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Molecular Genetic Analysis of Maize Starch Branching Isoforms: Modulation of Starch Branching Enzyme Isoform Activities in Maize to Produce Starch with Novel Branching Architecture and Properties

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EXECUTIVE SUMMARY: The main long-term objectives of this project were to understand the molecular and biochemical basis for starch biosynthesis and utilization in plants. Towards these goals, this project focused on the Starch Branching Enzymes (SBE) of maize, which are critical in determining the molecular fine-structure of starch. Supported primarily by the DOE, our work over the past decade has characterized the maize SBE gene family (SBEI, SBEIIa and SBEIIb) and begun to unravel the specific functions each isoform plays in plant development. The genes encoding each isoform were isolated, sequenced and their expression was studied. Using transposon mutagenesis, mutant *Sbe* lines were developed and used to study SBE function. Data indicate that each of the maize SBE genes plays unique, and partially overlapping functions important for normal maize development. The *Ae* gene (encoding SBEIIb) is the predominant SBE in seeds. In leaves, SBEIIa is the key if not only SBE, and in its absence, plants exhibit an extreme leaf senescence phenotype. In pollen, *ae* and *sbe2a* single mutants are normal, but recovery of double homozygous viable kernels is at a rate much lower than expected, suggesting a partially overlapping function of the class II SBEs. We have examined starch produced by our mutants using several approaches, including measurements of the branch chain lengths and distributions of granule size and shape. We have measured functional characteristics of the starches, including resistance to enzyme degradation and gelatinization physical parameters. Our mutant starches show profound changes in various parameters, which reflect changes in starch micro-structure and possibly changes in granule supra-molecular structure. Surprisingly, the double mutant combinations *sbe1; ae* and *sbe2a; ae* produce starch with increased branching as compared to *ae* starch alone, suggesting the possible existence of a feedback mechanism regulating the SBE activity during starch biosynthesis.

Backcrossing SBE Mutants: *sbe1::Mu* and *sbe2a::Mu*: Backcrossing to W64A has generated *sbe1::Mu* and *sbe2a::Mu* stocks that have been backcrossed seven and six generations, respectively.

Abnormal Segregation of the *sbe2a; ae* Double Mutant: The rate of double mutant recovery was accurately determined to be 5.7% from a population of ~700 seedlings, significantly lower than the theoretical 25% expected. This abnormal segregation ratio demonstrates that at least one isoform of SBE (either SBEIIa or IIb) is required for either kernel germination or normal reproduction. Kernels that failed to germinate were genotyped and shown to not be double mutants. Our data suggests that the double mutant is impaired in pollen tube growth and is not capable of competing for fertilization with pollen containing one or more of the functional *Sbe2a* or *2b* genes. This indicates that the SBEII class isoforms act redundantly during pollen tube development. While *sbe2* mutant pollen grains are viable, as evidenced by their 5.7% recovery, they are outcompeted by the growth of Wt or single *sbe2* mutant pollen grains, explaining the low recovery rate. We hypothesize that the reduced fitness of double mutant *sbe2* pollen is due to an altered

Fig. 5 SBE activity in mature pollen grains. A) Immunoblotting with anti-SBEIIa antibody; SBEIIa is absent in the *sbe2a* mutant. **B)** In-gel SBE activity assay; Individual activities missing in each mutant. **C)** *In vitro* SBE assay; SBE activity is ~50% reduced in *sbe2a* pollen as compared to W64A

starch structure such that the starch is more resistant to degradation than in Wt in a manner found in *ae* kernels or *sbe2a* leaves.

SBE Activity in Mature Pollen Grains: Western analysis, in-gel SBE assays and *in vitro* phosphorylase a stimulation assays of SBE proteins in W64A, *ae* and *sbe2a* pollen collectively demonstrate that SBEIIa and SBEIIb are both present and active in dehiscent wildtype pollen. Total SBE activity was reduced by half in *sbe2a* pollen as compared to wildtype.

Each of the 39 double mutants individuals identified and grown to maturity in this study produced tassels, anthers and pollen grains that were phenotypically indistinguishable from *ae/ae* single mutants. This demonstrates that the *sbe2a; ae* double mutant is not male sterile.

Effects of *sbe* Mutations on Vegetative Growth: The *sbe2a* mutation dramatically reduced plant height and leaf number, while the *sbe1* mutation slightly increased height, indicating the critical role of starch biosynthesis in plant growth and development. Field grown plants originating from progeny of selfed *Sbe1/sbe1*; *Sbe2a/sbe2a* plants exhibited height differences that correlated with *sbe* genotype. *sbe1* single mutants were the tallest (172.9 ± 3.5 cm; p-value < 0.02). *Sbe1*; *Sbe2a* wildtype plants (160.5 ± 5.5 cm) were significantly taller than the *sbe1* single (172.9 ± 3.5 cm) and the *sbe1*; *sbe2a* double (60.3 ± 19.8 cm) mutants (p-value < 0.01). The heights of the *sbe2a* single (96.2 ± 11.6 cm) mutants and the *sbe1*; *sbe2a* double mutants did not significantly differ but were significantly shorter than *sbe1* single mutant and wildtype plants (p-value < 0.01). In the same population of plants described above, leaf number varied with *sbe* genotype. Wildtype (14.1 ± 0.3) and *sbe1* mutant (14.8 ± 0.4) plants had a similar number of leaves, which was significantly higher than in the *sbe2a* single mutants (11.9 ± 0.6). Data was recorded for two yearly crops.

Starch Digestibility is Altered in *sbe* Mutants: Our work has more recently focused on studying the susceptibility of starch to degradation by various amylolytic enzymes as a measure of both structural differences, and also of functionality in resistant starch applications. *In vivo* digestibility of endosperm starches were mimicked using a novel *in vitro* technique of the modified official AOAC method. The rate of digestion and proportion of resistant starch from endosperm was measured via a 16-hour time course treatment with porcine pancreatic α -amylase. Mutation of the gene encoding SBEIIb, which results in the synthesis of high amylose or *ae* starch, dramatically increases resistance of the starch to amylolytic digestion (i.e., 60.7%). The *sbe1* mutation, but not the *sbe2a* mutation produced starch that also exhibited enhanced resistance to enzymatic degradation, somewhat intermediate between *ae* and wildtype controls. The finding that *sbe1* starch produces an appreciable amount of RS in endosperm could be of practical importance since these mutants have a higher starch yield in kernels as compared to *ae* mutants, which is the current commercial source for RS.

The effect of the *ae* mutation on RS amount is likely due to dramatic reduction in branch points throughout the starch. However, the *sbe1* mutation results in only very subtle fine-structural changes (see below). We hypothesize that this effect on enzyme resistance may be at least partly mediated by a larger-scale alteration of starch granule supra-molecular structure. These findings might have broad application in the production of resistant starch-containing materials for food and medical purposes, and they may also be pertinent to degradation of starch for the production of ethanol biofuel.

Digestibility of Maize Leaf Blade Starches Varying in Starch Branching Enzyme Isoform Activities: We have investigated the possible mechanisms by which the deficiency in SBEIIa activity in leaves leads to premature senescence. Our preliminary data suggest that:

- The *sbe2a* mutation leads to leaf starch more resistant to digestion (~47%).
- Starch granules in *sbe2a* mutant leaves do not cycle at night.
- *sbe2a* starch granules are approx. three times larger than normal.

Based on these observations, a series of experiments carried out to rigorously verify these findings. We hypothesize that a dramatic reduction of starch digestibility in the mutant leaves results in a greatly disturbed energy metabolism in which sugars are the main form of carbon currency utilized by the cells. It is further possible that this situation disturbs the normal energy relationships between

Table 1 Resistant Starch in Mutant Endosperm and Leaves

Genotype	Percent Resistant Starch	
	Endosperm	Leaf
W64A	0.3 ± 0.5 ¹	NA ²
<i>sbe1</i>	14.0 ± 3.4	NA
<i>sbe2a</i>	0.5 ± 0.8	NA
<i>sbe1, sbe2a</i>	14.1 ± 3.9	NA
<i>ae</i>	60.7 ± 0.7	NA
<i>Wx</i>	ND ³	ND
<i>sbe1, wx</i>	ND	25.7
<i>sbe2a; wx</i>	ND	47.6 ± 2.8
<i>sbe1; ae; wx</i>	NA	12.8
<i>ae; wx</i>	53.9 ± 4.1	ND

¹Standard deviation

²NA, not available

³ND, no resistant starch was detected (i.e., 0%).

⁴RS for this genotype was measured by the Gons method, which could potentially underestimate the RS value

Fig. 6 Time-course β -amylolysis of SBE-deficient endosperm starches. A) An early stage, treatment for 24 hr with 2.5 U β -amylase/ml. B) An intermediate stage, treatment for 2 hr with 25 U β -amylase/ml. (c) A late stage, treatment for 2 hr with 250 U β -amylase/ml.

source and sink tissues, resulting in a gradual decline and senescence of the leaf.

Fine Structure Analysis: Branching Pattern Differences among Maize Endosperm Amylopectins Varying in Starch Branching Enzyme Isoform Activities: By combining β -amylolysis over time with chromatographic techniques of branch chain length distributions, we have detected, for the first time, alterations resulting from the *sbe1* and *sbe2a* single mutants individually and in combinations with *ae*. Our data suggests that the proportion of closely associated branch points is in the order *sbe2a; wx* > *sbe1; wx* > *wx*. Starch from *sbe2a; wx* shows lower susceptibility to β -amylase than starches from *sbe1; wx* and *wx* in all the three stages of hydrolysis. These observations provide the first evidence for differences of amylopectin branching pattern among *wx*, *sbe1; wx*, and *sbe2a; wx* starches. Differences between *ae; wx* and *sbe1; ae; wx*, and between *ae*-containing and non-*ae*-containing mutants were also observed by this novel approach.

Changes in Experimental Plan: Our experimental plan included a series of experiments in which a set of dosage series containing 1, 2 or 3 mutant alleles for each of the SBE genes alone and in

double mutant combinations were constructed and starch isolated from endosperm for structural analysis. The results of preliminary experiments proved that only very minor, if any, differences were noted in the various dosage series, thus we terminated a further detailed analysis of these lines.

During the previous funding period we observed that *sbe2a; ae* double mutant plants were difficult to obtain from a selfed *Sbe2a/sbe2a; ae/ae* heterozygote. Although not proposed in the 2004 submission, during the final funding period we confirmed this observation and thus conducted a study of pollen tube function in the double mutants. Manuscripts describing this work are under preparation.

Publications Supported by this Project

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