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Genetics

A single gene mutation that increases maize seed weight

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ABSTRACT **The maize endosperm-specific gene *shrunken2* (*Sh2*) encodes the large subunit of the heterotetrameric starch synthetic enzyme adenosine diphosphoglucose pyrophosphorylase (AGP; EC 2.7.7.27). Here we exploit an *in vivo*, site-specific mutagenesis system to create short insertion mutations in a region of the gene known to be involved in the allosteric regulation of AGP. The site-specific mutagen is the transposable element *dissociation* (*Ds*). Approximately one-third (8 of 23) of the germinal revertants sequenced restored the wild-type sequence, whereas the remaining revertants contained insertions of 3 or 6 bp. All revertants retained the original reading frame 3′ to the insertion site and involved the addition of tyrosine and/or serine. Each insertion revertant reduced total AGP activity and the amount of the SH2 protein. The revertant containing additional tyrosine and serine residues increased seed weight 11–18% without increasing or decreasing the percentage of starch. Other insertion revertants lacking an additional serine reduced seed weight. Reduced sensitivity to phosphate, a long-known inhibitor of AGP, was found in the high seed-weight revertant. This alteration is likely universally important since insertion of tyrosine and serine in the potato large subunit of AGP at the comparable position and expression in *Escherichia coli* also led to a phosphate-insensitive enzyme. These results show that single gene mutations giving rise to increased seed weight, and therefore perhaps yield, are clearly possible in a plant with a long history of intensive and successful breeding efforts.**

Zea mays L., maize or corn, is the major, economically most important, domesticated plant in the United States and one of the major cereals of the world. Appropriately, it has received a considerable amount of attention from plant breeders, and yield increases, due to genetic improvement, have approximately doubled since the exploitation of hybrid vigor (1). [In virtually all economic situations, yield is defined as weight of seed harvested per unit area, and individual seed weight is a major determinant of yield (1)].

Starch comprises approximately 70% of maize seed dry weight and is therefore a major component of yield. Many of the biochemical and molecular studies involved in starch synthesis have focused on the identification of rate-limiting enzymes to genetically alter these important regulatory steps. The enzyme adenosine diphosphoglucose pyrophosphorylase (AGP) (EC 2.7.7.27) has received considerable attention primarily because of its allosteric properties and its position as the first unique step in starch synthesis (for reviews see refs. 2 and 3). This was recently demonstrated to be an important target by investigators at Monsanto (4), who expressed a bacterial-derived AGP in potato tubers and increased starch content 30%. The increase in starch came at the expense of water, the major component of the harvested tuber. In contrast, the mature maize seed contains a relatively small amount of moisture and therefore it is not clear whether altered AGPs would function in a manner similar to the case in potato.

AGP is composed of two large and two small subunits. The endosperm-specific maize *shrunken2* (*Sh2*) gene encodes the large subunit, whereas the small subunit is encoded by *brittle2* (*Bt2*) (5–7). Lack of AGP and a decrease from wild-type starch levels result in shrunken, brittle, or collapsed kernels at seed maturity. In the studies reported here, a series of wild-type *Sh2* isoalleles were synthesized by movement of the transposable element, *dissociation* (*Ds*) from the *sh2* locus. The mutant *sh2-m1* was isolated by Oliver Nelson (University of Wisconsin, Madison), who monitored populations for movement of *Ds* from the closely linked *A1* gene to *Sh2*. Subsequent work has shown that the *Ds* insertion (8) lies in the last of 16 exons, and only 20 of the 515 *Sh2* amino acids are encoded distal to the site of *Ds* integration. Alternative splicing leads to the synthesis of five transcripts. Whereas perfectly wild-type mature transcripts can be produced by splicing, structurally altered AGP is also synthesized (5, 9).

A series of revertants was isolated by means of *Ac*-mediated transposition of *Ds* from the *Sh2* locus. Within the 23 independently isolated revertants, 5 different alleles were synthesized. All revertants restored the wild-type open reading frame distal to the site of *Ds* insertion and all non-wild-type or imprecise excisions involved the insertion of only one or two amino acids. This strongly suggests that this carboxyl-terminal portion of the protein is critical to wild-type gene function. Because AGP is important to starch synthesis, starch and seed dry weight were monitored in mature kernels differing only by these changes at the *Sh2* locus. One revertant allele, which contains an additional tyrosine and serine, conditions an 11–18% increase in seed weight. Because the percentage of starch in this revertant does not differ from the wild-type counterpart, the increased seed weight cannot be attributed solely to increased starch content. Rather, it appears that several seed components, including starch, are increased. Associated with the increased seed weight is an insensitivity to phosphate inhibition of the altered AGP. Furthermore, insertion of the tyrosine and serine residues in the comparable position of the potato large subunit of AGP conditions phosphate insensitivity in this enzyme as well.

MATERIALS AND METHODS

Isolation of *Sh2* Isoalleles from *sh2-m1*. The inbred stock containing *sh2-m1*, the closely linked *a1-m3*, and the activating element, *Ac* was described (5). Plants were self-pollinated and plump or nonshrunken seed were collected. Remnant mutant seed from ears of corn containing at least one plump seed were saved for planting in subsequent generations. Most revertant

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Abbreviations: AGP, adenosine diphosphoglucose pyrophosphorylase; 3-PGA, 3-phosphoglyceric acid.

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alleles were recovered from advanced populations in which plump seed arose at a frequency of approximately 1 per 1000 mutant seed.

Putatively revertant, plump seed were grown, self-pollinated and also crossed to a stock (the sweet corn 'Florida Stay Sweet') containing nonfunctional alleles of *a1* and *sh2*. The presence of plump seed exhibiting *a1* mutability in the testcross progeny was used to eliminate cases of accidental outcrossing in the original population, which gave rise to plump seed. Legitimate homozygous revertants were then selected from the selfed progeny and used in the analysis below. Each revertant was derived from a separate selfed ear in the original population and therefore each represents an independent event.

Seed Weights and Starch Determinations. Crosses of each homozygous revertant onto the sweet corn 'Florida Stay Sweet' were made in 1992, 1993, 1994, and 1995. 'Florida Stay Sweet' contains the null *sh2* allele, *sh2-R* (10). The maternal parent of 'Florida Stay Sweet', is the result of a cross between two closely related inbreds, whereas the male parent is distantly related. Because the revertants were derived from one inbred population and the tester is effectively an F_1, seeds derived from the cross described above are in a common genetic background containing one dose of the altered *Sh2* allele. Ears were harvested at 22 days after pollination or at maturity with all resulting kernels dried at 65°C for 1 week, cooled in a desiccator, and immediately weighed. Starch was determined by use of amyloglucosidase digestion as described (11).

AGP Assays. AGP assays in the direction of ADPglucose synthesis of 22-day-old kernels were done as described (5, 12). Specifically, a 100-μl reaction mixture was 80 mM Hepes (pH 8.0), 2 mM glucose 1-phosphate, 2 mM ATP, 2 mM $MgCl_2$ and contained 50 μg of bovine serum albumin and variable amounts of [^{14}C]-labeled glucose 1-phosphate. 3-phosphoglyceric acid (3-PGA) was routinely added in doses of 20 mM. Approximately 5–10 units (1 unit = 1 μmol of ADPglucose formed per 10 min) of AGP per g fresh weight were routinely obtained from developing seeds. Phosphate inhibition studies employed preparations partially purified through a PEG purification procedure (13). Preparations were resuspended in 1/5 vol of original buffer in 15 mM KH_2PO_4/1 mM EDTA/1 mM DTT/10% glycerol. These were stored as aliquots, kept at −70°C and diluted with H_2O before assay.

SH2 Protein Quantitative Assays. The amount of SH2 in the developing kernels was assayed using Western blots containing equal amounts of protein soluble in SDS buffer. The samples were ground and added to standard SDS buffer and run on 10% polyacrylamide gels according to standard methods. Gels were transferred to nitrocellulose and developed using an SH2 (10) antibody and a secondary antibody linked to a horseradish peroxidase. Blots were developed using a chemiluminescent substrate kit (Amersham). Multiple lanes were run for each sample and several autoradiogram exposures were quantified using a laser densitometer (Molecular Dynamics).

The stability of SH2 in revertant kernels was assayed by grinding kernels in 50 mM KH_2PO_4/1 mM EDTA. Samples were incubated for various times at 30°C and aliquots were removed and added to SDS sample buffer containing 5% 2-mercaptoethanol then heated to 100°C for 3 min.

Sequence of Revertant Footprints. High molecular weight DNA isolated from leaves of *Sh2* revertants was used in PCR reactions. Direct sequencing of PCR amplification products determined the sequences surrounding the site of *Ds* insertion. *Ds* of *sh2-m1* is inserted after base 5930 (14). PCR primers LH71 (5′-GGGAAGAAGCTTCAAAGCTAC-3′) and LH72 (5′-GGATCCAAAGCAGGGTAATGG-3′) (corresponding to bases 5565–5583 and 6230–6212, respectively) were used for the initial amplification. The reaction conditions were 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min, and a final extension of 10 min at 72°C. Each 100-μl reaction contained approximately 0.4 μg of genomic DNA and 50 pmol of each primer. Following amplification, the PCR products were partially purified through Millipore minicolumns and the approximately 700 bp products were sequenced with primers LH 291 (5′-CTTGTGTGCTCATCTATC-3′; bases 5807–5824) and LH290 (5′-ATGCAATACCATTGTAGG-3′; bases 6042–6025).

RNA Isolation. RNA was isolated from kernels that had been quick frozen in liquid N_2. A mortar and pestle were used to grind the samples to a fine powder and the RNA was isolated by a LiCl method (15).

AGP Expression in *Escherichia coli*. AGP expression in *E. coli* followed the procedures of Iglesias *et al.* (16). Full-length cDNA clones of *Sh2* and *Bt2* transcripts were synthesized by reverse transcription (RT)-PCR as follows. First-strand cDNA synthesis was primed with gene-specific 3′ primers from developing maize endosperm poly(A)$^+$ RNA using Superscript reverse transcriptase (GIBCO/BRL) followed by RNase H digestion. Primers for amplification of the cDNA hybridize to positions 525–551 and 5968–5993 for *Sh2* (14) and 19–80 and 1432–1461 for *Bt2* (7). Primers incorporated *Nco*I and *Sst*I sites for cloning into vectors from pMON17335 and pMON17336. To incorporate the *Nco*I site into the 5′ *Sh2* primer, the codon GCC was added to the sequence after the ATG and the sequence GGGGCC was added 5′ to the ATG. In the case of the 5′ *Bt2* primer, an *Nco*I site surrounding the ATG already existed. An *Nco*I site further downstream was removed by use of a 60-base primer containing a C instead of the A of the *Nco*I site. *Sst*I sites were added to the 3′ primers distal to the stop codon. The pMON vectors containing the potato large and small subunit cDNAs (16) were cleaved with the enzymes listed above, vector fragments were isolated from agarose gels, and vector from pMON17336 was ligated to the *Sh2* cDNA whereas vector from pMON17335 was ligated to the *Bt2* cDNA. Resulting clones were then transformed into the *glgC* mutant AC70R1-504, and selection for the presence of both plasmids was done as described elsewhere (16). Approximately 26 units of maize endosperm AGP (assayed in the absence of 3-PGA) are obtained per gram of *E. coli* cells. Addition of the 6-bp insertion of the *sh2-m1*-Rev6 variant into the potato large subunit AGP gene used standard site-specific mutagenesis methodology. Units of AGP obtained from these cells are comparable to those obtained from expression of the maize endosperm AGP in *E. coli* and are comparable to previous studies (16)

RESULTS

Sequence of the Revertants Derived from *sh2-m1*. Twenty-three revertants were sequenced in the area surrounding the *Ds* insertion site. The sequences as well as that of the *Ds* mutation *sh2-m1* are shown in Fig. 1. Placement of the *Ds* element within the *sh2* locus is detailed elsewhere (8). All germinal revertants restored the original wild-type open reading frame. Eight revertants contained wild-type sequence (precise excisions) and 11 contained an insertion of TAC (tyrosine). Two revertants had a footprint of CCT (serine) whereas the remaining two contained the 6-bp insertions, TTACTA (tyrosine and tyrosine) and CGTACT (tyrosine and serine).

We asked if excision of the 1.7-kb *Ds* element from its site in exon 16 resulted in a reinsertion of the element elsewhere in the *Sh2* cistron or in other types of relevant genomic rearrangements. Southern blots of genomic DNA digested with *Eco*RI were probed separately with the 5′ and 3′ portions of the *Sh2* cDNA (Fig. 2 *A* and *B*, respectively). Each footprint class is represented. None of the revertants exhibited a rearrangement compared with the wild-type progenitor of *sh2-m1*. We conclude that transposition of *Ds* from exon 16 did not result in any subsequent change within this locus.

The data above show that *Ds* excision events giving rise to phenotypically wild-type kernels encompass only a few amino

Germinal Reversion Events

Progenitor of *sh2-m1* GGG TAC TAC ATA AGG

sh2-m1 GGG TAC TACtagggatgaaa-1.68 kb *Ds2*-tttcatccctaGG TAC TAC ATA AGG

Insert	Revertant Sequence							Frequency	
None	GGG	TAC	TAC			ATA	AGG	8/23	
Tyr	GGG	TAC	TAC	**TAC**		ATA	AGG	11/23	
Ser	GGG	**TCC**	**TAC**	TAC		ATA	AGG	2/23	
Tyr Tyr	GGG	TAC	TA**T**	**TAC**	**TAC**	ATA	AGG	1/23	(Rev 9)
Tyr Ser	GGG	TAC	**TCG**	**TAC**	TAC	ATA	AGG	1/23	(Rev 6)

FIG. 1. Sequences of the 23 revertants arising from movement of *Ds* from *sh2-m1*. Sequences surrounding the *Ds* insertion site in *sh2-m1* and its wild-type progenitor are also shown. The 8 bp duplicated by *Ds* are underlined, while the 11 bp inverted repeats of *Ds* are represented in lowercase letters. The sequence of the revertant footprints is given in boldface type along with the overall frequency and amino acid inserted.

acid changes and no alteration in the open reading frame distal to the site of insertion. Non-wild-type sequences conditioning a plump kernel involved the insertion of only tyrosine and/or serine. We asked whether there are constraints on the excision process with this *Ds* element in this location or whether only a small spectrum of the excision events condition the wild-type phenotype. Random excision events were isolated by PCR amplification of DNA from *sh2-m1* seedlings containing *Ac* that had been cleaved within the *Ds* element. These data (R. Okagaki and L.C.H., unpublished results) reveal that the excision events mimic the many different types of excision events that occur with other *Ds* elements. Most do not restore the wild-type open reading frame. We concluded that only a small fraction of the excision events give rise to an AGP with wild- type function. Furthermore, the site of *Ds* insertion lies 10 amino acids upstream of a lysine residue important in the binding of the allosteric activator 3-PGA (for review, see ref. 3). Because of the selected nature of these revertants and the possible importance of this region of the protein in allosteric activation, we characterized these isoalleles at the expression, protein, enzyme, and physiological levels.

Initially we determined whether these selected isoalleles affected seed starch content and seed dry weight. Crosses of each revertant were made each of 4 years onto the commercial sweetcorn variety, 'Florida Stay Sweet'. Because the revertant isoalleles were generated by selfing an inbred line, they most likely are genetically identical except for the alterations within the *Sh2* locus. Furthermore, the female parent is effectively an F_1, as described above, and thus these plants are genetically identical. 'Florida Stay Sweet' contains the reference allele of

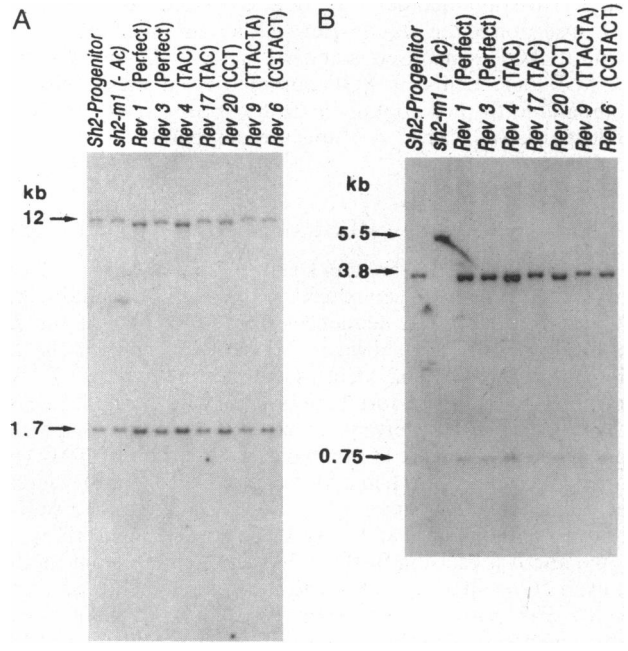

FIG. 2. Southern blot analysis of revertant genomic DNA probed with *Sh2* 5′ (*A*) and 3′ (*B*) cDNA probes. Approximately 5 mg of genomic DNA digested with *Eco*RI was electrophoresed in 1% agarose gels, transferred to nylon membrane (Hybond N, Amersham), and probed with radiolabeled *Sh2* cDNA probes. One or more members of each footprint class is shown. None of the 23 revertants contained detectable DNA rearrangements relative to the progenitor allele (*Sh2-Progenitor*).

Table 1. Dry weight and starch content of seed of the genotype, *Sh2-Rev-X/sh2-R/sh2-R*

	1992–1993			1994	1995
Class	Weight 22 dpp/g	Weight of material, g	Starch	Weight of material, g	Weight of material, g
Perfect					
Average	0.138	0.250	54.49%	0.240	0.219
SD	0.005	0.013	7.16	0.031	0.033
		(177)		(53)	(55)
TAC					
Average	0.128	0.238	55.16%	0.257	0.220
SD	0.011	0.025	13.33	0.019	0.020
% Change	−7.3	−4.8	+1.8	+6.8	+0.18
		(165)		(4)	(9)
CCT					
Average	0.130	0.261	55.35%	0.270	0.247
SD	0.01	0.014	6.92	0.017	0.028
% Change	−4.4	+4.4	+1.6	+12.2	+12.8
		(41)		(35)	(30)
TTACTA					
Average	0.126	0.223	48.77%	0.249	0.220
SD	0.008	0.016	3.39	0.029	0.034
% Change	−8.7	−10.8	−10.5	+3.5	+0.27
		(9)		(29)	(32)
CGTACT					
Average	0.141	0.286	55.31%	0.283	0.244
SD	0.008	0.013	9.57	0.048	0.051
% Change	+2.2	+14.4	+1.5	+17.8	+11.4
		(28)		(33)	(18)

The letter X in the genotype in the title references each of the various revertants. Kernels were harvested at 22 days after pollination or at maturity. Starch determinations were done on kernels grown in 1993. Number of mature ears is given in parenthesis. dpp, Days after pollination.

Table 2. Dry weight of seed of the genotype, *Sh2-Rev-X/Sh2-W64a/Sh2-W64a* and *Sh2-Rev-X/Sh2-182e/Sh2-182e*

Class	Dry weight, g	SD	No. ears	% Difference
Perfect	0.224	0.022	47	0
TAC	0.235	0.026	24	4.9
CCT	0.279	0.029	32	24.9
CGTACT	0.255	0.036	34	14.0

sh2, sh2-R, and produces no detectable SH2 protein (10). The effect then of one dose of each functional *Sh2* isoallele in identical genetic backgrounds was measured.

A preliminary experiment was performed in 1992 and was repeated on a larger scale in 1993. The 1994 and 1995 experiments focused on the increased seed weight seen in 1992 and 1993 with Rev6 (CGTACT). In the initial work, seed weight was determined at an early developmental stage, 22 days after pollination, as well as at seed maturity. Starch determinations were done on mature seed produced in 1993. Results are given in Table 1.

Rev6 (insertion of serine and tyrosine) in kernels of the genotype *Sh2-Rev6/sh2-R/sh2-R* increased mature seed weight 11–18% (Table 1) in comparison with the wild-type revertants in the same genetic background. This increase was seen in each of the four growing seasons in Florida as well as in material produced in Iowa (data not shown). The increase in seed weight is not due totally to an increase in starch content. The percentage of starch in Rev6 is not significantly higher or lower than that seen in the wild-type class (Table 1). Starch amounts were measured by enzymic hydrolysis and measurement of released glucose. Nondestructive, spectrophotometric measurements (D. Ertl, Pioneer Hi-Bred, and T. Rocheford, University of Illinois) also did not detect changes in percentage of starch content in Rev6 (personal communication). The increase in seed weight is not associated with a reduced number of seed per ear (data not shown).

Other isoalleles also affected seed weight. Noteworthy is the increase in mature seed weight seen with the serine insertion. The decreased seed weight observed with Rev9 (insertion of

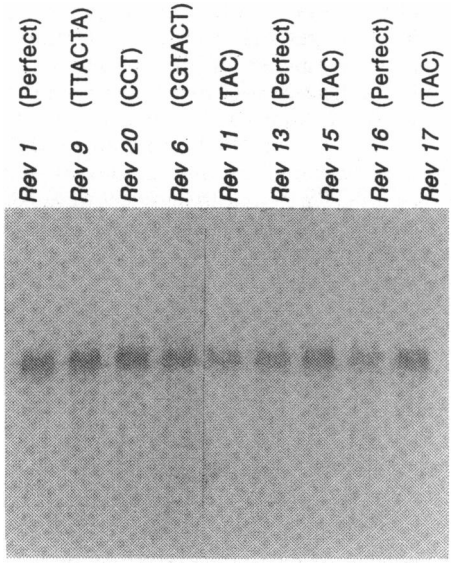

FIG. 3. Northern blot analysis of total RNA isolated from intact kernels 22 days after pollination. Total RNA (10 mg) from a representative of each revertant class was electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham), and probed with the *Sh2* cDNA. The blot was developed using Molecular Dynamics PhosphorImager equipment.

Table 3. AGP activity and SH2 subunit amount in developing kernels of various *Sh2* isoalleles

Class	Activity	SH2	Specific activity
Perfect			
AVE	100	100	1.0
SD	21	30	
TAC			
AVE	50	26	1.96
SD	19	7	
CCT			
AVE	51	31	1.64
SD	2	2	
TTACTA			
AVE	37	34	1.09
SD	3	2	
CGTACT			
AVE	41	28	1.46
SD	5	4	

Averages for AGP activity and SH2 amount were compiled from data obtained from more than 5 ears for each class and more than 10 replications for each of the 1992 and 1993 growing seasons. Specific activity was derived by dividing AGP activity by the amount of the SH2 protein. Activity obtained from the perfect excision events was equated to 100. SD, standard deviation.

two tyrosine residues) in 1992 and 1993 was not observed in the larger 1994 and 1995 experiments.

We also investigated whether Rev6 and the serine insertion increased seed weight in the presence of other, functional alleles of *Sh2* and in another genetic background. Accordingly, the allelic series was crossed onto the field corn F_1 hybrid, W64a × 182e, in 1994. In contrast to the kernels described in Table 1, the resulting seed here contained one dose of the revertant allele but also two, female-derived functional *Sh2* alleles of W64a or 182e origin. Both genotypes should occur with equal frequency on the same ear. Resulting seed weights are given in Table 2. Compared with kernels containing a perfect excision event, the two serine-containing insertions conditioned 14 and 25% increases in seed weight.

Because of the increased seed weight conditioned by Rev6, we attempted to decipher the biochemical or molecular mechanism underlying this physiological change. The amount of the *Sh2* transcript was monitored in each revertant. Northern blots containing equal amounts of total RNA were probed with a *Sh2* cDNA probe (Fig. 3). Changes in transcript amounts consistent with a given footprint class were not detected for the

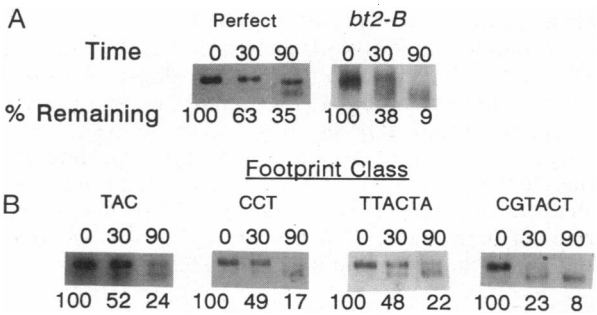

FIG. 4. Heat stability of the SH2 protein. Preparations were incubated at 30°C in the absence of reducing agents or proteolytic inhibitors. SDS proteins (5 mg) from developing kernels, 22 days after pollination, were used in preparing Western blots. Experiments were repeated four or more times with random kernel samples from several ears. The amounts of the intact SH2 protein were quantified by laser densitometry of autoradiographs of chemiluminescent Western blots. Numbers shown are the mean of at least four experiments. Values at 30 and 90 min at 30°C are given relative to the amount at time 0 for each genotype. Standard deviations averaged less than 20% of the mean.

START *Ds* Lysine STOP

ATG....491 aa.....GGG TAC TAC ATA AGG.....7 aa..AAG.... 10 aa.....TAG

Ds

Sh2	GGG	TAC	TAC	ATA	AGG	TCT	GGA	ATC	GTG	GTG	ATC	CTG	AAG	AAT	GCA	ACC	ATC
SH2	G	Y	Y	I	R	S	G	I	V	— V	I	L	**K**	N	A	T	I
S 51	*	*	F	*	K	*	*	*	*	T *	*	—	*	D	*	L	*
Rice	*	*	F	*	K	*	*	*	*	T *	*	—	*	D	*	L	L
BT2	*	*	F	*	K	G	*	*	*	T *	*	—	*	D	*	L	L
Potato	*	*	F	*	K	S	*	*	*	T *	*	—	*	D	*	L	I

FIG. 5. Putative AGP activator binding site. The region surrounding the putative site of activator binding in several different species is shown. The lysine residue believed to be important in activator binding is shown in boldface and conserved bases as asterisks. The site of *Ds* insertion in *sh2-m1* is shown along with the relative positions of the lysine residue in SH2. S51 is the 51-kDa subunit from spinach. The large subunits from rice and potato are also represented. The sequences were compared previously (18).

Sh2 transcript (Fig. 3) or for the other AGP-encoding transcript, *Bt2* (data not shown). The altered transcript sequences do not have any detectable effect on transcription or stability of *Sh2* message.

We measured the amount of AGP activity and SH2 protein conditioned by the new functional alleles. Isoalleles with altered sequences contained less total activity and less SH2 protein (Table 3). Each class was assayed for AGP activity in the absence of inhibitor, phosphate, and in the presence of saturating levels of activator, 3-PGA. Revertants with one or two additional amino acids conditioned enzyme levels 50% or less of that in the wild-type revertants. The relative amount of SH2 protein was assayed with western blots. Each revertant datum is based on several replicates and is expressed relative to the perfect excision class. Each non-wild-type isoallele contained only one-third of the SH2 protein amount found in the perfect excision events.

AGP specific activity was calculated from amounts of SH2 protein and total enzyme activity. Three of the four non-wild-type revertants had specific activities greater than that of wild-type. The specific activity of the revertant containing two additional tyrosines is indistinguishable from wild-type. These results suggest that several of the revertants condition AGP with a greater turnover number than the original wild-type gene. The data also suggest that the altered SH2 is less stable than the wild-type counterpart.

The possible instability of the SH2 subunit encoded by the various isoalleles as well as the role of the BT2 subunit in protecting the SH2 protein from degradation was addressed by *in vitro* studies. Previous work showed that maize endosperm AGP is susceptible to proteolytic attack leading to site-specific cleavage of the small subunit (13). Initially, we asked whether the large subunit of AGP also was susceptible to site-specific cleavage (Fig. 4*A*). Protein extracts from wild-type developing kernels (W64a × 182e) were incubated in the absence of proteolytic inhibitors or reducing reagents at 30°C for various periods of time. Western blot analysis (Fig. 4*A*, left panel) revealed that the SH2 subunit is initially cleaved giving rise to a peptide 2.5–3 kDa smaller than the intact protein. This cleavage, at least in a wild-type kernel, is seen only after 30 min of incubation. Prolonged incubation at 30°C leads to loss of both bands with no detectable degradation products. The stability of SH2 protein encoded by the four different *Sh2* isoalleles was measured (Fig. 4B). All revertants with insertions showed increased SH2 degradation, with SH2 from Rev6 (CGTACT) being particularly susceptible to cleavage.

Interestingly, the absence of a functional BT2 subunit significantly decreases the stability of the SH2 protein (Fig. 4*A*, right panel). Previously, we (17) monitored the amount of the SH2 protein through endosperm development in the presence or absence of a functional BT2 subunit. The absence of BT2 profoundly shortened the developmental lifespan of the SH2 subunit. The data reported here are in accord with our previous conclusion that the successful formation of functional polymeric AGP is an important parameter affecting subunit half-lives. Interpretation of the increased proteolytic cleavage of the Rev6 SH2 subunit therefore is complex. Among several possibilities, it is conceivable that the altered SH2 of Rev6 is less able to bind the BT2 subunit and is thereby rendered less stable.

The region of SH2 altered in these isoalleles lies 10 amino acids proximal to a lysine involved in binding of 3-PGA (ref. 18, Fig. 5). This lysine residue and the surrounding region are conserved across a number of species and in both AGP subunits. Thus, the inclusion of two amino acids might alter the kinetics of 3-PGA activation or phosphate inhibition. Accordingly, we partially purified AGP from the isoalleles (13), including proteolytic inhibitors, and monitored AGP activity as a function of 3-PGA amount. Differences were not detected (data not shown). We also measured inhibition of each AGP by phosphate. Assays were done in the presence of 3-PGA. Rev6 is less inhibited by phosphate in the presence of 3-PGA in comparison to other revertants (Table 4). However, in our

Table 4. Inhibition of AGP by phosphate

	KH_2PO_4, mM		
Excision class	0	5	10
Perfect	100	86	75
TTACTA	100	65	42
CGTACT	100	95	87

AGP was partially purified (13) from each of the three revertant types listed and assayed in the presence of 20 mM of 3-PGA in varying amounts of phosphate.

Table 5. Phosphate inhibition of plant AGP expressed in *E. coli*

	Maize AGP		Potato AGP	
Phosphate	wild type	CGTACT insert	wild type	CGTACT insert
Absent	1.00	1.00	1.00	1.00
Present	0.29	0.93	0.10	1.04

Maize large subunit AGP structural gene from Rev6 and from its wild-type progenitor were each expressed with the same small maize endosperm subunit (*Bt2*) in *E. coli*. Resulting AGP was assayed in the presence and absence of P_i. Activity in the presence of 30 mM P_i is expressed as a fraction of that observed in the absence of P_i in each case. The additional 6 bp of Rev6 were inserted into the potato large subunit and activity was monitored in the presence of 0.5 mM of 3-PGA and in the absence or presence of 5 mM phosphate.

hands, this purification procedure yields only a small percentage of total activity and the difference in phosphate inhibition, as noted above, was seen in only three of five replicated experiments and was never seen when total AGP activity was assayed using nonpartially-purified preparations diluted to a point at which activity is proportional to time of incubation and to protein content.

Because of the complex nature of the modification(s) of AGP occurring in the maize endosperm, we expressed the maize endosperm AGP structural genes in *E. coli* and monitored the resulting activity for phosphate inhibition (Table 5). Two genotypes were assayed: (*i*) Rev6 contains the 6-bp insertion derived from the *Ds* insertion, *sh2-m1*, and (*ii*) Sh2-Prog, the wild-type allele which served as the progenitor of *sh2-m1*. The *Bt2* cDNA in both cases was derived from the *Sh2-Prog* line. In the absence of 3-PGA, addition of 30 mM of phosphate led to a 70% reduction in AGP activity whereas the Rev6 variant exhibited almost no (7%) phosphate inhibition.

We also investigated whether the tyrosine-serine insertion would condition a reduction in phosphate inhibition in AGP derived from other plants. The large subunit of the potato AGP was modified to contain the two amino acids of Rev6, and wild-type and mutant AGPs were assayed in the presence of 0.5 mM of 3-PGA and in the presence or absence of 5 mM of P_i. As shown in Table 5, insertion of these amino acids into the potato large subunit gave rise to phosphate insensitivity in this enzyme as well.

DISCUSSION

An *in vivo*, site-specific mutagenesis system was used in maize to create defined mutations within the gene encoding the large subunit of the endosperm-specific starch-synthetic enzyme AGP. This approach identified an important protein motif of this subunit and one resulting change leads to an 11–18% increase in seed weight. Secondarily, these results complement those in potato (4), showing not only that AGP is an important enzyme but also that alterations in it can increase plant constituents above those found in wild-type plants. The work here extends this conclusion to a major cereal crop and to a harvested plant organ with low moisture content. Furthermore, the increase in seed weight we report is not attributable solely to an increase in starch content, although absolute starch content is increased in this variant. We suggest that enhanced starch synthesis caused by Rev6 creates a stronger sink within the seed leading to increased synthesis of other seed components.

Rev6 is likely to have major impact in maize improvement programs. Duvick (1) has noted that seed weight is a major parameter of corn yield. Whereas an increase in seed weight is associated with a reduction in seed number in many breeding populations, this does not appear to be the case with Rev6, at least in the genetic materials used in our analysis. While maize breeding programs have been successful and genetic improvement has played a significant role in increased corn yields, the genetic component to yield has led to only a doubling of this parameter since the 1930s (1). The increase in yield due to genetic improvement is less than 1% percent per year. Viewed in this context, the single-gene mutation that increases seed weight, Rev6, should have a significant impact on future maize improvement programs.

The significant change in the allosteric properties of Rev6 mediated by the insertion of two amino acids near the carboxyl terminus supports existing evidence that this region of the polypeptide comprises at least part of the allosteric domain. Ten residues distal to the insertion site is a lysine. In the spinach leaf AGP large subunit, this conserved lysine residue is specifically labeled by pyridoxal phosphate, which itself can serve as an activator in place of 3-PGA (19, 20). The presence of the activator 3-PGA or P_i prevents labeling of this lysine in the spinach leaf AGP large subunit, suggesting that the binding sites for these effector molecules overlap. This view is supported by the apparent allosteric properties of the Rev6 AGP, which still displays activation by 3-PGA but very little inhibition by P_i. Alterations in phosphate inhibition were not detected in all experiments involving endosperm-derived AGP, although this alteration was clearly noted in *E. coli*-expressed maize endosperm AGP and in *E. coli*-expressed potato AGP containing the Rev6 alteration. Furthermore, Rev6 conditions decreased amounts of the SH2 protein and AGP activity when extracted from the maize endosperm, whereas equal amounts of activity from wild-type and Rev6 were noted in *E. coli*. This is clearly consistent with our observations that endosperm-expressed Rev6 AGP is more susceptible to proteolytic attack, and therefore *in vitro* measurements of endosperm-derived Rev6 SH2 protein or AGP activity likely do not provide a meaningful measurement of the activity *in vivo*.

We conclude from this work that AGP is important to seed weight and that the two amino acid insertion of Rev6 causes the increased seed weight. If the inverse relationship between phosphate inhibition and seed weight is causal, the data presented here strongly suggest that the 6-bp insertion of Rev6 should be universally important since this change also conditions phosphate insensitivity in the potato AGP.

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