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Abstract: In rice, maize and barley, the lack of isoamylase 1 activity materially affects the composition of endosperm starch. Here, the effect of this deficiency in durum wheat has been characterized, using transgenic lines in which Isa1 was knocked down via RNAi. Transcriptional profiling confirmed the down-regulation of Isa1 and revealed a pleiotropic effect on the level of transcription of genes encoding other isoamylases and pullulanase. The polysaccharide content of the transgenic endosperms was altered from that of the wild type in a number of ways, including a marked reduction in the content of starch and an enhancement to that of both phytoglycogen and β-glucan. Some alterations were also induced in the distribution of amylopectin chain length and amylopectin fine structure. The amylopectin present in the transgenic endosperms was more readily hydrolyzable by weak hydrochloric acid, a treatment which disrupted its semi-crystalline structure. The conclusion was that in durum wheat, isoamylase 1 is important for both the synthesis of amylopectin and for determining its internal structure.

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Dear Editor,

we are submitting a paper entitled " **The effect of silencing the genes encoding Isoamylase 1 on the starch composition of the durum wheat grain"** by Francesco Sestili et al. to be considered for a possible publication on Plant Science. The manuscript reports, for the first time, a functional study of Isoamylase gene in wheat, providing new insights on the role of this enzyme in starch biosynthesis.

Thank you for your kind attention

Yours sincerely,
Domenico Lafiandra

Viterbo, April 26, 2016

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Highlights

- The silencing of Isa1 affects the gene expression of the other debranching enzymes
- RNAi ISA1 plants show a reduction in the content of starch
- RNAi ISA1 plants result in an increase of phytoglycogen and β-glucans
- The distribution of amylopectin chain length is altered in RNAi ISA1 plants

The effect of silencing the genes encoding Isoamylase 1 on the starch composition of the durum wheat grain

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Abstract

In rice, maize and barley, the lack of isoamylase 1 activity materially affects the composition of endosperm starch. Here, the effect of this deficiency in durum wheat has been characterized, using transgenic lines in which Isa1 was knocked down via RNAi. Transcriptional profiling confirmed the down-regulation of Isa1 and revealed a pleiotropic effect on the level of transcription of genes encoding other isoamylases and pullulanase. The polysaccharide content of the transgenic endosperms was altered from that of the wild type in a number of ways, including a marked reduction in the content of starch and an enhancement to that of both phytoglycogen and β-glucan. Some alterations were also induced in the distribution of amylopectin chain length and amylopectin fine structure. The amylopectin present in the transgenic endosperms was more readily hydrolyzable by weak hydrochloric acid, a treatment which disrupted its semi-crystalline structure. The conclusion was that in durum wheat, isoamylase 1 is important for both the synthesis of amylopectin and for determining its internal structure.

Keywords: amylopectin, isoamylases, RNAi, durum wheat

Abbreviations

CSLF6, Cellulose synthase-like F6; DP, Degree polymerization; HKW, Hundred grain weight; HPAEC-PAD, High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection; ISA1, Isoamylase 1; ISA2,Iisoamylase 2; ISA3, Isoamylase 1; PUL, Pullunanase; qRT-PCR, Quantitative real time PCR; RNAi, RNA interference; SEM, Scanning electron microscope; SUSY, Sucrose synthase;

1. Introduction

Most of the dry matter present in the cereal grain takes the form of carbohydrates, represented by sugars (mono- and disaccharides), oligosaccharides, starch and non-starch polysaccharides [1,2]. Typically, the combined contribution of the monosaccharides (glucose and fructose), disaccharides (sucrose and maltose) and oligosaccharides (raffinose and fructo-oligosaccharides) is of the order of 4%, that of the non-starch polysaccharides (cellulose, arabinoxylan, β-glucan and fructans) 10%, and that of starch is 65-75%. The content of phytoglycogen (a highly branched water-soluble glucan polysaccharide, structurally similar to animal glycogen [3] is typically below 1% [4]. Storage starch, which is packaged into water-insoluble granules comprising semi-crystalline growth rings alternating with amorphous regions, is a mixture of the polysaccharides amylopectin and amylose; their synthesis is mediated by ADP-glucose pyrophospholylases, starch-synthases, starch-branching enzymes and starch-debranching enzymes [5,6]. The latter have been classified into pullulanases (or limit dextrinases) and isoamylases; both are involved in the cleavage of α-1,6 linkages within branched polysaccharides, but show contrasting substrate preferences [7-9]: while pullulanases hydrolyze the α-1,6-glucosidic bonds in amylopectin and limit dextrins, and require the presence of two α-1,4 bonds adjacent to cleavage site, the isoamylases act on both amylopectin and glycogen, and cleave α-1,6 linkages where at least three α-1,4-linkages lie adjacent to the ramification [10]. Three groups of isoamylases have been recognized, designated ISA1, ISA2 and ISA3 [11,12].
The absence of ISA1 activity in potato tubers and the grain of maize, rice and barley produces the so-called *sugary* phenotype, in which starch granule morphology is radically altered, at the same as the phytoglycogen content being substantially increased [4,9,13-17]. ISA1 complexes with ISA2, forming a structure widely considered to be important for the starch granule initiation [7,14,16-19]. Utsumi et al. [20] demonstrated that in rice, endosperms lacking the ISA1 homo-oligomer are both deficient with respect to starch and have an overabundance of water-soluble malto-dextrins. According to both Kubo et al. [4] and Utsumi et al. [20], the absence of ISA2 is not associated with an increase in phytoglycogen content, but others have maintained that both ISA1 and ISA2 activity is required to produce normal starch [7,14,16,21]. In wheat the genes encoding ISA1 have been successfully isolated [8,22], but as yet neither loss-of-function mutants nor transgenically derived *Isa1* knockouts have been identified, so the effect if ISA1 absence on the wheat endosperm remains unknown. Here, the *RNA interference* (RNAi) method has been applied to durum (tetraploid) wheat, allowing for a first analysis of the phenotypic consequences of ISA1 deficiency in wheat.

2. Material and Methods

2.1 Plant culture

All plants were vernalized by holding them at 4°C for three weeks, after which they were transferred to a chamber delivering a 16 h photoperiod (intensity 300 µE m^{-2} s^{-1}), with a temperature regime of 28°C/16°C.

2.2 Biolistic bombardment of immature embryos

The sequence used for constructing the RNAi transgene was extracted from a preparation of total RNA obtained from immature (21 days post anthesis) grains of durum wheat cv. Svevo, following the protocol described by Sestili et al. [23]. The *Isa1* sequence from base 584 to 1071 (GenBank accession No. AF438329) was PCR-amplified using as primers 5'-CGACGGCACCTTTGCTCCTC/5'-TGGCATCACGCCCACAGTTT in a 50 µL reaction containing using 1 µL cDNA, 25 µL GoTaq®Hot Start Color-less Master Mix (Promega, Madison, WI, USA) and 0.5 µM of each primer. The reaction was initially denatured (95°C/3 min), then subjected to 30 cycles of 95°C/30 s, 61°C/30 s and 72°C/30 s and given a 72°C/10 min final extension step. The resulting amplicon was introduced in both the sense and antisense direction into pRDPT [24], using, respectively, the *SalI*/*KpnI* and *XbaI*/*PstI* restriction sites, to produce the construct pRDPT-ISA1(RNAi) (Fig. S1). An endosperm-specific promoter [25-26] was chosen to minimize any perturbation of sugar metabolism in the leaf of the transformants. The final construct was co-bombarded with the plasmid pAHC20, which carries the bar gene [27] in a 3:1 molar ratio. The bombardment target was immature cv. Svevo embryos harvested at 15 days post anthesis. The bombardment was effected using a Model PDS-1000/He Biolistic particle delivery system (Bio-Rad, Hercules, CA, USA) as described by Okubara et al. [28]. The presence of the RNAi construct and the bar gene in bialaphos resistant regenerants and their progeny was checked by amplifying genomic DNA extracted from leaf material, using as primer pairs PromDx5Fw/R and BarFw/R [25].

2.3 Quantitative real time PCR (qRT-PCR)

Total RNA, extracted from immature grains harvested 21 days post anthesis from T_4 transgenic and non-transformed cv. Svevo plants and processed as described above, was used as the template for synthesizing single stranded cDNA, used as template for qRT-PCRs. The reactions were performed in a CFX 96 Real-Time PCR Detection System device (Bio-Rad Hercules, CA, USA), using SsoAdvUniver SYBR GRN SMX (Bio-Rad Hercules, CA, USA) and involved three technical replicates per biological sample and three independent plants per transgenic line. The reactions were carried out in a volume of 15 µl using the following protocol: 94°C for 30 s and 40 cycles at 94°C

for 5 s, 60°C for 30 s and melt curve 65–95°C with 0.5°C increment 5 s/step. The quantification analysis were performed as described in Sestili et al. [25].

The primer pairs used for the detection of *Isa1*, *Isa2*, *Isa3* and *Pul* transcripts have been reported by Kang et al. [29]; the pair used for the gene encoding sucrose synthase (SUSY) (GenBank accession KJ769004.1) was 5'-GTGTGTCCGGCTACCACAT/5'-AGCTTCCAGGTGTACTTCTCCTC; those for *CELLULOSE SYNTHASE-LIKE F6* (CSLF6) and the reference sequence Ta2526 were taken from Nemeth et al. [30].

2.3 Measurement of total starch, amylose, glucose, phytoglycogen and β-glucan

The total starch content of grains produced by T_4 transgenic lines and non-transformed controls was obtained using a Total Starch Assay kit (Megazyme Pty Ltd., Wicklow, Ireland), following the manufacturer's protocol. Amylose contents were obtained from purified starch, following Sestili et al. [23]. Free glucose and phytoglycogen contents were determined from a 100 mg sample of wholemeal flour, following Hamada et al. [31]. The β-glucan content was measured from a 500 mg sample of wholemeal flour using a β-glucan assay kit (Megazyme Pty Ltd., Wicklow, Ireland) as directed. A minimum of three replicates per transgenic line was included.

2.4 Starch granule morphology, distribution and crystallinity

The morphology of the starch granule was assessed by scanning electron microscopy (SEM), as described elsewhere [23]. Granule size was estimated from 10 pictures at two different magnifications (500x and 2500x) per line. The SEM-derived images were captured as described by Sparla et al. [32] and the granules' major axes were measured using ImageJ software (http://imagej.net/). Measurements were obtained from a mean of ~300 granules per line, allowing them to be classified into A- and B-types, based on a threshold major axis length of 8 μm. The extent of crystallinity of the starch was determined from X-ray powder diffraction patterns, as described by Sparla et al. [32].

2.5 Acid hydrolysis of starch granules

A 50 mg sample of starch granules, isolated as described above, was hydrolyzed by the addition of 3 mL 2.2 M HCl, and holding at 38°C for 12 h with continuous shaking [33]. Subsequently, 15 mL cold distilled water was added and the reaction centrifuged (4,000 g, 10 min). The supernatant was discarded and the air-dried pellet was subjected to SEM analysis.

2.6 Amylopectin chain length distribution

A 5 mg aliquot of starch, obtained as above, was solubilized in 100 µL 0.5 M NaOH, neutralized by titration with 1 M HCl, diluted to 5 mg/mL by the addition of 10 mM sodium acetate (NaOAc) and adjusted to pH 4. The starch was de-branched by incubating with 1.0 U isoamylase (E-ISAMY, Megazyme Pty Ltd., Wicklow, Ireland) and 0.9 U pullulanase M2 (E-PULBL, Megazyme Pty Ltd., Wicklow, Ireland) for 4 h at 37°C with occasional mixing. Higher concentrations of enzymes and/or longer incubation times were tested to ensure that full debranching had been achieved. The reaction was then neutralized by adding 1 M Tris-HCl (pH 8.5) and the reaction stopped by boiling for 5 min. After centrifugation (5000 g, 1 min), the supernatant was diluted to 1 mg/mL and a 10 µL aliquot injected onto a CarboPac PA-100 4 x 250 mm column, using a Dionex ICS 3000 system equipped with an autosampler (Dionex, Thermo Fisher Scientific, Amsterdam, Netherlands). Amylopectin chain length distribution was determined by High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection (HPAEC-PAD). The sample was eluted by applying a linear 0-150 mM NaOAc gradient, dissolved in 50 mM NaOH, over 10 min, followed by a convex gradient of 150-350 mM NaOAc over the subsequent 120 min, by 1 M NaOAc for 5 min and finally by 0 mM NaOAc for 10 min. A 10 µg/mL sample of debranched oyster glycogen (Sigma-Aldrich, St. Louis, USA) was used as a control. After baseline subtraction, peak area was normalized to the total peak area of the injected sample.

3. Results

3.1 Production of RNAi transgenic lines

Altogether 1,000 immature cv. Svevo embryos were co-transformed with pRDPT-ISA1(RNAi) and pAHC20. The PCR assay confirmed the presence of both the RNAi construct and the bar gene in the leaf of the five T_0 transgenic plants recovered (data not shown). Three of these five plants were self sterile, but the other two (MJ59-5b and -44) were fertile and were advanced to the T_4 generation. Plant morphology was unaffected by the presence of the transgene, and the hundred grain weight (HKW) was unaltered (Table 1).

3.2 Molecular characterization of ISA1-RNAi transgenic lines

The transcription of *Isa1* genes was explored using qRT-PCR carried out on a template of cDNA prepared from immature T_3 grains harvested 21 days post anthesis. In both MJ59-5b and -44, the abundance of *Isa1* transcript was strongly reduced compared to that found in the wild type grain (Fig. 1A). When qRT-PCR was applied to assay for the transcription of *Isa2*, *Isa3* and *Pul*, the presence of the transgene was revealed to have also affected both *Isa3* and *Pul* transcription; while the abundance of the latter's transcript was reduced by up to 66%, that of the former was enhanced

by between 2.7 and 3.3 fold (Fig. 1). Meanwhile, the transcriptional behavior of *Isa2* was unaffected. The equivalent assays directed at *CSLF6* and *SUSY* showed that the transcription of the former (the gene product of which is important for β-glucan synthesis) was unaffected by the presence of the RNAi construct, while that of the latter (important for glucose and fructose allocation) was strongly reduced (Fig. 1B).

3.3 The effect of Isa1 suppression on starch properties

The total starch content was reduced by 7% (a statistically significant effect) in both MJ59-5b and -44 (Table 1), but the down-regulation of *Isa1* had no significant effect on the amylose/amylopectin ratio (Table 1). In both transgenic lines, the grains contained a higher level of free glucose (4.6 fold), phytoglycogen (1.8 fold) and β-glucan (1.3 fold) than did the wild type grains (Table 1). Based on SEM imaging, no drastic differences were seen between the starch granules present in the grains of either transgenic and the wild type lines, although the shape of A-type granules appeared less regular in the former (Fig. 2). In the transgenic grains, ellipsoidal granules occurred less frequently, and the granules exhibited a few small protuberances. A quantitative analysis of the A- and B-type granules produced a bimodal distribution in all three lines (Fig. 3). Applying the 8 μm threshold to distinguish between the A- and B-type granules revealed a slightly greater overall abundance of B-type granules (a mean of 41% A- and 59% B-types across the three lines). Although the A-type granules appeared to be somewhat larger (by 5-10%) in the transgenic grains (T_4 generation), this difference was not statistically verifiable. The mean major axis lengths were 17.5 μm (A-type granules) and 4.3 μm (B-type granules) (Tables 2 and S1).

3.4 Starch granule structure and crystallinity

When the starch granules were acid-hydrolyzed and then subjected to SEM, two major differences were revealed between the transgenic and wild type grains. In the latter, the characteristic alternation of crystalline and amorphous lamellae was evident; in contrast, it was impossible to distinguish the lamellae in either of the transgenic lines (Fig. 4). In addition, several granules in the transgenic grains were partially or completely dissolved by the hydrolysis treatment, indicating a greater accessibility to the acid solution and thus a looser degree of packing than in the wild type. Based on X-ray powder diffraction, the crystallinity of all three sources of starch was estimated to be around 28%. The type of crystallinity was also homogeneous (Figs 5 and S3). A-type crystal packing predominated (around 68%) with a minor contribution of V-type packing (Table 3). There was no evidence for any B-type crystallinity.

3.5 Distribution of amylopectin chain length

The HPAEC-PAD oligosaccharide analysis revealed only minor alterations to the amylopectin chain length profiles, although the difference between the transgenic and the wild type profiles was highly reproducible between the T_3 and the T_4 material (Figs 6 and S4). The polyglucan fraction in both transgenic lines was enriched with respect to intermediate degree polymerization chains (10-40 DP) and impoverished with respect to both very short (<10 DP) and long chains (>40 DP).

4. Discussion

The role of *Isa1* has been widely investigated in rice, maize and barley, but as yet no equivalent characterization has been made in wheat, largely because it is less straightforward to produce *null* mutants in a polyploid species than it is in a diploid. Here, RNAi technology was used to simultaneously silence both durum wheat homeologs of *Isa1*, allowing for an analysis of the role of ISA1 on starch synthesis and the determination of its composition. The absence of *Isa1* transcription was shown to pleiotropically influence the transcript abundance of the genes encoding the starch debranching enzymes ISA3 and PUL: *Pul* was drastically down-regulated, while *Isa3* was up-regulated, thereby compensating for the deficiency in *Isa1* transcript. Yun et al. [34] have shown that ISA1 and ISA3 are not functionally redundant: in rice, the over-expression of *Isa3* in an *Isa1* loss-of-function mutant did not rescue the phenotype, leaving the endosperms with a high content of water-soluble phytoglycogen. A major drastic reduction in PUL activity has been noted in both maize and rice *Isa1* mutants [13, 15, 18], but this effect is not replicated in barley [16]. Here, the level of *Isa2* transcription was the same in both the *Isa1* knockdown lines and the wild type, matching the outcome in rice [35].

The down-regulation of *Isa1* had a pronounced effect on the carbohydrate composition of the durum wheat endosperm. Although the transformants' amylose content was similar to that in the cv. Svevo endosperm - which is also the case for potato and maize *Isa1* mutants [4, 17] - their total starch content was reduced and their content of free glucose and phytoglycogen was increased. An induced increase in the free glucose content has similarly been observed in the rice *sugary-1* mutant endosperm [31], although the extent of the increase was more modest than in durum wheat (about 2 fold vs 4.5 fold). The phytoglycogen content of the wheat transformant endosperms was double that of the non-transformed cv. Svevo endosperms, an increase which matches that seen in equivalent mutants/transgenic lines of barley, maize, rice, potato, *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* [7, 13, 14, 16, 17, 19, 31, 36, 37]. In *C. reinhardtii Isa1* mutants, starch is completely replaced by phytoglycogen [37], whereas in the other species, starch and phytoglycogen coexist. Although the observed marked increase in free glucose raised the possibility of a boost to the

synthesis of β-glucan, at the transcriptional level, at least, no significant variation was seen for any of the main genes involved. Nevertheless, the endosperms of the *Isa1* knockdown transgenics contained significantly more (28-34%) β-glucan than did the wild type ones, perhaps reflecting cross-talk between the starch and β-glucan synthesis pathways [38].

SUSY is an important enzyme involved in the regulation of sink growth through the provision of substrate for respiration and for starch synthesis in storage sinks [39]. Its activity has also been correlated with the synthesis of cellulose in roots suffering from hypoxia [40]. The abundance of *SUSY* transcript was significantly lower in the *Isa1* knockdown transgenics than in cv. Svevo, reflecting most probably a negative feedback mechanism driven by an excess of free glucose. Similarly, Zhou et al. [41] have shown that both SUSY activity and *SUSY* transcription are enhanced by the provision of sucrose, whereas they are markedly reduced by that of glucose or fructose. Although the transgenics' starch granules appeared less regular than those in the wild type, there was no evidence for any change in their size distribution. Similarly, in rice, *Isa1* mutants produce starch granules as large as those produced by the wild type, but with less regular shape [35]. In both potato and barley *sugary-1* mutants, compound starch granules have been observed [16, 17].

When X-ray diffraction was used to detect differences in the structure of the starch in the wild type's and *Isa1* knockdown transgenics' endosperms, neither the degree of crystallinity nor the diffraction pattern was altered by the presence of the transgene. In contrast, rice *Isa1* mutants produce less crystalline starch in their grain than do the wild type plant [42]. The acidic hydrolysis treatment of the starch granules revealed that the amylopectin's internal structure was less compact in the transgenic lines than in cv. Svevo. The indications are therefore that, in durum wheat, ISA1 contributes to starch, and in particular to amylopectin, synthesis, and is important for assuring the correct packaging of the amylopectin within the starch granule. There were some notable differences between the two transgenic lines and the non-transformed control with respect to the distribution of amylopectin chain length. The loss of ISA1 activity was associated with a reduced representation of both the smaller (DP <10) and larger (DP>39) chains, balanced by a rise in that of the intermediate length (DP: 13-40) ones. In the equivalent rice mutant, a similar loss of long chain amylopectins has been reported [7, 35], but a different behavior was described for the other chains. Overall, the loss of ISA1 activity in durum wheat had fewer phenotypic consequences than is the case in rice, barley or maize mutants for the corresponding gene (e.g. *sugary-1*). The basis for this contrasting behavior could be species-specific, or may rather reflect the tetraploidy of the durum wheat genome. Although the global abundance of *Isa1* transcript was very low in the endosperm of both *Isa1* RNAi transgenics, there were still observable changes induced to the properties of the

starch, implying an important role for ISA1 in amylopectin synthesis and in starch granule assembly.

5. References

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Tables

Table 1. Hundred grain weight (HKW) and the starch, amylose, phytoglycogen, free glucose and β-glucan content of the grain of cv. Svevo and two RNAi Isa1 knock-down lines. Data are given in the form mean ± standard error. The values shown in parentheses represent the % of the wild type value. Asterisks indicate that the value differs significantly (P≤0.05) from that of the wild type.

Line	**HKW**	**Total Starch**	**Amylose**	**Phytoglycogen**	**Free Glucose**	**Beta-glucan**
	g	**%**	**%**	**μg/100mg**	**μg/100mg**	**g/100g**
Svevo	3.32±0.31 (100)	59.58±0.71 (100)	25.51±0.35 (100)	439.45±68.75 (100)	30.40±2.66 (100)	0.47±0.01 (100)
MJ59-5b	3,59±0.27 (108)	55.53±0.60* (93)	23.66±1.21 (92)	822.02±111.66* (187)	141.48±28.34* (465)	0.63±0.01* (134)
MJ59-44	3,21±0.25 (97)	55.64±0.23* (93)	23.89±1.33 (93)	816.04±60.70* (185)	122.71±5.84* (403)	0.60±0.05* (128)

Table 2. Length of major axis (µm) of A- and B-type starch granules in the two RNAi *Isa1* knock-down (T_4 generation) lines and in cv. Svevo.

	Type A (> 8 µm)	**Type B (< 8 µm)**
Svevo	16.9 ± 6.2	4.5 ± 1.9
MJ59-5b	18.1 ± 6.3	4.1 ± 1.8
MJ59-44	17.6 ± 5.9	4.4 ± 1.7

Table 3. Crystallinity and A-type crystal packing in the two RNAi *Isa1* knock-down (T_4 generation) lines and in cv. Svevo.

	Crystallinity [%]	**A type [%]**
Svevo	28.3	65.5
MJ59-5b	28.1	68.3
MJ59-44	28.6	65.6

Figure legends

Fig. 1. Transcriptional behavior of genes encoding (A) debranching enzymes (isoamylases and pullulanase) and (B) CSLF6 and SUSY. Each bar represents the mean of three biological replicates, each of which was derived from three technical replicates. The data are given in the form of fold differences in transcript abundance between the non-transformed cv. Svevo and the two transgenic lines. Dotted line indicates the relative transcription value of the control (cv. Svevo). Standard errors are shown above each bar, along with an asterisk to indicate where the transgenic value differed significantly ($P<0.05$) from that of the wild type.

Fig. 2. SEM image of starch granules extracted from the mature grain of the two RNAi *Isa1* knock-down lines and of non-transformed cv. Svevo.

Fig. 3. The distribution of starch granule size in the the mature grain of the two RNAi *Isa1* knock-down lines (T_4 generation) and of non-transformed cv. Svevo.

Fig. 4. Scanning Electron Microscopy analysis of starch granules treated with a 8% hydrochloric acid solution.

Fig. 5. X-ray diffraction patterns of native starch extracted from (a) non-transformed cv. Svevo, (b,c) the two RNAi Isa1 knock-down lines (T4 generation). The characteristic peaks of the A- and V-type crystals are indicated. Intensity is reported in arbitrary units (a. u.).

Fig. 6. Characterization of amylopectin in non-transformed cv. Svevo and the two RNAi *Isa1* knock-down lines MJ59-5b and -44 (T_4 generation). (A) Chain length distribution, (B) differences in chain length distribution between the transformants and non-transformed cv. Svevo.

Supplementary materials

Supplementary Table 1. Length of major axis in A- and B-type starch granules in the two RNAi *Isa1* knock-down lines (T_3 generation) and in non-transformed cv. Svevo.

Supplementary Table 2. Crystallinity and A-type crystal packing in the two RNAi *Isa1* knock-down lines (T_3 generation) and in non-transformed cv. Svevo.

Supplementary Figures

Supplementary Fig. 1. Structure of the pRDPT-ISA1(RNAi) construct used for transformation.

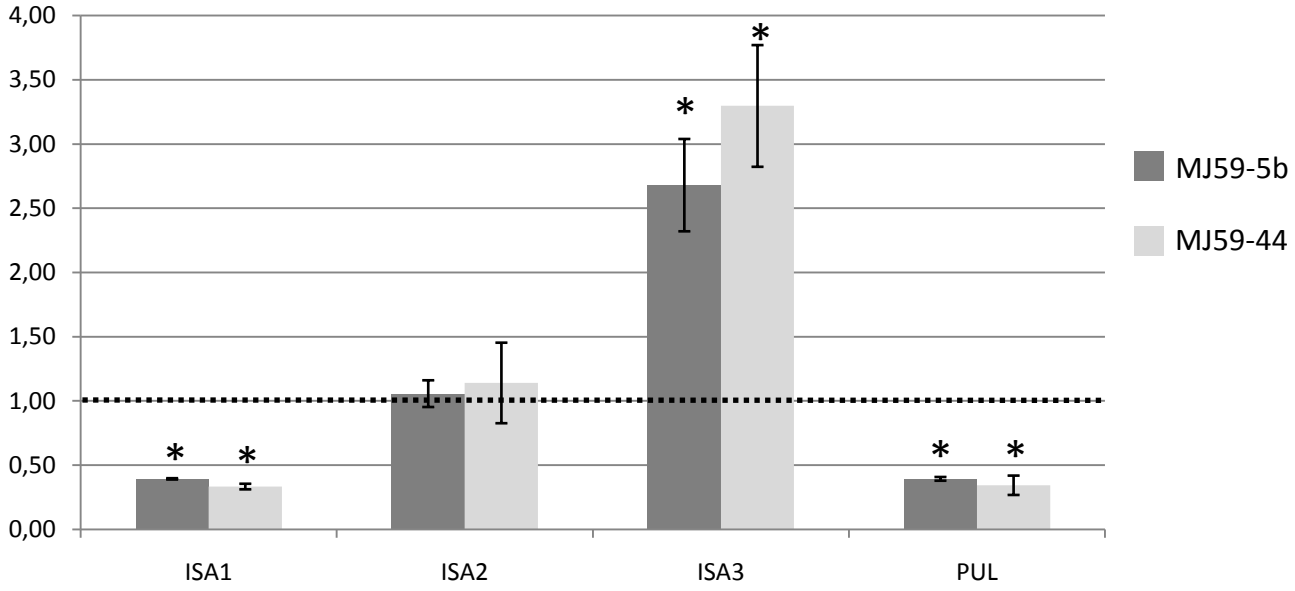
Supplementary Fig. 2. Distribution of starch granule size in the two RNAi *Isa1* knock-down lines (T_3 generation) and in non-transformed cv. Svevo.

Supplementary Fig. 3. X-ray diffraction patterns of native starch extracted from (a) non-transformed cv. Svevo, (b,c) the two RNAi *Isa1* knock-down lines (T3 generation). The characteristic peaks of the A- and V-type crystals are indicated.

Supplementary Fig. 4. Characterization of amylopectin in non-transformed cv. Svevo and the two RNAi *Isa1* knock-down lines MJ59-5b and -44 (T_3 generation). (A) Chain length distribution, (B) differences in chain length distribution between the transformants and non-transformed cv. Svevo.

Figure 1

A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ISA2
ISA3
PUL
MJ59-5b
MJ59-44

B

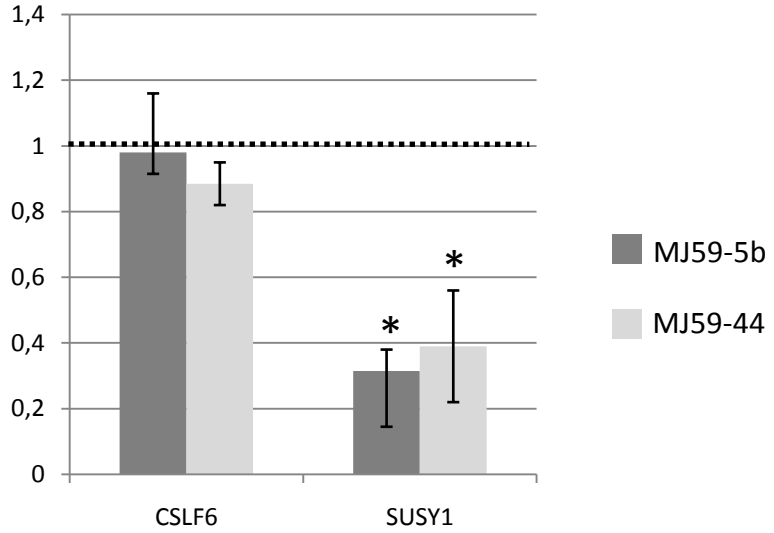
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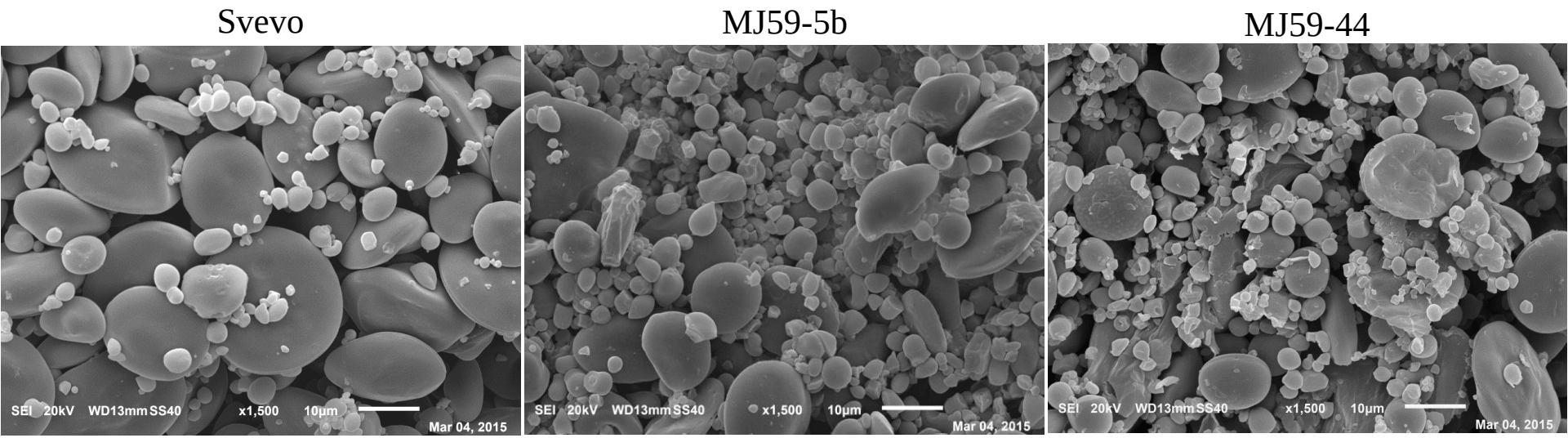
Figure 2


Svevo
MJ59-5b
MJ59-44
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SEI 20kV WD13mm SS40 x1,500 10µm Mar 04, 2015
SEI 20kV WD13mm SS40 x1,500 10µm Mar 04, 2015

Figure 3

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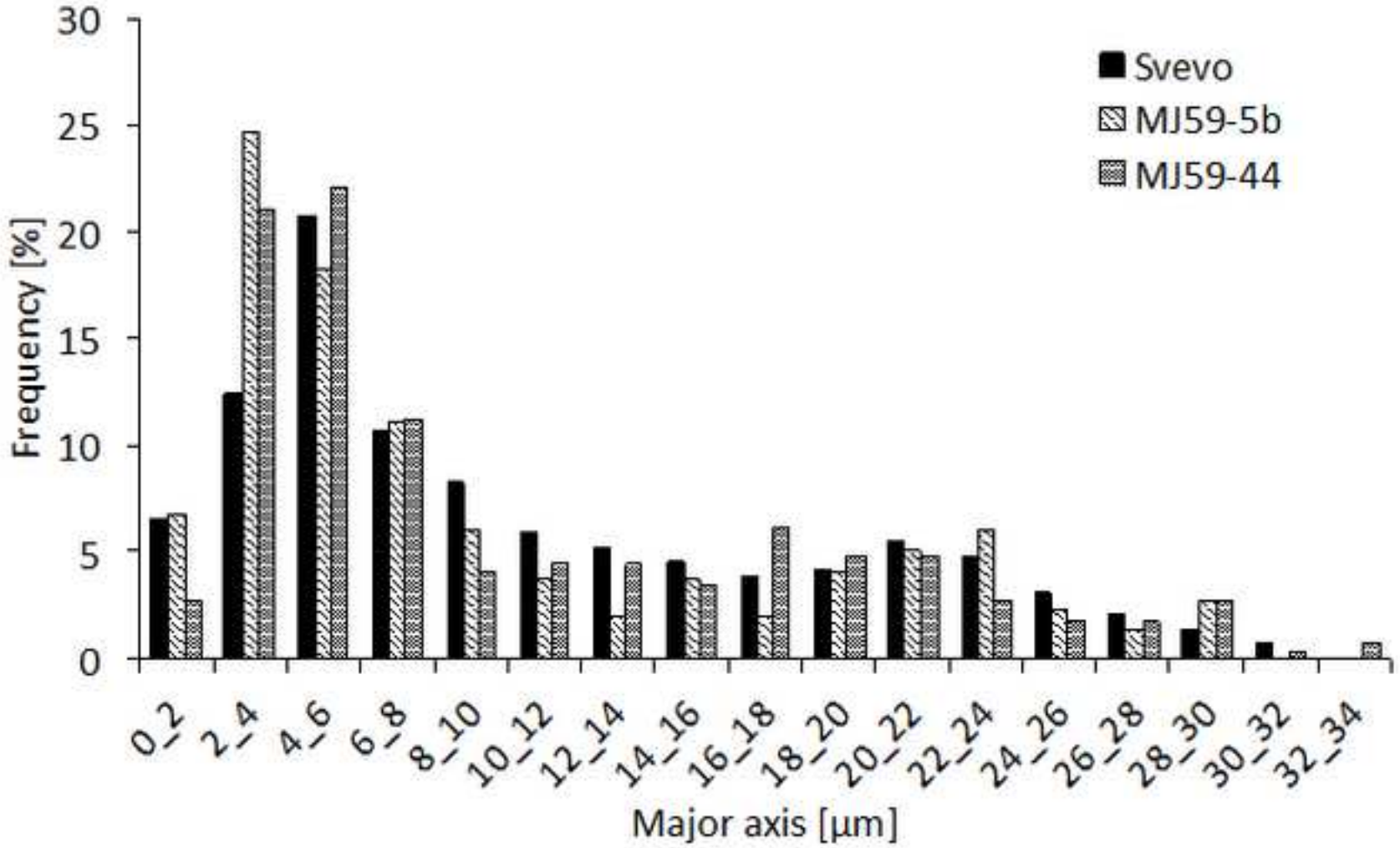
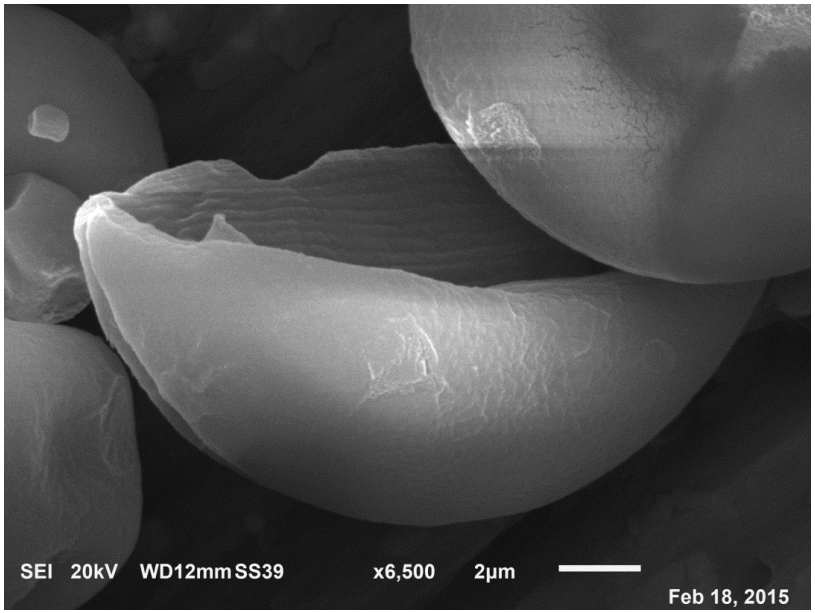


Figure 4

Svevo



MJ59-44

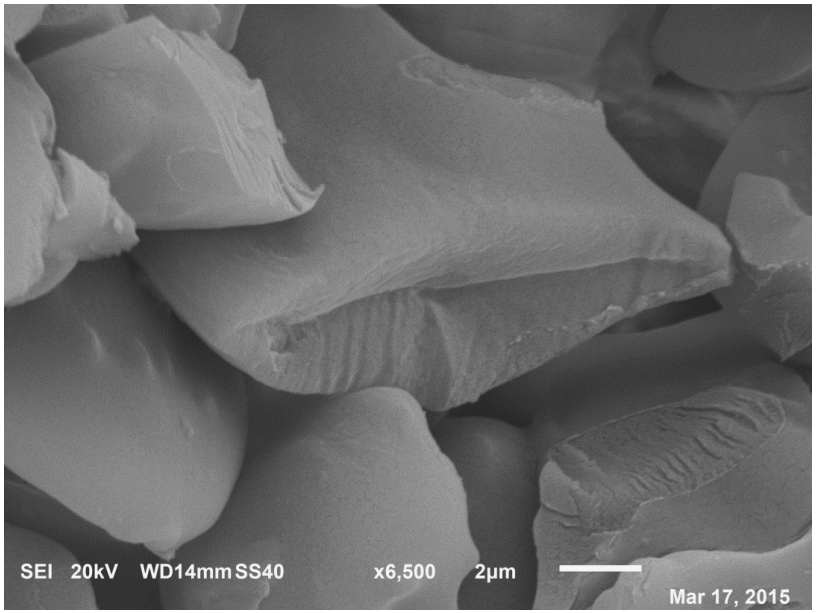


Figure 5

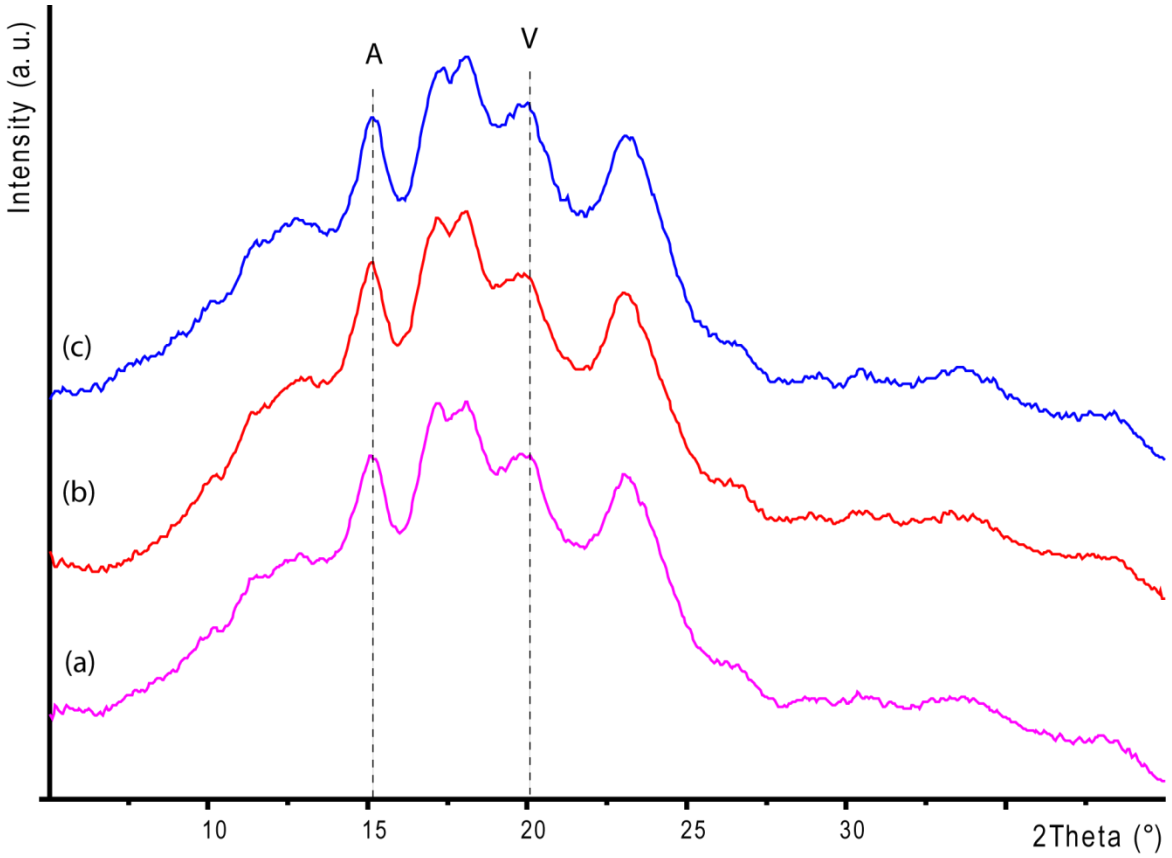


Figure 6

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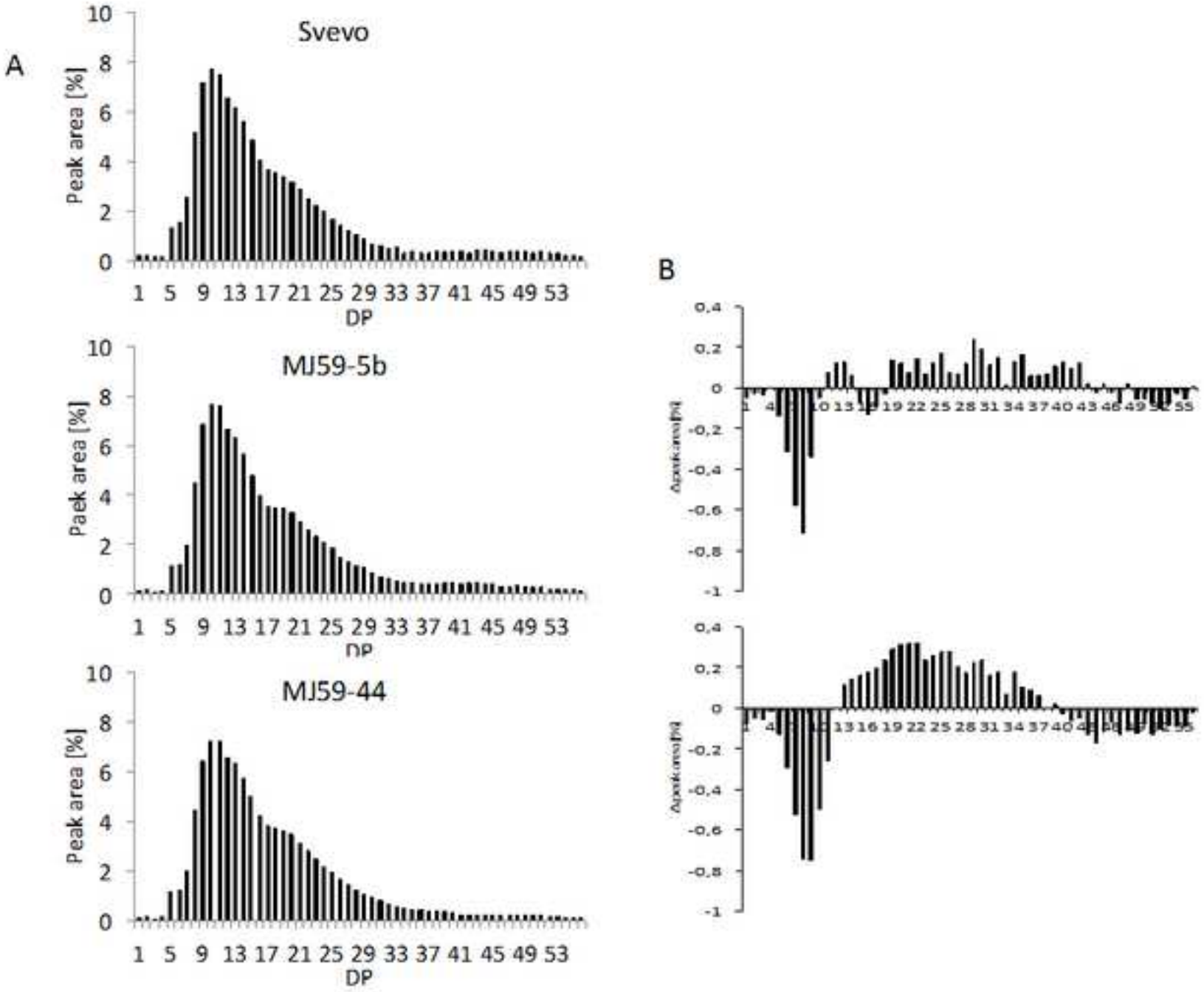

Table S1

	Type A (> 8 µm)	Type B (< 8 µm)
Svevo	16.5 ± 5.9	4.4 ± 1.6
MJ59-5b	18.7 ± 7.0*	4.4 ± 1.8
MJ59-44	17.6 ± 5.7	4.2 ± 1.7

Table S1.

Table S2

	Crystallinity [%]	A type [%]
Svevo	28.5	66.2
MJ59-5b	28.6	68.5
MJ59-44	28.4	68.8

Table S2.

Figure S1

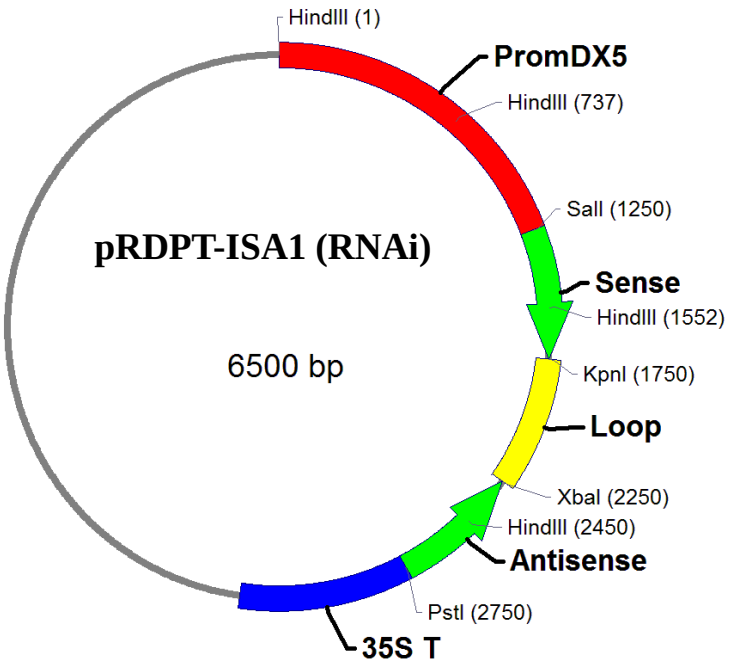

Figure S1.

Figure S2

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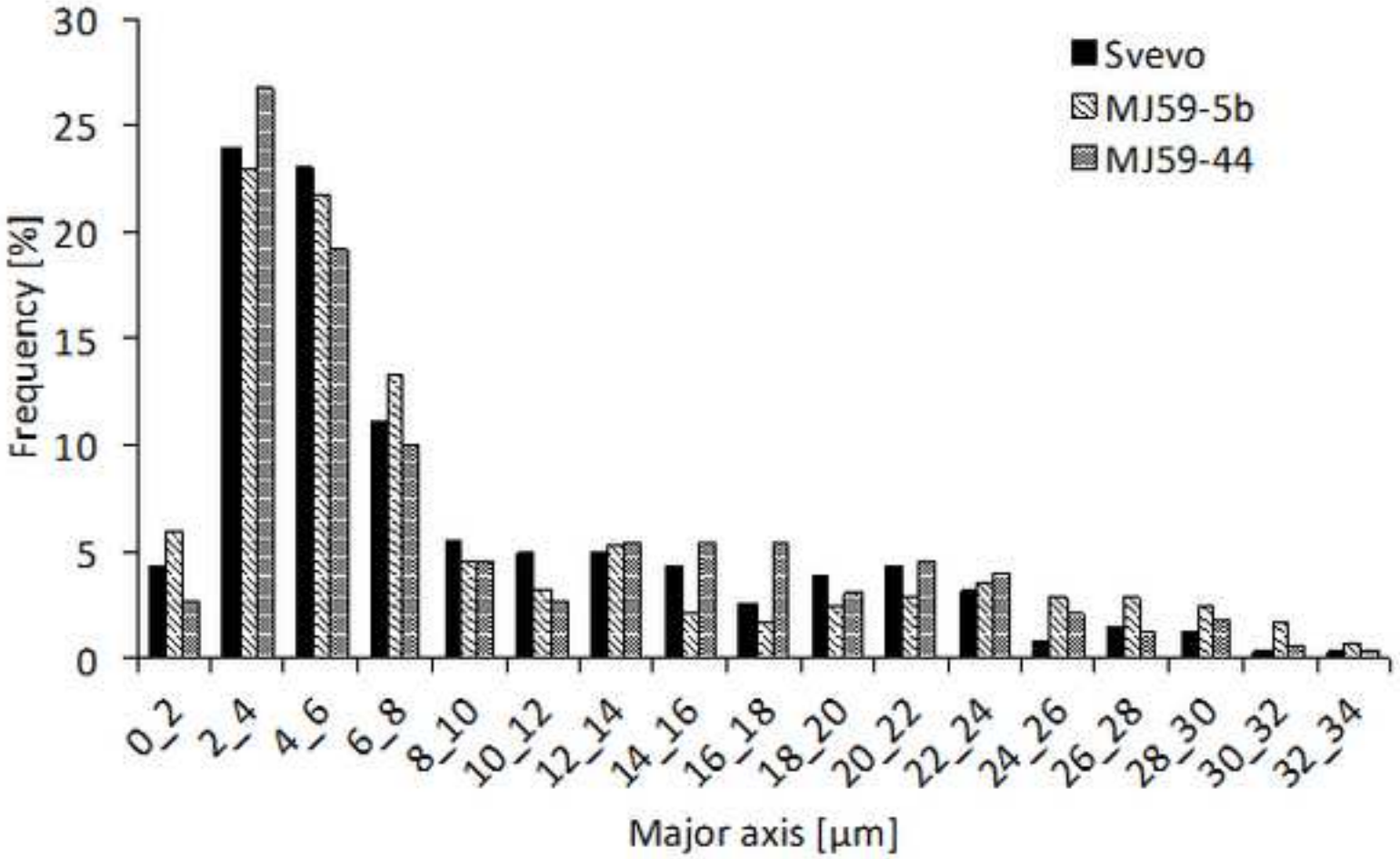

Figure S2.

Figure S3

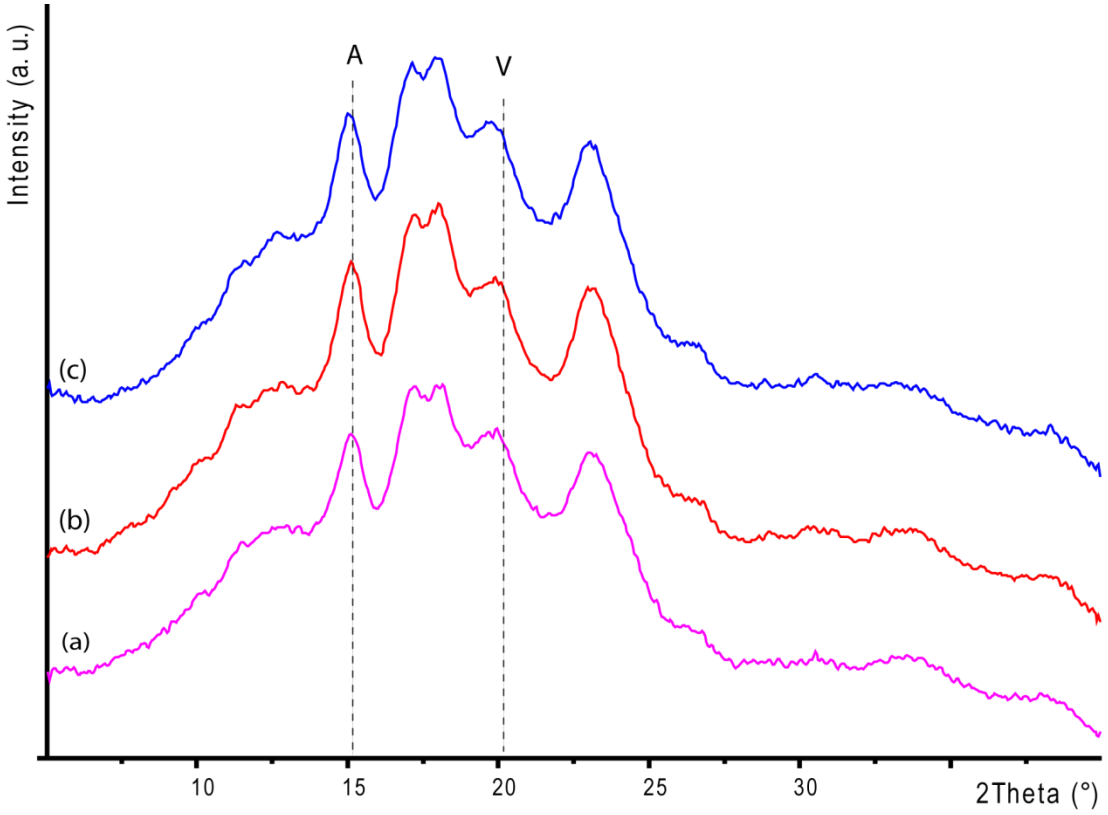

Figure S3.

Figure S4

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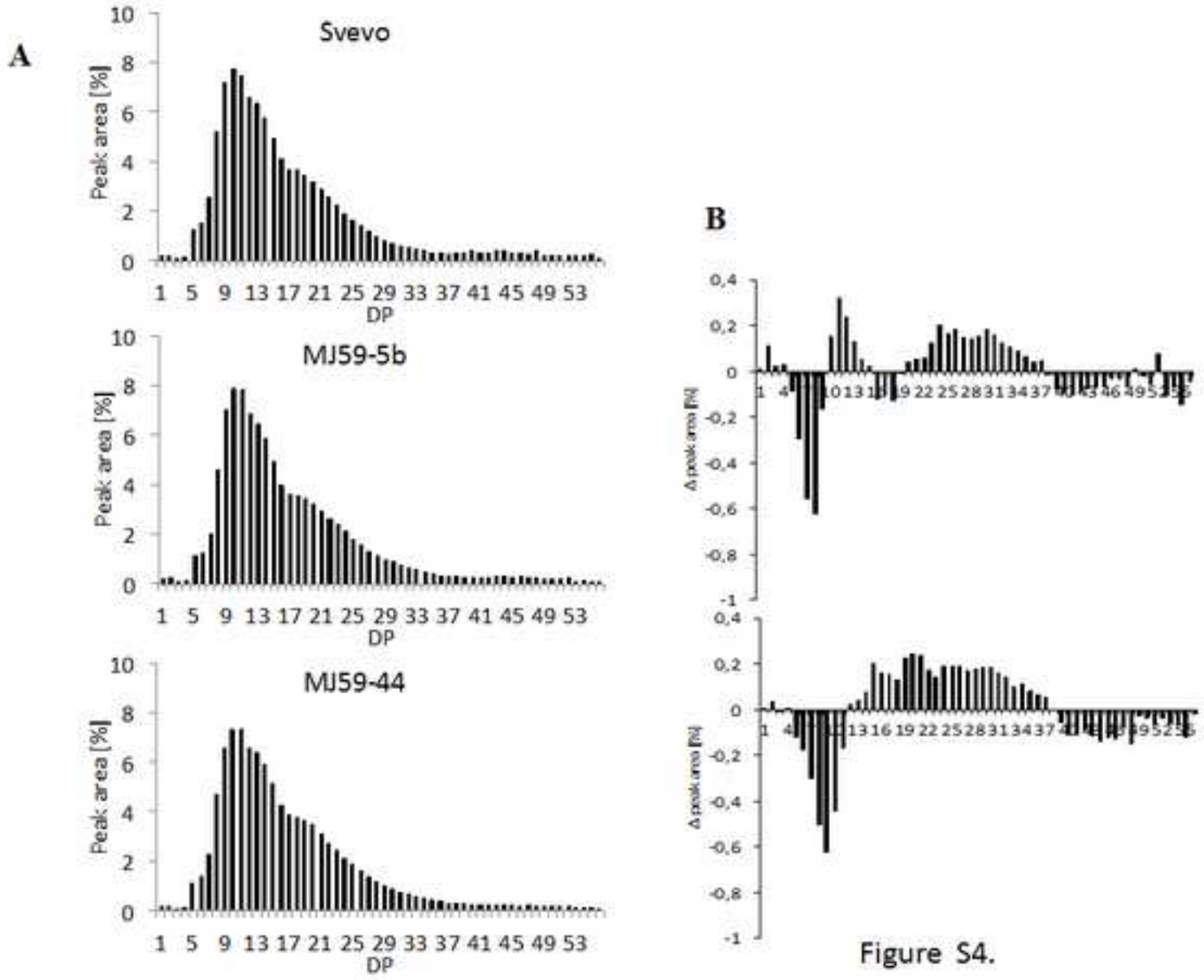

Figure S4.