Replacement of the Endogenous Starch Debranching Enzymes ISA1 and ISA2 of Arabidopsis with the Rice Orthologs Reveals a Degree of Functional Conservation during Starch Synthesis

Sebastian Streb*, Samuel C. Zeeman
Institute for Agricultural Sciences, Department of Biology, ETH Zurich, Zurich, Switzerland

Abstract
This study tested the interchangeability of enzymes in starch metabolism between dicotyledonous and monocotyledonous plant species. Amylopectin - a branched glucose polymer - is the major component of starch and is responsible for its semi-crystalline property. Plants synthesize starch with distinct amylpectin structures, varying between species and tissues. The structure determines starch properties, an important characteristic for cooking and nutrition, and for the industrial uses of starch. Amylopectin synthesis involves at least three enzyme classes: starch synthases, branching enzymes and debranching enzymes. For all three classes, several enzyme isoforms have been identified. However, it is not clear which enzyme(s) are responsible for the large diversity of amylpectin structures. Here, we tested whether the specificities of the debranching enzymes (ISA1 and ISA2) are major determinants of species-dependent differences in amylpectin structure by replacing the dicotyledonous Arabidopsis isoamylase enzymes (AtISA1 and AtISA2) with the monocotyledonous rice (Oryza sativa) isoforms. We demonstrate that the ISA1 and ISA2 are sufficiently well conserved between these species to form heteromultimeric enzymes (ISA1 homomultimeric complex in Arabidopsis isa1isa2 mutants. This homomultimer was able to facilitate normal rates of starch synthesis. The resulting amylpectin structure had small but significant differences in comparison to wild-type Arabidopsis amylpectin. This suggests that ISA1 and ISA2 have a conserved function between plant species with a major role in facilitating the crystallization of pre-amylpectin synthesized by starch synthases and branching enzymes, but also influencing the final structure of amylpectin.

Introduction
Starch is the major storage carbohydrate in plants and an important renewable resource for both the food and non-food industry sectors. Starch is comprised of two glucose polymers (amylopectin and amylose) and accumulates in plant tissues as semi-crystalline granules. Amylopectin accounts for the majority of the granule mass (around 60% to 90%, depending on the botanical source). It is a branched molecule in which α-1,4-linked glucan chains are connected via α-1,6-bonds [1,2] resulting in a tree-like structure. On average, there is one branch point every 20–25 glucose residues. However, the arrangement of branch points is thought to be non-random, such that linear chain segments can align together to form double-helical structures that pack into stable, semi-crystalline lamellae. The branch points are concentrated in the amorphous regions between these crystalline lamellae [1]. The higher-order structures adopted by amylpectin are thought to occur in all wild-type starches and underlie the water-insoluble granular characteristics of starch. Nevertheless, there is considerable variation in starch granule morphology, structure and composition between plant sources. These factors are important due to the impact they have on starch properties, which are relevant for downstream functional applications. A detailed understanding of how different biosynthetic enzymes influence amylpectin structure has been hindered by the fact that it is currently not possible to determine the exact structure of amylpectin. Also, we can only partially relate the physical properties of starch to its structure, and hence it is difficult to predictably control these characteristics through manipulation of enzyme abundance and/or specificities.

Starch synthesis is mediated by three enzyme classes. Starch synthases extend α-1,4-linked chains by transferring new glucose units from ADP-glucose to the non-reducing end. Branch points are introduced by branching enzymes, which cleave an existing α-1,4-bond of a linear chain and transfer the cut end to another chain, creating an α-1,6-bond. Both starch synthases and branching enzymes exist as multiple isoforms, thought to have different specificities [3-5]. A third class, the debranching enzymes, are involved in the removal of α-1,6-bonds. This hydrolytic step is obviously required during starch re-mobilization,
but is interestingly also required for normal starch synthesis [5–8]. Debranching enzymes can be divided into two classes: isoamylase (ISA) and limit-dextrinase (LDA). The isoamylase class is subdivided into proteins designated as ISA1, ISA2 and ISA3 [9,10]. ISA3 and LDA are mainly involved in starch breakdown [11–13], whereas ISA1 and ISA2 participate in starch synthesis. Current evidences suggest that ISA1 is an active enzyme, whereas ISA2 proteins are non-catalytic due to changes in 6 out of 8 key amino acids within the active site [9,14,15]. In all plant species studied, ISA1 and ISA2 are found in heteromultimeric complexes, in which the ISA2 subunit is proposed to have a regulatory function or confer substrate specificity (Arabidopsis: [16]; Potato: [17]; Chlamydomonas reinhardtii: [10]; maize: [19]; rice: [20]). In addition, ISA1 homomultimers have been shown to occur in rice and maize endosperm [19,20] and proposed for C. reinhardtii [18]. Thus far, homomultimer forms of ISA1 have not been reported in leaves. Therefore, it is still unknown whether this reflects a tissue-specific feature (heterotrophic versus autotrophic) or an evolutionary difference between monocot and dicot plants.

The role of these isoamylase complexes in starch biosynthesis has been determined through phenotypic analysis in a range of plant species and tissues in which ISA1 or ISA2 gene expression has been reduced or abolished. The loss of ISA1 results in a reduction of granular starch in endosperms of maize [21], rice [6] and barley [22], in C. reinhardtii cells [18,29,24], in Arabidopsis leaves [13,16] and in potato tubers [17]. In all instances, the starch was partially replaced by a water soluble glucose polymer called phytoglycogen, which had shorter chain lengths compared to amylopectin in chain length distribution (CLD) analyses, and a higher degree of branching. The impact of mutations in ISA2 is more variable. In endosperms of maize and rice, loss of ISA2 had no measurable effect, explained by the fact that these tissues contain still the active ISA1 homomultimeric complexes [19,25]. In Arabidopsis, loss of ISA2 causes the same phenotype as the loss of ISA1 [13,16]. This observation is explained by the fact that Arabidopsis leaves appear to contain only the heteromultimeric ISA1/ISA2 complex, and the loss of either protein subunit results in a loss of the enzymatic activity destabilization of the remaining subunit [15]. Overall, the differing phenotypic severity caused by the loss of ISA1 and the existence of both homomultimeric ISA1 and heteromultimeric ISA1/ISA2 complexes means that function of isoamylases in amylopectin biosynthesis is still not fully understood.

In this study, we wanted to address three hypotheses arising from our current knowledge. First, are differences in starch structure between different plant species due to different catalytic specificities of ISA1 and ISA2 protein complexes? Second, are the functions between the ISA complexes from different plant species conserved? Third, are the subunits of the ISA complexes adequately conserved such that they are interchangeable between species? To answer these questions we replaced the endogenous ISA proteins in the dicot Arabidopsis (AtISA1 and AtISA2) with the respective monocot rice isoforms (OsISA1 and OsISA2). Our data show that active heteromultimeric complexes can be formed between AtISA1 and OsISA2 as well as AtISA2 and OsISA1. Additionally, we were able to reconstitute the OsISA1 homomultimeric complex as found in rice endosperm. The resulting transgenic lines synthesize comparable amounts of starch to wild-type Arabidopsis plants. This illustrates that rice isoforms can replace the endogenous Arabidopsis proteins. Furthermore, in addition to playing a major role in facilitating starch biosynthesis, differences in the final structure of amylopectin between the transgenic lines suggests that the isoamylases enzymes composed of ISA1 and ISA2 subunits also contribute to the species-specific differences in amylopectin structure.

**Results**

**Transient and storage starch structure in monocot and dicot plants**

Despite the conservation in starch biosynthetic enzymes between species (SBs, BEs and DBEs), the reported amylopectin structures differ considerably between different plant species and tissues [26]. This may be due to differences in enzyme specificities and/or relative expression levels. To identify species with significant differences in amylopectin structure and exclude variability resulting from the different techniques used between different laboratories, we analysed the structure of amylopectin from starch extracted from two monocot species (maize and rice) and two dicot species (Arabidopsis and potato). Although the plants were grown under different conditions appropriate for each species (see Material and Methods), the starch analysis was done in parallel. We used the well-established method of chain length distribution (CLD) analysis, where the α-1,6-bonds of amylopectin are enzymatically hydrolysed, resulting in linear chains that can be separated and quantified by HPAEC-PAD (high performance anion exchange chromatography with pulsed amperometric detection). The CLD profiles had distinct species-specific features and varied between tissues (Fig. 1).

In the CLDs of leaf starch from all four species, chains with a degree of polymerisation (d.p.) of 12 were most abundant, but the profiles differed in other ways. The CLD of potato, had a prominent peak of chains of d.p. 6 and d.p. 7 (Fig. 1C), which was also visible in Arabidopsis, but less prominent in maize and rice (Fig. 1). The Arabidopsis leaf starch CLD also had a discontinuity at d.p. 18, suggestive of a subpopulation of chains of between d.p. 5 and d.p. ~20, overlapping with a population of longer chains up to d.p. 35. This CLD discontinuity was also visible for potato leaf starch but much less apparent for the monocot leaf starches. Similarly, the amylopectin structure of storage starch from the endosperms of rice and maize, and from potato tuber differed from one another (Fig. 1B–D). Potato amylopectin still had an early peak of chains at d.p. 6 and d.p. 7. This was also visible in maize, but not in rice. Otherwise, the amylopectin CLDs from the storage starches had smooth distributions up to d.p. ~35, with no discontinuity at d.p. 18. None of the three crop species had the same amylopectin within their transient and storage starches (Fig. 1B–D). These structural differences between species and tissues indicate that the biosynthetic machinery in each case is not identical.

**Generation of transgenic Arabidopsis lines expressing OsISA1 and OsISA2**

We investigated the role of the isoamylases (ISA1 and ISA2) in determining the starch structure between plants using a gene-swap experiment. We chose the rice, which had the largest difference in starch structure compared to Arabidopsis (Fig. 1). The latter is highly amenable to genetic transformation and mutants in ISA1 and ISA2 have been characterised, together with the double mutant [13,16].

We cloned the protein-coding region of OsISA1 and OsISA2 into the pB7WG2 plant Gateway-compatible expression vector [27]. OsISA2 was expressed in the Arabidopsis isa2 single mutant under the cauliflower mosaic virus (CaMV) 3SS promoter. The OsISA1 construct was transformed into the Arabidopsis isa1 single mutant and the isa1isa2 double mutants. In contrast to OsISA2, which can only form a heteromultimeric complex with OsISA1, the OsISA1
protein is reported to also form a homomultimeric complex [20]. Therefore, we hypothesized that the expression of OsISA1 might be able to complement both of the missing Arabidopsis proteins.

The three Arabidopsis mutants used for transformation all have the same reduced starch, phytoglycogen-accumulating phenotype, due to the complete loss of the heteromultimeric isoamylase activity. The altered glucan composition of these mutants makes it possible to distinguish between them and wild-type plants by simple iodine staining. Amylopectin of wild-type plants stain dark brown whereas the phytoglycogen in the mutants stains a more red-brownish colour (Fig. 2A). We isolated a minimum of ten independent lines per transformation from the T1 generation. We then screened these lines by iodine staining to search for those in which the loss of the endogenous isoamylase activity was complemented by the expression of the rice gene. Half or more of the T1 transformants stained like the wild type, suggesting complementation. We confirmed that this phenotype was stable in the T2 generation of the three most promising candidate lines per genotype (Fig. 2A). These lines were analysed further and were named as follows: isa1 mutants transformed with OsISA1, isa1-35S::OsISA1 A, B and C; isa2 mutants transformed with OsISA2, isa2-35S::OsISA2 A, B and C; isa1isa2 double mutants transformed with OsISA1, isa1isa2-35S::OsISA1 A, B and C.

Quantitative starch and phytoglycogen measurement

Iodine staining is suitable for rapid screening but is largely qualitative. Therefore we measured the starch and phytoglycogen in all of the transformed lines at the end of a 12-h photoperiod. As expected, wild-type plants had around 8 mg starch per gram plant fresh weight and contained only trace amounts of soluble glucan. In contrast, the isa1, isa2 and isa1isa2 mutants contained both starch and phytoglycogen and synthesized less glucan in total than the wild type (Fig. 2B). All three mutants had the same phenotype, as reported earlier [13,16]. The starch measurements for the three isa1-35S::OsISA1 lines confirmed the results from the iodine staining (Fig. 2A) as they contained wild-type levels of starch and no phytoglycogen. Interestingly, full complementation was also achieved in the isa1isa2-35S::OsISA1 plants despite the fact that no ISA2 protein is present (neither AtISA2 nor OsISA2). Comparable results were also recently described for these isa mutants transformed with the Maize ISA1 gene [28].

The three isa2-35S::OsISA2 lines showed a marked change in phenotype compared with the parental line, but complementation was incomplete. Although all three lines contained only starch, with little or no phytoglycogen, the starch content varied significantly between the lines (Fig. 2B). The line isa2-35S::OsISA2 B contained only half as much starch as the wild-type (comparable to the sum of starch and phytoglycogen in the isa2 parental line),

Figure 1. Amylopectin structure in different plant species and tissues. Comparison of chain length distributions (CLDs) of starch in different tissues from Arabidopsis thaliana, Oryza sativa (rice), Solanum tuberosum (potato) and Zea mays (maize). A) CLD of Arabidopsis leaf starch, harvested at the end of the light period. B) CLD of rice leaf starch (filled grey circle) and seed storage starch (open grey circle). C) CLD of potato leaf starch (filled red circle) and tuber storage starch (open red circle). D) CLD of maize leaf starch (filled blue circle) and seed storage starch (open blue circle). Note that in all cases, the CLDs differ between the tissues from the same plant species. Values are means ± SE (n = 3). doi:10.1371/journal.pone.0092174.g001
whereas isa2-35S::OsISA2 C contained almost wild-type amounts of starch.

**Rice isoamylase complex formation in Arabidopsis**

The complementation of the Arabidopsis mutants suggests that the rice proteins are functional and can substitute the loss of the endogenous proteins. To validate this further we used native PAGE with gels containing beta-limit dextrin, a good substrate to visualize isoamylase enzyme activity. The three isoamylase mutants, isa1, isa2 and isa1isa2 lacked the debranching activity found in wild-type plants (Fig. 3 and [16]). In isa1isa2-35S::OsISA1 lines two new activities were visible compared with the isa1isa2 parental line (Fig. 3B). This is consistent with the idea that two homomultimeric forms of OsISA1 with different electrophoretic mobilities are formed [29]. Interestingly, in isa1-35S::OsISA1 lines, three new enzyme activities were visible compared with the isa1 parental line. Two migrated at positions comparable to the activities observed when OsISA1 was expressed in the isa1isa2 double mutant (i.e. likely homomultimers of OsISA1) while the third ran at the same height as the endogenous Arabidopsis ISA1/ISA2 complex (Fig. 3A). This is most likely a chimeric isoamylase heteromultimer comprised of OsISA1 and AtISA2 subunits. Surprisingly, in the isa2-35S::OsISA2 lines, no additional enzyme activities were detected compared to the isa2 parental line. This was unexpected because even though there was phenotypic variability, these plants clearly differed from the isa2 mutants in respect to starch and phyto-tycogen content and more closely resembled the wild type (See Fig. 2).

**Effect of the rice isoamylases on endogenous isoamylase protein levels**

In Arabidopsis, only the heteromultimeric ISA1/ISA2 complex occurs and, in mutant plants lacking either ISA1 or ISA2, the activity is abolished (see Fig. 3). Furthermore, evidence suggests that the remaining protein is unstable in the absence of its partner. Thus, ISA1 protein is strongly reduced in amount in isa2 mutants and ISA2 protein is undetectable in isa1 (Fig. 3 D and E). Given this profound effect of one protein on the stability of its interaction partner, we tested what impact the expression of the rice isoamylases has on the levels of the endogenous Arabidopsis proteins.

The same protein extracts used for the native PAGE were analysed by SDS-PAGE and immunoblotting, probing with specific antibodies raised against AtISA1 [16] and AtISA2 [15]. We confirmed that the ISA1 protein was reduced in isa2 mutants and the absence of ISA2 protein in isa1 (Fig. 3 D and E). In isa1-
Rice Isoamylases Replacing Arabidopsis Enzymes

35S::OsISA1 lines, the Arabidopsis ISA2 protein reappeared, although not to wild-type levels (Fig. 3D). This observation is in agreement with the native PAGE (Fig. 3A), where a putative chimeric OsISA1/AtISA2 heteromultimer was detected. It is worth noting that the activity of this putative chimeric enzyme was less intense than that of the endogenous ISA1/ISA2, consistent with the lower levels of AtISA2 protein. Nevertheless, these observations strongly suggest that, in these plants, the OsISA1 protein interacts with and stabilises the Arabidopsis ISA2 protein.

In contrast, no increase in the AtISA1 protein could be detected in isa2-35S::OsISA2 compared to the isa2 parental line (Fig. 3E). It is possible that the residual AtISA1 in isa2 is available to form an active chimeric heteromultimer composed of AtISA1 and OsISA2 subunits. This would explain the observed partial complementation of the isa2 phenotype in these lines (Fig. 2B). However, our inability to detect isoamylase activity in these lines renders this explanation somewhat speculative.

Starch structure in Arabidopsis isoamylase mutants expressing rice isoamylases

In addition to the accumulation of phytoglycogen, loss of the ISA1/ISA2 isoamylase in Arabidopsis results in alterations of the CLD of the residual starch (Fig. 4A and [13,16]). We analysed starch from plants harvested at the end of the day (the same samples as used for the starch measurements; Figure 2B). CLD profiles were calculated by averaging the three biological replicates of each genotype. As previously reported, the amylopectin CLDs from the starch from all three Arabidopsis isa mutants (isa1, isa2 and isa1isa2) were the same, having more very short chains (d.p. 4 to 7) but less medium-length chains (d.p. 10 to 16) compared to the wild type (only isa1isa2 is shown). We compared the amylopectin CLDs of the starch synthesized in our Arabidopsis mutants complemented with the rice isoamylases to the wild type, to the isa1isa2 double mutant, and to the rice starches. For each transformation, the three independent lines had very similar CLD profiles (Fig. S1). Both the isa1-35S::OsISA1 and the isa2-35S::OsISA2 lines synthesized starch more similar to wild-type Arabidopsis starch than to isa1isa2 mutant (Fig. 4 B and D). Interestingly, the isa1isa2-35S::OsISA1 CLD profile was very different from the isa1isa2 mutant, and was intermediate between wild type Arabidopsis starch and rice leaf starch (Fig. 4C).

Discussion

Interchangeability of rice and Arabidopsis isoamylase subunits

In this work, we demonstrated the interchangeability of enzymes in starch metabolism between monocotyledonous and dicotyledonous plants. Arabidopsis mutants lacking the ISA1/ISA2 complex have reduced capacity to synthesise crystalline starch - instead they accumulate water-soluble phytoglycogen. Here, we show that the rice isoamylase proteins are able to rescue this phenotype. All the transgenic lines analysed contained exclusively starch (Fig. 2B), even though the respective isoamylase complex differed in each case. In isa1isa2 expressing OsISA1, we observed two complexes (Fig. 3B). In these plants OsISA1 is the sole ISA protein present in these plants, suggesting that these two are homomultimeric OsISA1 complexes. The formation of an OsISA1 complex was expected, as a homo-pentameric form in rice

Figure 3. Reconstitution of isoamylase complexes in Arabidopsis isoamylase mutants expressing OsISA1 and OsISA2. A) Soluble protein extracts of the wild type (WT), isa1, isa2, and isa1isa2 mutants, and two isa1 lines complemented with OsISA1 (isa1-35S::OsISA1 A and B), analyzed by native PAGE in beta-limit dextrin-containing gels. After electrophoresis and incubation, gels were stained with iodine to reveal bands of activity where the glucan was hydrolyzed. White arrows; the endogenous heteromultimeric Arabidopsis isoamylase (AthISA1/AthISA2). Black arrows; the homomultimeric rice isoamylase (OsISA1). B) Soluble protein extracts of controls (as in A) and two isa1isa2 lines complemented with OsISA1 (isa1isa2-35S::OsISA1 A and B). Arrows as described in A. C) Soluble protein extracts of controls (as in A) and two isa2 lines complemented with OsISA2 (isa2-35S::OsISA2 A and B). Arrow as described in A. D) AthISA2 (arrow), detected by immunoblotting (same sample order as in A). Note that AthISA2 is not detectable in isa1, isa2, isa1isa2 mutants, but is visible in the transformed lines. E) AthISA1 (arrow), detected by immunoblotting (same sample order as in C). Note that AthISA1 amount is reduced in the isa2 mutant, but does not increase in the transformed lines.
doi:10.1371/journal.pone.0092174.g003
Endosperm has been reported [20]. The observation of two complexes in our plants suggests that the OsISA1 protein can also adopt a different oligomeric state, or is post-translationally modified in a way that affects its migration in our gels (i.e. by changing surface charge or substrate affinity). Consistent with our observations, early studies in which the homomultimer was purified to homogeneity from rice endosperm also yielded an enzyme which migrated in several positions on a native gel and in two positions during isoelectric focusing [29]. Importantly, these data show that starch synthesis in Arabidopsis is not dependent on the presence of ISA2, even though this subunit is critical for the stability of the endogenous enzyme.

In the isa1 lines expressing OsISA1, we observed an additional enzymatic activity besides the two homomultimeric OsISA1 complexes (Fig. 3A). The difference compared to isa1isa2 expressing OsISA1 is the intact AtISA2 gene, so the additional activity is most probably a chimeric heteromultimeric isoamylase complex composed of OsISA1 and AtISA2 subunits. This is in agreement with the increased AtISA2 protein content in these lines, compared to the isa1 mutants, in which AtISA2 protein is not detectable (Fig. 3D; [15]). The formation of a complex between OsISA1 and AtISA2 presumably stabilizes the AtISA2 protein. This result implies that the ISA1 and ISA2 proteins are sufficiently conserved between the dicot Arabidopsis and the monocot rice to allow assembly into an enzymatically functional complex. This is consistent with the ability of the maize ISA1 to form an active chimeric complex with AtISA2 in vitro [28].

In the isa2 lines expressing OsISA2, the isa2 phenotype was rescued and the plants produced exclusively starch and no phy toglycogen. The most likely explanation for this would be that AtISA1 and OsISA2 assemble into an active enzyme in these plants, as seen for OsISA1 and AtISA2. However, unexpectedly, we were not able to detect an additional isoamylase activity compared to isa2 mutants (Fig. 3C). It is possible that the activity may be below the detection limit of our native gel assay. Although we tried increasing the protein content five-fold and prolonging the incubation time from 2 h to 12 h, no activity band was observed even under these conditions. We also did not observe a
stabilising effect of OsISA2 expression on AtISA1 protein levels (in contrast to the stabilisation of AtISA2 by overexpressing OsISA1). However, there is residual AtISA1 protein in the isa2 mutants, which was comparable in lines overexpressing OsISA2. This might mean that there is sufficient protein to partner OsISA2. It is also possible that a chimeric complex is formed which has an altered migration such that it co-migrates with another starch degrading activity and is therefore masked. Alternatively, the chimeric enzyme may not resolve well under our experimental conditions or may be very labile during extraction.

The idea that a chimeric AtISA1/OsISA2 isoamylase complex may be present at a very low abundance in our isa2-33S::OsISA2 plants would suggest that extremely low levels of a functional isoamylase complex are sufficient to facilitate starch synthesis. Although all three lines had a similar starch structure it is worth noting that the starch level was not the same as in the wild type (Fig. 2B). The fact that very low levels of isoamylase may be functional was also suggested from a study of potato plants in which either SISAI or SISAI2 were silenced. Transgenic lines were identified which showed no detectable isoamylase activity on native PAGE (as used here) but still accumulated almost exclusively starch and only tiny amounts of phytyoglycogen [17]. This study differs from most, as intermediate levels of the wild-type isoamylase activity were obtained. In contrast, when mutants are used, they generally result in a complete loss of isoamylase activity or an altered isoamylase enzyme, which is typically accompanied by a phytyoglycogen-accumulating phenotype (e.g. [6,16,18,21,22]). These results lead us to speculate that as isoamylase activity is reduced and becomes limiting, the initial phenotype may be a drop in starch production before further reduction in the enzyme results in phytyoglycogen accumulation. However, this is an aspect of the phenotype that we cannot fully explain at present.

The fact that the rice proteins were able to functionally replace the Arabidopsis proteins does not necessarily mean that the converse is true, and that the Arabidopsis proteins would be able to complement the phenotypes of the corresponding rice mutants. This may be especially the case for ISA1, because Arabidopsis ISA1 seems unable to form an active homomultimer. This may be important as it is specifically the ISA1 homomultimer that is thought to facilitate starch synthesis in the rice endosperm [25]. Mutants lacking ISA2 have only the homomultimer and produce near-normal starch. In contrast, plants overexpressing ISA2 possess the heteromultimer, but lack the ISA1 homomultimer. These plants produce only half as much starch as the wild type and the starch is aberrant in structure, suggesting that the specificity of the heteromultimer is not ideally matched to the glucan produced by the other starch biosynthetic enzymes of the endosperm [25].

A role for isoamylase in determining species-specific starch structure?

The replacement of Arabidopsis ISA1 and ISA2 with the rice orthologs enabled us to study the effect of distinct isoamylase complexes on the fine structure of starch. Given that the isoamylases of different subunit compositions have been shown to have distinct functionalities in cereal endosperm, it is reasonable to hypothesise that the differences in amylopectin between species may be partly dependent on the isoamylases they contain. All three transgenic lines had starch structures that were similar, but not identical, to Arabidopsis wild-type starch (Fig. 4). The line possessing only the rice homomultimers had the most divergent amylopectin structure. The increased abundance of short chains between d.p. 3 and d.p. 8 in the isa1isa2 mutant CLD (compared to wild-type Arabidopsis amylopectin) was lost. Instead an increased abundance of longer chains between d.p. 6 and d.p. 14 was seen. This structure is somewhat intermediate between Arabidopsis and rice transient starch. In the isa1-33S::OsISA1 lines the amylopectin CLD was much more similar to wild-type Arabidopsis amylopectin, but the same tendencies towards increased number of chains between d.p. 6 and d.p. 14 was seen. In this line, the homomultimeric OMSA1 is accompanied by a chimeric Arabidopsis/rice isoamylase complex - AtISA1/OsISA2. This chimeric enzyme presumably functions in a similar way to the endogenous Arabidopsis enzyme. Plants putatively containing the other chimeric Arabidopsis/rice isoamylase complex - AtISA1/OsISA2 also had starch with modest increases in the numbers of chains between d.p. 6 and d.p. 14. It has been proposed that the catalytically inactive subunit ISA2 has a regulatory function, provides substrate specificity, or increased stability to the catalytic ISA1 subunit [5]. These results suggest that the replacement of the AtISA2 with OsISA2 causes a change in substrate specificity, but this conclusion needs to be treated with caution since no activity was directly measured. Further analysis, such as X-ray scattering, scanning electron microscopy and differential scanning calorimetry could be used to study in more depth the structure and properties of starch produced in plants with different chimeric isoamylase complexes.

Conclusion

Our results are consistent with the findings of other studies. The wheat ISA1 (TaISA1) protein was able to revert the phytyoglycogen-accumulating phenotype of the rice sugary-1 mutant (lacking OISA1) back to a starch-accumulating phenotype [30]. However, rice and wheat are much more closely related to each other than rice and Arabidopsis used, and wheat may also have the two types of isoamylase complex (i.e. homo- and heteromultimers). Nevertheless, after complementation with TaISA1, the amylopectin CLD was similar – but not identical – to the wild type rice amylopectin. Recently, it was shown that the maize ISA1 (ZmISA1) protein expressed in the Arabidopsis isa1 and isa1isa2 mutants were able to restore exclusive starch synthesis in Arabidopsis, similar to our observations. Interestingly, the authors observed a greater variability of the starch content, with some transgenic lines producing even more starch than the wild type, which was not the case in our plants. However, this raises the possibility that the isoamylase may somehow determines not only the starch structure but also the amount produced. Interestingly, the ZmISA1 expressing plants produced starch with the same amylopectin CLD as the Arabidopsis wild type [28]. This is in contrast to our results, but might be explained by the fact that the amylopectin CLDs of maize and Arabidopsis are more similar to each other than Arabidopsis and rice (Fig. 1). It is tempting to conclude from all of these studies that isoamylase can contribute to species-specific amylopectin structure. However, it is important to note that the differences in structure that we measure are relatively small. Therefore, it seems likely that species-specific differences in the starch synthases and branching enzymes might also contribute.

Furthermore, it is important to emphasise that differences in expression of these enzymes between tissues or between species may have an equally large impact on the final structure of amylopectin. This was strikingly illustrated recently through RNA-seq analysis of the myrmecophytic tropical tree Cecropia peltata, where differential expression of the starch-biosynthetic apparatus results in amylopectin production in leaves and
glycogen biosynthesis in food bodies produced to feed mutualistic ants [31].

Materials and Methods

Plants and growth conditions

*Arabidopsis thaliana* plants were grown in Percival AR95 climate chambers at constant 20°C, 60% relative humidity, 12-h photoperiod and a light intensity of 150 μmol photons m⁻² sec⁻¹. Seeds were sown on soil and seedlings transferred to individual pots two weeks after germination. Mature plants were harvested three weeks later, weighed, frozen in liquid N₂ and kept at −80°C until use. Single *isa* mutants used were described in earlier studies: *isa1*, *isa1-1*, SALK_042704 [16]; *isa2*, *isa2-1* (initially called *dbi1-1* [32] and the double mutant *isa1isa2*, *isa1-1/isa2-1* [16].

*Potato* (*Solanum tuberosum cv. Agata*) tubers were obtained from a local market and used for storage starch purification. Potato (*S. tuberosum*) plants were grown in a chamber (Ka¨lte 3000 AG, Landquart, Switzerland) at constant 22°C, 10% [w/v] KI).

Materials and Methods

Plants and growth conditions

*Arabidopsis thaliana* plants were grown in Percival AR95 climate chambers at constant 20°C, 60% relative humidity, 12-h photoperiod and a light intensity of 150 μmol photons m⁻² sec⁻¹. Seeds were sown on soil and seedlings transferred to individual pots two weeks after germination. Mature plants were harvested three weeks later, weighed, frozen in liquid N₂ and kept at −80°C until use. Single *isa* mutants used were described in earlier studies: *isa1*, *isa1-1*, SALK_042704 [16]; *isa2*, *isa2-1* (initially called *dbi1-1* [32] and the double mutant *isa1isa2*, *isa1-1/isa2-1* [16].

*Potato* (*Solanum tuberosum cv. Agata*) tubers were obtained from a local market and used for storage starch purification. Potato (*S. tuberosum*) plants were grown in a chamber (Ka¨lte 3000 AG, Landquart, Switzerland) at constant 22°C, 10% [w/v] KI).

Rice (*Oryza sativa cv. Taipei 309*) plants were grown in greenhouse at 23°C, 70% relative humidity at night and 26°C, 80% relative humidity at day with a minimum 14-h photoperiod and minimum light intensity of 250 μmol photons m⁻² sec⁻¹.

Leaves for the extraction of transient starch were harvested from four-week-old plants. Mature, dry seeds from these plants were used for storage starch purification. Potato (*S. tuberosum*) leaves for the extraction of transient starch were harvested from four-week-old plants. Purchased seeds were used directly for storage starch purification.

Extract and measurement of glucose polymers

The extraction procedure was the same for all plant species and tissues and was performed using perchloric acid as described previously [16]. Glucose polymers (starch and phytoglycogen) were quantified by enzymatic digestion with amyloglucosidase (*Aspergillus niger*; Roche) and α-amylase (*pig pancreas; Roche*), and released glucose was measured spectrophotometrically [33].

Structural analysis of starch and phytoglycogen

Starch and phytoglycogen in the insoluble and soluble fractions from the perchloric acid-extracted plant material, respectively, were used for glucan structural analysis. Volumes containing 100 μg of starch or phytoglycogen were used from each of four individually extracted plants. Samples were boiled for 10 min prior to enzymatic digestion. Debranching reactions with *Pseudomonas amylolectrina* isomylase (Sigma-Aldrich, Buchs, Switzerland) and *Klebsiella planticola* pullulanase (Megazyme) were carried out for 2 h at 37°C in 10 mM Na-acetate, pH 4.8. The resulting linear glucans were analysed by HPAEC-PAD as described previously [8].

Construction of *OsISA1* and *OsISA2* overexpression vectors and plant transformation

*OsISA1* and *OsISA2* coding sequences were amplified by PCR using attB-site containing primers (Table S1). The templates were pGEM-T Easy vectors containing *OsISA1* and *OsISA2* cDNA sequences [25]. The PCR fragments were cloned into pDONR 221 via the BP reaction of the Gateway recombination cloning technology (Invitrogen, LifeBioScience GmbH, Lucerne, Switzerland).

After sequence confirmation, inserts were cloned into the plant vector pB7WG2 [27] using the Gateway LR reaction. Stable *Arabidopsis thaliana* transgenic lines were generated through *Agrobacterium tumefaciens*-mediated transformation using the floral dip method [34]. Independent transformants were selected on soil by spraying two-week-old plants with 0.1% (v/v) BASTA herbicide.

Supporting Information

Figure S1. Amylopectin structure of starch produced in independent *isa1-35S::OsISA1*, *isa2-35S::OsISA2* and *isa1isa2-35S::OsISA1* lines. Comparison of the three independent transformants lines of *isa1-35S::OsISA1*, *isa1isa2-35S::OsISA1* and *isa2-35S::OsISA2* illustrating the uniformity in starch structure produced. Raw data used to calculate the mean normalized CLD (± SE, n = 3) was also used to calculate the difference plots in Fig. 5 B to D. A) CLDs of the lines *isa1-35S::OsISA1 A* (open circle), *isa1-35S::OsISA1 B* (open square) and *isa1-35S::OsISA1 C* (open triangle). B) CLDs of the lines *isa1isa2-35S::OsISA1 A* (open circle), *isa1isa2-35S::OsISA1 B* (open square) and *isa1isa2-35S::OsISA1 C* (open triangle). C) CLDs of the lines *isa2-35S::OsISA2 A* (open circle), *isa2-35S::OsISA2 B* (open square) and *isa2-35S::OsISA2 C* (open triangle). (TIF)

Table S1. Primers used for Gateway cloning of *OsISA1* and *OsISA2*. (DOCX)

Acknowledgments

We thank our trainee technicians Markus Reichlin and Raphaela Göchwind and our under-graduate student Stefan Oberlin for experimental help. Furthermore, we thank Yasunori Nakamura for the full length clones of *OsISA1* and *OsISA2*.

Author Contributions

Conceived and designed the experiments: SS. Performed the experiments: SS. Analyzed the data: SS. Contributed reagents/materials/analysis tools: SS SCZ. Wrote the paper: SS SCZ.
References

1. Buleon A, Colonna P, Planchot V, Ball S (1998) Starch granules: structure and biosynthesis. Int J Biol Macromol 23: 83–112.

2. Zeeman SC, Smith SM, Smith AM (2007) The diurnal metabolism of leaf starch. Biochem J 401: 13–28.

3. Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, et al. (2006) Function and characterization of starch synthase I using mutants in rice. Plant Physiol 140: 1070–1084.

4. Jeon JS, Ryo N, Hahn TR, Walia H, Nakamura Y (2010) Starch biosynthesis in cereal endosperm. Plant Physiol Biochem 48: 383–392.

5. Streb S, Zeeman SC (2012) Starch Metabolism in Arabidopsis. The Arabidopsis Book; e0180.

6. Nakamura Y, Kubo A, Shimamura T, Matsuda T, Harada K, et al. (1997) Correlation between activities of starch debranching enzyme and alpha-polygalacturan structure in endosperms of sugary-1 mutants in rice. Plant Journal 12: 143–153.

7. Myers AM, Morell MK, James MG, Ball SG (2000) Recent progress toward understanding biosynthesis of the amylopectin crystal. Plant Physiol 122: 989–997.

8. Streb S, Delatte T, Unhang M, Ecke S, Schorderet M, et al. (2008) Starch granule biosynthesis in Arabidopsis is abolished by removal of all debranching enzymes but restored by the subsequent removal of an endosomylase. Plant Cell 20: 3440–3456.

9. Hussain H, Manu A, Scale R, Zeeman S, Hinchliffe E, et al. (2005) Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. Plant Cell 15: 2013–2024.

10. Roughan S, Nakamura Y, Li Z, Clarke B, Ecke S, et al. (2003) The sugary-type isoamylase gene from rice and Aegilops tauschii: characterization and comparison with maize and Arabidopsis. Genome 46: 496–506.

11. Streb S, Ecke S, Zeeman SC (2012) The simultaneous abolition of three starch hydrolases blocks transient starch breakdown in Arabidopsis. J Biol Chem 287: 41745–41756.

12. Delatte T, Unhang M, Trevisan M, Ecke S, Thorneycroft D, et al. (2006) Evidence for distinct mechanisms of starch granule breakdown in plants. J Biol Chem 281: 12650–12659.

13. Watcheblod F, Dong Y, Dumez S, Delvalle D, Planchot V, et al. (2005) Mutants of Arabidopsis lacking a chloroplastic isoamylase accumulate phytoglycogen and an abnormal form of amylopectin. Plant Physiol 138: 104–115.

14. MacGregor EA (1993) Relationships between Structure and Activity in the Alpha-Amylase Family of Starch-Metabolizing Enzymes. Starch-Stärke 45: 232–237.

15. Sundberg M, Pfister B, Fulton D, Bischof S, Delatte T, et al. (2013) The heteromultimeric debranching enzyme involved in starch synthesis in Arabidopsis requires both Isoamylase1 and Isoamylas2 subunits for complex stability and activity. PLoS ONE, in press.

16. Delatte T, Trevisan M, Parker ML, Zeeman SC (2005) Arabidopsis mutants Atisa1 and Atisa2 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.

17. Bustos R, Fahy B, Hyton CM, Scale R, Nebane NM, et al. (2004) Starch granule initiation is controlled by a heteromultimeric isoamylase in potato tubers. Proc Natl Acad Sci USA 101: 2215–2220.

18. Daviville D, Collconi C, Mouille G, Morell MK, d’Hulst C, et al. (2001) Biochemical characterization of wild-type and mutant isoamyloses of Chlamydomonas reinhardtii supports a function of the multimeric enzyme organization in amylopectin maturation. Plant Physiol 125: 1725–1731.

19. Kubo A, Collconi C, Dinges JR, Lin Q, Lappe RR, et al. (2010) Functions of heteromeric and homomeric isoamylase-type starch-debranching enzymes in developing maize endosperm. Plant Physiol 153: 956–969.

20. Usunmi 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.

21. James MG, Robertson DS, Myers AM (1993) Characterization of the maize gene sugary1, a determinant of starch composition in kernels. Plant Cell 7: 417–429.

22. Burton RA, Jenner H, Carrangis I, Fahy B, Finch PB, et al. (2002) Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. Plant J 31: 97–112.

23. Mollie G, Maddelein ML, Libesart N, Talaga P, Decq A, et al. (1996) Preamylopectin Processing: A Mandatory Step for Starch Biosynthesis in Plants. Plant Cell 8: 1353–1366.

24. Posewitz MG, Smolinski SL, Kanakagiri S, Melis A, Seibert M, et al. (2004) Hydrogen photoproduction is attenuated by disruption of an isoamylase gene in Chlamydomonas reinhardtii. Plant Cell 16: 2151–2163.

25. Utsumi Y, Usunmi C, Sawada T, Fujita N, Nakamura Y (2011) Functional diversity of isoamylase oligomers: the BAI homo-oligomer is essential for amylopectin biosynthesis in rice endosperm. Plant Physiol 156: 61–77.

26. Morell MK, Samuel MS, O’Shea MG (1998) Analysis of starch structure using fluorophore-assisted carbohydrate electrophoresis. Electrophoresis 19: 2603–2611.

27. Karimi M, Inze D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7: 193–195.

28. Facon M, Lin Q, Azaz AM, Hennex-Bierwagen TA, Myers AM, et al. (2013) Distinct functional properties of isoamylase-type starch debranching enzymes in monocot and dicot leaves. Plant Physiol 163: 1363–1373.

29. Fujita N, Kubo A, Francisco PB Jr., Nakakita M, Harada K, et al. (1999) Purification, characterization, and cDNA structure of isoamylase from developing endosperm of rice. Planta 208: 283–293.

30. Kubo A, Rahman S, Usunmi Y, Li Z, Mukai Y, et al. (2005) Complementation of sugary-1 phenotype in rice endosperm with the wheat isoamylase1 gene supports a direct role for isoamylase1 in amylopectin biosynthesis. Plant Physiol 137: 43–56.

31. Bischof S, Unhang M, Ecke S, Streb S, Qi W, et al. (2013) Cecropia peltata Accumulates Starch or Soluble Glycogen by Differentially Regulating Starch Biosynthetic Genes. Plant Cell 25: 1400–1413.

32. Zeeman SC, Umemoto T, Lau WL, Au-Yeung P, Martin C, et al. (1998) A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phyto glycogen. Plant Cell 10: 1699–1712.

33. Hostetler C, Kelling K, Santelia D, Streb S, Kotting O, et al. (2011) Analysis of starch metabolism in chloroplasts. Methods Mol Biol 775: 387–410.

34. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743.

Rice Isoamyloses Replacing Arabidopsis Enzymes