The solution was stirred for 30 min and then centrifuged for 35 min at 20,000g. The pellet was resuspended in buffer E, desalted with a G-50 column, and stored at 4°C until use.

**HSDH Assay**

HSDH activity was measured in the forward direction by monitoring the oxidation of NADPH at 340 nm with a Beckman DU-65 spectrophotometer (Walter et al., 1979). The 1-mL reaction solution contained the following: 200 mM potassium phosphate (pH 7.0), 1.4 mM β-mercaptoethanol, 0.2 mM NADPH, 6 mM ASA (the 167-mM stock solution was neutralized with 4 n NaOH just before use), and 30 µL enzyme. The decrease in A₃₄₀ was recorded for 1 to 5 min at an interval of 1 min. The control assay solution contained all components except ASA. One enzyme unit was defined as the amount required for the oxidation of 1 nmol of NADPH per min at room temperature (25°C). For the Thr inhibition assay, 5, 10, and 20 mM Thr was added to the reaction solution.

**DHDPs Assay**

Enzyme activity was measured in 1.5-mL centrifuge tubes containing 100 mM Tris-HCl (pH 8.0), 10 mM pyruvate, 4 mM ASA (the 167-mM stock solution was neutralized with an equal volume of 4 n NaOH just before use), 20 to 60 µL of enzyme, and sterile double-distilled water to a final volume of 250 µL. The tubes were incubated at 37°C for 30 to 60 min, and the reaction was stopped by addition of 1 mL of stop buffer (0.22 M citric acid and 0.55 mM sodium phosphate [pH 5.0]) containing 0.25 mg mL⁻¹ o-amino-benzaldehyde (Sigma). The color was allowed to develop for 3 to 6 h at 37°C. Maximal color formation occurred after 3 h at 37°C, and the color remained stable for an additional 10 h. After color formation, the samples were centrifuged at 10,000g for 5 min and the absorbance was read at 520 nm with a DU-65 Beckman spectrophotometer. The control assay solution contained all the reaction components except pyruvate. One unit of enzyme activity was defined as the amount required for an increase of 0.001 A₅₂₀ min⁻¹ at 37°C. Inhibition assays were conducted with 10, 20, 30, 40, 50, and 100 µM Lys.

**LKR Extraction and Assay**

Five grams of developing endosperm was ground with the Polytron homogenizer in buffer F (100 mM Tris-HCl [pH 7.4], 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF, 15% [v/v] glycerol, and 5% [w/v] insoluble PVPP) and centrifuged at 20,000g for 20 min. The supernatant was brought to 33% (NH₄)₂SO₄ by adding a half volume of 100% saturated (NH₄)₂SO₄ and centrifuged at 20,000g for 20 min. Solid (NH₄)₂SO₄ was added to bring the solution to 60% saturation. This mixture was stirred for 30 min and then centrifuged at 20,000g for 30 min. The pellet was resuspended in 1 mL buffer F without PVPP, desalted with a Sephadex G-20 column (Pharmacia Biotech) in buffer F without PVPP, and then stored at 4°C until use.

The reaction mixture had a final volume of 1 mL and contained 20 mM Lys, 10 mM α-ketoglutaric acid (neutralized to pH 7.0 with KOH), 0.1 mM NADPH, 0.2 mM Tris-HCl (pH 7.4), and 0.04 to 0.1 mg of protein. Oxidation of NADPH was monitored at 340 nm with a Beckman DU-65 spectrophotometer at room temperature. The control assay solution contained all components except Lys. One unit of activity was defined as the amount of enzyme required for
the oxidation of 1 nmol of NADPH per min at room temperature.

Determination of Protein Concentration

Protein was measured by the Bradford (1976) method with bovine serum albumin as a standard.

Statistical methods

Analysis of variance was performed with the software package provided with Excel (Microsoft, Redwood, WA).

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