1994 REPORT
GRANT DE-FG02-88ER13863
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During the past year there has been progress in several areas relevant to maize endosperm development. These areas are 1] The tentative identification of the enzymatic deficiency in a previously unknown endosperm mutant, sugary3-1 (su3-1) recently reported by Philip Stinard (1992). The evidence leading to this conclusion will be presented below. 2] the recognition that the endosperm mutant that produces an interesting starch resembling some starches that have been chemically modified is actually an unusual, hypomorphic allele (8132) at the brittle2 (bt2) locus; 3] the orange endosperm color present in some progenies derived from a cross between the original bt2-8132 and W22N apparently results from an interaction between two genes, one of which behaves as though linked to the bt2 locus. In the orange endosperm derivative, our limited evidence suggests that the quantity of all the carotenoids present in the yellow endosperm stocks appear to be increased proportionally.

1] In 1992, Philip Stinard reported the identification of a previously undetected locus affecting endosperm development. The mutant kernels at this locus have a vitreous appearance much as sul kernels but with less wrinkling. For this reason, the locus was designated as su3. Mr. Stinard shared his stock of this mutant with us, and we believe that the enzymatic deficiency responsible for this phenotype is a second debranching enzyme. The initial observation was that the mature kernels of this mutant have a considerable quantity of a water-soluble polysaccharide just as do sul-1 kernels. However, this polysaccharide is clearly not the phytoglycogen typical of sul kernels. In some backgrounds, it produces a clear aqueous solution rather than the opalescent solution typical of sul phytoglycogen. Whether the solution is clear or opalescent, its absorption maximum after reaction with a KI/I₂ reagent is 485 rather than the 510 that is characteristic of sul phytoglycogen (Fig. 1). This difference while small is reproducible. Less water-soluble polysaccharide is produced by su3 kernels than by sul kernels as is shown in Figure 2 although this may be a consequence of the different genetic backgrounds in which these mutations are present.

When we had an opportunity to examine developing kernels
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(homozygous su3 and a dosage series from crosses by and on the W22N inbred) following the 1994 growing season, we found that the su3 kernels had substantially less debranching (alpha-1,6-glucosidase) activity than the nonmutant kernels measured as nmols maltotriose released from the fungal polysaccharide, pullulan and that the debranching activity above a basal level was roughly proportional to the number of copies of the nonmutant allele present (Fig. 3). We had reported the same finding for developing sul kernels (Pan and Nelson, 1984) and showing that there are three peaks of debranching enzyme activity in the nonmutant extracts following DEAE 52 and hydroxyapatite column chromatography. Two of these are reduced in sul extracts while the third peak is absent. The converse of this activity spectrum is seen for the su3 extract following the column chromatography (Fig. 4). The debranching enzyme peaks that are present but reduced in sul extracts are also reduced in the su3 extracts while the peak missing from sul extracts is present in su3 extracts at close to the level observed for the nonmutant control. Peak I from the su3 mutant appears to be quite different from the nonmutant Peak I apart from having much less activity on pullulan. It has less activity on sul WSP than on su3 WSP.

Figure 5 shows the increase in glucose, sucrose, and reducing sugars in developing endosperms (22 DAP) of su3 homozygotes as compared to a nonmutant control.

The location of su3 is not known at the present time, and the possibility has recently been raised that su3 may in fact be a duplicate factor pair (Stinard & Schnable, 1994). Our recent results from Florida show that su3 kernels appear in the F2 progeny of a cross of su3 by a several nonmutant stocks in even fewer numbers than would be expected for a duplicate factor pair.

The double mutant, sul;su3, which would be obviously very interesting, has not yet been identified positively. We have, however, found in seed sent by Stinard a plant that is su3/su3; sul/Sul. When selfed, this plant produced seeds segregating for a reduced kernel that had phytoglycogen but virtually no starch. It is possible that these reduced kernels are the double mutant, but that remains to be tested.

2] For the past several years, we have been interested in a mutant received originally from the Maize Coop that was obviously a mutable gene because patches of nonmutant tissue formed erup-
tions on the surface of the seeds. The mutable system involved
is not Ac/Ds nor Spm/dSpm as shown by making the requisite cross-
es. The transposable element system might be Mu since we have
yet to recover a nonmutant derivative from the selfed progeny.
We also found that all the 8132 kernels recovered from
outcrosses to W22N still showed evidence of mutability, but we
could select away from mutability in subsequent selfed progenies.
That the system is Mu has not been demonstrated, however.

There has been considerable interest on the part of American
Maize Products, a wet milling concern, that has been testing
samples of the mutant seeds we have grown for them for several
years. The specific attribute of the mutant starch is that when
sheared with sufficient force while being heated, the starch does
not increase in viscosity while cooling. This characteristic is
desirable for certain food products and is revealed by a conven-
tional Brabender test. This past summer American Maize has grown
larger quantities of the mutant so that their tests can be scaled
up in order to assess more accurately the commercial possibili-
ties of this starch.

The mutant seeds in most backgrounds are easily distin-
guished from nonmutant seeds on the same ear by the deep dimple
in the crown of the kernel (Fig. 6). The mutant kernels average
about 80% of the weight of nonmutant kernels on the same ear. We
knew from allele tests that the mutant was not ae, du, or su2,
which were the possibilities suggested by the phenotype of the
mutant seeds. Tests of enzymatic activity suggested that the
mutant had only scant activity of a glucosidase-transferase that
was highly active in the inbred line with which it was being
compared leading to the tentative conclusion that the locus
encoding this enzyme activity was the one affected by the muta-
tion. Attention was, however, forcibly directed elsewhere by
accumulating evidence. We had found that the locus in question
was located on the short arm of chromosome 4 since it was uncov-
ered by TB-4Sa. Attempts to isolate other alleles at the locus
by EMS mutagenesis were successful, and the first two alleles so
isolated, 9101 and 9201, conditioned the production of kernels
that are considerably more shrunken than the original mutant and
with phenotypes indistinguishable from those mutants, brittle1,
brittle2, and shrunken2, that have the most severe effect on
starch production. Although allele tests with these mutants
should have been made earlier, late is better than never. It was
not surprising to find that brittle1 and shrunken2 complemented
the new mutants, but they were allelic to brittle2 which was
known to be on the same chromosome arm, 4S. However, it was a
surprise to find that the original mutant, which is much less
defective than any other bt2 mutant and which had been found in
our tests to have considerable ADPGLc pyrophosphorylase activity
(1/3 to 1/2 of nonmutant activity), is a bt2 allele, which is
designated as bt2-8132. The intermediate enzymatic activity of
8132 has been confirmed in the laboratory of L.C. Hannah at the
University of Florida. Although almost all of the mutants in-
duced by EMS have been very shrunken, we have detected one appar-
ent, high grade intermediate that is currently being investigat-
ed.

The identification of this mutant as a bt2 allele raises
several interesting questions. Why should a mutant allele (bt2-
8132) of a locus encoding one of the two sub-units of ADPGLc
pyrophosphorylase, which synthesizes the principal (only?) sub-
strate for the starch synthases, condition the production of
starch that is demonstrably different from the starch produced by
nonmutant plants. The obvious expectation is that homozygous
mutant seeds should produce a lesser quantity of starch that is
not distinguishable from nonmutant starch. A second question is
why with enzyme activity that is often measured as being more
than that produced by a single copy of a Bt2 allele that produces
a phenotypically nonmutant endosperm, homozygous 8132 endosperms
are not also nonmutant.

3] An interesting outcome of our research with bt2-8132 is
the appearance of kernels with orange endosperms in the F₂ proge-
ny from crosses of bt2-8132 times W22N, both of which have yellow
endosperms. Thus the appearance of the orange kernels appears
to be a classic example of transgressive segregation. This
deeper endosperm color can be noted in some of the 8132 kernels
in Figure 6. While the investigation of the genetic basis of
orange endosperm is only really beginning, there are data that
suggest a hypothesis.

When the orange endosperm bt2-8132 is crossed by W22N either
as a male or as a female, the F₁ kernels are all yellow. When
the F₁ plants are backcrossed in either direction by the orange
bt2-8132, one-half of the kernels are orange and one-half are
yellow. The orange kernels are almost entirely bt2-8132 while
the yellow kernels are almost all Bt2. By contrast, when the
yellow endosperm bt2-8132 is crossed by the orange endosperm bt2-
8132, all the F₁ kernels are orange. When backcrossed by the
yellow parent, one-half the resultant kernels are orange and one-
half are yellow.

These data suggest a hypothesis that invokes the existence of two hypothetical loci. One is the locus, orange (ore) at which an allele, Ore conditions the production of the orange endosperm although there is no evidence that the functional allele is not an allele at y or some other locus known to affect carotinoid production. The second hypothetical locus is suppressor of orange (spo) with a dominant allele, Spo, that effectively suppresses the production of the orange phenotype in Ore/-/- endosperms.

If one then assumes that the genotype of the original, yellow endosperm bt2-8132 is ore/ore; spo/spo with spo being rather tightly linked to bt2 and that the genotype of W22N is Ore/Ore; Spo/Spo, this would explain the genetic data gathered to date. It is evident that the orange phenotype is not expressed only in bt2/bt2 seeds since orange nonbrittle seeds have also been recovered in F$_2$ progenies of orange·8132 stocks times W22N.

There has also been an effort to elucidate the changes in carotinoid composition in orange endosperm seeds as compared to yellow endosperm seeds in genetic backgrounds that are as close as possible. The expectation (hope?) was that we would find that beta carotene is increased at the expense of one or more of the usual carotinoid components of yellow endosperm maize. The results to date indicate that this is not the case but that all of the usual carotinoids are increased proportionately.

In preparing for the assay of the carotinoids, 0.2g of maize meal was extracted under reduced light with methanol using apo-8-carotenal as an internal standard. The total carotinoid content was estimated by reading the absorption at A$_{450}$ and using the extinction coefficient of lutein, which is the predominant carotinoid at that wavelength. This is not the most precise method since each carotinoid has a slightly different absorption maximum, and the extinction coefficient differs for each carotinoid, but it is widely used.

A single sample of each type was analyzed by HPLC (Weber, 1987) for efficiency of extraction using the internal standard as a reference. The inability to analyze more than a single sample at that time resulted from our use of an HPLC in another
laboratory, and there was insufficient time available to do more. Lutein was then calculated by comparison to a known concentration of authentic lutein. Although the efficiency of extraction was estimated to be only about 70%, the data (Table 1) indicate that there is an increase in total carotenoids.

There are also clearly differences in carotenoid contents in different genetic backgrounds, as has been reported (Brunson and Quackenbush, 1962; Grogan et al., 1963). It is possible, however, that the low carotenoid content of W22N may be due in part to the fact that the W22N kernels tested were from the 1992 crop while all other samples tested were from the 1993 crop. Weber (1987) has reported that corn kernels lose carotenoid content during storage. It is also possible that the relatively high values found for the bt2-8132 kernels, both yellow and orange, may be due partly or wholly to a specific interaction with the 8132 allele owing possibly to the greater quantity of CH_2O being available for carotenoid synthesis since there is less being used for starch synthesis. The samples listed as OP-Bt2 yellow and orange were obtained from the open-pollinated F_2 progeny of a cross of W64A X W182E grown from seed treated with EMS times orange 8132 plants. The object of the cross was to produce more intermediate bt2 mutants with phenotypes similar to 8132. Kernels with nonmutant phenotypes resulting from this cross were planted in 1993 in a large block and allowed to open-pollinate in order to reveal other mutations that affect endosperm development. A number of such were found, but among the nonmutant kernels on each ear were a few with an orange phenotype in contrast to the great majority of nonmutant kernels, which were yellow.

Although as noted previously we had hoped that we would find that the orange endosperms were due to the enhanced synthesis of beta-carotene at the expense of other carotenoids, and this appears not to be so, these orange endosperm stocks may still be useful if the trait is incorporated into productive hybrids. The greater quantity of carotenoids including beta-carotene should be useful in feeds.

We should be able to bring much of the above research to the point at which the results can be published within another year. We will, in fact, make a preliminary report of the su3 work to the 1995 Maize Conference in Asilomar this coming March.
LITERATURE CITED


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Table 1. Estimate of carotenoid content in yellow and orange endosperms from nonmutant and \textit{bt2-8132} kernels from comparable genetic backgrounds.

<table>
<thead>
<tr>
<th>Stock</th>
<th>Carotenoid (ug/g)</th>
<th>Extraction Efficiency (%)</th>
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<tbody>
<tr>
<td>W22N</td>
<td>18 ± 0.2</td>
<td>71</td>
</tr>
<tr>
<td>OP-Bt2* yellow</td>
<td>35.5 ± 5</td>
<td>80</td>
</tr>
<tr>
<td>OP-Bt2* orange</td>
<td>62.5 ± 2.5</td>
<td>68</td>
</tr>
<tr>
<td>8132 yellow</td>
<td>52.5 ± 5</td>
<td>77</td>
</tr>
<tr>
<td>8132 orange</td>
<td>82.5 ± 2</td>
<td>84</td>
</tr>
</tbody>
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* Derivation described in the text.
FIGURE LEGENDS

Figure 1. The spectra of su1 and su3 WSPs from mature seeds following reaction with a KI/I₂ reagent.

Figure 2. The WSPs from mature seeds of su1, su3, and a nonmutant inbred.

Figure 3. The debranching activity on pullulan of a dosage series, su3/su3/su3, su3/su3/Su3+, Su3/Su3/su3, Su3/Su3/Su3. The enzymatic activity was measured in 30-55% ammonium sulfate fractions of endosperm extracts (21 DAP).

Figure 4. The debranching activity on pullulan of su3 and Su3 endosperm (21 DAP) extracts following DEAE 52 and hydroxyapatite column chromatography.

Figure 5. The reducing sugars and sucrose present in su3 and Su3 endosperms 21 DAP.
Water-soluble Polysaccharide Absorption

$su1$ vs $su3$

Absorption Peaks

$su1$: 510 nm  $su3$: 485 nm

Figure 1
Water-soluble Polysaccharides

WSP (mg/endosperm)

Su3   su3   su1

Genotype

Figure 2
Debranching Enzyme Activity

Maltotriose (uMole/mg protein)

Time (min.)

Figure 3
Debranching Enzyme Activity

Su3 vs su3

Maltotriose (uMole/100 ul/hr)

Fraction Number

Figure 4
Sugars per Endosperm

Su^3 vs su^3

Carbohydrate (mg/endosperm)

Glucose | Fructose | Sucrose | Tot. Red. Sugar

Su^3 | su^3

Figure 5