Final Progress Report

Summary statement: The major scientific contributions developed during the previous project period are as follows.

1) The SSIII isoform coded for by Du1 is required for the formation for two distinct groups of chain lengths in Ap of maize endosperm, DP 7-9, and DP 37-55. Decrease in these chains is accompanied by an increase in chains of DP 11-15. A hypothesis consistent with these data is that one of the other SS isoforms produces chains in the range of DP 11-15, and these are then elongated by SSIII to the range of DP 37-55.

2) SSIII affects Ap structure by an as yet unidentified mechanism in addition to its enzymatic ability to elongate glucan chains. This is evident from the finding that transgenic expression of a non-catalytic fragment of SSIII causes alterations in Ap structure.

3) Transgenic alteration of SSIII expression can cause structural changes in Ap different than those of any known classical mutation.

4) The N terminal region of the SSIII isoform coded for by Du1 interacts with BEIIa, as indicated by positive two hybrid interactions in yeast.

5) Disruption of SSIII expression does not cause any observable change in the activities of known BEs, in contrast to previous literature reports that used a different method to observe BE activity (3). A potential allele-specific effect on DBE activity was detected, however, which may explain the synthetic genetic interactions of *du1* mutations with certain mutations of the *sul* gene.

6) Contrary to expectation at the beginning of the previous project period, there does not appear to be a significant amount of diversity conditioned by distinct *du1* mutations. The effects of ten different naturally occurring *du1* alleles on both Ap structure and starch biosynthetic enzyme expression were characterized, and in all instances the phenotypic consequences were very similar.

Hypothesis 1 - SSIII is responsible for constructing glucan chains in Ap of specific lengths: The project was successful in providing a detailed description of the Ap chain length distribution that results from ten allelic mutations of the *du1* locus. Figure 4 presents the data for six different alleles, in the form of differences between the mutant and wild type for the relative abundance of each chain length between 6 and 55 glucosyl units. The data shown derive from mature kernels, although similar results were observed for kernels harvested 20 DAP. Panels B and D indicate the variation observed between independent samples of the same genotype. Comparing the extent of this variation to the differences from wild type observed for the six alleles shown in the other panels illustrates the significance of the changes induced by the *du1* mutations. Furthermore, all ten alleles analyzed (including four not shown) gave very similar results. Taken together the data demonstrate definitively that specific groups of chains are reduced when SSIII is mutated, and certain other groups of chains are increased in abundance. The pattern is complex. Chains of DP 6-9 are present less frequently in the absence of SSIII, those of DP 11-16 are present at higher frequency, DP 17-37 are either unchanged or slightly elevated, and chains of DP 38-55 are reduced in
Figure 4. Differences in the relative abundance of specific chain lengths between mutant and wild type Ap. Whole starch from mature kernels of W64A mutants and wild type was debranched with isoamylase and resultant linear chains were fluorescently labeled at the reducing end. Chains were separated by capillary electrophoresis and quantified. The percentage of total fluorescence for each chain among the total fluorescence for chains from DP 6 - 55 was calculated. In panels A, C, D-H the value for each chain in wild type starch was subtracted from that for the indicated mutant. Values for each chain are the average of two or three independent repetitions of the assay. In Panels B and D the values for one repetition were subtracted from those for another of the same genotype.
frequency. Precisely how these changes result from loss of SSIII activity cannot be discerned clearly, although part of the explanation may be that chains in the range of 11-16 are substrates of SSIII, which catalyzes elongation by approximately 30-40 units. It is clear from these data that SSIII does play a very specific role in Ap synthesis that cannot be replaced by any of the other classes of SS or any other isoform of the SSIII class that may exist.

The prediction that different alleles of du1 would condition distinct changes in the chain length distribution was not borne out by the results of the project. All ten du1' mutations examined caused approximately the same changes in change length distribution as those shown in Figure 4. This is despite the fact that some alleles cause loss of SSIII protein whereas others do not. Thus, the critical factor in the chain length distribution may be SSIII activity, such that all of the alleles cause loss of the enzyme even if the protein remains. There is some slight degree of diversity in the range DP 17-37, in which some alleles exhibit a slight increase in abundance whereas others do not. Whether these changes are significant and reproducible remains to be determined.

**Hypothesis 2 - SSIII interacts physically with other components of the starch biosynthetic pathway and regulates their activity(s).** The results of the previous project period are consistent with this hypothesis, although the data are not necessarily definitive nor do they clearly reveal a specific mechanism. SSIII was tested for interactions with approximately ten other components of the starch biosynthetic system using the yeast two-hybrid system (Table 1). A portion of the N terminus of SSIII yielded a positive signal in combination with full-length BEIIa, and this result was observed in both orientations of the two-hybrid test. Thus, a relatively firm conclusion can be drawn that SSIII and BEIIa interact directly. Significant efforts were made to seek confirming data by immunoprecipitation experiments, however, to date these experiments have not yielded definitive results.

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<td>SU1</td>
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<td>ZPU1</td>
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<td>BEIIa</td>
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* A 1.1 kb region of the N-terminus of the DU1 protein was expressed, comprising approximately amino acids 500 to 803

* Negative control plasmids, expressing unfused binding domain (BD) or activation domain (AD)

* Positive control plasmids

The second proposed approach to seek interactions between SSIII and other starch biosynthetic enzymes was comparison of the fractionation behavior and activity level of each SS, BE, and DBE among wild type and du1' mutants. These experiments were completed successfully for the BEs and DBEs, however, analysis of the SS complement in du1' mutants remains to be completed. Zymogram analysis effectively allowed determination of the effects of various du1' mutations on BEs and DBEs. In the one-dimensional zymogram (Figure 5A), crude soluble protein extracts are fractionated by native PAGE, and the gel is then blotted to a second gel impregnated with solubilized starch. Enzyme activity that acts on the starch is visualized by staining the gels with iodine solution, because distinct colors result from different chemical structures. In the two-dimensional zymogram (Figure 5B), the extracts are first fractionated by anion exchange chromatography, and those fractions are then subjected to native PAGE.
A goal of the project during the previous funding period was to find the explanation for the previously published observation that a BE activity was affected by altered SSIII expression, owing to the du1-Ref mutation (3). The affected enzyme was thought to be a BEII isoform based on its anion exchange properties. The data shown in Figure 5C, however, show clearly that both BEIIa and BEIIb are present at apparently the same level as wild type in all of the du1+ mutants examined. Thus, the nature of the pleiotropic effect of SSIII on the BEII class remains undetermined.

One instance of allele-specific change in a DBE was observed, as the result of mutation du1-8801. The major band of isoamylase-type DBE activity, visualized by bright blue color, was reduced in both 1D and 2D zymograms (Figure 5A,B). This DBE was not affected by the null allele du1-M5 (Figure 5A,B) consistent with the hypothesis that SSIII regulates other starch biosynthetic activities by direct interactions. The affected DBE is the enzyme coded for by the sul1 gene. Synthetic genetic interactions between du1 and sul1 (40) were the initial basis for undertaking these experiments. The results further our understanding of this interaction, by showing that DBE activity can be dependent on the state of SSIII. These observations provide strong support for the concept of a centrally organized starch biosynthetic
apparatus, as opposed to a series of independent steps. Mechanistic detail is still lacking, however, because evidence of direct binding of these two components was not observed.

Initial experiments to characterize the full complement of SSs in the du1 mutants made use of anion exchange chromatography as the means of fractionation. These analyses were found to be insufficient to clearly distinguish specific effects, owing to problems with resolution and reproducibility. Thus, the project has not yet completed the specific aim of analyzing all SS activities in the lines deficient in SSIII. Recent implementation of a 2D zymogram technique, however, has made a major improvement the resolution of this analysis. These assays are similar to the 2D procedure described for BE and DBE activity, however, the second gel contains a low amount of glycogen instead of starch. The gels are incubated in the presence of ADPGlc, so that SS activity extends the glycogen chains. Iodine staining produces brown bands in a yellow background. Using this method, six different bands of SS activity were identified (Figure 5D). Analysis of kernel extracts from the du1-M5 null mutant revealed that the slowest-migrating band was missing, thus identifying SSIII activity (data not shown), and another of the bands has been identified as SSIIAs by similar genetic analysis (58). The project can now apply this method to many of the du1 alleles in the study set, and examine for reproducible changes in the mobility or level of any of the other SSs. These experiments will continue through the fall of 2003.

Hypothesis 3 - Transgenic expression of SSIII can cause changes in Ap structure that would not occur by classical mutation: This aspect of the project has proven successful, as novel Ap structures were observed in transgenic plants. Three transgenes were constructed. Construct P183 expresses the N-terminal 770 amino acids of the SSIII coded for by Du1, P184 express the N-terminal 1228 amino acids, and P185 expresses the full-length SSIII protein. Figure 2 illustrates that the first two constructs contain overlapping regions of the non-catalytic N-terminal extension. The intent of these experiments was to test the effects of producing in plants a region of SSIII that potentially could interact with the in vivo binding partners, but that would be disconnected from the catalytic activity. Interference with the putative starch biosynthetic assembly complex in this way was proposed as a means of causing subtle changes that would have novel affects on Ap structure.

Transformation directly into inbred Oh43 was successful. At least five independent integration events, with 10 regenerated plants for each event, have produced a series of lines containing the three transgenes. PCR analysis using primer pairs specific to the transgene verified the presence of the recombinant construct in the maize genome. DNA gel blot hybridization analysis identified plants with a low copy number of the transgene, although further work is needed to precisely determine this parameter. Analysis to date has been limited to kernels from self-pollinated ears of the primary regenerated plants. These lines are now being propagated in the field, and two subsequent generations will be available for analysis later in the summer of 2003.

The results obtained for the primary regenerant plants indicate that the transgenes have indeed resulted in a novel Ap structure (Figure 6). The analyses are the same as those described for hypothesis #1 earlier in this section. Starches from the transgenic plants will be a major focus during the proposed project period, so a more detailed description of the data is included in the description that follows of objective #1. The data in Figure 6 show, however, that expressing an N terminal fragment of SSIII causes and effect that differs from that of a null mutation at the du1 locus. This distinct chemical phenotype makes it unlikely that the effects are the result of RNA interference reducing SSIII expression.

Further work during fall 2003 and spring 2004 will better characterize the transgenic plants, using immunoblot analysis and RT-PCR to measure transgene expression. Also, additional repetitions of the Ap chain length profiles from independent samples are needed in order to verify the reproducibility of the results shown in Figure 6 as well as their heretibility in subsequent generations.
Figure 6. Differences in the relative abundance of specific chain lengths between Ap from various sources. The analysis is as described in the legend to Figure 4.