Distinct Functional Properties of Isoamylase-Type Starch Debranching Enzymes in Monocot and Dicot Leaves

Maud Facon, Qiaohui Lin, Abdelhamid M. Azzaz, Tracie A. Hennen-Bierwagen, Alan M. Myers, Jean-Luc Putaux, Xavier Roussel, Christophe D’Hulst, and Fabrice Wattebled*

Unité de Glycobiologie Structurale et Fonctionnelle, UMR 8576 du Centre National de la Recherche Scientifique-Université Lille 1, Sciences and Technologies, F-59655 Villeneuve d’Ascq, France (M.F., X.R., C.D., F.W.); Roy J. Carver Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011 (Q.L., A.M.A., T.A.H.-B., A.M.M.); and Centre de Recherches sur les Macromolécules Végétales-CNRS, Université Joseph Fourier, Institut de Chimie Moléculaire de Grenoble and Institut Carnot PolyNat Domaine Universitaire de Grenoble-Saint Martin d’Hères, 38041 Grenoble, France (J.-L.P.)

ORCID ID: 0000-0002-5556-9099 (C.D.)

Da Silva et al., 2013 American Society of Plant Biologists. All Rights Reserved. 1363-4546/13/

Isoamylase-type starch debranching enzymes (ISA) play important roles in starch biosynthesis in chloroplast-containing organisms, as shown by the strict conservation of both catalytically active ISA1 and the noncatalytic homolog ISA2. Functional distinctions exist between species, although they are not understood yet. Numerous plant tissues require both ISA1 and ISA2 for normal starch biosynthesis, whereas monocot endosperm and leaf exhibit nearly normal starch metabolism without ISA2. This study took in vivo and in vitro approaches to determine whether organism-specific physiology or evolutionary divergence between monocots and dicots is responsible for distinctions in ISA function. Maize (Zea mays) ISA1 was expressed in Arabidopsis (Arabidopsis thaliana) lacking endogenous ISA1 or lacking both native ISA1 and ISA2. Analysis of recombinant enzyme function showed that Arabidopsis ISA1 requires ISA2 as a partner for enzymatic function, whereas maize ISA1 is active by itself. The electrophoretic mobility of recombinant and native maize ISA1 differed, suggestive of posttranslational modifications in vivo. Sedimentation equilibrium measurements showed recombinant maize ISA1 to be a dimer, in contrast to previous gel permeation data that estimated the molecular mass as a tetramer. These data demonstrate that evolutionary divergence between monocots and dicots is responsible for the distinctions in ISA1 function.

Semicrystalline starch enables photosynthetic eukaryotes to store large quantities of Glc over extended time periods compared with other species, in which the soluble polymer glycogen functions to store carbohydrate reserves (Ball and Morell, 2003). Eukaryotes gained the capacity to photosynthesize after the capture of a cyanobacterial endosymbiont by a glycogen-metabolizing host cell. In the lineage that evolved subsequently, known as the Archaeplastida, select glucan-storage enzymes encoded within the host nucleus, the endosymbiont, and potentially a prokaryotic parasite located within the host cell developed so as to generate the branched glucan polymer amylopectin (Ball et al., 2011, 2013). Such molecules are highly similar to glycogen in terms of chemical structure, but the molecular architecture of amylopectin enables the formation of semicrystalline structures (Buléon et al., 1998). These latter then assemble into higher order structures leading to starch granule formation. The advent of starch granules is likely to have been critical for the evolution of chloroplast-containing organisms, including the spread of land plants on the Earth’s surface, because they enable the storage of photosynthetically generated Glc for many hours in tissues such as leaves during diurnal cycles or for months to years in seeds.

An important aspect of the evolutionary change from glycogen to starch is the use of particular α(1→6)-glucosidases, referred to as isoamylase-type starch debranching enzymes (ISA), in the production of amylopectin (Ball et al., 1996; Myers et al., 2000; Hennen-Bierwagen et al., 2012). A suite of genes encoding the enzymes that accomplish starch biosynthesis was established early in the evolution of chloroplast-containing
organisms (i.e. the Chloroplastida) prior to the divergence of distantly related groups including green algae and land plants. Included in this gene set are three paralogs that encode the proteins ISA1, ISA2, and ISA3, each of which is highly conserved in chloroplast-containing species. ISA1 of vascular plants and bryophytes, for example, are approximately 70% identical over more than 600 residues, and between land plants and prasinophyte algae this value is about 60%. ISA1 or ISA2 deficiencies in potato (Solanum tuberosum) tuber, Arabidopsis (Arabidopsis thaliana) leaf, Chlamydomonas reinhardtii cells, and cereal endosperms result in reduced starch content, altered amylopectin structure, and the appearance of soluble, branched glucans similar to native glycogen (James et al., 1995; Mouille et al., 1996; Nakamura et al., 1996; Bustos et al., 2004; Delatte et al., 2005; Wattebled et al., 2005). Such soluble polymers, referred to as phytoglycogen, have not been observed in wild-type plants. Thus, ISA1 and ISA2 functions are important determinants of whether storage glucans are semicrystalline or soluble. ISA3, in contrast, functions primarily in starch catabolism (Wattebled et al., 2005; Delatte et al., 2006).

ISA1 and ISA2 appear to function together in Arabidopsis leaf as a single entity, because essentially identical phenotypes are observed in single mutants lacking either protein or double mutants lacking both of them (Zeeman et al., 1998; Delatte et al., 2005; Wattebled et al., 2005). Biochemical analysis of native and recombinant proteins has shown directly that ISA1 and ISA2 function together in a complex. ISA activity was first purified from potato tuber and found to contain two distinct polypeptides identified as ISA1 and ISA2 (Ishizaki et al., 1983; Hussain et al., 2003). Heteromultimers containing these two proteins were also purified from rice (Oryza sativa) and maize (Zea mays) endosperm (Utsumi and Nakamura, 2006; Kubo et al., 2010). Finally, a mixture of native and recombinant rice proteins demonstrated directly that specific enzymatic activities are provided by ISA1 and ISA2 functioning together in a heteromultimeric complex (Utsumi and Nakamura, 2006). ISA1 is the catalytic subunit within this complex, whereas ISA2 is noncatalytic, owing to amino acid substitutions at residues that are essentially invariant in the GH13 family of glycoside hydrolases (i.e. the α-amylase superfamily), several of which participate in the catalytic mechanism (Hussain et al., 2003; Utsumi and Nakamura, 2006). Despite lacking catalytic activity, ISA2 proteins are conserved in all chloroplast-containing species that have been examined, which rules out recently evolved mutations and, to the contrary, suggests a functional selective advantage.

The necessity for the ISA1/ISA2 heteromultimer is not obvious in light of the fact that, in some instances, ISA1 by itself can condition normal levels of starch and the suppression of phytoglycogen accumulation. Cyanidioschyzon merolae, a species within the Rhodophyta lineage of the Archaeplastida family, contains semicrystalline starch and amylopectin with physical characteristics similar to that of Chloroplastida species (Hirabaru et al., 2010). The C. merolae genome contains elements that encode ISA1 and ISA3 yet lacks a homolog encoding ISA2 (Coppin et al., 2005). Thus, in some instances, starch can be generated, and phytoglycogen accumulation suppressed, without an ISA2 protein. Cereal endosperms provide additional evidence that ISA2 is not strictly required for normal starch levels and the suppression of phytoglycogen accumulation. Mutants or transgenic lines lacking ISA2 are known in rice (Utsumi et al., 2011) and maize (Kubo et al., 2010). Endosperm from these plants exhibits normal starch levels, with amylopectin structure essentially the same as the wild type, and lacks phytoglycogen. ISA activity presumably is provided in the endosperm of these mutants by a homomultimeric enzyme containing only ISA1.

The reason why ISA2 is strictly conserved in the Chloroplastida is not understood yet. Two explanations can be considered. One possibility is that the inherent structure of ISA1 in cereals, resulting from mutations accumulated specifically in this evolutionary lineage, allows it to act without ISA2. Another possibility is that metabolic differences in specific tissues (e.g. leaf versus endosperm) require specialized enzymatic properties of the ISA1/ISA2 heteromer that ISA1 by itself does not provide. To test these hypotheses, this study combined maize and Arabidopsis ISA1 and ISA2 isoforms both in vitro and in vivo. Maize ISA1 was found to be active without any ISA2 protein, either in vitro or in Arabidopsis leaves, whereas Arabidopsis ISA1 required an ISA2 partner in all instances. Thus, ISA1 appears to have evolved in the cereal lineage so that it no longer requires ISA2 for enzymatic activity or metabolic function in the generation of starch and the suppression of phytoglycogen accumulation.

RESULTS

Expression and Purification of Recombinant Arabidopsis and Maize ISA1 and ISA2

Recombinant ISA from Arabidopsis or maize were generated to enable subsequent comparison of their biochemical properties. The coding sequences of the ISA1 and ISA2 complementary DNAs (cDNAs) from both species were synthesized with codon usage optimized for Escherichia coli and cloned into an expression vector containing the phage T7 promoter for the control of transcription and eight His codons at the 3′ end of the open reading frame. The N-terminal codons in the synthetic genes were selected using the ChloroP algorithm to predict the extent of the plastid transit peptide (Emanuelsson et al., 1999). Four plasmids encode individual plant proteins referred to as AtISA1, AtISA2, ZmISA1, and ZmISA2 to indicate Arabidopsis and maize sequences, respectively, all of which possess C-terminal 8× His tags. Two additional plasmids contain synthetic operons with both ISA1 and ISA2 open reading frames separated by a ribosome-binding site. In these instances, the coexpressed proteins are referred to as AtISA1/AtISA2
or ZmISA1/ZmISA2 and the 8× His tag is present only on ISA2. Expression plasmid details, including the particular codons present in each synthetic gene, are shown in Supplemental Figure S1.

Recombinant ISA were expressed singly in E. coli or coexpressed and collected from soluble extracts by affinity to nickel-nitrilotriacetic acid agarose. The purity of the fractions was assessed by separation in SDS-PAGE followed by Coomassie blue staining or immunoblot analysis using affinity-purified rabbit IgG from sera raised against peptide fragments of maize ISA1 or ISA2 (Kubo et al., 2010). ZmISA1 was obtained essentially pure, whereas ZmISA2 coeluted from the affinity matrix with several other proteins and was present in the purified fraction in low abundance to the extent that it could be detected only by immunoblot (Fig. 1A) or by loading large amounts for SDS-PAGE (Fig. 1B). When ZmISA2 was coexpressed together with ZmISA1, however, the two proteins were present in apparently equal abundance (Fig. 1A). ZmISA1 and ZmISA2 must copurify in a complex because the affinity tag is present only on the latter. These data indicate that ZmISA1 and ZmISA2 assemble into a complex and also that ZmISA2 is unstable in E. coli extracts unless it is present in that complex.

AtISA1 and AtISA2 behaved differently from the maize proteins during purification from E. coli. Singly expressed AtISA1 and AtISA2 copurified with the same set of contaminant proteins (Coomassie blue-stained gel; Fig. 1C) and were present in approximately the same abundance in the affinity-purified fraction (Fig. 1C). Since the antibody raised against the ZmISA1 protein cross-reacts with AtISA1, we were able to detect this protein in the purified E. coli soluble extract (Fig. 1C).

Enzymatic Activities of Recombinant ISA

The activities of recombinant ISA were compared in zymograms, initially using total soluble E. coli extracts. Constant amounts of total protein were separated by native-PAGE on a gel impregnated with 0.3% β-limit dextrin (final concentration). After incubation in physiological buffer, the gel was stained with

![Figure 1. Purified ISA proteins. A, ZmISA1 and ZmISA2 expressed singly or coexpressed. ZmISA1 and ZmISA2 were expressed singly as 8× His-tagged proteins or coexpressed from an operon with the 8× His tag only on ZmISA2. The indicated amounts of purified proteins were separated by SDS-PAGE, and duplicate lanes were either stained with Coomassie blue or probed in immunoblot analysis with the indicated IgG fraction. The asterisk indicates ZmISA2. B and C, Maize (B) and Arabidopsis (C) ISA1 and ISA2 expressed singly as 8× His-tagged proteins. Analysis was as in A. Asterisks indicate ISA2 proteins.](image-url)
iodine-potassium iodide solution (I₂/KI) so that changes in color of the glucan-iodine complex revealed the presence of ISA and other β-limit dextrin-modifying enzymes (Fig. 2A). No exogenous activity was observed in extracts containing AtISA1 or ZmISA2 expressed singly, as expected considering that ISA2 proteins lack conserved catalytic residues. Singly expressed AtISA1 and ZmISA1 behaved differently in this assay. Whereas no exogenous activity was found in the AtISA1 lysate, at least three bands of β-limit dextrin-modifying activity were observed with the ZmISA1 lysate. Coexpression of AtISA1/AtISA2, in contrast, yielded two clearly discernible activity bands (Fig. 2A, green arrows). In the ZmISA1/ZmISA2 lysate, the three activity bands detected in the ZmISA1 lysate were again observed, and in addition, a fourth band dependent on the presence of both proteins was detected (Fig. 2A, yellow arrow). These data indicate that ZmISA1 and AtISA1 act differently in the regard that the maize protein can provide enzyme activity on its own whereas the Arabidopsis protein cannot. At this stage, we checked for the putative presence of the endogenous bacterial debranching enzyme protein (GlgX) that could combine with the plant enzyme for the formation of an active complex. Thus, nanoliquid chromatography-tandem mass spectrometry analysis was performed on the major debranching enzyme activity observed in zymograms in the ZmISA1-expressing cell extract. Bands corresponding to ZmISA1 activity were cut out of the polyacrylamide gel and submitted to in-gel trypsin digestion before liquid chromatography-tandem mass spectrometry analysis. Although peptides corresponding to the maize enzyme were identified in the sample, none was detected for the GlgX enzyme, ruling out the possibility of heterocomplex formation.

Singly expressed recombinant maize ISA were further characterized in zymograms involving native-PAGE in the absence of substrate and subsequent electrophoretic transfer to starch-containing gels. ZmISA1 and coexpressed ZmISA1/ZmISA2 were compared with native ISA in maize endosperm extracts. Previous analyses identified three forms of in vivo ISA activity, specifically form I homomultimer containing only ISA1 and forms II and III heteromultimers containing ISA1 and ISA2 (Kubo et al., 2010). Recombinant ZmISA1 migrated near but slightly faster than in vivo form I (Fig. 2B, right panel). Recombinant ZmISA1/ZmISA2 generated three activity bands, similar in mobility to the three in vivo forms but in each instance running slightly faster in native-PAGE (Fig. 2B, left panel). The identities of the three bands from ZmISA1/ZmISA2 as one homomeric and two heteromeric forms were revealed by immunoblot analysis, confirming correspondence with in vivo forms I, II, and III. The increased mobility of all three ISA activities from recombinant proteins relative to the endosperm forms was reproducible in multiple biological replicates (data not shown).

Singly expressed recombinant proteins were mixed to seek to reconstitute enzymatic activity. As noted, AtISA1 by itself does not possess enzymatic activity. The mixture of affinity-purified AtISA2 with purified AtISA1 in increasing concentration generated an enzymatically active complex observed by zymogram (Fig. 3A), consistent with the comparison of singly expressed and coexpressed proteins in total soluble E. coli extracts (Fig. 2A). Two heteromeric activities were generated from the recombinant proteins, as had also been observed for ZmISA1/ZmISA2 (Fig. 2B). A monocot-dicot cross-species mixture was also tested with affinity-purified proteins, specifically titration of a constant amount of ZmISA1 with increasing AtISA2. In this instance, a new mobility form of ISA activity was obtained in addition to the ZmISA1 homomer (Fig. 3B). Thus, AtISA2 can constitute a single functional heteromeric form with
ZmISA1. Note that the ratio of ISA1 and ISA2 was chosen empirically in the unique objective to get visible activity on the gel. This in vitro experiment does not necessarily reproduce the physiological ratio or indicate that the physiological ratio should be 3:1. Further work would be needed to determine that value. These preliminary experiments engendered the following in vivo experiments involving transgenic Arabidopsis lines in which the endogenous ISA1 was replaced with ISA1 from maize.

**Molecular Mass of ZmISA1**

The availability of essentially pure, enzymatically active ZmISA1 afforded the opportunity to measure the molecular mass of the complex by sedimentation equilibrium. The activity and purity of freshly isolated ZmISA1 were confirmed by zymogram and SDS-PAGE (data not shown). Purified enzyme at three concentrations was centrifuged at three different speeds in a Beckman XL-A analytical ultracentrifuge. A$_{280}$ was measured as a function of the radius, and repeated scans over a 3-h time period without any change confirmed that the samples were at equilibrium. UltraScan Analysis software (Demeler, 2005) was used to plot equilibrium protein distribution as a function of the square of the scanned radius, and the observed curves were compared with those predicted for various molecular association models (Supplemental Fig. S2). The model with the lowest statistical variance from the observed data was that of a single component sedimenting with a molecular mass of 158 kD, and the observed variance from the prediction for a 170-kD single component was also a good fit (Supplemental Table S1). Considering that the monomer molecular mass of ZmISA1 is 85 kD, the results indicate that the homomerich complex purified from E. coli is a dimer. No other molecular association models fit the observed data (e.g. monomer or tetramer). Full enzyme activity measured by zymogram was recovered at the end of the longest centrifugation (data not shown), indicating the intactness of the complex throughout the analysis. The entire procedure was repeated twice from separate E. coli cell pellets with essentially identical results.

**Expression of Maize ISA1 in Arabidopsis Leaves**

Binary plasmids were constructed to express either ZmISA1 or AtISA1 as a positive control in transgenic plants (Supplemental Fig. S3). Both transgenes contained the first 43 codons of the Arabidopsis ISA1 cDNA, which are predicted by the ChloroP algorithm to encode a chloroplast transit peptide. In one instance, the transit peptide coding sequence is followed by codons 50 to 789 of the ZmISA1 cDNA, which constitute the mature protein after the maize transit peptide is removed. The hybrid cDNA was cloned into the entry vector pENTR D-TOPO and then sequenced to ensure integrity. Transfer to the destination vector pMDC32 generated plasmid pZmISA1, which included the 235S promoter and NOS terminator for transcriptional controls as well as a selectable marker gene conferring hygromycin B resistance and transfer DNA (T-DNA) elements that direct integration into the plant genome (Curtis and Grossniklaus, 2003). The second binary plasmid, pAtISA1, is the same except that it contains the full-length AtISA1 cDNA sequence in place of the ZmISA1 cDNA.

Each plasmid was used for Agrobacterium tumefaciens-mediated transformation of Arabidopsis carrying the null mutation isa1-1 or a double mutant carrying both isa1-1 and a second null mutation, isa2-2 (Wattebled et al., 2005, 2008). A total of 276 transformed plants were generated from seeds selected on Murashige and Skoog medium containing hygromycin B, and the presence of the transgene was confirmed on a subset of 40 individuals by PCR amplification from genomic DNA of a

---

**Figure 3.** A, Reconstitution of Arabidopsis ISA. The indicated amounts of purified AtISA1 and AtISA2 were mixed, incubated at 0°C overnight, and then subjected to native-PAGE zymography with transfer to a 0.3% starch substrate gel. Purified, coexpressed ZmISA1/ZmISA2 and AtISA1/AtISA2 were included on the same gel as controls. B, Reconstituted ISA activity from ZmISA1 and AtISA2. Analysis was as in A except that ZmISA1 was mixed with AtISA2. The arrow indicates reconstituted ZmISA1/AtISA2 activity. [See online article for color version of this figure.]
portion of the hygromycin B resistance gene. The transgenes in each of these plants represents an independent integration event. The seeds of these 40 individuals were then collected, and only hygromycin-resistant plants were used in the rest of the work.

Expression of ISA1 from the transgenes was demonstrated by the observation in zymograms of enzyme activities not present in the parental lines. Total soluble leaf extracts were analyzed by zymograms with β-limit dextrin on the separation gel as described for E. coli cell extracts (Fig. 4). As expected, the major band of ISA activity (which is actually composed of two bands of slightly different mobility in the wild-type sample; Fig. 4, blue arrow) was absent in extracts of either isa1-1 single mutants or isa1-1 isa2-2 double mutants. An ISA activity band (again in the form of two bands of slightly different mobility; Fig. 4 red arrows) was recovered when AtISA1 was expressed from a transgene in the isa1-1 single mutant host but not when the host was the isa1-1 isa2-2 double mutant. These results were expected from the fact that isa2 single mutants lack ISA activity (Zeeman et al., 1998). The ZmISA1 transgene was functional in Arabidopsis, because an ISA activity band was recovered in the isa1-1 single mutant host. ISA activity was not detected in total soluble extracts of isa1-1 isa2-2 double mutants containing the ZmISA1 transgene; however, the expression of the transgene was confirmed by reverse transcription-PCR of a specific portion of the ZmISA1 transgene (Supplemental Fig. S4). Moreover, genetic function was indicated by the starch phenotype in such transformants, as shown in the following section.

Note that the following analyses were performed on at least three independent hygromycin-resistant transgenic lines regardless of the host mutant background (isa1-1 or isa1-1 isa2-2) and the debranching enzyme (AtISA1 or ZmISA1) used. At least two complementing lines were obtained for each construction. Although some slight variations were observed from line to line, the same phenotype was basically observed. Only the results of one representative transgenic line per type of transformation are presented below, for better clarity of the paper. Other results are compiled in Supplemental Figure S5.

Leaf Starch and Water-Soluble Polysaccharide Content

The starch accumulation phenotype of the transformants was first investigated by iodine staining of leaves harvested at the end of the illuminated period (Fig. 5). Wild-type leaves stain dark brown in this assay, whereas both isa1-1 and isa1-1 isa2-2 mutant leaves stain yellow/orange, indicating low starch content. Leaves of hygromycin-resistant plants stained dark brown when AtISA1 was transgenically expressed in the isa1-1 host, indicating the expected restoration of starch accumulation. The yellow/orange iodine-staining phenotype of isa1 isa2 double mutant leaves was unchanged by the AtISA1 transgene donated from pAtISA1 in any of 84 hygromycin-resistant plants tested, in agreement with previous data indicating the requirement for ISA2. The presence of the ZmISA1 transgene from pZmISA1 in the isa1-1 mutant host resulted in a range of iodine-staining phenotypes, with leaves of some transgenic plants staining dark brown, others yellow/orange, and most displaying an intermediate phenotype. The ZmISA1 transgene expressed in isa1 isa2 double mutant leaves resulted in some plants with an intermediate iodine-staining phenotype (Fig. 5).

The quantity of leaf starch and water-soluble polysaccharide (WSP) normalized to fresh weight was determined in selected transformant lines (Fig. 6). Soluble and granular glucans were extracted from mature leaves harvested at the end of a 16-h illuminated period. Consistent with previous analyses, isa1-1 and isa1-1 isa2-2 mutants accumulated less starch than the wild type and also contained WSP at levels approximately equal to those of the granular glucans. WSP was not detected at appreciable levels in wild-type leaves. Starch accumulation was fully restored in an isa1-1 host expressing AtISA1 (line P6C3), and WSP accumulation was repressed to the residual level typical of the wild type. Starch accumulation was also restored in the isa1-1 host expressing ZmISA1 (line P2D7). In that instance, the starch level was significantly higher than normal, whereas the WSP content was negligible in this transformant line. ZmISA1 supported granular starch accumulation to a greater level than the wild type when expressed in the isa1-1 isa2-2 double mutant host (line P4D3; Fig. 6). This is in contrast to the double mutant host expressing AtISA1, which never exhibited starch accumulation as judged by iodine staining. The WSP content was insignificant in the isa1 isa2 host line expressing ZmISA1 (P4D3), indicating full complementation of

![Figure 4. β-Limit dextrin-modifying activities in total soluble leaf extracts. Analysis was as in Figure 2A. Arabidopsis isa1-1 single mutants or isa1-1 isa2-2 double mutants were transformed with either full-length AtISA1 or the hybrid protein ZmISA1 fused to the Arabidopsis ISA1 transit peptide. The blue arrow indicates native ISA activity in the wild type, and red arrows indicate ISA activities dependent on the presence of a transgene. The black arrows indicate a β-limit dextrin-modifying activity that is variably present in leaf extracts including wild-type lines. [See online article for color version of this figure.]]
the isa1-1 isa2-2 double mutant phenotype by exogenous expression of ZmISA1 alone.

Amylopectin Structure and Starch Granule Morphology

The linear chain length distribution (CLD) of leaf starch from the parental and transformant lines was determined. Polymers within purified granules were solubilized and completely debranched with commercial α(1→6)-glucosidases, then linear chains were separated by high-performance anion-exchange chromatography at high pH and quantified by pulsed amperometric detection. The CLD profiles of starch from all transformants shown in Figure 6 were identical within technical limits to that of the wild-type line (Supplemental Fig. S6). Thus, the increased short chain frequency characteristic of isa1 single mutants and isa1 isa2 double mutants (Delatte et al., 2005; Wattebled et al., 2005) was not observed in any of the complementing lines.

Starch granule morphology, size, and quantity per chloroplast were characterized in situ by transmission electron microscopy (TEM) performed on ultrathin sections of Arabidopsis leaves harvested at the end of the illuminated period (Fig. 7). The wild-type phenotype regarding granule size and shape within palisade mesophyll chloroplasts was restored when AtISA1 was expressed in the isa1-1 host (line P6C3). This phenotype is obviously different from that of the isa1-1 and isa1-1 isa2-2 host lines, which exhibited only small starch granules less than 1 μm in diameter within stroma filled with WSP and surrounded by thylakoid structures. The accumulation of larger starch granules was also restored when ZmISA1 was expressed in the isa1-1 mutant (line P2D7). Granule size and morphology were different from the wild type, however, when ZmISA1 was expressed in the isa1-1 isa2-2 double mutant background (line P4D3). In this instance, granules were smaller than in the wild-type line but bigger than those found in the isa1-1 isa2-2 double mutant.

Starch granule size distributions were determined by scanning electron microscopy (SEM) of purified particles. The size distribution of the particles was determined by the analysis of the most representative SEM images taken at the same scale. For each sample, up to 600 particles were analyzed using the ImageJ software. To limit bias, the analysis was carried out independently by two researchers on the same set of images. Basically, the same distributions were obtained (Fig. 8). Granules in an isa1-1 host line expressing ZmISA1 (line P2D7) were larger than those of the isa1-1 mutant, suggesting a complementation of the deficiency by the maize enzyme. Some granules in these transformant lines were 4.5 μm or more in diameter, which typically does not occur in wild-type plants. In agreement with the TEM data, ZmISA1 transformants of the isa1-1 isa2-2 host (line P4D3) contained a heterogeneous granule size population, including a majority of small, rounded, irregular granules with a diameter of 1 μm or less and some larger particles with an average diameter close to that of the wild type.

DISCUSSION

Inherent Properties of Maize and Arabidopsis ISA1

Taken together, the in vivo and in vitro data presented here support the conclusion that the gene encoding ISA1...
has evolved in the Poaceae branch of monocotyledonous plants so that it possesses biochemical activity without the participation of an ISA2 partner. This enzymatic activity provides metabolic functions within endosperm or leaf tissue to support the accumulation of starch granules.

ZmISA1 was obtained in essentially pure form after it was generated in *E. coli*, so that no other maize protein was present (especially the bacterial endogenous GlgX isoamylase), nor were there any post-translational modifications that potentially could occur in maize plants. These results are consistent with the observation of enzymatically active protein complexes in rice and maize endosperm extracts that possess only ISA1 and extend those observations by proving that activity is inherent to the polypeptide itself. Recombinant AtISA1 was capable of enzymatic activity if mixed with recombinant AtISA2 but did not display such function on its own. Thus, the ZmISA1 polypeptide possesses inherent α(1→6)-glucosidase activity, whereas AtISA1 does not.

The maize ISA1 protein, when expressed in Arabidopsis leaves lacking both endogenous ISA1 and ISA2,
was able to support the accumulation of starch to nearly wild-type levels. However, the apparent modified morphology and number per plastid of these granules as seen by TEM and SEM was distinct from the wild type. It is not known whether such differences were due to the activity of ZmISA1, which did not function identically to the native leaf enzyme, or whether such modifications were the consequence of various expression levels of the maize enzyme due to ectopic integration of the transgene (or both).

It is not clear why the activity of ZmISA1 was not observed on the zymogram. It could be that ZmISA1 expressed alone in the isa1-1 isa2-2 double mutant, and possibly organized in the form of homomer if we assume that it does not interact with AtISA3 and AtPU1, two other debranching enzymes of Arabidopsis involved in starch metabolism (Wattebled et al., 2005), is unstable (at the molecular or the activity level or both) when extracted from the Arabidopsis leaves. This could explain why the phenotype of the isa1-1 isa2-2 double mutant is complemented but the ZmISA1 activity is not seen on the zymogram of Arabidopsis leaf extracts. The situation is different when ZmISA1 is expressed in *E. coli*, since the activity is easily visible on the zymogram. The recombinant protein could be more stable in the specific context of bacterial expression (e.g. no posttranslational modification) or could be simply expressed at such a higher level compared with Arabidopsis leaves that the activity is visible on the zymogram.

The CLD in the transformant was identical to the wild type, so the differences in granule morphology could result from altered branch placement or from higher order assemblies of primary crystallization units. Maize ISA1 by itself also supported near-normal starch metabolism in maize leaves (Lin et al., 2013). These results indicate that homomeric ISA functions to support nearly normal starch accumulation in the context of leaf metabolism in which the native enzyme is heteromeric. These observations are again consistent with the conclusion that evolutionary divergence in the line including maize and rice has altered ISA1 so that it has gained enzymatic and biological function without the participation of ISA2. This result excludes the hypothesis of the existence of a molecular factor specifically present in maize or rice endosperm that controls ISA1 activity even in the absence of ISA2.

**Structure of Recombinant Enzymes**

Previous characterizations of ISA enzymes from rice or maize endosperm identified two ISA1/ISA2 heteromeric enzyme complexes that separate in native-PAGE in addition to a single ISA1 homomultimer (Utsumi and Nakamura, 2006; Kubo et al., 2010). Two heteromeric enzymes were also observed in this study when recombinant ISA1 and ISA2 proteins produced from either maize or Arabidopsis cDNA sequences were combined. This observation implies that the two activity bands identified from native-PAGE are not explained by different posttranslational modification states in the plant. The two heteromeric ISA activity bands may have different quaternary structure (i.e. they each possess a different assortment of ISA1 and ISA2 subunits).
relative abundance of the two heteromer mobility forms produced in *E. coli* was similar to the native enzymes, consistent with different assembly states that exist owing to association constants inherent to the folded polypeptides. Another potential explanation is that ISA2 can fold stably into different conformations in complexes with ISA1, so that alternative charge-to-mass ratios could exist. This is not typical; however, a pullulanase-type starch debranching enzyme, which is a distantly related homolog of ISA, is known to exhibit such folding behavior (Henker et al., 1998; Renz et al., 1998).

Distinctions were noted between the native maize ISA complexes and the recombinant forms of these enzymes purified from *E. coli*. Specifically, the electrophoretic mobility of all three recombinant ISA complexes was greater than that of the corresponding assembly state from endosperm. A possible explanation for these properties is that the enzymes are post-translationally modified in vivo and this affects their mobility in native-PAGE by changing their charge to molecular mass ratios. Such alterations would apply equally to all three ISA complexes and, as noted, would not be responsible for the existence of any particular activity band.

Analysis of the recombinant enzymes also indicates that heteromeric ISA is in equilibrium between associated and dissociated states, such that the ISA1 homomer can assemble from subunits released from the ISA1/ISA2 heteromer. This conclusion follows from the fact that only heteromeric forms are obtained initially when ZmISA1/ZmISA2 is purified, owing to the affinity tag specifically on ZmISA2. Thus, the recovery of active ZmISA1 homomer requires the dissociation of the heteromeric complex and reassociation into a homomeric enzyme. These data suggest a dynamic equilibrium between the assembly states of ISA1 and ISA2.

The determination that recombinant ZmISA1 is a homodimer provides evidence that the enzyme complex exhibits an elongated structure. Homomeric ISA1 from rice was estimated based on gel permeation chromatography (GPC) data to have a molecular mass of 420 to 480 kD (Utsumi and Nakamura, 2006), and the maize endosperm ISA1 homomer had an apparent mass of approximately 300 kD as also judged by GPC (Kubo et al., 2010). This is in contrast to the 160-kD molecular mass demonstrated directly for recombinant ZmISA1 homomer by sedimentation equilibrium. Such determinations from GPC data are approximate, however, because this method separates molecules based on hydrodynamic volume, so rod-shaped or otherwise elongated molecules will appear to have artifically large molecular masses when compared with spherical standards. From these considerations, the data shown here indicate ZmISA1 homomer to be an elongated structure containing two oblong monomers connected end to end. The oblong shape of each monomer subunit is presumed based on the known structure of *Pseudomonas* species isoamylase, which is approximately 40% identical to both ISA1 and ISA2.

This conclusion is supported by observing an apparent molecular mass greater than 400 kD when recombinant ZmISA1 was analyzed by GPC (data not shown) and by crystallographic data showing an elongated dimeric structure for recombinant ISA1 encoded by a *C. reinhardtii* cDNA (L. Sim and M. Palic, personal communication).

The shape of an active ISA complex as shown here could contribute to the acquisition of a starch biosynthetic function from a primordial catabolic enzyme. ISA present in the progenitor of the Archaeplastida presumably were degradative and acted as a monomer with a single active site, as do current ISA3 enzymes and prokaryotic isoamylase relatives. The formation of a dimer or other multimeric structure, with an end-to-end arrangement of oblong subunits, may have spaced the hydrolytic active sites a fixed distance from each other. The action of such an enzyme on a glycogen-like molecule potentially could contribute to the generation of products that are capable of crystallization, by generating a regular spacing of densely branched and infrequently branched regions of the molecule according to the model reviewed elsewhere (Hennen-Bierwagen et al., 2012).

Data regarding recombinant ZmISA1 homodimer do not imply that heteromeric forms of ISA are necessarily dimers as well. These enzymes from rice or maize endosperm consistently elute later than homomultimeric ISA1 on GPC columns, so they likely have a different subunit structure. Inclusion of a noncatalytic subunit (i.e. ISA2) in a multimeric structure potentially could allow alternatives for the spacing of the active sites and thus provide variability in the fine structures that could be generated from a precursor glucan prior to crystallization and the assumption of subsequent higher order structures. Such variability in the architecture of molecules generated as the result of ISA complex activity may, in turn, explain evolutionary selection for ISA2.

**MATERIALS AND METHODS**

Expression and Purification of Recombinant ISA Proteins

Two operon sequences that specify AtISA1 and AtISA2 or ZmISA1 and ZmISA2 were synthesized chemically (GenScript; Supplemental Fig. S1). The order of the elements in the synthetic operons starting from the 5′ end is as follows: (1) BglII restriction site, (2) ATG codon, (3) ISA1 coding region beginning at the predicted N terminus, (4) HindIII site, (5) stop codon, (6) ribosome-binding site (i.e. the Shine-Dalgarno sequence), (7) BamHI site, (8) ATG codon, (9) ISA2 coding region beginning at the predicted N terminus, (10) Xhol site, ISA3 genes were excised from the synthetic operons as BglII/HindIII fragments and cloned into the unique BglII/HindIII sites of pBE1343 immediately downstream of the ribosome-binding site sequence of the expression vector. In these constructs, the ISA1 coding regions are followed by 15 codons derived from pBE1343 that specify the sequence KLLAA-LEHHHHHHHHH, then a stop codon. ISA2 genes were excised from the synthetic operons as BamHI/Xhol fragments and cloned into the unique BglII/Xhol sites of pBE1343. In these instances, the C-terminal nucleotides from the vector specify the sequence LEHHHHHHHHH. For coexpression, the ISA1/ISA2 sequences, separated by a ribosome-binding site, were cloned as BglII/Xhol fragments into pBE1343, so that the ISA1 protein is not tagged and the ISA2 protein contains an 8× His tag.
For the analysis of ISA activities in total *Escherichia coli* extracts, expression plasmids were introduced into thermo competent *E. coli* BL21 DE3 cells (Invitrogen). Cultures containing 10 mL of Luria-Bertani medium supplemented with 50 μg mL⁻¹ kanamycin were seeded with 1 mL of overnight preculture and grown at 37°C with shaking to optical density at 600 nm of 0.5. Expression from the T7 promoter of pBE1345 was induced by the addition of 1 mL of 1 M isopropylthio-β-galactosidase, and cultures were grown for 4 h at 30°C. Cells were recovered by centrifugation (2,000g; 10 min, 4°C), and the pellet was suspended in 500 μL of buffer (50 μL Tris-HCl, pH 7.0). Cells were lysed by pulse sonication (two times for 20 s each, on ice), and the lysate was centrifuged at 25,000 × g for 2 min at 4°C. Protein concentration in the supernatant was assayed by the Bradford method (Bio-Rad; Bradford, 1976) prior to zymogram analysis.

For protein purification, the same plasmids were introduced into *E. coli* host strain Rosetta BL21 (DE3) pLysS (EMD Biosciences), and fresh single colonies were inoculated into 10-mL cultures of Luria-Bertani medium supplemented with 50 mg mL⁻¹ kanamycin and 34 mg mL⁻¹ chloramphenicol. After growth at 37°C overnight, the entire pregrowth culture was added to a 1-L culture of the same medium, which was grown at 37°C with shaking for about 4 h until *A. maxima* of 0.8. Cultures were then cooled to 16°C, isopropylthio-β-galactoside was added to a final concentration of 0.1 mM, and growth was continued at 16°C overnight. Cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C, suspended in 20 mL of sonication buffer (20 mM sodium phosphate, pH 7.5, 0.5 mM NaCl, 1 mg mL⁻¹ lysozyme, and 1× protease inhibitor cocktail [Sigma]), and stored at −80°C in approximately 10-mL portions until further use.

Cell pellets were thawed and sonicated 10 times for 30 s each, cooling on ice between treatments. Crude lysates were centrifuged at 16,000 rpm for 30 min at 4°C, then the supernatant was passed through a 0.2-μm syringe filter and loaded onto a 1-mL bed volume HiTrap FF column (GE Healthcare) pre-equilibrated in buffer A (20 mM sodium phosphate, pH 7.5, and 0.5 mM NaCl). In this and subsequent chromatography steps, the flow rate was 1 mL min⁻¹. The column was washed successively with 20 mL of buffer A containing 30 mM imidazole, then 20 mL of buffer A containing 50 mM imidazole, then 5 mL of buffer A containing 90 mM imidazole. Proteins were eluted in 5 mL of elution buffer (buffer A containing 300 mM imidazole), concentrated to 0.5 mL by centrifugal filtration, and used immediately for further analysis or stored at −20°C in elution buffer adjusted to 1 mM dithiothreitol (DTT) and 10% (v/v) glycerol. ZmISA1 retained activity for up to 1 week in this storage condition; however, coexpressed ZmISA1/ZmISA2 was not stable upon freezing.

**Zymogram Assays**

Zymography was performed either with substrate in the running gel or with electrophoretic transfer to a gel impregnated with substrate. Proteins (100 μL) preloaded on a native 7.5% (w/v) polyacrylamide gel containing 0.3% (w/v) β-limit dextrin (Megazyme) at final concentration. After migration in an electric field of 15 V cm⁻¹ for 3 h at 4°C in separation buffer (25 mM Tris, 200 mM Gly, and 1 mM DTT), the gel was incubated overnight at room temperature in fresh separation buffer. Glucan-modifying activities were revealed by soaking the gel in fresh 1/3Xki. E. coli extracts for these assays were prepared as described in the previous sections (Isoamylase function in monocots and dicots). Proteins (100 μL) preloaded on a native 7.5% (w/v) polyacrylamide gel containing 0.3% (w/v) β-limit dextrin (Megazyme) at final concentration. After migration in an electric field of 15 V cm⁻¹ for 3 h at 4°C in separation buffer (25 mM Tris, 200 mM Gly, and 1 mM DTT), the gel was incubated overnight at room temperature in fresh separation buffer. Glucan-modifying activities were revealed by soaking the gel in fresh 1/3Xki. E. coli extracts for these assays were prepared as described in the previous sections (Isoamylase function in monocots and dicots) extracts for zymogram analyses were prepared from two to three leaves harvested at midday. Leaves were homogenized in 50 μL of ice-cold 50 mM Tris-HCl, pH 7.0. The homogenate was centrifuged at 10,000 × g for 5 min at 4°C, and proteins in the supernatant were measured by the Bradford method as described previously for the Bradford method (Bio-Rad; Bradford, 1976) prior to zymogram analysis. Western-blot analysis was performed as described previously for the Bradford method (Bio-Rad; Bradford, 1976) prior to zymogram analysis. Western-blot analysis was performed as described previously for the Bradford method (Bio-Rad; Bradford, 1976) prior to zymogram analysis. Western-blot analysis was performed as described previously for the Bradford method (Bio-Rad; Bradford, 1976) prior to zymogram analysis. Western-blot analysis was performed as described previously for the Bradford method (Bio-Rad; Bradford, 1976) prior to zymogram analysis. Western-blot analysis was performed as described previously for the Bradford method (Bio-Rad; Bradford, 1976) prior to zymogram analysis.
Extraction and Quantification of Starch and WSP

Arabidopsis leaves were harvested at the end of the day, immediately frozen in liquid nitrogen, and stored at −80°C before extraction. Starch and WSP were extracted by the perchloric acid method (Delatte et al., 2005) adapted as follows: about 1 g of leaves was homogenized with a polytron blender in 5.5 mL of 0.7 M perchloric acid. The crude lysate was centrifuged at 10,000g for 10 min at 4°C to separate the pellet, which contains starch, and the supernatant, which contains WSP. The pellet was rinsed three times with sterile deionized water. An aliquot was collected for the glucan assay (described below), and the rest of the sample was treated to further purify the starch. To this end, the pellet was resuspended in sterile deionized water and filtered through two layers of Miracloth (Calbiochem). The starch pellet was further purified by isopinic centrifugation in Percoll (GE Healthcare) at 10,000g for 1 h at 4°C. The pellet was rinsed twice with sterile deionized water and stored in 20% (v/v) ethanol at 4°C until use.

The supernatant obtained from the crude lysate was neutralized with 2 M KOH, 0.4 M MES, and 0.4 M KCl. The potassium perchlorate precipitate was removed by centrifugation at 10,000g for 15 min at 4°C. The supernatant was stored at −20°C until use.

Insoluble (starch) and soluble (WSP) polysaccharides were assayed, as described (Wattelbled et al., 2005), by the spectrophotometric method after complete digestion of the polysaccharides by amyloglucosidase following the instructions (R-Biopharm).

**Nanoliquid Chromatography-Tandem Mass Spectrometry Analysis of In-Polyacrylamide Gel Proteins**

Bands corresponding to ZmISA1 activity observed on zymograms were cut out of the polyacrylamide gel. Iodine was removed by three successive baths of 10 min at 4°C in Tris-Gly separation buffer (as described in “Zymogram Assays”). Then in-gel trypsin digestion of proteins was performed with trypsin (V511A; Promega); conditions were slightly modified to optimize the proteolysis of native and complex proteins analyzed on glycoprotein-containing gels. Reduction time was 2 h in 10 mM DTT at 56°C; gel pieces were incubated 1 h in 100 mM iodoacetamide and then for 30 min at 37°C with another denaturating agent (0.1% Rapigest; Waters SAS) in ammonium bicarbonate buffer. Then, 0.3 μg of trypsin was put on gel pieces and incubated 24 h at 37°C. Elution was performed with 45% acetonitrile (liquid chromatography-mass spectrometry grade) in 0.1% formic acid (both from Biosolve). The samples were then separated by online reverse-phase chromatography using a Thermo Scientific Proxeon Easy-nLC system equipped with a Proxeon trap column (100 μm i.d. × 2 cm; Thermo Scientific) and a C18 packed tip column (100 μm i.d. × 10 cm; Thermo Scientific). Elution was carried out using an increasing gradient of acetonitrile (5%-30% (v/v) over 110 min) and a flow rate of 300 nL min⁻¹. A voltage of 1.6 kV was applied to the needle of the nanospray source. The chromatography system was coupled to a Thermo Scientific LTQ Orbitrap XL mass spectrometer programmed to acquire data-dependent mode. The survey scans were acquired in the Orbitrap mass analyzer operated at 60,000 (full width at half maximum) resolving power. A mass range of 300 to 1,600 mass-to-charge ratio and a target of 1 × 10⁶ ions were used for the survey scans. Precursor ions observed with an intensity over 500 counts were selected “on the fly” for ion-trap collision-induced dissociation fragmentation with an isolation window of 4 atomic mass units and a normalized collision energy of 35%. A target of 5,000 ions and a maximum injection time of 200 ms were used for tandem mass spectrometry spectra. The method was set to analyze the top 10 most intense ions from the survey scan, and a dynamic exclusion was enabled for 60 s.

**Starch CLD**

Debranching reactions and starch CLD determination by high-performance anion-exchange chromatography at high pH and quantified by pulsed amperometric detection using a PA200 CarboPac column (Dionex) were performed according to the procedure described previously (Roussel et al., 2013).

**TEM and SEM**

Arabidopsis or maize leaves harvested at the end of the light period of the diurnal cycle were cut with a fresh razor blade into small pieces a few millimeters on edge and immediately immersed in fixative (0.1 M cacodylate, pH 7.2, 2% (v/v) paraformaldehyde, and 2% (v/v) glutaraldehyde). Vacuum was applied for 30 min. Leaf samples were embedded in Spurr’s resin, then postfixed with 1% (v/v) osmium tetroxide, sectioned to a thickness of 80 nm, affixed to grids, and stained with uranyl acetate and lead citrate. TEM observation was performed with a JEOL 2100 microscope operating at 200 kV. SEM was performed as follows. Droplets of starch granule suspensions were allowed to dry on freshly cleaned mica. After coating with gold/palladium, they were observed in secondary electron mode using a JEOL JSM6300 microscope operating at 8 kV. Size distribution histograms were determined by measuring the apparent diameter of 600 particles per sample from the SEM images using the ImageJ software.

**Sedimentation Equilibrium**

ZmISA1 was expressed in E. coli and purified as described by Ni²⁺-affinity chromatography. Proteins were diluted in the affinity column elution buffer at concentrations of 3.76, 6.47, or 8.47 μM and loaded into a six-channel cell in an An 60 Ti rotor. The samples were centrifuged in a ProteomeLab XL-A analytical centrifuge (Beckman Coulter) at 8,500 rpm for 38.25 h until equilibrium was attained, as shown by A₉₀₀ scans performed over a 3-h time period. Speed was then increased to 10,200 rpm for an additional 18.25 h until a new equilibrium was attained. A third equilibrium distribution was then recorded after increasing the speed to 12,000 rpm and centrifuging for an additional 10.75 h. Equilibrium scans of A₉₀₀ across the radius of the centrifuge cell were compared using UltraScan Analysis software (Demeler, 2005) with the distributions predicted for single or multiple component systems of given molecular mass components.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP_181522 (AtISA1), NP_973751 (AtISA2), and ACG43008 (ZmISA1).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Plasmid maps and sequences of synthetic genes for the expression of maize and Arabidopsis ISA1 and ISA2.

**Supplemental Figure S2.** Curve fitting of sedimentation equilibrium data.

**Supplemental Figure S3.** Maps of binary vectors for the transgenic expression of maize or Arabidopsis ISA1.

**Supplemental Figure S4.** Expression of the ZmISA1 transgene in the isa1-1 isa2-2 Arabidopsis mutant host line.

**Supplemental Figure S5.** Starch and WSP contents and SEM of purified starch samples from host and transgenic plants expressing Arabidopsis or maize ISA1 protein.

**Supplemental Table S1.** Curve fitting to calculated sedimentation equilibrium distributions.

**ACKNOWLEDGMENTS**

We thank Dr. Christine Lancelon-Pin (Centre de Recherche sur les Macromolécules Végétales) for the SEM observation of purified starch granules, Marie-Christine Sloimanny (Unité de Glycobiologie Structurale et Fonctionnelle) for mass spectrometry analysis, and Adeline Courseaux (Unité de Glycobiologie Structurale et Fonctionnelle) for her excellent technical assistance. Nanoliquid chromatography Orbitrap experiments were performed by Clinical Chemistry Imaging, the mass spectrometry facility of the Laboratory of Fondamental et Applied Biological Mass Spectrometry laboratory at Université Lille 1. Received July 25, 2013; accepted September 6, 2013; published September 16, 2013.

**LITERATURE CITED**


Delatte, T., Trevisan, M., Parker, M. L., Zeeman, S. C. (2005) Arabidopsis mutants Atis1 and Atis2 have identical phenotypes and lack the same multimeric isoamylase, which influences the branch point distribution of amylopectin during starch synthesis. Plant J 41: 815–830


