Distinct functional properties of isoamylase-type starch debranching enzymes in monocot and dicot leaves

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One-sentence summary:

Starch metabolism in maize and Arabidopsis involve starch debranching enzyme ISA1 that have evolved separately so that the monocot protein possesses enzymatic activity by itself whereas the dicot protein requires an ISA2 partner, which is an intrinsic property of ISA1.
Footnotes:

1 This work was supported by:

Centre National de la Recherche Scientifique (CNRS), Agence Nationale de la Recherche ("Glycoballs" and "CaSta DivA" grants, ANR-09-CP2D-07-01 and ANR-11-BSV6-003-02 respectively) the Université Lille 1, sciences & technologies. M.F. was supported by the Ministère de la Recherche et de l’Enseignement Supérieur.

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ABSTRACT

Isoamylase-type starch debranching enzymes (ISA) play important roles in starch biosynthesis in chloroplast-containing organisms as shown by strict conservation of both catalytically active ISA1 and the non-catalytic 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 near-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 thaliana lacking endogenous ISA1, or lacking both native ISA1 and ISA2. The maize protein functioned in Arabidopsis leaves to support near normal starch metabolism, in the absence of any native ISA1 or ISA2. Analysis of recombinant enzymes showed that Arabidopsis ISA1 requires ISA2 as a partner for enzymatic function whereas maize ISA1 was active by itself. Electrophoretic mobility of recombinant and native maize ISAs differed, suggestive of post-translational 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. The data demonstrate that evolutionary divergence between monocots and dicots is responsible for distinctions in ISA1 function.
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58 INTRODUCTION
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60 Semi-crystalline starch enables photosynthetic eukaryotes to store large quantities of glucose over extended time periods, compared to 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 amyllopectin (Ball et al., 2011; Ball et al., 2013). Such molecules are highly similar to glycogen in terms of chemical structure, but the molecular architecture of amyllopectin enables formation of semi-crystalline structures (Buleon 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 storage of photosynthetically-generated glucose for many hours in tissues such as leaves during diurnal cycles, or for months to years in seeds.

61 An important aspect of the evolutionary change from glycogen to starch is the use of particular \( \alpha(1 \rightarrow 6) \)-glucosidases, referred to as isoamylase-type starch debranching enzymes (ISA), in the production of amyllopectin (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 tuber, Arabidopsis leaf, Chlamydomonas cells, or cereal endosperms result in reduced starch content, altered amyllopectin structure, and 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 semi-crystalline 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 and maize endosperm (Utsumi and Nakamura, 2006; Kubo et al., 2010). Finally, 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 non-catalytic 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 infers 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 suppression of phytoglycogen accumulation. Cyanidioschyzon merolae, a species within the Rhodophyta lineage of the Archaeplastida family, contains semi-crystalline starch and amyllopectin 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 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 amyllopectin structure essentially the same as wild type, and lacks
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phytoglycogen. ISA activity presumably is provided in 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 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 generation of starch and suppression of phytoglycogen accumulation.

RESULTS

Expression and Purification of Recombinant Arabidopsis and Maize ISA1 and ISA2

Recombinant ISAs from Arabidopsis or maize were generated to enable subsequent comparison of their biochemical properties. The coding sequences of the ISA1 and ISA2 cDNAs from both species were synthesized with codon usage optimized for E. coli, and cloned into an expression vector containing the phage T7 promoter for control of transcription and eight histidine codons at the 3' end of the open reading frame. The amino 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, or ZmISA2 to indicate Arabidopsis or maize sequences, respectively, all of which possess C-terminal 8X 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 co-expressed proteins are referred to as AtISA1/AtISA2 or ZmISA1/ZmISA2, and the 8X 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 ISAs were expressed singly in *E. coli*, or co-expressed, and collected from soluble extracts by affinity to Ni\(^{2+}\)-NTA 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 co-eluted 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 (Figure 1A) or by loading large amounts for SDS-PAGE (Figure 1B). When ZmISA2 was co-expressed together with ZmISA1, however, the two proteins were present in apparently equal abundance (Figure 1A). ZmISA1 and ZmISA2 must co-purify 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 than the maize proteins during purification from *E. coli*. Singly expressed AtISA1 and AtISA2 co-purified with the same set of contaminant proteins (Coomassie stained gel; Figure 1C), and were present in approximately the same abundance in the affinity-purified fraction (Figure 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 (Figure 1C).

**Enzymatic Activities of Recombinant ISAs**

The activities of recombinant ISAs were compared in zymograms, initially using total soluble *E. coli* extracts. Constant amounts of total protein were separated by native-PAGE in a gel impregnated with 0.3% \(\beta\)-limit dextrin (final concentration). After incubation in physiological buffer the gel was stained with iodine-potassium iodide solution (I\(_2\)/KI) so that changes in color of the glucan-iodine complex revealed the presence of ISA and other \(\beta\)-limit dextrin-modifying enzymes (Figure 2A). No exogenous activity was observed in extracts containing AtISA2 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 \(\beta\)-limit dextrin-modifying activity were observed with the ZmISA1 lysate. Co-expression of
AtISA1/AtISA2, in contrast, yielded two clearly discernable activity bands (Figure 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 (Figure 2A, yellow arrow). The 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 DBE protein (GlgX) that could combine to the plant enzyme for the formation of an active complex. Thus NanoLC MS/MS analysis was performed on the major DBE activity observed in zymogram 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 LC MS/MS 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.

Purified recombinant maize ISAs were further characterized in zymograms involving native-PAGE in the absence of substrate and subsequent electrophoretic transfer to starch-containing gels. ZmISA1 and co-expressed ZmISA1/ZmISA2 were compared to native ISAs 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 (Figure 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 (Figure 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. Mixture of affinity purified AtISA2 with purified AtISA1 in increasing concentration generated an enzymatically active complex observed by zymogram (Figure 3A), consistent with the comparison of singly expressed and co-expressed proteins in total soluble E. coli extracts (Figure 2A).
heteromeric activities were generated from the recombinant proteins, as had also been observed
for ZmISA1/ZmISA2 (Figure 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 (Figure 3B). Thus, AtISA2 can constitute a single functional heteromeric
form with ZmISA1. Note that the ratio of ISA1 and ISA2 were chosen empirically in the unique
objective to get visible activity on the gel. This \textit{in vitro} experiment does not necessarily
reproduce the physiological ratio or indicates that the physiological ratio should be three to one.
Further work would be needed to determine that value. These preliminary experiments
engendered the following \textit{in vivo} experiments involving transgenic \textit{Arabidopsis} lines in which
the endogenous ISA1 was replaced with ISA1 from maize.

\textbf{Molecular Mass of ZmISA1}

Availability of essentially pure, enzymatically active ZmISA1 afforded the opportunity to
measure the molecular mass of the complex by sedimentation equilibrium. Activity and purity of
freshly isolated ZmISA1 was 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 function of the radius, and repeated scans
over a three hour time period without any change confirmed 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 to those
predicted for various molecular association models (Supplemental Figure 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 kDa, and the observed variance from the prediction for a 170 kDa
single component was also a good fit (Supplemental Table S1). Considering that the monomer
molecular mass of ZmISA1 is 85 kDa, the results indicate that the homomeric complex purified
from \textit{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 intactness of the complex throughout the
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...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 Figure 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 2X 35S promoter and NOS terminator for transcriptional controls as well as a selectable marker gene conferring hygromycin B resistance and 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*-mediated transformation of *Arabidopsis* carrying the null mutation *isa1-1* or double mutant carrying both *isa1-1* and a second null mutation, *isa2-2* (*Wattebled et al., 2005; Wattebled et al., 2008*). A total of 276 transformed plants were generated from seeds selected on MS medium containing hygromycin B, and presence of the transgene was confirmed on a subset of 40 individuals by PCR amplification from genomic DNA of a 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 observation in zymograms of enzyme activities not present in the parental lines. Total soluble leaf extracts were analyzed by zymograms with β-limit dextrin in the separation gel as described for *E. coli* cell extracts (Figure 4). As expected, the major band of ISA activity (which is actually composed of two bands of slightly different mobility in the WT 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 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 RT-PCR of a specific portion of the ZmISA1 transgene (Supplemental Figure 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 whatever the host mutant background (isa1-1 or isa1-1 isa2-2) and the DBE 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 were presented below for a better clarity of the manuscript. Other results were 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 (Figure 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 requirement for ISA2. Presence of the ZmISA1 transgene from pZmISA1 in isa1- the 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 \textit{isa1- isa2-} double mutants resulted in some plants with an intermediate iodine-staining phenotype (Figure 5).

The quantity of leaf starch and water-soluble polysaccharide (WSP) normalized to fresh weight was determined in selected transformant lines (Figure 6). Soluble and granular glucans were extracted from mature leaves harvested at the end of a 16 h-illuminated period. Consistent with previous analyses, \textit{isa1-1} and \textit{isa1-1 isa2-2} mutants accumulated less starch than 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 \textit{isa1-1} host expressing \textit{AtISA1} (line P6C3), and WSP accumulation was repressed to the residual level typical of wild type. Starch accumulation was also restored in the \textit{isa1-1} host expressing \textit{ZmISA1} (line P2D7). In that instance the starch level was significantly higher than normal whereas the WSP content was negligible in this transformant line.

\textit{ZmISA1} supported granular starch accumulation to greater level than wild type when expressed in the \textit{isa1-1 isa2-2} double mutant host, (line P4D3; Figure 6). This is in contrast to the double mutant host expressing \textit{AtISA1}, which never exhibited starch accumulation as judged by iodine staining. The WSP content was insignificant in the \textit{isa1- isa2-} host expressing \textit{ZmISA1} (P4D3), indicating full complementation of the \textit{isa1-1 isa2-2} double mutant phenotype by exogenous expression of \textit{ZmISA1} alone.

**Amylopectin Structure and Starch Granule Morphology**

The linear chain length distributions (CLD) of leaf starch from the parental and transformant lines were determined. Polymers within purified granules were solubilized and completely debranched with commercial $\alpha$(1→6)-glucosidases, then linear chains were separated by high-performance anion exchange chromatography at high pH and quantified by pulsed amperometric detection (HPAEC-PAD). 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 Figure S6). Thus, the increased short chain frequency characteristic of \textit{isa1-} single mutants and \textit{isa1- isa2-} double mutants (\textit{Delatte et al., 2005}; \textit{Wattebled et al., 2005}) was not observed in any of the complementing lines.
Starch granule morphology, size, and quantity per chloroplast were characterized \textit{in situ} by transmission electron microscopy (TEM) performed on ultrathin sections of \textit{Arabidopsis} leaves harvested at the end of the illuminated period (Figure 7). The wild-type phenotype regarding granule size and shape within palisade mesophyll chloroplasts was restored when AtISA1 was expressed in the \textit{isa1-1} host (line P6C3). This phenotype is obviously different from that of the \textit{isa1-1} and \textit{isa1-1 isa2-2} host lines that exhibited only small starch granules less than 1 \textmu m in diameter within stroma filled with WSP and surrounded by thylakoid structures. Accumulation of larger starch granules was also restored when ZmISA1 was expressed in the \textit{isa1-1} mutant (line P2D7). Granule size and morphology were different from the wild type, however, when ZmISA1 was expressed in the \textit{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 \textit{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 pictures 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 pictures. Basically, the same distributions were obtained (Figure 8). Granules in \textit{isa1-1} host lines expressing ZmISA1 (line P2D7) were larger than those of the \textit{isa1-1} mutant suggesting a complementation of the deficiency by the maize enzyme. Some granules in these transformant lines were 4.5 \textmu m or more in diameter, which typically does not occur in wild type plants. In agreement with the TEM data, ZmISA1 transformants of the \textit{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 \textmu m or less and some larger particles with an average diameter close to that of the wild type.

**DISCUSSION**

**Inherent Properties of Maize and \textit{Arabidopsis} ISA1**

Taken together the \textit{in vivo} and \textit{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 participation of an ISA2 partner. This enzymatic activity
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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. The results are consistent with 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 accumulation of starch to near wild type levels. However, the apparent modified morphology and number per plastid of these granules as seen by TEM and SEM was distinct from wild type. It is not known whether such differences were due to the activity of ZmISA1 that 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 doesn’t interact with AtISA3 and AtPU1 two other DBE 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 not seen on the zymogram. The situation is different when ZmISA1 is expressed in E. coli since the activity is easily visible on zymogram. The recombinant protein could be more stable in the specific context of bacterial expression (no post-translational modification for example) or could be simply expressed at such higher level compared to Arabidopsis leaves that the activity is visible on zymogram.

The CLD in the transformant was identical to 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 also suppressed accumulation of phytoglycogen in
Arabidopsis leaves (Lin et al., 2013). The results indicate that homomeric ISA functions to
support near 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 participation of ISA2. This result excludes the
hypothesis of the existence of a molecular factor specifically present in maize or rice endosperm
that control ISA1 activity even in the absence of ISA2.

Structure of Recombinant Enzymes

Previous characterization 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 the current 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
post-translational 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. The 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 (i.e. subunit number) 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 ISA1 homomer can assemble from subunits released from 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, recovery of active ZmISA1 homomer requires dissociation of the heteromeric complex and re-association 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 GPC data to have a molecular mass of 420-480 kDa (Utsumi and Nakamura, 2006), and the maize endosperm ISA1 homomer had an apparent mass of approximately 300 kDa also judged by GPC (Kubo et al., 2010). This is contrast to the 160 kDa 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 and so rod-shaped or otherwise elongated molecules will appear to have artifactually large molecular masses when compared to spherical standards. From these considerations the data shown here indicate ZmISA1 homomer to be an elongated structure containing two oblong-shaped monomers connected end to end. The oblong shape of each monomer subunit is presumed based on the known structure of Pseudomonas isoamylase, which is approximately 40% identical to both ISA1 and ISA2. The conclusion is supported by observing an apparent molecular mass greater than 400 kDa 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 Chlamydomonas cDNA (personal communication).

The shape of an active ISA complex as shown here could contribute to acquisition of a starch biosynthetic function from a primordial catabolic enzyme. ISAs 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. Formation of a dimer or other multimeric structure, with an end to end arrangement of oblong shaped subunits, may have
spaced the hydrolytic active sites a fixed distance from each other. 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 non-catalytic 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 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, Piscataway, USA) (Supplemental Figure 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) XhoI site. ISA1 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 KLAAALEHHHHHHH, then a stop codon. ISA2 genes were excised from the synthetic operons as BamHI/XhoI fragments and cloned into the unique BglII/XhoI sites of pBE1343. In these instances the C terminal nucleotides from the vector specify the sequence LEHHHHHHHH. For co-expression, the ISA1/ISA2
sequences, separated by a ribosome-binding site, were cloned as *BglII/XhoI* fragments into pBE1343, so that the ISA1 protein is not tagged and the ISA2 protein contains an 8X His-tag.

For analysis of ISA activities in total *E. coli* extracts, expression plasmids were introduced into thermocompetent *E. coli* BL21 DE3 cells (Invitrogen, Saint Aubin, France). Cultures containing 10 mL of LB medium supplemented with 50 µg/mL kanamycin were seeded with 1 mL of overnight preculture and grown at 37°C with shaking to OD$_{600}$ ≈ 0.5. Expression from the T7 promoter of pBE1343 was induced by addition of 1 mM IPTG and cultures were grown for 4 h at 30°C. Cells were recovered by centrifugation (2,000 g, 10 min, 4°C) and the pellet was suspended in 500µL of buffer (50 mM 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 (Biorad, Marnes-la-Coquette, France) (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 LB medium supplemented with 50 mg/mL kanamycin and 34 mg/mL chloramphenicol. After growth at 37°C overnight the entire pre-growth culture was added to a 1 L culture of the same medium, which was grown at 37°C with shaking at 200 rpm for about 4 h until the A$_{600}$ ≈ 0.8. Cultures were then cooled to 16°C, IPTG 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 sonication buffer (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl, 1 mg/mL lysozyme, 1X protease inhibitor cocktail [Sigma catalog no. P-2714]) and stored -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 HisTrap FF column (GE Healthcare catalog no. 17-5319-01) pre-equilibrated in Buffer A (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl). In this and subsequent chromatography steps the flow rate was 1 mL/min. The column was washed successively with 20 mL Buffer A containing 20 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 DTT and 10% glycerol. ZmISA1 retained activity for up to 1 week in this storage condition, however, co-expressed ZmISA1/ZmISA2 was not stable upon freezing.

**Zymogram Assays**

Zymograms were performed either with substrate in the running gel or with electrophoretic transfer to a gel impregnated with substrate. Proteins (100 μg) were loaded on a native 7.5% polyacrylamide gel containing 0.3% of β-limit dextrin (Megazyme, Wicklow, Ireland) 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 glycine, 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 I₂/KI solution. *E. coli* extracts for these assays were prepared as described in the previous section. *Arabidopsis* 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 quantified by Bradford assay. Zymogram analysis of recombinant proteins purified from *E. coli* was performed as described previously using transfer to substrate gels containing 0.3% solubilized starch (Kubo et al., 2010).

**Western Blots Analysis**

Western blots analysis carried out on recombinant maize or *Arabidopsis* debranching enzymes expressed in *E. coli* were performed as described in (Kubo et al., 2010) with antibodies raised against specific peptide sequences selected from the maize enzyme sequences (Kubo et al., 2010). Note that the specific antibody rose against the ZmISA1 protein cross-reacted with the AtISA1 enzyme but not the ZmISA2 specific antibody.

**Arabidopsis thaliana** Lines, Growth Conditions and Media

*Arabidopsis thaliana* lines of the Columbia ecotype were used in this study. The wild type line was indicated as Col-0. The single mutant lines *isa1-1* and *isa2-2* were engendered by
T-DNA insertion mutagenesis (Alonso et al., 2003; Wattebled et al., 2005) and were provided by the Nottingham Arabidopsis Stock Centre. The *isa1-1, isa2-2* double mutant line was obtained by a cross between the two corresponding single mutants and screening of the progeny by PCR amplification of genomic DNA using a T-DNA end primer and gene-specific primers.

Seeds were stratified for 48 hours in the dark at 4°C in Phytagel solution 0.1% (p/v) and were sown subsequently on loam « FloraFleur » (TREF®) pre-treated with Trigard (Syngenta®). Plants were grown in greenhouse with a photoperiod of 16 h light/8 h dark at 20°C during the illuminated period and 18°C during the night.

**Construction of Binary Vectors for Protein Expression in *Arabidopsis thaliana***

A chimeric DNA construction comprising the coding sequence of the AtISA1 transit peptide and the mature ZmISA1 protein was created by PCR. The first 43 codons of the AtISA1 cDNA were used as the transit peptide according to the ChloroP algorithm prediction (Emanuelsson et al., 1999). Primers 2For (CACCATGGATGCAATCAAATGCAGTTCCAGTTTC) and 4Rev (CCGCCTGCACCGCCTCGGCCACGGAAATCGAAATCGGACGGAAG) were used to amplify this sequence from an AtISA1 cDNA clone and to add a 3’ sequence overlapping the 5’ end of the ZmISA1 coding sequence. Conditions for this PCR reaction were: 94°C 5 min, [94°C 45 sec, 59°C 45 sec, 72°C 45 sec] x 35, 72°C 5 min. The amplified fragment was mixed with a DNA fragment including the ZmISA1 cDNA sequence (10 ng each) and subjected to one cycle of extension by DNA polymerase by incubation at 94°C for 5 min, 59°C for 45 sec, 72°C for 5 min. The chimeric fragment was then amplified by PCR using primers 2For and 6Rev (TCAGACATCAGGGCGCAATACAAGGATG) carried out in the following conditions: 94°C 5 min, [94°C 45 sec, 59°C 45 sec, 72°C 3 min 20 sec] x 35, 72°C 5 min. PCR reactions were carried out with Kappa HiFi polymerase (CliniSciences, Nanterre, France) following supplier’s instructions. The same PCR protocol was used to amplify the full-length sequence coding AtISA1, including the transit peptide, from a cDNA clone using primers 2For and 5Rev (TCAGGGGTCTTTAATTGGTGAAAGAAGGA).

After the final PCR amplification, the fragments of interest, i.e., the full length coding sequence of AtISA1 or the mature sequence of ZmISA1 with the transit peptide of AtISA1, were purified with the NucleoSpin ExtractII kit (Marcherey-Nagel EURL, Hoerdt, France) and ligated.
into the pENTR-D-TOPO vector by the TOPO cloning method (Invitrogen, Saint Aubin, France). Recombinant plasmids were selected, amplified and stored in the E. coli TOP10 strain. The constructions were verified by restriction enzyme mapping and DNA sequencing. The ISA coding regions were then transferred from the pENTR-D-TOPO vector to the pMDC32 destination vector (Curtis and Grossniklaus, 2003) using LR Clonase II following supplier’s instructions (Invitrogen, Saint Aubin, France). These final vectors were stored at -80°C in E. coli TOP10 cells.

**Arabidopsis Transformation**

Prior Arabidopsis transformation, the pMDC32 vectors were introduced in Agrobacterium tumefaciens strain GV3101 by a freeze and thaw method as described in Jyothishwaran et al. (Jyothishwaran et al., 2007). An adaptation of the Floral-Dip method was used to transform isa1-1 and isa1-1, isa2-2 mutants of Arabidopsis thaliana (Martinez-Trujillo et al., 2004; Zhang et al., 2006). Harvested seeds were then analysed for the presence of the construction. Seeds were sown on Murashige and Skoog 1% medium (1% agar) containing 20µg.mL⁻¹ of Hygromycin B (Sigma-Aldrich, Lyon, France) and germinated in growth cabinets (22°C, 80% of humidity and a specific photoperiod according to Harrison et al. (Harrison et al., 2006). The plants harbouring the hygromycin B resistant phenotype were then transferred to soil in greenhouse (16h light/ 8h night). The presence of the transgene in the selected hygromycin resistant individuals was verified by PCR amplification of a region of the hygromycin B resistance gene using primers HygrRFor (GATGTAGGAGGGCGTGGATA) and HygrRRev (GATGTTGGCGACCTCGTATT) with the following reaction conditions: 94°C 5 min, [94°C 45 sec, 60°C 45 sec, 72°C 60 sec] x 36, 72°C 5 min.

**Iodine Staining of Leaves**

Leaves harvested at the end of the light period were immediately immersed in 70% ethanol and heated at 70°C for 30 min with regular shaking to remove pigments. This operation was repeated until bleaching was complete. Leaves were then rinsed with water, immersed in 1% KI, 0.1% I₂ (I₂/KI) until homogenous staining, rinsed again in water, and photographed.
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 10,000 g for 10 min at 4°C to separate the pellet, which contains starch, and the supernatant that contains WSP. The pellet was rinsed three times with sterile deionized water. An aliquot was collected for glucan assay (described hereafter) 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, Darmstadt, Germany). The starch pellet was further purified by isopicnic centrifugation in Percoll (GE Healthcare, Velizy-Villacoublay, France) at 10,000 g for 1 h at 4°C. The pellet was rinsed twice with sterile deionized water and stored in 20% ethanol at 4°C until use.

The supernatant obtained from the crude lysate was neutralized with 2 M KOH, 0.4 M MES, 0.4 M KCl. The potassium perchlorate precipitate was removed by centrifugation at 10,000 g 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 by Wattebled et al. (Wattebled et al., 2005), by the spectrophotometric method after complete digestion of the polysaccharides by amyloglucosidase following supplier’s instructions (R-Biopharm, Saint Didier au Mont d’Or, France).

NanoLC MS/MS analysis of In-Polyacrylamide Gel Proteins

Bands corresponding to ZmISA1 activity observed on zymogram were cut out of the polyacrylamide gel. Iodine was removed by 3 successive baths of 10 min at 4°C in Tris-glycine separation buffer (as described in zymogram section). Then in-gel trypsin digestion of proteins was performed with trypsin (ref V511A from Promega, Lyon, France); conditions were slightly modified to optimize proteolysis of native and complex proteins analyzed in glycogen-containing gel. Reduction time was 2 hours in DTT 10 mM at 56°C; gel pieces were incubated 1 hour in iodoacetamide 100 mM and then for 30 min at 37°C with another denaturing agent (RapiGest,
Waters SAS, Guyancourt, France) 0.1% in buffer ammonium bicarbonate. Then 0.3 µg of trypsin was put on gel pieces and incubated 24 hours at 37°C. Elution was performed with acetonitrile (AcN, LC-MS grade) 45% in formic acid (FA) 0.1% (AcN and FA were from Biosolve, Dieuze, France). The samples were then separated by online reversed-phase chromatography using a Thermo Scientific Proxeon Easy-nLC system equipped with a Proxeon trap column (100 µm ID x 2 cm, Thermo Scientific) and C18 packed tip column (100 µm ID x 10 cm, Thermo Scientific). Elution was carried out using an increasing gradient of AcN (5% to 30% over 110 minutes) and a flow rate of 300 nL/min. 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 in data dependent mode. The survey scans were acquired in the Orbitrap mass analyzer operated at 60 000 (FWHM) resolving power. A mass range of 300 to 1600 m/z and a target of 1E6 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 (CID) fragmentation with an isolation window of 4 a.m.u. and a normalized collision energy of 35%. A target of 5000 ions and a maximum injection time of 200 ms were used for MS² spectra. The method was set to analyze the top 10 most intense ions from the survey scan and a dynamic exclusion was enabled for 60s.

Starch Chain Length Distribution

Debranching reactions and starch CLD determination by HPAEC-PAD using a PA200 CarboPac column (Dionex, Courtaboeuf, France) were performed according to the procedure described previously (Roussel et al., 2013).

Transmission and Scanning Electron Microscopy

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 millimetres on edge and immediately immersed in fixative (0.1 M cacodylate, pH 7.2, 2% paraformaldehyde, 2% glutaraldehyde). Vacuum was applied for 30 min. Leaf samples were embedded in Spurr's resin, then postfixed with 1% osmium tetroxide, sectioned to a thickness of 80 nm, affixed to grids and stained with
uranyl acetate and lead citrate. Transmission electron microscopy (TEM) observation was performed with a Jeol 2100 microscope operating at 200 kV. Scanning electron microscopy (SEM) was performed as follows: droplets of starch granule suspensions were allowed to dry on freshly cleaved mica. After coating with Au/Pd, 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 Ni$^{2+}$-affinity chromatography. Proteins were diluted in the affinity column elution buffer at concentrations of 3.76 μM, 6.47 μM, or 8.47 μM and loaded into a 6-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 A$_{280}$ 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 A$_{280}$ across the radius of the centrifuge cell were compared using UltraScan Analysis software (Demeler, 2005) to the distributions predicted for single or multiple component systems of given molecular mass components.

ACKNOWLEDGEMENTS

The authors thank Dr. Christine Lancelon-Pin (CERMAV) for the SEM observation of purified starch granules, Marie-Christine Slomianny (UGSF) for mass spectrometry analysis, and Adeline Courseaux (UGSF) for her excellent technical assistance. NanoLC-Orbitrap experiments were performed by CLIC-Imaging, the Mass Spectrometry facility of the FABMS laboratory in the Université Lille 1. CLIC-Imaging is funded by the SIRIC ONCOLILLE, the Région Nord-Pas de Calais (France), and the Université Lille 1.

LITERATURE CITED
Isoamylase-type DBE function in monocots & dicots


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rhodophyte Cyanidioschyzon merolae contains a semiamylopectin-type, but not an amylose-type, alpha-glucan. Plant and Cell Physiology 51: 682-693


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Utsumi Y, Nakamura Y (2006) Structural and enzymatic characterization of the isoamylase1 homo-oligomer and the isoamylase1-isoamylase2 hetero-oligomer from rice endosperm. Planta 225: 75-87


FIGURE LEGENDS

Figure 1. Purified ISA proteins. A, ZmISA1 and ZmISA2 expressed singly or co-expressed. ZmISA1 and ZmISA2 were expressed singly as 8X His-tagged proteins, or co-expressed from an operon with the 8X 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 and Arabidopsis ISA1 and ISA2 expressed singly as 8X His-tagged proteins respectively. Analysis as in panel A. Asterisks indicate ISA2 proteins.

Figure 2. Zymogram analysis of recombinant ISA activities. A, Total soluble E. coli extracts. Soluble fractions from cells expressing the indicated single protein, or co-expressing ISA1 and ISA2 from Arabidopsis or maize, were separated by native-PAGE in a gel containing 0.3% β-limit dextrin, then the gel was stained with I2/KI solution. “Control” indicates extract from the host strain lacking a plasmid. Orange arrows indicate activities dependent on expression of ZmISA1 alone, the yellow arrow indicates an activity dependent on co-expression of ZmISA1 and ZmISA2, and green arrows indicate activities dependent on co-expression of AtISA1 and AtISA2. B, Purified maize proteins. Proteins were separated by native-PAGE then transferred to a gel impregnated with 0.3% starch. ISA activities were identified by I2/KI staining. Duplicate native-PAGE lanes were analyzed by immunoblot using ISA1- or ISA2-specific IgG as indicated. Soluble maize endosperm extract was included as a control. Native ISA activities I, II, and III are labeled for reference to the text. The amount of protein loaded was 50 μg for endosperm extract, 0.1 μg for ZmISA1 and 1.0 μg for ZmISA1/ZmISA2.

Figure 3. A, Reconstitution of Arabidopsis ISA. The indicated amounts of purified AtISA1 and AtISA2 were mixed, incubated at 0°C overnight, then subjected to native-PAGE zymogram with transfer to a 0.3% starch substrate gel. Purified, co-expressed ZmISA1/ZmISA2 and AtISA1/AtISA2 were included on the same gel as controls. B, Reconstituted ISA activity from ZmISA1 and AtISA2. Analysis as in panel A except that ZmISA1 was mixed with AtISA2. The arrow indicated reconstitutes ZmISA1/AtISA2 activity.
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 wild type and red arrows indicate ISA activities dependent on presence of a transgene. The black arrow indicates a β-limit dextrin modifying activity that is variably present in leaf extracts including wild type lines.

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Figure 6. Starch and WSP contents determined in host and transgenic lines (as indicated in the figure). Contents are expressed in mg of carbohydrate per g of fresh weight (FW) and are the means of three independent biological replicates. Black and grey vertical bars indicate starch and WSP content respectively. Vertical lines on bars stand for standard error. Typically no or very low amount of WSP were detected for WT and transgenic lines expressing AtISA1 or ZmISA1 protein.

Figure 7. Transmission electron microscopy. Leaves were harvested at the end of the illuminated period and fixed immediately. Images show chloroplasts within palisade mesophyll cells. The scale bar applies equally to all images. The host genotype (above pictures) and transgenically expressed ISA protein (in bold below pictures) are indicated.

Figure 8. Scanning electron microscopy images of purified starch granules (host and transgenic lines are indicated) and corresponding size distribution histograms (X-axis = size in μm; Y-axis = % of particles of the indicated size in the population analyzed). The starch samples were prepared from Arabidopsis leaves harvested at the end of the illuminated period. The histograms were determined by measuring the apparent diameter of 600 particles per sample using the ImageJ program. Scale bars = 10μm.
SUPPLEMENTAL DATA

Supplemental Figure S1. Plasmid maps and sequences of synthetic genes for 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 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 scanning electron microscopy of purified starch samples from host and transgenic plants expressing Arabidopsis or maize ISA1 protein.

Supplemental Figure S6. Chain Length Distribution (CLD) of starch-forming glucans of host and transgenic plants expressing Arabidopsis or maize ISA1 protein.

Supplemental Table S1. Curve Fitting to Calculated Sedimentation Equilibrium Distributions.
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<th>isa1-1 + ZmISA1</th>
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