Functions of Heteromeric and Homomeric Isoamylase-Type Starch-Debranching Enzymes in Developing Maize Endosperm

Akiko Kubo, Christophe Colleoni, Jason R. Dinges, Qiaohui Lin, Ryan R. Lappe, Joshua G. Rivenbark, Alexander J. Meyer, Steven G. Ball, Martha G. James, Tracie A. Hennen-Bierwagen, and Alan M. Myers*

Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011 (A.K., C.C., J.R.D., Q.L., R.R.L., J.G.R., A.J.M., S.G.B., M.G.J., T.A.H.-B., A.M.M.); and Unité de Glycobiologie Structurale et Fonctionnelle, UMR8576 CNRS, Université des Sciences et Technologies de Lille, Villeneuve d’Ascq cedex 59655, France (S.G.B.)

Functions of isoamylase-type starch-debranching enzyme (ISA) proteins and complexes in maize (Zea mays) endosperm were characterized. Wild-type endosperm contained three high molecular mass ISA complexes resolved by gel permeation chromatography and native-polyacrylamide gel electrophoresis. Two complexes of approximately 400 kD contained both ISA1 and ISA2, and an approximately 300-kD complex contained ISA1 but not ISA2. Novel mutations of sugary1 (su1) and isa2, coding for ISA1 and ISA2, respectively, were used to develop one maize line with ISA1 homomer but lacking heteromeric ISA and a second line with one form of ISA1/ISA2 heteromer but no homomeric enzyme. The mutations were su1-P; which caused an amino acid substitution in ISA1, and isa2-339, which was caused by transposon insertion and conditioned loss of ISA2. In agreement with the protein compositions, all three ISA complexes were missing in an ISA1-null line, whereas only the two higher molecular mass forms were absent in the ISA2-null line. Both su1-P and isa2-339 conditioned near-normal starch characteristics, in contrast to ISA-null lines, indicating that either homomeric or heteromeric ISA is competent for starch biosynthesis. The homomer-only line had smaller, more numerous granules. Thus, a function of heteromeric ISA not compensated for by homomeric enzyme affects granule initiation or growth, which may explain evolutionary selection for ISA2. ISA1 was required for the accumulation of ISA2, which is regulated posttranscriptionally. Quantitative polymerase chain reaction showed that the ISA1 transcript level was elevated in tissues where starch is synthesized and low during starch degradation, whereas ISA2 transcript was relatively abundant during periods of either starch biosynthesis or catabolism.

Starch biosynthesis is a central function in plant metabolism that is accomplished by a multiplicity of conserved enzymatic activities. Two known activities are starch synthase, which catalyzes the polymerization of glucosyl units into α(1→4)-linked “linear” chains, and starch-branching enzyme, which catalyzes the formation of α(1→6) glycoside bond branches that join linear chains. Acting together, the starch synthases and starch-branching enzymes assemble the relatively highly branched polymer amylopectin, with approximately 5% of the glucosyl residues participating in α (1→6) bonds, and the lightly branched molecule amyllose. Amylopectin and amyllose assemble into semi-crystalline starch granules, which in land plants and green algae are located in plastids.

A third activity necessary for normal starch biosynthesis is provided by starch-debranching enzyme (DBE), which hydrolyzes α(1→6) linkages. Two DBE classes have been conserved separately in plants (Beatty et al., 1999). These are referred to here as pullulanase-type DBE (PUL) and isoamylase-type DBE (ISA), based on similarity to prokaryotic enzymes with particular substrate specificity. ISA function in starch production is implied from genetic observations that mutations typically result in reduced starch content, abnormal amylopectin structure, altered granule morphology, and accumulation of abnormally highly branched polysaccharides similar to glycogen. Applicable amounts of phytoglycogen have been detected only in plants with compromised ISA function, including maize (Zea mays), rice (Oryza sativa), and barley (Hordeum vulgare) endosperm (James et al., 1995; Kubo et al., 1999; Burton et al., 2002), Arabidop-
sis (*Arabidopsis thaliana*) leaves (Zeeman et al., 1998), and *Chlamydomonas* (Mouille et al., 1996). The primary role of PUL is in starch mobilization; however, a biosynthetic function also is indicated, because mutations affect starch level and structure in plants with compromised ISA function (Kubo et al., 1999; Dinges et al., 2003; Wattebled et al., 2005, 2008; Streb et al., 2008). The specific biosynthetic function(s) of DBE is not yet known, although data have been presented consistent with (1) editing preamylopectin structure to enable crystallization, (2) elimination of soluble glucans that compete with crystalline starch, and/or (3) regulation of granule initiation (Ball et al., 1996; Zeeman et al., 1998; Myers et al., 2000; Burton et al., 2002; Nakamura, 2002; Bustos et al., 2004).

Four genes coding for DBE proteins are strictly conserved in the Chlorophyta lineage, three for ISA proteins and one for a PUL protein (Deschamps et al., 2008). Evolutionary maintenance indicates functional significance; however, specific roles for each DBE protein are not fully understood. In potato (*Solanum tuberosum*) tuber, antisense down-regulation of either ISA1 or ISA2 caused the same abnormal starch phenotype (Bustos et al., 2004), and these two proteins exist together in a heteromeric complex (Ishizaki et al., 1983; Hussain et al., 2003). Similarly, in Arabidopsis leaf, elimination of ISA1, ISA2, or both conditioned the same phenotype of starch deficiency and phytoglyco-gen accumulation, and both proteins were required for the presence of the same enzymatic activity (Delatte et al., 2005; Wattebled et al., 2005). Biochemical characterization of ISA in developing kidney bean (*Phaseolus vulgaris*) seeds also identified a heteromeric complex containing ISA1 and ISA2 (Takashima et al., 2007). Thus, it appears that in tuber, leaf, and cotyledon, the ISA1 and ISA2 proteins function together in a complex. ISA2 is likely regulatory, because it contains variant amino acids at essential catalytic residues of the α-amylase superfamily that render it enzymatically inactive (Hussain et al., 2003). Such amino acid changes are present in ISA2 of all species characterized to date (Deschamps et al., 2008), indicating functional selection for a noncatalytic protein beginning very early in the divergence of the Chlorophyta. Mutational analysis of ISA3 function in Arabidopsis indicated a primary role in starch mobilization but also a partially overlapping function with ISA1/ISA2 in biosynthesis (Streb et al., 2008; Wattebled et al., 2008).

Endosperm tissue from rice contains multiple ISA complexes (Fujita et al., 1999; Utsumi and Nakamura, 2006). An ISA1/ISA2 heteromer is present, as in tuber and leaf, and in addition rice endosperm contains a homomeric complex containing only ISA1. Whether the two classes of ISA complex have specific functions in endosperm, and how these may differ from starch biosynthesis in leaf or tuber, are not known. In this study, homomeric ISA1 and heteromeric ISA1/ISA2 complexes were identified in maize endosperm and found to be analogous to the rice ISAs, and their functions were investigated using mutations that specifically prevent the formation of each class of complex. The results showed that either heteromeric or homomeric ISA is sufficient for near-normal starch synthesis but also that both forms have a function(s) that is not fulfilled by the other.

### RESULTS

#### Complete Maize Gene Set Coding for DBE Proteins

Three genes coding for ISA proteins and one coding for a PUL protein are evident in the maize genomic databases (Yan et al., 2009). ISA1 and *Zea mays* PUL1 (ZPU1) had been previously characterized from cDNA and genomic sequences (GenBank accession nos. EU970890, AF303882, and AF080567; James et al., 1995; Beatty et al., 1999). In this study, PCR primers based on rice ISA gene sequences were used to amplify full-length cDNAs coding for ISA2 and ISA3 (GenBank accession nos. AY172633 and AY172634). Each of the four proteins is greater than 30% identical only to the other three members of the group among all predicted maize polypeptides. Thus, ISA1, ISA2, ISA3, and ZPU1 constitute the complete set of DBEs present in maize. The genes that code for these proteins are denoted *sugary1* (*su1*; James et al., 1995), *isa2*, *isa3*, and *zpu1* (Dinges et al., 2003), respectively. Summary information about maize DBE genes and proteins is presented in Supplemental Table S1.

Phylogenetic analysis showed that each maize DBE sequence falls into one of the four conserved families previously identified in green algae and land plant species (Hussain et al., 2003; Deschamps et al., 2008; data not shown). Like ISA2 of potato, Arabidopsis, and other species, maize ISA2 exhibits amino acid variation at six of the eight residues that are normally strictly conserved in the α-amylase superfamily and are deemed critical for activity (Jespersen et al., 1991, 1993; Hussain et al., 2003; Supplemental Table S2).

#### DBE Expression at the Level of mRNA Accumulation

Previous reverse transcription (RT)-PCR results indicated that *ISA2* and *ISA3* transcripts are present in endosperm tissue throughout development (Yan et al., 2009). To provide further insight into the potential functions of each DBE, the relative levels of *ISA1*, *ISA2*, *ISA3*, and *ZPU1* mRNA were measured using quantitative PCR (qPCR). Expression was first measured in whole kernels harvested from 4 to 40 d after pollination (DAP), which includes the period of maximal starch deposition (Prioul et al., 1994). The abundance of all four DBE transcripts was highest at 20 DAP (Fig. 1A), thus correlating with maximal starch deposition. *ISA1* was present in the highest relative abundance, followed by *ISA2* and *ZPU1*, and then by *ISA3* at a very low level. Analyses of separated endosperm and embryo tissue from kernels harvested 20 DAP showed that the great majority of the transcripts detected in
whole kernels are from endosperm (Supplemental Fig. S1A).

Transcripts were quantified in germinating seeds to examine for potential functions in starch mobilization (Fig. 1B). Dry kernels were incubated at 30°C on moist filter paper, and qPCR was conducted over 8 d. ISA2 was the only mRNA detected in mature kernels (0 d of germination). The most abundant transcript throughout germination was ZPU1, which accumulated 4 to 6 d post germination to levels 2- to 3-fold higher than any of the other three mRNAs. ISA2, ISA3, and ZPU1 were prevalent during the most active starch degradation period, from 4 to 7 d of germination, while ISA1 was present only at trace levels.

Adult leaf tissue harvested at midday from field-grown plants was analyzed (Supplemental Fig. S1A) to compare transient starch metabolism with storage starch synthesis. All four transcripts were present in leaf, with the distinctions compared with endosperm that (1) ISA2 rather than ISA1 was in highest abundance, (2) appreciable amounts of ISA3 accumulated, and (3) ZPU1 was present at a low level. Transcript levels also were measured over a 16-h-light/8-h-dark photoperiod in seedling leaves grown in a controlled-environment chamber (Supplemental Fig. S1B), again with the result that ISA2 was present in excess of ISA1, in this instance throughout the diurnal period.

Mutations Affecting ISA1 or ISA2

Mutations of su1 and isa2 were characterized and backcrossed into the W64A inbred background. Two previously characterized su1 alleles were used: su1-4582 results from a Mutator (Mu) transposon in exon 1 and is a null allele that fails to produce any ISA1 (James et al., 1995; Rahman et al., 1998); su1-Ref is a nucleotide substitution that changes the Trp residue at position 578 to an Arg (Dinges et al., 2001; Remington et al., 2001; GenBank accession no. AAB97167). Two other alleles were characterized in this study, su1-am (Mangelsdorf, 1947) and su1-P (Garwood and Creech, 1972). Neither mutation by itself conditions a noticeable kernel phenotype, yet they failed to fully complement su1-Ref with regard to kernel morphology and water-soluble polysaccharide (WSP) accumulation (Cameron, 1947; Mangelsdorf, 1947; Garwood and Creech, 1972). The su1 genomic locus of su1-am or su1-P homozygotes was sequenced after PCR amplification. Identical sequences were observed, with a single substitution compared with the wild type that changes the Arg residue at position 308 to an Ile. Thus, su1-am and su1-P appear to be the same allele, and subsequent analyses utilized the latter mutation. The observation that su1-P by itself supports apparently normal kernel development yet fails to fully complement the point mutant su1-Ref is most likely explained by quaternary structure interactions of the two abnormal proteins. This conclusion is supported by the observation that su1-P is dominant to the null allele su1-4582 (i.e. su1-P/su1-4582 compound heterozygotes exhibit normal kernel morphology identical to su1-P homozygotes; data not shown).

Transposon mutagenesis using the Trait Utility System for Corn (Bensen et al., 1995) was used to obtain an isa2- null mutation. A large population was screened by PCR for individuals containing Mu located within isa2. Positive PCR signals generated using the Mu-end primer 9242 and the isa2 gene-specific primer Ak2r identified the allele isa2-339 (Fig. 2A). The sequence of a junction fragment containing Mu and genomic DNA revealed that the transposon was located between nucleotides 880 and 881 of the ISA2 coding sequence. During backcrosses into the W64A background, isa2-339 was identified by PCR amplification with primer pair 9242/Ak2r, and wild-type Isa1 was identified by primer pair Ak1f/Ak2r that flanks the transposon insertion site (Fig. 2, A and B).
Gene expression from the mutant alleles was analyzed at the level of mRNA accumulation in 20-DAP endosperm (Fig. 2C). ISA1 was undetectable by RT-PCR analysis of a su1-4582 homozygote, in agreement with previous RNA-blot hybridization data (James et al., 1995). Similarly, ISA2 transcript was not detected in a homozygous isa2-339 mutant. The point mutations su1-Ref and su1-P both produced ISA1 transcript detected by RT-PCR with results indistinguishable from the wild type.

Accumulation of ISA1 and ISA2 proteins was also measured. Antisera were generated using synthetic peptides with unique sequences from either ISA1 or ISA2 as the antigen. The same peptides were also used to bind IgG from crude sera and generate affinity-purified fractions designated as αISA1 and αISA2. Escherichia coli strains expressing recombinant ISA1 or ISA2 fused to glutathione S-transferase (GST) provided a test to demonstrate that each IgG fraction is isofrom specific (Fig. 3A). Soluble endosperm extracts were then probed with αISA1 or αISA2 in immunoblot analyses (Fig. 3B). ISA1 was not detected in su1-4582 mutants, whereas reduced amounts of the protein compared with the wild type were observed in both su1-P and su1-Ref endosperm. Heterozygous isa2-339 endosperm lacked detectable ISA2. The protein and mRNA expression data together confirmed that su1-4582 and isa2-339 are null alleles and that su1-P and su1-Ref produce abnormal forms of ISA1 protein.

Effects on ISA2 of mutations that alter ISA1, and vice versa, were analyzed similarly. Absence of ISA2 in isa2-339 mutants had no effect on the accumulation of
ISA1 mRNA or protein (Figs. 2C and 3B). In contrast, loss of ISA1 in su1-4582 endosperm secondarily prevented ISA2 protein accumulation (Fig. 3B). This is a posttranscriptional effect, because the ISA2 mRNA level was not altered in a su1-4582 line when measured either by standard RT-PCR (Fig. 2C) or qPCR (Supplemental Fig. S1C). The point mutations su1-P and su1-Ref were distinct from the null allele because they did not affect the ISA2 protein level (Fig. 3B). Homozygous su1-Ref did condition a change in ISA2, however, because in addition to the normal protein a variant of higher apparent $M_r$ was also observed (Fig. 3B). This effect was reproducible in multiple biological replicates (data not shown).

Characterization of Maize ISA Complexes

Multiple ISA complexes had been previously observed in maize endosperm total extracts; however, these were partly obscured by other starch-modifying activities (Dinges et al., 2001, 2003). To improve the resolution, total extracts were first fractionated by anion-exchange chromatography (AEC) on MonoQ resin as described previously (Colleoni et al., 2003). Proteins in each fraction were separated by native-PAGE and then electrophoretically transferred to starch-containing gels. After incubation and staining with $I_2/KI$ solution, these zymogram analyses revealed three blue-colored bands in a specific elution peak, which based on their color can be presumed as ISA activities (Fig. 4A). The bands are referred to as ISA complexes I, II, and III, in order from highest to lowest mobility in native-PAGE. The same analysis applied to su1-4582 endosperm failed to generate any blue bands, confirming their identities as ISAs and indicating that ISA1 is required for all three activities (Fig. 4A; Dinges et al., 2001; Colleoni et al., 2003).

ISA protein compositions were characterized using isoform-specific IgG fractions. The complexes were concentrated from wild-type 20-DAP endosperm by AEC on Q Sepharose. Immunoblots showed that ISA1 and ISA2 bound completely to the column in low NaCl concentration and eluted in the same sharp peak (data not shown). Those fractions were pooled, concentrated, and analyzed in zymograms. Duplicate gels run simultaneously in the same apparatus were trans-

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**Figure 4.** Identification of ISA activities in 20-DAP endosperm extracts. A, Zymogram activity gels following MonoQ separation. Equal volumes of each fraction were analyzed. B, Correspondence of ISA proteins with ISA enzyme activities. Extracts were concentrated by AEC on Q Sepharose and separated by native-PAGE. ISA activities were visualized in zymograms, and ISA proteins were identified by immunoblot analysis. Zymograms are from a single gel, and immunoblots are from a second gel run simultaneously in the same apparatus. Numbers below each lane indicate micrograms of protein loaded. C, Independent repeat analysis of ISA activities in wild-type and isa2-339 endosperm. Details are as in B. D, Correspondence of ISA proteins with ISA enzyme activities in su1-P endosperm. Details are as in B.
ferred to nitrocellulose and probed with αISA1 or αISA2 in immunoblots. Three ISA activities were again visualized in the zymogram, and immunoblot results showed that ISA1 comigrates with all three complexes, whereas ISA2 comigrates with complexes II and III but is not present in form I (Fig. 4, B and D).

ISA complexes were further purified by gel permeation chromatography (GPC) of the proteins concentrated by AEC. ISA1 and ISA2 in the GPC fractions were detected by SDS-PAGE and native-PAGE followed by immunoblot. The two proteins eluted in distinct but overlapping GPC peaks at volumes indicative of molecular mass between approximately 200 and 400 kD, with ISA2 in the larger complex (Fig. 5A). Neither ISA1 nor ISA2 was detected as a monomer. Native-PAGE revealed which ISA complexes were present in the two GPC peaks (Fig. 5B). The lower molecular mass peak (concentrated in fractions C3–C5) contained predominantly a complex that migrated in native-PAGE at the position of ISA complex I and that contained ISA1 but lacked ISA2. The higher molecular mass peak (concentrated in fractions C1–C3) contained a complex with both ISA1 and ISA2 that migrated at the position of ISA form II. After long exposure, a weak immunoblot signal was also observed in the higher molecular mass GPC peak using αISA1 IgG at the position expected for ISA complex III (data not shown).

Taken together, these data indicate that ISA1 and ISA2 sort into multiple high M, complexes in maize endosperm. ISA complex I contains only ISA1 and is the smallest of the complexes. This form is a homodimer, at least concerning the DBE protein present. ISA complexes II and III are heteromultimers containing both ISA1 and ISA2 and are larger than form I, as judged by GPC. These properties match closely with the ISA complexes previously characterized from rice endosperm (Utsumi and Nakamura, 2006); however, the maize complexes have not yet been purified to homogeneity, so at this point the presence of proteins in addition to the ISAs cannot be ruled out.

**Effects of ISA1 and ISA2 Mutations on ISA Activities**

The composition of the ISA complexes was confirmed by determining the effects of su1- or isa2- mutations that cause known deficiencies in the ISA1 and/or ISA2 proteins. Total soluble extracts of 20-DAP endosperm from wild-type W64A and congenic lines homozygous for su1-4582, su1-P, or isa2-339 were fractionated by AEC. Immunoblot analysis of the fractions showed that the ISA1 and ISA2 elution profiles were the same in su1-P and the wild type and that the ISA1 elution profile was the same in isa2-339 and the wild type, thus confirming that no complexes were lost in the mutants as the result of altered AEC behavior (data not shown). The ISA-containing fractions or their equivalents were pooled and concentrated and then analyzed by native-PAGE for DBE activity and the presence of ISA1 and ISA2 in each complex. The su1-4582 control lacked any detectable ISA activities in the zymograms, and immunoblot signal was not detected for ISA1 or ISA2 at the position of any of the three ISA complexes (Fig. 4B). The isa2-339 line exhibited ISA complex I in both the zymogram and ISA1 immunoblot (Fig. 4B). ISA complexes II and III were missing in the isa2-339 mutant, as indicated by lack of ISA1 or ISA2 immunoblot signal at those native gel positions even on long exposure (Fig. 4B) and loss or major reduction of the blue activity bands of ISA complexes II and III (Fig. 4, B and C). A slight activity at the position of ISA complex II was detected, however, in the isa2-339 mutant, despite the absence of ISA2 protein (Fig. 4, B and C). These data confirm that ISA1 is a component of all three forms of the enzyme and ISA2 is a component of forms II and III but not the form I complex.

The su1-P mutant was unique because it lacked homomeric ISA complex I but contained heteromeric enzyme (Fig. 4D). The prominent blue band of ISA complex I activity was absent from su1-P endosperm, as was ISA1 immunoblot signal at that position on the native gel. Heteromeric ISA complex II also was apparently missing or defective in su1-P endosperm, again as judged by both zymogram and immunoblot analysis. ISA complex III was detected in su1-P extracts when gels were loaded with a relatively large

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**Figure 5.** GPC separation of ISA complexes. AEC fractions containing ISA1 and ISA2 were concentrated and purified further by GPC. ISA1 and ISA2 in the GPC eluate were detected by immunoblot analysis with αISA1 or αISA2. Equal volumes of each fraction were loaded. The elution positions of standards of known molecular mass are indicated. A, SDS-PAGE. B, Native-PAGE. Lanes probed for ISA1 and ISA2 are from the same gel.
amount of protein, and immunoblot signals for both ISA1 and ISA2 comigrated with that activity. In addition, blue color indicating ISA activity was evident as a diffuse signal migrating between the wild-type positions of complexes II and III. Thus, the amino acid substitution at ISA1 residue 308 apparently prevents the assembly of homomeric ISA (complex I) and the heteromeric complex II. The second heteromeric complex, form III, does assemble into an active enzyme in su1-P endosperm, and the diffuse region of ISA activity in the zymogram may indicate abnormal assembly states that occur only in the mutant.

Absence of a functional ISA1 homomeric complex in the su1-P line suggested that the mutant ISA1 protein in su1-P endosperm requires ISA2 in order to support starch biosynthesis. This was tested by self-pollinating a su1-P/+; +/isa2-339 heterozygote. Approximately one-sixteenth of the kernels on the ears exhibited the shrunken, translucent appearance typical of ISA1-null plants (24 mutant kernels, 358 normal kernels), and PCR analysis and DNA sequencing confirmed that these kernels were homozygous for both su1-P and isa2-339 (Supplemental Fig. S2). These data support the conclusion that the abnormal ISA1 produced from su1-P functions only as part of a heteromeric complex containing ISA2.

**Effects of Changes in ISA Complexes on Carbohydrate Content**

Carbohydrate content in the endosperm was compared between the wild type and lines lacking either the homomeric or heteromeric ISA complexes. Specifically, comparisons were made between (1) the wild type containing all three ISA complexes, (2) isa2-339 mutants containing only homomeric ISA complex I, and (3) su1-P mutants that contain heteromeric ISA complex II. Endosperm tissue collected 20 DAP was analyzed from multiple biological replicates using plants grown in the 2009 summer nursery (Fig. 6A; Supplemental Table S3). Granular starch content quantified on a dry weight basis did not vary significantly between wild-type, su1-P, and isa2-339 lines. Thus, either heteromeric or homomeric ISA is sufficient to produce normal quantities of starch at mid development. One significant difference was noted between the wild-type and isa2-339 lines, specifically an approximately 10-fold increase in WSP in the mutant (Fig. 6A). Elevated WSP in isa2-339 lines compared with the wild type was also observed in two independent plants grown in the 2008 summer nursery (data not shown). This effect of the ISA2-null mutation on WSP, however, is far less severe than that resulting from the ISA1-null mutation su1-4582. In the latter instance, in agreement with previous results (Dinges et al., 2001), the starch content was reduced approximately 6-fold compared with the wild type and WSP was elevated 100-fold (Fig. 6A). Furthermore, the approximately 10-fold elevation in Suc typical of the ISA1-null mutant was not observed in isa2-339 lines.

The only other statistically significant difference compared with the wild type was slightly elevated Glc content in isa2-339 endosperm (Fig. 6A).

WSP that accumulates at mid development in isa2-339 endosperm was analyzed by GPC on Sepharose CL-2B to estimate its molecular mass. Chromatography standards were amylopectin from wild-type starch granules, phytoglycogen from su1-4582 endosperm, and free Glc. WSP from 20-DAP isa2-339 endosperm eluted from the column at a volume that overlapped the phytoglycogen peak (Fig. 7). Thus, the WSP accumulating during isa2-339 kernel development is a polysaccharide rather than a collection of maltooligosaccharides, and the size of the polymer matches the smaller range of phytoglycogen molecules from su1-4582 endosperm.

Carbohydrates were also quantified in endosperm tissue from mature, dried kernels (Fig. 6B). At this stage, the WSP content in isa2-339 was comparable to the wild type, in contrast to mid development, when WSP in the mutant was significantly elevated (Fig. 6A). This is again distinct from su1-4582, where the high WSP content and low abundance of granular starch persisted through kernel maturity. The simple
sugar content in mature kernels was comparable in the wild type, isa2-339, and su1-P. Again the ISA1-null endosperm carbohydrate content was clearly distinct from the ISA2-null or su1-P mutants, in the sense that simple sugar contents were highly elevated at maturity. Finally, there appears to be a slight reduction in total starch content in mature endosperm in the isa2-339 and su1-P lines, but this effect is far less severe than the starch deficiency in the ISA1 null mutant su1-4582 (Fig. 6B).

Summary points from the carbohydrate content data are as follows. First, the levels of simple sugars and starch, either at mid development or maturity, are not drastically altered by loss of ISA2 or the su1-P mutation of ISA1. Second, loss of ISA2 conditions an abnormal accumulation of WSP at mid development that is not observed at maturity. Third, the phenotypes caused by loss of ISA1 compared with loss of ISA2 are clearly distinct.

discussion
Distinct ISA Complexes in Maize Endosperm

This study characterized roles of conserved ISA proteins in maize endosperm starch biosynthesis and

Effects of Changes in ISA Complexes on Starch Structure

Starch structure was characterized to determine whether the ISA complexes provide specific functions in the determination of amylose-amylopectin ratio, granule morphology, or amylopectin chain length distribution. To determine amylose-amylopectin ratio, granular starch from mature kernels was dissolved in dimethyl sulfoxide (DMSO), precipitated with ethanol, suspended in 10 mm NaOH, and separated by GPC on Sepharose CL-2B. Quantification of Glc in the fractions revealed the amylose and amylopectin peaks. The amylopectin content for W64A, isa2-339, and su1-P granules was 75.1%, 71.4%, and 73.1%, respectively. Thus, either the heteromorphic or homomorphic ISA complex(es) functions to generate the wild-type amylose-amylopectin ratio in mature kernels.

Granule morphology from 20-DAP or mature endosperm starch was analyzed by scanning electron microscopy. At 20 DAP, the isa2-339 granules were noticeably smaller than those from the wild type or su1-P, although their overall spherical morphology was not altered (Fig. 8). ISA2-null granules appeared distinct from ISA1-null starch, which displayed distinctive misshapen, flattened granules. Similar effects were observed at maturity, with isa2-339 granules smaller than the wild type and distinct in form from su1-4582 granules. The su1-P mutant has nearly normal granule morphology at 20 DAP or maturity (Fig. 8). These effects were observed in independent replicates from different growing seasons (data not shown).

The linear chain length distribution in 20-DAP amylopectin was compared between the four genotypes. As a control, su1-4582 starch exhibited the severe change in amylopectin structure observed previously (Dinges et al., 2001), with significant increases in the frequencies of degree of polymerization (DP) 5 to 12 chains compared with the wild type and decreases in DP 16 to 25 chains (Supplemental Fig. S3). Amylopectin from su1-P endosperm showed a small but consistent change from the wild type, reproducible in four biological replicates. In this instance, chains of DP 11 to 13 were moderately increased in abundance, such that their frequency as a percentage of all chains analyzed was 0.5% to 0.75% higher than the corresponding wild-type value (Supplemental Fig. S3). These values are well above background scatter for biological replicates. This is a far smaller effect, however, than the changes in su1-4582 amylopectin (Supplemental Fig. S3; Dinges et al., 2001). Amylopectin chain length frequency distribution from six biological replicates of isa2-339 starch varied compared with each other, with differences of up to 0.4% of total for given chain lengths (Supplemental Fig. S4). The same degree of variation was observed in comparison between isa2-339 and the wild type (Supplemental Fig. S4). Thus, loss of ISA2 does not condition an obvious change in chain length distribution.
found differences from previously identified functions in dicots. In earlier studies, ISA1 and ISA2 were separately eliminated from Arabidopsis leaves or potato tubers, and the resultant essentially identical phenotypes indicated that the two proteins work together at the same step in amylopectin synthesis, presumably in a heteromeric complex (Bustos et al., 2004; Delatte et al., 2005; Wattebled et al., 2005, 2008). The roles of ISAs appeared to be different in maize and rice endosperm based on two lines of evidence. First, the presence of both ISA1 homomeric and ISA1/ISA2 heteromeric complexes in rice suggested that ISA1 functions in some instances independent of ISA2. Second, spontaneous su1 alleles have been detected repeatedly in maize, owing to a distinctive kernel phenotype, yet mutations affecting ISA2 have not appeared. Thus, ISA2 most likely is not necessary for a wild-type kernel phenotype, which implies normal or near-normal starch granules. In this study, ISA complexes in maize endosperm were characterized, and their functions separated by genetic means, to test directly whether ISA1 and ISA2 requirements differ between specific tissues and/or species.

Independent lines of evidence demonstrated that ISA1 and ISA2 are components of the same enzyme complexes: (1) both proteins comigrated with specific ISA activities in AEC, GPC, and native-PAGE; (2) functional su1 and isa2 were both required for the same ISA activities; (3) coupling the isa2-339 and su1-P mutations conditioned a synthetic phenotype indicative of the ISA-null condition; and (4) ISA2 failed to accumulate, owing to a posttranscriptional effect, when ISA1 was completely absent. The evidence is also clear that some ISA activity in maize endosperm does not require ISA2. First, in wild-type tissue, ISA2 did not comigrate with ISA1 in the most active ISA band (complex I). Second, complex I activity was unaffected by loss of ISA2. Thus, the composition of endosperm ISA complexes is similar in maize and rice (Utsumi and Nakamura, 2006). In both species, three ISA complexes were detected in zymograms, with ISA1 and ISA2 both in the lower mobility complexes (II and III) and ISA1 exclusively in the highest mobility form (complex I). The heteromeric maize ISA complexes are larger than the homomeric form, as judged by GPC and as also seen in rice. The explanation for two different heteromeric complexes is not known. Possibilities include different subunit compositions and/or posttranslational modification of one or more components.

Coordinate Regulation of Protein Abundance

A likely explanation for the fact that ISA2 accumulation requires ISA1 is that free ISA2 is readily proteolyzed. This predicts that all ISA2 is present as part of a complex, and this was shown by the fact that no monomeric ISA2 was detected in wild-type extracts by GPC and SDS-PAGE. A corollary is that the ISA2 accumulating in su1-P or su1-Ref mutants, which produce aberrant ISA1 proteins, should be found in a high Mr complex, and this was demonstrated by GPC analysis (data not shown). In addition to stability, molecular interactions between ISA1 and ISA2 appear to influence posttranslational modification of the latter. This is suggested by the fact that in su1-Ref endosperm, ISA2 is reproducibly detected both at its predicted size and as a second band with lower mobility in SDS-PAGE. Both ISA2 bands were observed in total extracts and also in purified ISA complexes from su1-Ref endosperm (T.A. Hennen-Bierwagen, unpublished data). A possible explanation is that the conformation of ISA2 in complex with ISA1 affects its posttranslational modification state. In contrast to the instability of ISA2 in the absence of ISA1, the latter protein accumulated to normal levels
in isa2-339 mutant endosperm. In other words, ISA2 in maize endosperm requires ISA1 in order to accumulate, but ISA1 does not require ISA2. This relationship is different from that of Arabidopsis leaf, where ISA1 was found to be severely reduced in abundance in an Atisa2 mutant (Delatte et al., 2005), even though ISA2 transcript was present. A second difference was noted with regard to transcriptional regulation in potato, where ISA1 mRNA failed to accumulate when ISA2 was specifically targeted by antisense transcription, and visa versa (Bustos et al., 2004). Such regulation was not evident in maize endosperm, because loss of ISA1 had no effect on ISA2 mRNA accumulation, as demonstrated by qPCR, and standard RT-PCR indicated that loss of ISA2 had no effect on the ISA1 mRNA level.

Requirements for Homomeric and Heteromeric ISA

The study proceeded to test for effects on starch biosynthesis resulting from loss of one or the other of the ISA complexes. The isa2-339 allele completely eliminated ISA2, and accordingly, there were no ISA1/ISA2 heteromeric complexes in this mutant endosperm. The resultant phenotype was distinct from the effects of mutations affecting ISA2 in Arabidopsis. Atisa2 mutations caused decreased leaf starch content to 20% to 30% of the wild type, identical to the effect of an ISA1-null mutation (Delatte et al., 2005; Wattebled et al., 2005). In contrast, in isa2-339 endosperm, no decrease in starch content was observed at mid development and there was only a minor effect at maturity. A second result of the Atisa2 mutations was accumulation of phyto-glycogen to about 60% of total leaf carbohydrate, again identical to the ISA1 mutation. In maize endosperm, a slight increase in phytoglycogen to about 7% of total carbohydrate resulted from the ISA2-null mutation at mid development, about 10-fold higher than the wild type. At maturity, however, the WSP content in isa2-339 was the same as in the wild type, less than 1% of total carbohydrate. This is in contrast to phytoglycogen accumulation in the maize ISA1-null line at about 60% of total carbohydrate at either mid development or maturity. Also, the ISA1 and ISA2 mutations in Arabidopsis had identical, severe effects on amyllopectin chain length distribution. Loss of ISA1 in maize endosperm had similar effects, but loss of ISA2 did not significantly alter this aspect of amyllopectin structure.

These observations are summarized as follows. Mutation of either ISA1 or ISA2 in Arabidopsis leaf conditions identical, severe effects. Loss of ISA1 in maize endosperm has effects similar to those seen in the dicot leaf, but these are not observed in the ISA2-null line. Thus, the ISA1/ISA2 heteromeric complexes II and III are not required in maize endosperm for normal starch content and structure, and the ISA1 homomeric complex is sufficient for most ISA functions.

The converse experiment was provided by su1-P, which allows the formation of an ISA1/ISA2 heteromer but not the ISA1 homomer. The remaining ISA activity in su1-P plants was conclusively identified as a heteromeric complex by comigration with both ISA1 and ISA2 and the fact that su1-P, isa2-339 double mutants possess no ISA activity, as judged by kernel phenotype. Detailed characterization of carbohydrates in the su1-P mutant indicated normal metabolism (i.e. no phytoglycogen), and starch content and granule morphology were both nearly normal. A small change in amyllopectin chain length distribution was observed; however, this effect was much less severe than the alteration in ISA1-null plants. Thus, just as the heteromeric ISA was not required for most functions, neither was the homomeric ISA. Taken together, these analyses revealed that either heteromeric or homomorphic ISA in maize endosperm is able to support near-normal starch biosynthesis.

Two possibilities can be considered to explain why only heteromeric ISA is present in the su1-P mutant. One is that the mutation at residue 308 results in altered protein-protein interactions such that the mutant ISA1 has lost a critical contact that allows formation of the homotrimmer. Modeling of the ISA1 structure by comparison with the known structure of Pseudomonas isoamylase using the Phyre program (Katsuya et al., 1998; Kelley and Sternberg, 2009) showed residue 308 located on the protein surface within an exposed pocket, consistent with the suggestion that the su1-P mutation could affect protein-protein interactions (Supplemental Fig. S5). According to this hypothesis, interactions of the mutant ISA1 with ISA2 do not require Arg-308, allowing formation of one of the heteromeric complexes. The second possibility is that the mutant ISA1 in su1-P endosperm is present at abnormally low abundance. If ISA1 were to have higher affinity for ISA2 than for itself, heteromeric ISA would be the only complex to form. The observation that su1-P isa2-339 double mutants display a kernel phenotype similar to that of the ISA1-null mutant suggests that the mutant ISA1 protein cannot productively interact with itself even in the absence of ISA2, thus favoring the former hypothesis.

These conclusions raise the question of why ISA2 has been retained in evolution, notably as a nonenzymatic protein, if ISA1 alone can support amyllopectin biosynthesis. One hypothesis is based on the observation of altered granule morphology in the isa2-339 mutant. At mid development, the starch level is normal in the mutant but granules are smaller, indicating increased granule number. Analogous effects were observed in potato tuber and barley endosperm, and from those data ISA activity was proposed to directly repress granule initiation (Burton et al., 2002; Bustos et al., 2004). Alternatively, a reduced rate of crystallization of preamyllopectin in the absence of ISA may slow granule growth. Granules would thus be smaller and more precursor glucan would be available to allow the as yet uncharacterized initiation event to
occur more frequently than normal. Here, we suggest that a function provided specifically by an ISA1/ISA2 heteromer affects granule number and size, and this cannot be fully compensated by the ISA1 homomeric enzyme. Determination of granule number in part through the activity of the ISA1/ISA2 enzyme may have provided a selective advantage during evolution of the Chlorophyte lineage, thus explaining why ISA2 is so highly conserved. Maintaining an appropriate number of granules through activity of the heteromeric complexes may be particularly important in leaves, because starch forms de novo in a regular diurnal pattern and the deposition period is a single daylight phase.

The presence of the ISA1 homomer in monocot endosperm may have arisen owing to selection for bulk starch synthesis as granules enlarge. This may have occurred during the cultivation of rice and maize and selection for large, starchy seeds, or potentially from a selective advantage in monocot seeds in general. Quantification of maize transcript abundance showed the ISA1 level in endosperm to be 2- to 3-fold higher than ISA2, whereas in leaf the ratio is reversed such that ISA2 predominates. Assuming that protein level is coordinate with transcript abundance, excess ISA1 protein in endosperm could explain the presence of both heteromeric and homomeric ISA, in contrast to leaf, where all of the available ISA1 may be committed to the heteromeric form. According to this hypothesis, a change in tissue-specific activity of the su1 promoter could have provided an evolutionary advantage to cereals by introducing the homomeric ISA in endosperm. Another possibility is that sequence drift in the monocot lineage introduced changes in ISA1 structure that allowed it to also function as a homomer. For example, variation in ISA1 sequence may have resulted in stability of the protein in the absence of ISA2, in contrast to Arabidopsis, where ISA1 is unstable in the absence of ISA2 (Delatte et al., 2005).

Transcript Levels

Predictions can be made from the transcript level data regarding the roles of ISA1 and ISA2 in starch catabolism. Unlike developing endosperm, where ISA1 predominates, in germinating seed the ISA1 transcript level is low compared with ISA2 and ISA3. ISA1 transcript is also present at comparatively very low levels in leaves during the dark phase (Supplemental Fig. S1B). Starch is rapidly degraded both in germinating seeds and leaves at night, so it appears that ISA1 is not involved in catabolism or has a minor role. ISA2, in contrast, is present in conditions of both starch biosynthesis and degradation in endosperm, in the latter condition expressed at the same level as ISA3. The ISA3 protein in Arabidopsis leaves is known to be required for starch degradation (Wattebled et al., 2005; Delatte et al., 2006). These observations are consistent with a proposed function of ISA2 in starch degradation, in addition to its known biosynthetic role. These suggestions presume that the transcript levels are indicative of protein accumulation, which may not be the case, so further investigation is necessary to test for a catalytic function of ISA2.

Finally, elimination of ISA1/ISA2 heteromeric complexes in isa2-339 plants allowed resolution of a minor, previously unidentified form of ISA activity seen in zymograms. The ISA proteins in that complex are not known at this time. One possibility is that the minor form is a different type of ISA1 homomeric complex visible only when heteromeric form II is absent and that the low abundance of this assembly prevented detection in αISA1 immunoblots. Another possibility is that the activity could involve ISA3, because ISA3 transcript is present in endosperm, even though at much lower relative abundance than ISA1 or ISA2.

CONCLUSION

Isoamylase-type DBE in maize endosperm functions to support starch synthesis either as a heteromeric multisubunit complex containing both ISA1 and the noncatalytic protein ISA2 or as a homomeric complex containing only ISA1. This is in contrast to Arabidopsis leaf, where ISA2 is required and only a heteromeric complex can support normal starch production. Relatively slight alterations from the wild type suggest that heteromeric ISA in maize endosperm suppresses granule initiation or is required for normal granule growth and that the ISA1 homomer cannot provide this function. Homomeric ISA has specific functions that determine amylopectin structure that are not provided by heteromERIC ISA. Tissue-specific changes in relative levels of ISA1 and ISA2 transcripts, or functional changes in the ISA1 protein, could explain how maize endosperm acquired the homomeric enzyme.

MATERIALS AND METHODS

Plant Materials

Maize (Zea mays) plants were field grown in summer nurseries at Iowa State University. Developing kernels from self-pollinated ears were collected at specified DAP, frozen in liquid N2, and stored at -80°C. All lines used were generated by backcrossing at least five times into the W64A inbred genetic background.

cDNA Cloning

Total RNA was isolated from W64A maize endosperm harvested 20 DAP using the Concert Plant RNA Extraction Reagent (Invitrogen 12322-012). Approximately 1 µg of RNA was treated with DNase (Promega RQ1) in a volume of 10 µl for 30 min at 37°C, followed by heat inactivation at 65°C for 10 min. Random hexamer-primed cDNA synthesis of the DNase-treated RNA was performed using the TAGman Reverse Transcription Reagents (Applied Biosystems N808-0234). RT-PCR was performed using the SuperScript One-Step system (Invitrogen 10928-034). Primers J3D7 (5'-AAAGCACTTATTTYCCNTAYCA-3') and J3D4 (5'-CCACATCTATCCCTATRTNTGAC-3') amplified the central portion of the ISA2 cDNA. Primers J2D1 (5'-CATCAGCACCACCTTCTCCATATTG-3') and J2D4 (5'-GAACCTGCGTTAAAGNG-TNGAR-3') amplified the central portion of the ISA3 cDNA.
Roles of Multiple Isoamylase-Type DBEs in Maize Endosperm

Harlow and Lane, 1988; Hermanson, 2008). The antigen for ISA1 was peptide ZmISA1-a (5'-CVDHYVNYFRWDKKEE-3'). The Cys residue is not part of the ISA1 sequence but was added to allow conjugation to the carrier protein Keyhole limpet hemocyanin. Two peptides were used simultaneously as the antigen for ISA2, ZmISA2-a (5'-KDRSSRLADNAAGTC-3') and ZmISA2-b (5'-CLHIAELDEKLPSD-3'). In both instances, the Cys residue is not part of the ISA2 sequence. Rabbits were immunized on day 0 with 200 μg of antigen in incomplete Freund's adjuvant and boosted with 100 μg in incomplete Freund's adjuvant on days 14, 28, 42, and 56. Sera were collected on days 49 and 63.

IgG fractions were purified from crude sera using the peptides as affinity reagents. Peptide disulfides were used reducing Immobilized TCEP Disulfide Reducing Gel (Pierce 77712) and covalently linked to SUltoLink Coupling Gel (Pierce 40041). For ISA2-reactive IgG purification, a mixture of both ZmISA2-a and ZmISA2-b was used. Crude sera were applied to the column, and IgG was purified as described previously (Hennen-Bierwagen et al., 2008). The IgG fractions raised against ISA1 and ISA2 are referred to as αISA1 and αISA2, respectively.

NATIVE-PAGE and SDS-PAGE were performed on Criterion precast 7.5% polyacrylamide Tris-HCl gels (Bio-Rad 345-0006). Proteins were transferred to nitrocellulose filters and probed with affinity-purified IgG fractions diluted 1:1,000 as described (Ausubel et al., 1989). Signals were detected by chemiluminescence (GE Healthcare RPN2209).

Protein Extraction and Partial Purification

Extraction and purification steps were performed on ice or at 4°C. Endosperm tissue (2–10 g wet weight) was dissected from frozen kernels and ground in a mortar and pestle under 0.5 to 1.0 mL g⁻¹ tissue extraction buffer (50 mM Tris-acetate, pH 7.5, 20 mM dithiothreitol [DTT], 10 mM EDTA, 1% protease inhibitor cocktail; Sigma P-2714). Lysates were centrifuged at 5,000 rpm for 10 min. Supernatants were transferred to thick-walled polypropylene tubes and centrifuged for 30 min at 40,000 rpm. These supernatants were used as total soluble endosperm extract after determining protein concentration (Ausubel et al., 1989).

For AEC, a 1-mL HiTrap Q HP column (GE Health Sciences) was equilibrated in buffer A (50 mM Tris-acetate, pH 7.5, with 10 mM DTT added immediately prior to use). Total soluble extract was applied to the column in a 5-mL volume of extraction buffer at a flow rate of 1 mL min⁻¹. The column was washed with 10 mL of buffer A, and bound proteins were eluted in a 20-mL linear gradient of 0 to 1 NaCl in the same buffer while collecting 1-mL fractions. Two fractions in the conductivity range of approximately 28 to 38 mS cm⁻¹ were pooled and concentrated 5- to 10-fold by centrifugal filtration.

For CPC, a Superdex 200 10/300 column was equilibrated in buffer A containing 150 mM NaCl. Concentrated AEC fractions were applied in a volume of 0.5 mL, and the column was eluted in the same buffer at a flow rate of 0.25 mL min⁻¹ while collecting 0.4-mL fractions.

Zymogram Analyses

Native-PAGE gels were run in standard Laemmli gel buffer (25 mM Tris and 192 mM Gly) adjusted to pH 8.0, with 1 mM DTT added prior to use. Gels were run with the apparatus on ice at 60 V for approximately 5 h. Proteins on native gels were electrophoresed at room temperature overnight at 10 V to a 7.5% acrylamide gel in 375 mM Tris-HCl, pH 8.8, containing 0.375% starch (Sigma S4251). After transfer, the starch-containing gels were stained for 5 min in iodine solution (1% KI and 0.1% I₂). Isoamylase-type DBE activity was visualized by light blue color on a purple background. Duplicate gels run in the same apparatus were transferred to nitrocellulose and analyzed by immunoblot to allow identification of specific proteins comigrating with each ISA activity.

Expression and Immunoblot Analysis of Recombinant Proteins

Recombinant GST-ISA2 fusion protein was expressed in Escherichia coli from plasmid pEXP15-ZmISA2, which was constructed as follows. ISA2 nucleotides 139 to 2,537 were amplified by PCR and cloned into pDONR221 using Gateway technology (Invitrogen). This region contains codons 35 through the native C terminus at codon 799 and the native stop codon. The first 34 codons, predicted to specify a plastid transit peptide, were omitted. The 5’ PCR primer included six additional codons preceding the ISA2 coding

Immunological Methods

Polyclonal antisera were raised in rabbits against synthetic peptides with sequences unique to either ISA1 or ISA2, following standard procedures
region that specify a threonin cleavage site. The resultant Gateway entry clone was used to transfer the modified ISA2 coding region to expression plasmid pDEST15, producing pEXPI-5ZmISA2. This plasmid contains a recombinant gene driven by the T7 promoter that codes for a fusion protein comprising GST at the N terminus, followed by the threonin site, followed by the ISA2-cDNA sequence. A portion of the starch pellet was dissolved by boiling for 30 min in 101 M DTT, and 0.1% Triton X-100, and lysed by sonication. Lyases were centrifuged at 39,000 $g$ for 30 min, and the supernatant was filtered

Carbohydrate Characterization

Starch, Glc, Fru, Suc, and WSP content as a function of endosperm dry weight was determined as described previously (Dinges et al., 2001). Briefly, endosperm harvested 20 DAP was dissected from five kernels that had been stored at $80^\circ\mathrm{C}$ and ground together in water in a mortar and pestle. All manipulations were performed on ice or at $4^\circ\mathrm{C}$ until subsequent boiling steps. A 1-mL sample of the crude lysate was dialyzed to allow determination of the dry weight of tissue analyzed. The remainder of the crude lysate was centrifuged for 10 min at 10,000 $g$ to separate soluble and granular glucans. The pellet was washed once in water, and the supernatant was pooled with that from the initial centrifugation. The soluble fraction was boiled for 30 min to inactivate enzymes. A portion of the starch pellet was dissolved by boiling for 30 min in 100% DMSO and then diluted 100-fold in water for analysis. Glc, Suc, Fru, and WSP were precipitated with 5 volumes of 100% ethanol overnight. Cells were collected by centrifugation, suspended in 1 mL of 10 mM NaOH, and applied to the column. Glc equivalents were precipitated with 5 volumes of 100% ethanol overnight. Glc, Fru, and Suc, and WSP in each fraction were quantified as described (Dinges et al., 2001). The following materials are available in the online version of this article.

Supplemental Figure S1. DBE transcript levels determined by qPCR.
Supplemental Figure S2. Kernel phenotype of su1-1, Isa2-339 double mutants.
Supplemental Figure S3. Differences in amylopectin chain length distribution (I).
Supplemental Figure S4. Differences in amylopectin chain length distribution (II).
Supplemental Figure S5. Predicted structure of ISA1.
Supplemental Table S1. Maize DBE genes and predicted proteins.
Supplemental Table S2. Sequence variation in ISA2.
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Supplemental Table S4. Primers for qPCR.

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Supplemental Data

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Supplemental Table S5. Primers for qPCR.

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