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# OsttaSBEIII expression alters granule size and increases starch levels and its degradability in *Arabidopsis*

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ABSTRACT

The physicochemical properties of starches from different botanical origin, such as viscosity, gelatinization temperature, solubility and degradability depend on the amylose/amylopectin ratio and the length and frequency of the α-1,6-glycosidic bonds in amylopectin. These branches depend on the action of the starch branching enzymes (SBEs), which are highly structurally conserved in plants. We recently identified a novel gene from *Ostreococcus tauri* (*OsttaSBEIII*) which codes for a protein showing starch branching activity (OsttaSBEIII) and with a different structure than other known SBEs from plants, containing two *in-tandem* carbohydrate binding modules (CBM41-CBM48) at its N-terminus. OsttaSBEIII overexpression in *A. thaliana* plants resulted in a higher starch content and smaller granules with an increased degradability. OEOsttaSBE lines showed also an increase in the expression and activity of starch degradative enzymes and a higher content of glucose and inorganic phosphate, which suggests a remodeling of the granule structure in response to the expression of OsttaSBEIII. These results allow us to propose the use of OsttaSBEIII as a new strategy to obtain starches with greater degradability that would be useful for different biotechnological applications.

## 1. Introduction

Starch is the most abundant storage polysaccharide in the living world and it is composed of two distinct fractions: amylose, a linear α-1,4 glucan and typically constitutes 15–20 % of starch, and amylopectin, a larger molecule with α-1,4 and α-1,6 glycosidic bonds and the major component of starch (Ball and Morell, 2003; Martin and Smith, 1995; Preiss et al., 1991; Zeeman et al., 2010).Linear adjacent chain segments within amylopectin pack into layers named as crystalline lamellae, which alternate with amorphous lamellae containing the amylopectin branch points. Thus, the resulting insoluble semi-crystalline matrix is organized into higher-order structures to form starch granules(Ball and Morell, 2003; Santelia and Zeeman, 2011). Two main crystalline structures of starch have been reported, the "A" and "B" types, which contain different proportions of amylopectin. The A type starches are found in cereals, while the B type is found in tubers and amylose-rich starches. A third type called "C type" appears to be a mixture of both A and B forms and is found in legumes (Tetlow and Emes, 2014).

Linear polysaccharides such as amylose have a highly regular conformation, so they can form crystalline structures that are poorly soluble or insoluble in water. In contrast, branched polysaccharides, such as amylopectin, in general presented an increased solubility because the branched structure could weaken intramolecular interactions due to steric effects between the molecules, and, in addition, it could also decrease the excluded volume compared to polysaccharides of similar molecular weight, which potentially increases the critical concentration and therefore improves water solubility (Guo et al.,

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2017). Thus, it was reported that the amylose/amylopectin ratio contributes to differences in the properties of starch from different botanical sources, such as granule size distribution and other functional properties such as gelatinization temperature, viscosity, solubility and degradability. These properties are important for its applications in food and non-food related industries, such as biofuel production (Santelia and Zeeman, 2011; Song and Jane, 2000; You and Izydorczyk, 2002).

The amylopectin fraction has a better solubility than amylose due to its branched structure and the amount, frequency and type of branching in amylopectin depends mainly on the action of starch branching enzymes (SBEs, 1,4-α-glucan: 1,4-α-glucan 6-glucosyl transferase, E.C. 2.4.1.18), which have a pivotal role in starch granule assembly. These enzymes influence the structure of starch by catalyzing the formation of α-1,6-branch points with varied frequency and branch chain length (Tetlow and Emes, 2014). It was reported that the various SBE isoforms in plants direct impact the structural and functional properties of starches (Nakamura, 2002; Tetlow and Emes, 2014). Thus, the modification or the introduction of new SBEs in plants could lead us to obtain starches with advantageous characteristics such as better digestibility and solubility for specific biotechnological applications (Tetlow and Emes, 2014). SBEs belong to the glycoside hydrolase family 13 (GH13) and all known plant SBEs contain three domains in their structure, a N-terminal carbohydrate binding module from family 48 (CBM48), and a C-terminal region containing the catalytic domain (CD) followed by a C-terminal extension that controls the glucan substrate preference (Abad et al., 2002; MacGregor et al., 2001).

Recently, we identified a novel SBE from *Ostreococcus tauri*, named OsttaSBEIII (Hedin et al., 2017). *O. tauri* belongs to the group of Prasinophyceae, one of the oldest within the lineage that gave rise to green plants, and it is characterized by its minimal cellular organization, a very compact genome and a structural complexity with respect to the metabolism of polysaccharides comparable to that of higher plants (Busi et al., 2014; Kuhn et al., 2009; Ral et al., 2004; Sorokina et al., 2011). This organism contains three SBEs, OsttaSBEI (OT_ostta04g03940), OsttaSBEII (OT_ostta03g00870) and OsttaSBEIII (OT_ostta01g03050) (Hedin et al., 2017; Sorokina et al., 2011). While OsttaSBEI and II contain a unique CBM48 at their N-terminal region (similar to plant SBEs), OsttaSBEIII showed a two in-tandem CBMs (CBM41-CBM48) at its N-terminal end (residues 64–403), followed by the CD (residues 426–1057). Analysis of truncated isoforms showed that the CBMs bind differentially to whole starch, amylose or amylopectin and that both CBMs seem to be essential for branching activity(Hedin et al., 2017). Thus, taking into account the direct effect of SBE isoforms on the structural and functional properties of starch, it is possible that the expression of this novel enzyme confers different properties to this polysaccharide.

Here, we characterized two *Arabidopsis thaliana* lines overexpressing OsttaSBEIII. These lines accumulate a higher level of starch compared to wild-type plants. Using scanning electron microscopy, we found that starch granules from OEOsttaSBE lines are smaller and have a different size distribution and also a greater degradability respect to wild-type granules. These findings allow us to postulate the use of OsttaSBEIII as a biotechnological tool for the generation of different starches in plants of agronomic interest.

## 2. Materials and methods

### 2.1. Plant material, molecular cloning and growth conditions

Two independent *Arabidopsis thaliana* transgenic lines OEOsttaSBE1 and OEOsttaSBE2, and a wild-type line (var. Columbia Col-0) were used in this study. To generate both transgenic lines, the gene coding for OsttaSBEIII (OT_ostta01g03050) was cloned using standard *Pfu* polymerase chain reaction and the following primers: OsttaSBEpCHF3-Fw ACCCGGGAAATGTCGGTCACCCACACGTC (SmaI site underlined) and OsttaSBEpCHF3-Rv CAACTGCAGCTATCGACACTGCACGAATACGGC (PstI site underlined). The resulting PCR product was cloned into the pCHF3 plasmid using SmaI and PstI restriction sites. *Arabidopsis thaliana* (var. Col-0) were grown until floral bud development (ca. 4 weeks) and wild type Arabidopsis plants were transformed with *Agrobacterium tumefaciens* GV3101 containing pCHF3::OsttaSBEIII, using the standard floral dip transformation protocol(Clough and Bent, 1998).

For plant growth, surface-sterilized seeds were plated on Murashige & Skoog medium supplemented with kanamycin (50 μg/ml) and transformants were selected after 2 weeks. Seedlings were grown in soil ina greenhouse at 25 °C under fluorescent lamps (Grolux, Sylvania, Danvers, MA, USA and Cool White, Philips, Amsterdam, The Netherlands) using a 16 h light/8h dark photoperiod (intensity was 150 μmol $m^{-2}$ $s^{-1}$) as described (Grisolia et al., 2017). Kanamycin selected plants were confirmed by PCR using the primer pair OsttaSBEpCHF3-Fw/OsttaSBEpCHF3-Rv and homozygous lines were selected.

### 2.2. Sequence alignment and phylogenetic studies

The sequence similarity search of OsttaSBEIII (Genbank code: OT_ostta01g03050, formerly Ot01g03030; XP_003074364.1, 1058 amino acid residues) whole amino acid sequences was performed with PSI-BLAST (Altschul et al., 1997) with the default parameters (threshold = 0.005) until convergence, using no redundant databases. Nineteen sequences were retrieved and aligned with the program CLUSTALW and the evolutionary history was inferred using the Neighbor-Joining method(Saitou and Nei, 1987), The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (Zuckerkandl, 1965). Analyses were performed with MEGA X (Kumar et al., 2018).

### 2.3. Starch content determination and degradability assay

Starch content was determined according to Zhang et al. with some modifications (Zhang et al., 2005). Leaves of 35-days-old plants (about 3 g) were boiled in 100 ml of 80 % ethanol. Decolored leaves were grounded with mortar and pestle using liquid nitrogen and resuspended in 80 % ethanol, centrifuged 15 min at 12,000*g*. and the pellet washed in 80 % ethanol 3 times. The insoluble material was suspended in water and incubated for 45 min at 100 °C. After cooling, the sample was incubated with a mixture of α-amylase/amyloglucosidase dissolved in buffer 50 mM Sodium acetate, pH = 5,1 to achieve the complete digestion of starch. Glucose obtained after starch degradation was quantified using a commercial kit (Glicemia enzimática AA, Wiener Lab, Argentine). The amylose/amylopectin ratio was measured using a commercial kit (Megazyme, Ireland), following the manufacturer's instructions.

Starch degradability was assayed as follows. Starch extracted from at least 20 plants as indicated in the previous paragraph was pooled, resuspended in 100 mM potassium phosphate buffer (pH = 7.0), 1 mM $CaCl_2$, brought to OD450 nm = 1.2 and incubated with 1 mU of α-amylase (Roche, #10102814001) for at least 18 h. After centrifugation for 15 min at 12,000*g* and incubation with the α-amylase/amyloglucosidase solution, glucose levels in the supernatant was determined as described above, with a commercial kit following the manufactureŕs instructions (Glicemia enzimática AA, Wiener Lab, Argentine). All assays were performed in triplicate, and measurement data were expressed as the mean ± SD.

### 2.4. Analysis of granule morphology

Starch preparations were obtained as described above, without enzymatic digestion of the samples. Images were obtained using a scanning electron microscope Quanta 200F (FEI, Hillsboro, Oregon, USA) coupled to a FEG electron source. Samples were not metal covered and amplification between 10,000X and 40,000X was used with the following setting: 2,5 kV energy, LFD large field detector and working

distance (WD) of 5,8 – 7,6 mm. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The circularity parameter (C) was calculated as reported previously(Ingkasuwan et al., 2012).

### 2.5. Quantitative RT-PCR assay

Total RNA from 35-days-old leaves of the different lines was extracted using TRIzol reagent (Invitrogen)in the middle of the light period. RNA integrity and genomic absence was checked on a 1 % (w/v) agarose gel, and the concentration was determined by measuring absorbance at 260 nm while protein contamination was evaluated by the measurement of absorbance at 260/280 nm. The complementary DNA was synthesized using random hexamers and the Access RT-PCR system first strand protocol (Promega). qPCR was performed using a Mini-Opticon2 equipment (Bio-Rad), SYBRGreenI (Invitrogen, Carlsbad, CA, USA), and GoTaq polymerase (Promega, Madison, WI, USA)(Busi et al., 2011). Primers were designed using the online tool PrimerBlast (Ye et al., 2012). The protocol used was the following: one cycle at 94 °C (2 min.), 40 cycles at 94 °C (10 s), 63 °C (15 s), and 72 °C (20 s) and finally one cycle for 10 min at 72 °C. Fluorescence detection was measured at the end of each annealing step for 1 s. Individual melting curves were determined by the decrease in fluorescence with increasing temperature (from 65 to 98 °C). The *ACTIN2* transcript levels (At3g18780) was used as an internal standard and quantification was performed using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Oligonucleotides used are shown in Supplementary Table 1.

### 2.6. Analysis of RNA-sequencing data

FASTQ raw-data files were downloaded from NCBI – SRA (Sequence Read Service). The paired-end data was analyzed with FASTQC for quality control (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were mapped against *A. thaliana* genome and reference annotations obtained from TAIR (TAIR 10 – https://www.arabidopsis.org) (CITA: https://doi.org/10.1002/dvg.22877) using HISAT2 (CITA: https://doi.org/10.1038/nmeth.3317). StringTie was used to assemble and quantify transcripts (CITA: https://doi.org/10.1038/nmeth.3317).

### 2.7. Enzymatic assays

Two grams of fresh leaves collected at the middle of the light period from all the lines under study were grinded with a mortar and pestle with 2 ml of 100 mM MOPS buffer, pH 7.5 and 1 mM PMSF. The crude extract was centrifuged at 12,000*g* for 15 min. at 4 °C and the soluble phase was desalted with a Sephadex G-75 column (Gomez Casati et al., 2000).

SBE activity was assayed using 20 mM Tris-HCl pH = 7.4 and amylopectin (Fluka #10118) to a final concentration of 0 – 40 mg/ml and different concentrations of protein from the crude extract, in a final volume of 33 μl. Reactions were performed at 37 °C during 30 min. and stopped by the addition of 100 μl of $H_2O$ and 865 μl of iodine reagent as previously described(Hedin et al., 2017). Enzymatic activity was determined monitoring the decrease of absorbance at 520 nm. One unit of enzymatic activity (U) is defined as the amount of enzyme that decreases the absorbance 1 U/min at 520 nm.

Total starch synthase activity was determined by measuring the released Pi after a glucose moiety is added to a growing polysaccharide chain. Malachite green reagent was used as previously describe (Barchiesi et al., 2017), to determine total Pi released by the proteins on the crude extract. ADPGlcPPase was determined according to(Gomez Casati et al., 2000); soluble starch synthase (SSS) and granule bound starch synthase (GBSS) activities were assayed as described by Szydlowski et al. (Szydlowski et al., 2009). Glucan phosphatase activity was assayed using amylopectin as described(Carrillo et al., 2018). Alpha-amylase activity was measured using a commercial kit (Amilokit, Wiener Lab). Briefly, the protein extracts were incubated in the presence of a reagent A consisting of a 500 mg/l starch solution, pH = 7.0. The reaction is stopped by adding reagent B (0.01 eq/l 20 mM iodine solution in HCl). The activity was measured as the decrease in absorbance at 640 nm.

### 2.8. Metabolic profile analysis

*Arabidopsis thaliana* leaves were used to obtain its metabolic profile through protocols developed in our laboratory and already cited in previous publications (Grisolia et al., 2017; Lisec et al., 2006). Hundred milligrams (100 mg) of tissue was grounded to a powder. Sixty microliters of ribitol (0.3 mg/ml) and 750 μl of chloroform were added to each sample. A centrifugation at 2200*g* for 15 min was performed, and derivation was done as previously described with minor modifications (Lisec et al., 2006).Three biological replicates per treatment and three technical replicates were used. GC–MS analysis was performed with a GC and a Perkin Elmer Turbo Mass Spectrometer as described (Grisolia et al., 2017). Briefly, one microliter split injection (split ratio 1:40) was injected at 280 °C in a capillary VF-5 ms column (Varian, Darmstadt, Germany) with helium as the carrier gas and a constant flow of 1 ml/min. The program was set as follows: 5 min at 70 °C, 5 min ramp-up to 310 °C and final heating for 2 min at 310 °C. The transfer line to the MS was set to 280 °C. Spectra were monitored in the mass range $m/z$ = 70–600. Chromatograms were obtained with TurboMass-4.1 software (Perkin Elmer) and the software platform used was the NIST98mass spectral search program (NIST, Gaithersburg, MD, USA). Retention time index and MS were compared with those values found in the Golm Metabolome Database (Kopka et al., 2004). MS matching was manually curated, only matches higher than 650 (being 1000 the maximum) and retention index deviation < 1.0 % were accepted. Peak heights determination, metabolite content and one-way ANOVA statistical analyses were performed as described (Grisolia et al., 2017).

### 2.9. Statistical analyses

The significance of differences was determined using Student's *t*-test or one-way ANOVA and Tukey's test. Asterisks denote values statistically different from the control ($P < 0.05$).

## 3. Results

### 3.1. Phylogenetic analysis

The alignment of the protein sequence of OsttaSBEIII with homologous SBEs identified with PSI-Blast, allowed us to build a phylogenetic tree (Supplementary Fig. 1). To perform this analysis, we used the protein sequence of OsttaSBEIII and 16 and 3 sequences from plant SBEs (monocot and dicots) and bacterial GBEs (glycogen branching enzymes), respectively. Results show three different groups of enzymes: Class I SBEs (6 sequences), Class II SBEs (10 sequences) and the GBEs group. It is interesting to note that OsttaSBEIII is not included in any group, but closer to bacterial GBEs, mainly due to the conservation of the CD as described previously(Hedin et al., 2017).Moreover, the Figure S1 shows that SBEs from Class I and II clusters together, and among each of these groups, monocot and dicot species also form discrete clusters. It is interesting to note that at least the CD of OsttaSBEIII probably have evolved independently from higher plants SBEs, and for this reason the presence of a different CBM configuration might be possible. As mentioned above, OsttaSBEIII possess a tandem of two CBMs (CBM41-CBM48) and this protein domain organization was not described for any of the other SBE proteins.

It was described that the final structure of the starch produced by a species is strongly dependent on the unique kinetic, structural and regulatory properties of the enzymes present in each species. Thus, this

novel structural feature of OsttaSBEIII protein could determine the production of starch granules with distinctive properties.

### 3.2. Analysis of OEOsttaSBE lines

In order to evaluate the effect of overexpression of OsttaSBEIII in *Arabidopsis*, we generated two starch SBE overexpressing lines named OEOsttaSBE1 and OEOsttaSBE2. The construct was obtained as indicated in Materials and Methods, by cloning the *OSttaSBEIII* gene into the pCHF3 plasmid under a CaMV35S double promoter. This system allows us to achieve a constitutive expression of the OsttaSBEIII (Jarvis et al., 1998). Total DNA from *A. thaliana* leaves was isolated using hexadecyltrimethylammonium bromide (CTAB) and the presence of *OsttaSBEIII* was verified using specific primers (Supplementary Fig. 2A). The mRNA levels of *OsttaSBEIII* on both, OEOsttaSBE1 and −2 lines is shown in Supplementary Fig. 2B. Both transgenic lines showed high expression of the OsttaSBEIII gene, whereas, as expected, there was a lack of expression of this gene in the wild-type line.

The transgenic plants appear to have a larger rosette size than the wt line (Supplementary Fig. 3A), however, all the plant lines analyzed showed a similar fresh weight and dry weight (Supplementary Fig. 3B). In addition, about 45 to 50 seeds per siliqua were found in both OEOsttaSBE lines and the wild-type plants (Supplementary Fig. 3C).

### 3.3. Starch content and granule morphology

We have first analyzed the starch content in all the *Arabidopsis* lines studied. Thus, starch granules were isolated from rosette leaves from 35-days-old plants at the end of the day and at the end of the dark period. The granules were hydrolyzed using amylase and amyloglucosidase and the glucose released was determined using a commercial kit. The results show that both OEOsttaSBE lines accumulate around 30 and 21 % more starch compared to the wild-type line at the end of the light or dark periods, respectively (Fig. 1A and B). Although an increase in total

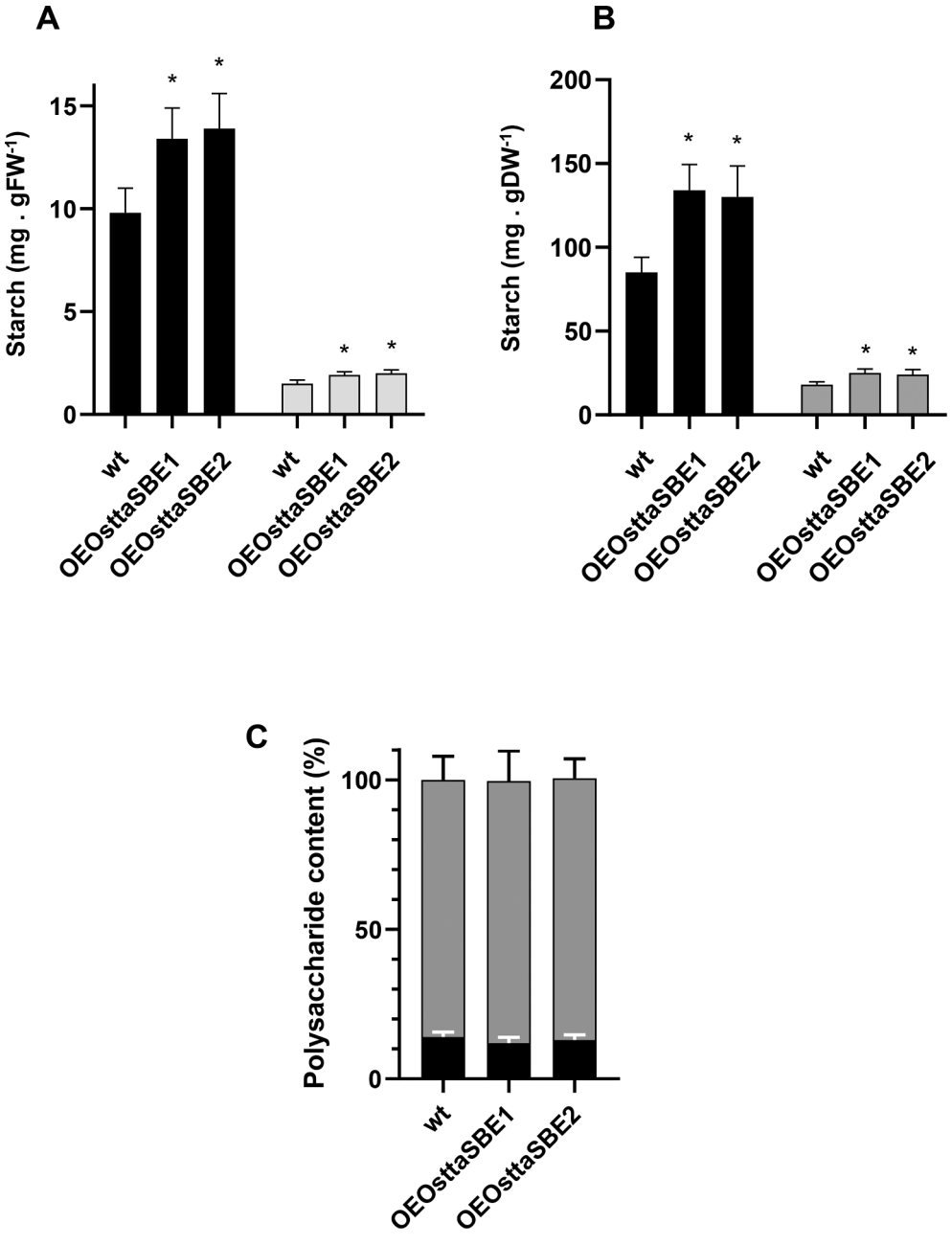



**Fig. 1. (A)** Starch content of *Arabidopsis* leaves from wild-type (wt), OEOsttaSBE1 and OEOsttaSBE2 lines. Starch was isolated from rosette leaves from 35-days-old plants at the end of the day (black columns) or at the end of the night period (grey colums). Values are the mean ± SD of three independent replicates. **(B)** Quantification of amylose (black columns) and amylopectin (grey columns) levels in 35-days-old *Arabidopsis* leaves from wild-type and transgenic lines. The amount of each starch fraction is plotted on a logarithmic scale to better visualize changes in amylose content.

starch content was observed, we did not observe significant differences in the amylose:amylopectin ratio (Fig. 1C).

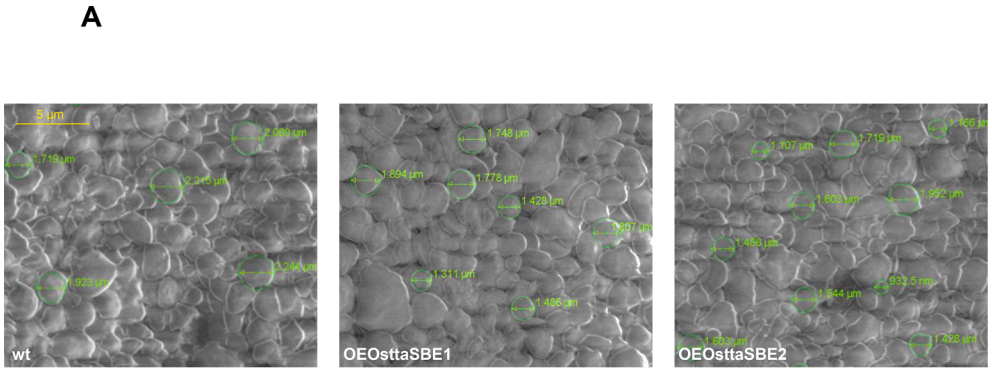
Based on these data, we decided to continue with the characterization of the morphology and the degradability analysis of the starch granules. First, we evaluated the morphology of the starch granules extracted from all the *Arabidopsis* plant lines using scanning electron microscopy. To obtain detailed micrographs, a magnification of 10,000× to 40,000× was employed. Fig. 2A shows the images obtained from the granules of the three lines under study. In order to compare the granules, we measured the diameter of at least 50 granules from at least five micrographs and we divided them into 3 groups, group 1: granules of diameter between 0 and 1 μm; group 2: between 1 and 2 μm and group 3: higher than 2 μm. Thus, we observed that the percentage of starch granules in group 3 decreased between 4 and 6-fold in the OEOsttaSBE plants, also showing an enrichment in the number of smaller granules corresponding to group 2. On the other hand, we also found in both transgenic lines between 5 % and 6 % of group 1 granules, which are not significantly represented in wild-type plants. These results indicate that there is a significant decrease in the size of the starch granules in the transgenic lines with respect to the Col-0 line (Fig. 2B).

Second, we evaluate the resistance to degradation of the starch purified from the plant leaves under study by treatment with an α-amylase. This enzyme is capable of hydrolyzing the α-1,4 glycosidic bonds of insoluble starch releasing maltose and glucose. After 18 h of incubation, a total hydrolysis of the starch from both OEOsttaSBE lines was observed, while the starch from wild-type plants was only partially hydrolyzed (Fig. 3A). We also determined the glucose levels after incubating the supernatant of the previous mixture (incubated for 18 h) with an α-amylase/amyloglucosidase solution as described in Materials and Methods. Results show an about 2-fold increase in glucose levels in both transgenic lines compared to the wild-type line (Fig. 3B). These results indicate that the transient starch from the lines that overexpress OsttaSBEIII is more easily degradable than that from the control.

Third, we calculated the circularity parameter (C) proposed by Ingkasuwan et al. (Ingkasuwan et al., 2012). This parameter evaluates the shape of the granule, being 1 for perfectly round shaped granules, and values other than 1 indicate distortion of circularity. We have not found significant differences in this parameter between the wild-type lines and overexpressing lines ($C_{Col\text{-}0}$ 0.900 ± 0.048, $C_{OEOsttaSBE1}$ 0.919 ± 0.060 and $C_{OEOsttaSBE2}$ 0.943 ± 0.029). These data indicate that the general structure of the granule is maintained. From the data shown in this section, we can conclude that the OEOsttaSBE1 and −2 lines would have a greater amount of starch accumulated in smaller granules and that these would degrade more easily than the granules from wild-type plants.

### 3.4. Transcriptional response and protein activity

To assess the expression levels of genes encoding enzymes involved in starch metabolism in wild-type and transgenic plants, we performed an *in silico* analysis of *Arabidopsis thaliana* RNASeq data. Fig. 4 shows the expression levels of starch synthesis (Fig. 4A) and degradation (Fig. 4B)



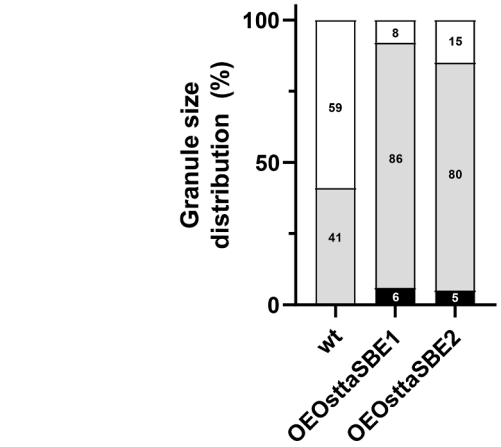




**Fig. 2. (A)** Scanning electron microscopy of starch granules from wild-type (wt), OEOsttaSBE1 and OEOsttaSBE2 lines. The diameter of several starch granules is indicated in each panel. **(B)** Distribution of granule sizes from wt and transgenic lines. The starch granules were grouped into three groups according to their diameter: group 1 (from 0 to 1 μm, black bars), group 2 (between 1 and 2 μm, grey bars) and group 3 (between more than 2 μm, white bars). The values are expressed as a percentage of granules from each group over the total number of starch granules measured.

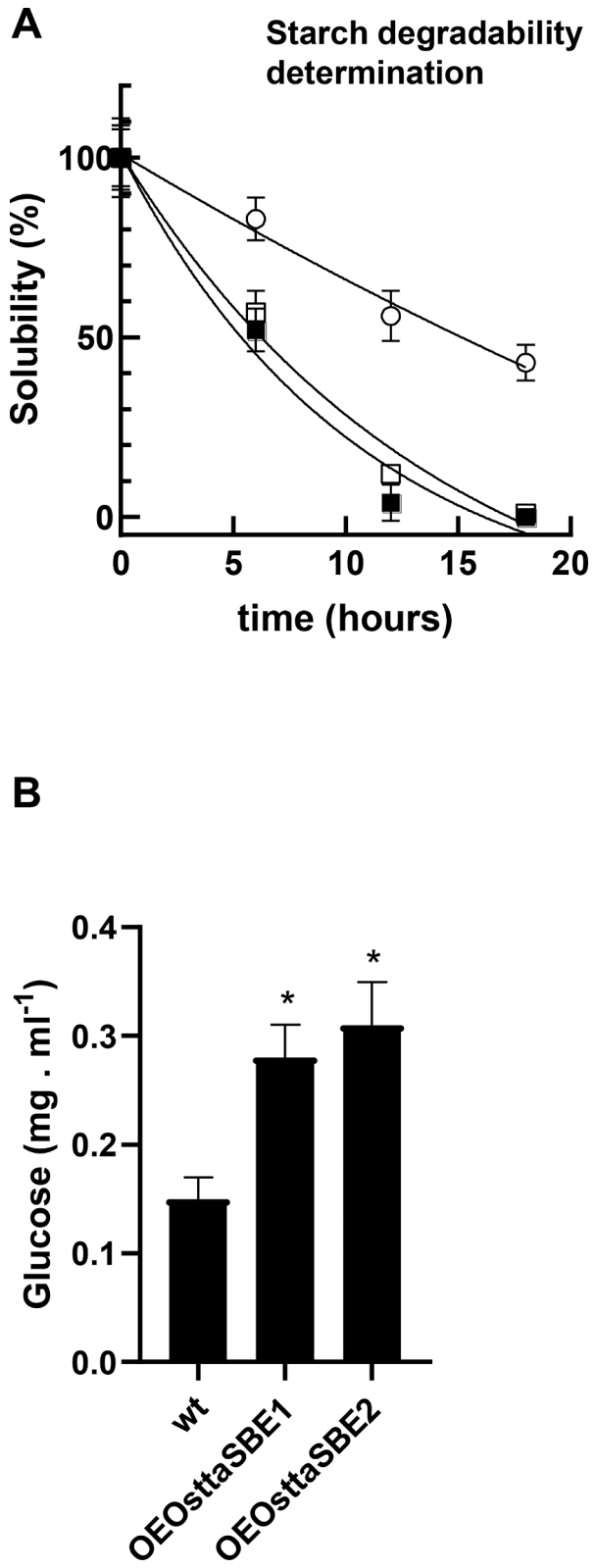



**Fig. 3.** **(A)** Determination of the degradability of starch *in vitro*. Starch isolated from 35-days-old rosette leaves from wild-type (white circles) and OEOsttaSBE1 (white squares) or OEOsttaSBE2 (black squares) plants was incubated in the presence of a mixture of α-amylase/amyloglucosidase solution and the absorbance was measured at different times. **(B)** Determination of glucose levels in the supernatant of tubes incubated for 18 h (panel A) using a colorimetric method (see Materials and Methods). Values are the mean ± SD of three independent replicates.

genes. The results show that in the middle of the day the difference in the levels of transcripts under study can be adequately evaluated. To confirm these data, we measured by qPCR the level of transcripts involved in starch synthesis and degradation in the middle of the light period using 35-days-old plant leaves from wild-type and the OEOsttaSBE lines (Materials and Methods section).

First, we determined the mRNA amounts of *APS* and *APL*, which code for the small and large subunits of ADPGlcPPase, respectively. The key step in the starch synthesis pathway is the generation of the sugar nucleotide ADPGlc by the ADPGlcPPase (Ball and Morell, 2003; Preiss et al., 1991; Zeeman et al., 2010). At this point it is determined whether the carbon fixed by the plant will be used for the accumulation of starch or will have another destination within the cells. Therefore, it is important to establish if the transcripts that code for this enzyme shows any alteration in their levels. Results show an about of 15–30 % increase inthe levels of *APS* and *APL* in both overexpressing lines with respect to wild-type plants (Fig. 5A). Furthermore, we found that ADPGlcPPase activity increased between 2 and 2.5-fold in the OEOsttaSBE lines, which is in agreement with the increase in starch content observed in these plants (Fig. 5B).

We also measured the transcript levels of different genes coding for granule-bound starch synthase (*GBSS*) and soluble starch synthases (*SSI-IV*). GBSSs are required for amylose synthesis and are founded mainly within the granule matrix, while SSS participates in the synthesis of amylopectin(Ball and Morell, 2003; Myers et al., 2000). As shown in Figure 5A, a decrease of about 50 % was observed in the mRNA levels of *GBBS*, *SSI* and *SSIV*, while the mRNA levels of *SSII* did not show significant differences in both OEOsttaSBE lines with respect to the wild-type line. In contrast, the levels of transcripts coding for SSIII increased between 1.8 and 2.1-fold in the OEOsttaSBE1 and −2 lines, respectively (Fig. 5A). Furthermore, total SS activity was found to be increased by around 2 to 2.5-fold in the OEOsttaSBE lines compared to the control Fig. 5B). Thus, to investigate if there was a variation in the enzymatic activities of the different SSs, we also determined the total SSS and GBSS activities. We found that the SSS activity increased around 2.5 and 3.5-fold in the OEOsttaSBE1 and −2 lines, respectively, while the GBSS activity decreased between 25 and 50 % in the overexpressing lines compared to the wild-type line (Fig. 5B).

We also determined the transcript levels of SBEs. It is worth mentioning that *Arabidopsis* only presents SBEs from family II (SBE2.1 and SBE2.2) whose members transfer chains of shorter length than SBEs from family I. Although it is not possible to differentiate the activity of endogenous SBEs and OsttaSBEIII, the results show a slight increase in *SBE2.1* expression in contrast to *SBE2.2* mRNA levels, where not changes were observed (Fig. 5A). However, a 5-fold increase in total SBE activity was observed in both OEOsttaSBE lines compared to wild-type plants, which could be attributed mainly to the overexpression of OsttaSBEIII (Fig. 5B). Finally, we also found an increase of about 2-fold of *ISA2* (isoamylase 2) mRNA levels in both transgenic lines. The function of ISA2 is important during starch synthesis: while it has been proposed that ISA2 is catalytically inactive, it forms a heterodimeric complex with ISA1 which works by hydrolyzing bonds in amylopectin molecules that are unfavorable in the formation of the semi-crystalline structure of the granule(Delatte et al., 2005; Pfister and Zeeman, 2016).

On the other hand, we also analyzed the transcript levels of genes coding for different enzymes involved in starch degradation (Fig. 6A). Thus, the mRNA of the phosphorylating protein PWD (phosphoglucan water dikinase) showed an increase of about 3-fold in both OEOsttaSBE lines compared to the Col-0 line, while the mRNA level that codes for the GWD (glucan water dikinase) enzyme was slightly increased, about 10 and 15 % in OEOsttaSBE1 and −2 lines, respectively. On the other hand, the transcript encoding SEX4 (starch excess 4) showed a 3- and 4-fold increase in the OEOsttaSBE1 and −2 lines, respectively. Moreover, we detected an increase between 1.5 and 2-fold of the mRNA levels of *LSF2* (like SEX4 phosphoglucan phosphatase 2). LFS2 is a phosphoglucan phosphatase specific for C3-glucosyl residues of starch. It was described

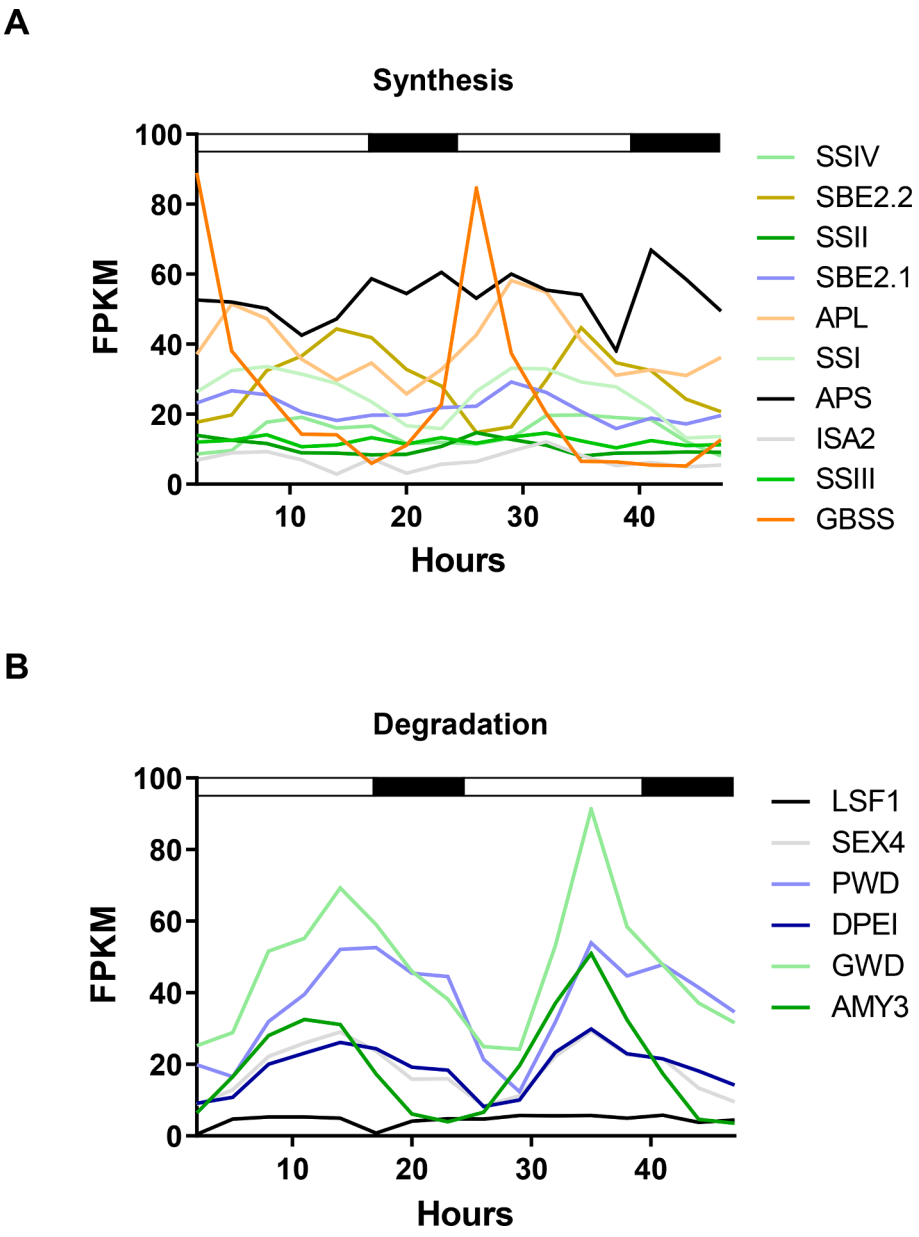



**Fig. 4.** Expression levels of *Arabidopsis thaliana* genes involved in starch biosynthesis **(A)** or degradation **(B)**. Fragment Per Kilobase Million (FPKM) is presented during 47 h period of plants exposed to long day conditions (16 h light / 8 h dark). **Ref: (A)** APS (ADPGlcPPase small subunit, At5g48300); APL (ADPGlcPPase large subunit, At519220); GBSS (granule bound starch synthase, At1g32900); SSI – SSIV (starch synthases I to IV, At5g24300, At3g01180, At1g11720, At4g18240, respectively); SBE2.1 and SBE2.2 (starch branching enzyme 2.1 and 2.2, At5g03650 and At2g36390, respectively) and ISA2 (isoamylase 2, At1g03310). **(B)** PWD (phosphoglucan water dikinase, At5g26570); GWD (glucan water dikinase, At1g10760); SEX4 (starch excess 4, At3g52180); AMY3 (amylase 3, At1g69830); LSF1 (like SEX4 phosphoglucan phosphatase, At3g01510) and DPEI (disproportionating enzyme I, At5g64860).

that LSF2 together with SEX4 play an important role in the remobilization of transient starch at night (Santelia et al., 2011).

In addition, the mRNA levels of *AMY3* (amylase 3) and *DPE1* (disproportionating enzyme 1) were also analyzed. AMY3 is the only *endo*-amylase with a known function in starch granule degradation (Streb et al., 2012). Although this enzyme is not one of the most relevant in this process, it was involved in altering polysaccharide levels in debranched mutant lines (Delatte et al., 2006). The DPE1enzyme is responsible for recycling maltotriose, avoiding a blockage of starch degradation due to product accumulation(Critchley et al., 2001). Our results showed that the mRNA levels of *DPEI* and *AMY3* are increased in both OEOsttaSBE lines, between 2- and 3-fold, compared to the Col-0 line (Fig. 6A).

Finally, we found an increase of total amylase and glucan phosphatase activities (about 5 and 1.2-fold, respectively) in both OEOsttaSBE lines, suggesting that there would be a greater degradation of the polysaccharide (Fig. 6B). All together these data indicate that there is an increase in the degradation of the starch granule during the day, which suggests that there would be a restructuring of the starch *in planta* due to the expression of OsttaSBEIII.

### 3.5. *Metabolic profile of leaf extracts*

To evaluate the metabolic changes that occurs in OEOsttaSBE plants, we analyzed the profile of primary metabolites in 35-day-old plant leaves of all lines studied by gas chromatography coupled to mass spectrometry (GC–MS). The heat map of Fig. 7 shows the relative metabolite levels in OEOsttaSBE plants. Compounds whose levels were found decreased compared to the Col-0 control line were represented in green, while those in which an increase in their concentration was observed compared to control, were represented in red. The black color indicates similar metabolite levels compared to the control.

Among the sugars, OEOsttaSBE plants showed an increase between 1.5 and 2-fold in free glucose content. In contrast, fructose levels were decreased by about 20 % in OEOsttaSBE1 and 40 % for OEOsttaSBE2 plants; while we did not observe any significant changes in sucrose levels in the analyzed lines. Other metabolites such as serine, shikimate, and two saturated fatty acids, hexadecanoic (C16:0) and octadecanoic (C18:0) acid, were also increased. This is in agreement with higher lipid accumulation due to high glucose levels(Li et al., 2015). Moreover, the

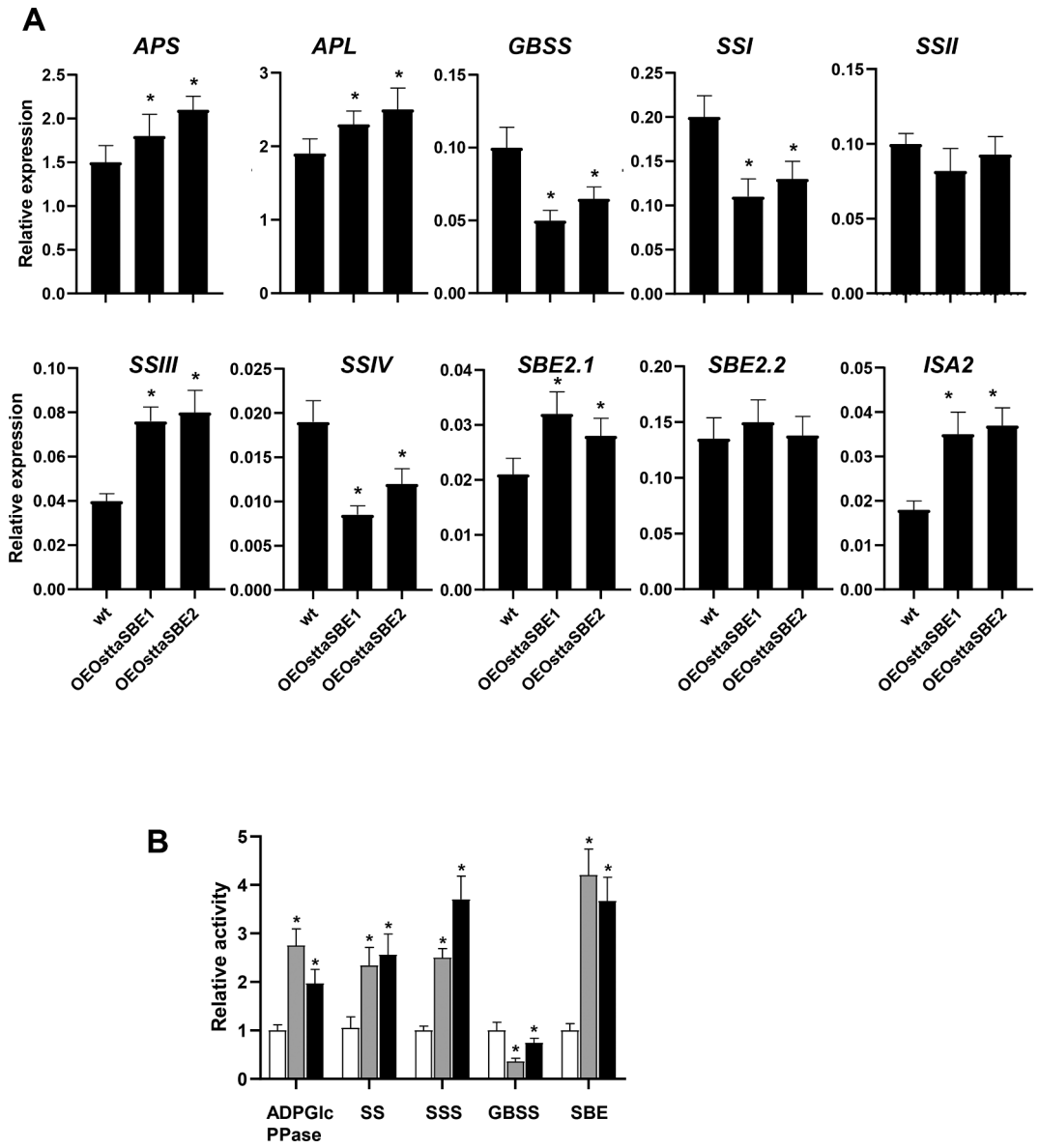



**Fig. 5. (A)**qRT-PCR analysis of genes involved in starch synthesis: *APS* (ADPGlcPPase small subunit, At5g48300); *APL* (ADPGlcPPase large subunit, At5g19220); *GBBS* (granule bound starch synthase, At1g32900); *SSI – SSIV* (starch synthases I to IV, At5g24300, At3g01180, At1g11720, At4g18240, respectively); *SBE2.1* and *SBE2.2* (branching enzymes 2.1 and 2.2, At5g03650 and At2g36390, respectively); *ISA2* (isoamylase 2, At1g03310). RNA was extracted from 35-days-old rosette *Arabidopsis* leaves. Asterisks show a statistically different result from the control value ($P < 0.05$). Columns represent mean values ± SD of three independent experiments. Relative expression levels are shown as fold change values with respect to *ACTIN2* mRNA levels. **(B)** Enzymatic activity of ADPGlc pyrophosphorylase (ADPGlcPPase), total starch synthase (SS), soluble starch synthase (SSS), granule bound starch synthase (GBSS) and branching enzyme (SBE) in crude extracts of 35-days-old rosette leaves of wild-type (white bars), OEOsttaSBE1 (grey bars) and OEOsttaSBE2 (black bars) lines. The activity of each enzyme in wild-type plants was used as a reference value. Values are the mean ± SD of three independent replicates and asterisks show a statistically different result from the control value ($P < 0.05$).

levels of some organic acids such as citrate and fumarate were slightly decreased in OEOsttaSBE plants compared to wild-type plants. However, no changes were observed in malate or succinate levels in both transgenic lines compared to control plants.

As previously described, in addition to amylose and amylopectin, native starches contain also small amounts of phosphate groups monosterified to glucose residues, mainly those from the amylopectin fraction (Blennow et al., 2002). We found an about 2-fold increase in phosphate-TMS (trimethylsilylated) content in the overexpressing lines. The rise in Pi levels is in accordance with the increase of glucose content due to the higher activity of starch degradative enzymes. These data reinforce our hypothesis where a greater degradation and/or remodeling of the starch granules occur upon expression of OsttaSBEIII.

## 4. Discussion

Numerous works have been reported on the modification of the expression level of SBEs *in vivo* in order to improve the quantity and quality of starch within plants. As described above, the final quality of the starch depends on structural characteristics, such as the ratio of amylose and amylopectin, the length and frequency of the branches, as well as the molecular and structural organization of the starch granules. These characteristics depend mainly on the activity and the particular properties of the SBEs. Therefore, SBEs are considered to be the major determinants of starch physical properties and structure(Li and Gilbert, 2016).

The model organism *Arabidopsis thaliana* has three genes that code

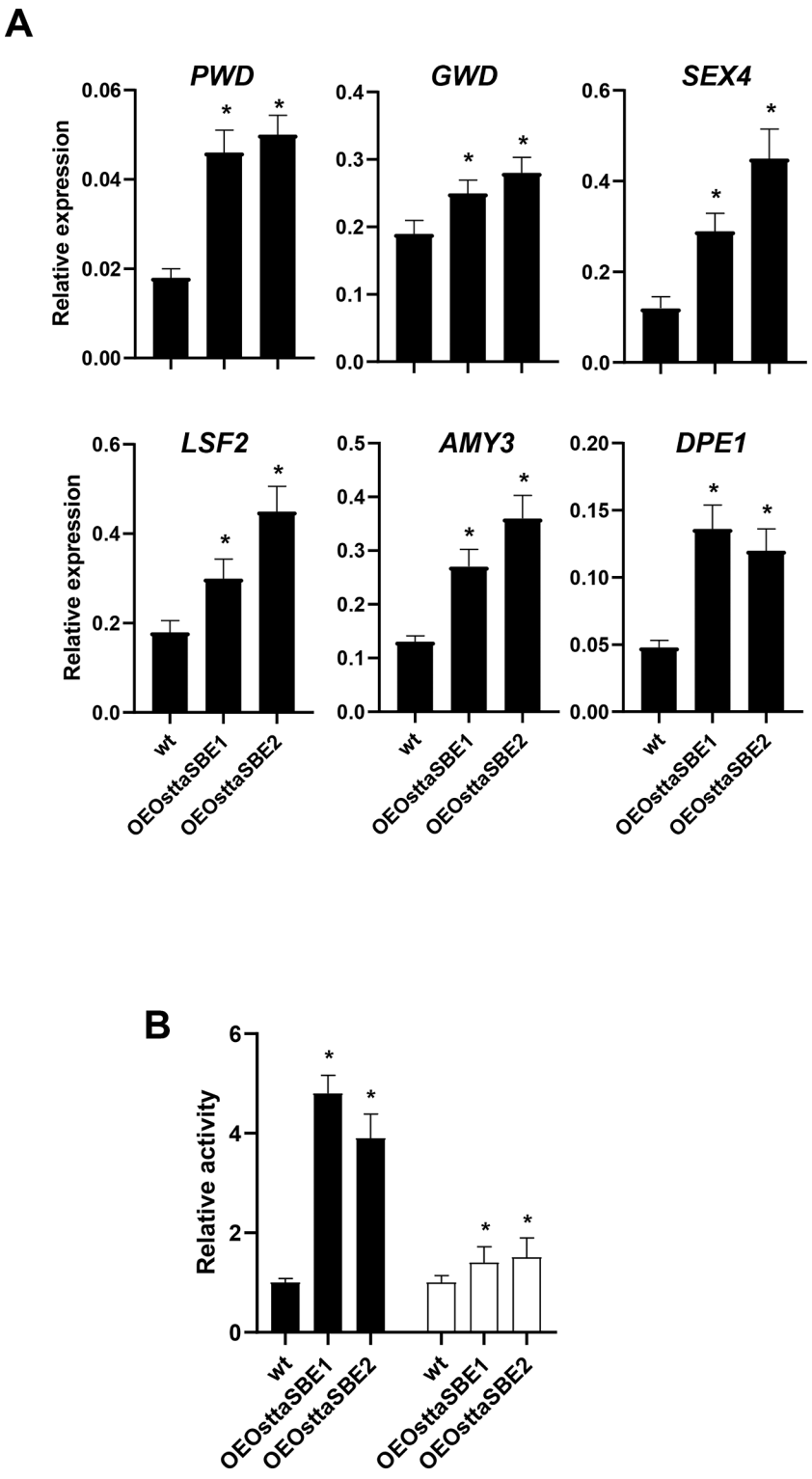



**Fig. 6. (A)**qRT-PCR analysis of genes involved in starch degradation: *PWD* (phosphoglycan water dikinase, At5g26570); *GWD* (glucan water dikinase, At1g10760); *SEX4* (starch excess 4, At3g52180); *AMY3* (amylase 3, At1g69830), *LSF1* (like SEX4 phosphoglucan phosphatase, At3g01510) and *DPE1* (disproportionating enzyme 1, At5g64860). RNA was extracted from 35-days-old rosette *Arabidopsis* leaves. Asterisks show a statistically different result from the control value ($P < 0.05$). Columns represent mean values ± SD of three independent experiments. Relative expression levels are shown as fold change values with respect to *ACTIN2* mRNA levels. **(B)** Total amylase (black bars) and glucan phosphatase activities (white bars) in crude extracts of 35-days-old rosette leaves of wild-type (wt) and transgenic lines. The activity of each enzyme in wt plants was used as a reference value. Values are the mean ± SD of three independent replicates and asterisks show a statistically different result from the control value ($P < 0.05$).

for branching enzymes, *AtBE1-3*; however, only two of them, *AtBE2* and *AtBE3*, which codes for SBE2.2 and SBE2.1, respectively, would produce functional enzymes classified as Class II SBEs, while *AtBE1*codes for an inactive protein (Dumez et al., 2006; Fisher et al., 1996; Han et al., 2007a, 2007b; Wang et al., 2010). It was reported that the absence of any of the SBE2 isoforms leads to a slight modification of the amylopectin structure, which suggests the superposition of functions between both isoforms. On the other hand, there is no accumulation of starch in the double mutant that lacks both enzymes (Dumez et al., 2006). The complementation of this double mutant line with SBEs from plants or of prokaryotic origin allowed to restore the synthesis of starch, obtaining insoluble granules composed of amylose and amylopectin. However, it was shown that the origin of the enzyme and its level of expression influence the structure of the starch produced (Boyer et al., 2016; Liu et al., 2016).

The classification of SBEs in two groups is in agreement with their different functions in amylopectin synthesis. *In vitro* analyzes of corn, rice and potato SBEs suggest that Class I enzymes act preferentially on

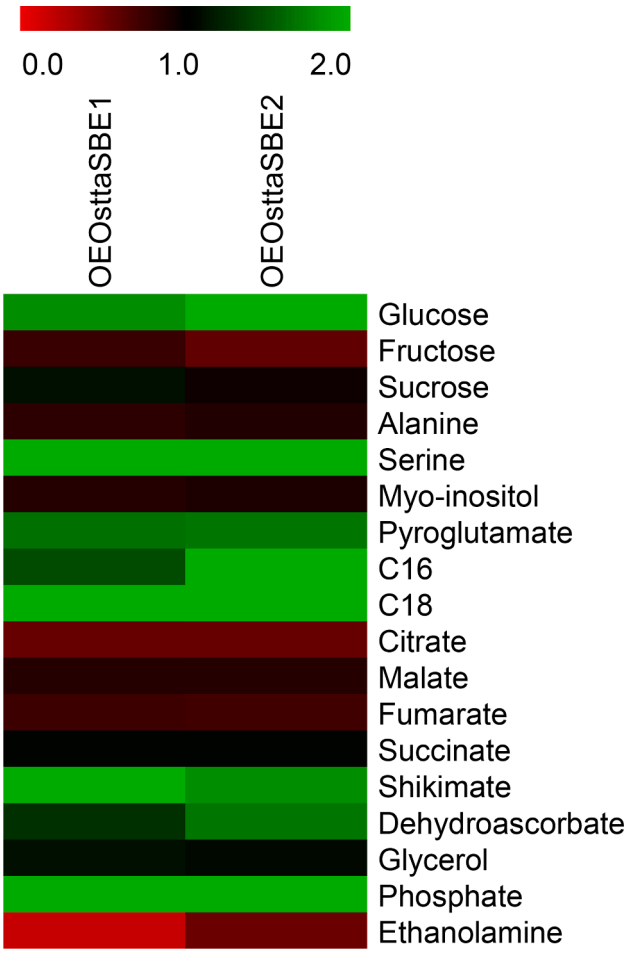



**Fig. 7.** GC–MS metabolite profiling in *Arabidopsis thaliana* 35-days-old rosette leaves OEOsttaSBE1 and OEOsttaSBE2 respect to the wild-type line. Green color show higher metabolite levels and red color show lower metabolite levels compared to wild-type plants. The data presented correspond to a mean of four biological replicates, and the visualization was performed using TM4:MultiExperiment Viewer 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

amylose by transferring longer glycosidic chains, while Class II enzymes act preferentially on branched substrates such as amylopectin, transferring shorter oligosaccharide chains (Guan and Preiss, 1993; Nakamura et al., 2010; Rydberg et al., 2001). Different models were proposed where both BE classes participate in determining the cluster structure of amylopectin. Thus, Class I BEs start branching by transferring long or branched glucan chains. Subsequently, Class II proteins act to create shorter and more numerous chains which are elongated by SSs to create clusters (Nakamura et al., 2010). Following the BEs reactions, a certain degree of debranching occurs. This fact would be related to the control the number of branches and position by promoting amylopectin crystallization (Myers et al., 2000; Zeeman et al., 2010).

It has been reported that despite the high structural conservation of the two SBE classes, significant alterations in starch properties are only observed when the genes coding for Class II enzymes are mutated or repressed (Lu et al., 2015). The loss of SBEI activity in rice or corn endosperm but also in potato tubers caused only small differences in the distribution of chain lengths or branch points of amylopectin without major alterations in starch properties and content (i.e. gelatinization or digestibility) (Blauth et al., 2002; Satoh et al., 2003; Xia et al., 2011). On the other hand, the loss of SBEs from Class II in species such as rice, potato, pea and barley, among others, resulted in a decrease in starch amount but with a high apparent amylose content (Bhattacharyya et al., 1990; Jobling et al., 1999; Mizuno et al., 1993; Stinard et al., 1993; Regina et al., 2006; Regina et al., 2010). This apparent high amylose levels was possibly due to the accumulation of amylopectin with a lower degree of branching (Boyer et al., 1976). A more marked phenotype was reported in potato tubers and barley by suppressing both, SBE1 and SBE2 (Schwall et al., 2000; Carciofi et al., 2012).

As mentioned above, a large number of publications reported the characterization of SBE null mutants in several plant species. It was shown that SBE deficiency results in a lower content of starch with a high amylose phenotype (more than 80 % amylose) and also alterations in plant growth and development (Regina et al., 2006; Schwall et al., 2000; Shaik et al., 2016). In contrast, there are few studies on the overexpression of these enzymes and their effect on the quality of the starch accumulated *in planta* (Brummell et al., 2015; Tanaka et al., 2004). Plant SBEs have a particular structure, composed of a CBM48 in their N-terminal region (Abe et al., 2014), which would be responsible for the association of the enzyme with the starch granule. As mentioned above, *O. tauri* possesses three branching enzyme isoforms: OsttaSBEI, OsttaSBEII, and OsttaSBEIII (Hedin et al., 2017; Xia et al., 2011). While both OsttaSBEI and II present a single CBM48; the OsttaSBEIII isoform contains two CBMs at its N-terminal end, one belonging to CBM41 family and the other from CBM48 family, that is conserved with plant SBEs. Thus, we postulated that the presence of a tandem repeat of two CBMs in OsttaSBEIII would have important consequences on the functional properties of the enzyme (Hedin, et al., 2017). For this reason, we decided to carry out this study with this particular SBE isoform and evaluated the consequences of the expression of this novel algal enzyme on the content and quality of starch in *Arabidopsis* plants.

The results showed that overexpression of OsttaSBEIII resulted in higher starch accumulation at the end of the day, and a small granule size with an increase degradability, without significant alterations in amylose:amylopectin ratio. The higher amount of starch detected at the end of the day is in agreement with the higher SS and ADPGlcPPase activities. It was described that ADPGlcPPase is the key enzyme in the regulation of starch synthesis (Ball and Morell, 2003; Kang et al., 2013). Moreover, although our results showed a decrease in the transcript levels of some genes coding for SSSs, such as *SSI* and *SSIV*, we found an increase in the expression of *SSIII* with no significant changes in the expression of *SSII*, resulting in an increase of total SS and SSS activities in both transgenic lines. It was previously reported that the absence of some SS isoforms increased total SS activity in different species such as corn and rice (Fujita et al., 2007; Singletary et al., 1997; Zhang et al., 2008). Moreover, it was reported that the overexpression of SSIII in tomato plants produced a greater amount of starch and an alteration of the granule shape (Miao et al., 2017). On the other hand, it was described that starch metabolism enzymes interact to form a multienzyme complex, and that when the amount of one of the isoforms is reduced, it could be complemented by another *in vivo* (Hennen-Bierwagen et al., 2008). Thus, the decrease in the amount of a particular enzyme or the overexpression of another isoform could alter the conformation and / or stoichiometry of this complex, which would result in a different activity and an alteration of the final structure of the granule.

Although the transgenic plants showed higher levels of starch than the control plants, we also found an increase in the expression and activity of starch degradative enzymes. It was previously observed that there is simultaneous synthesis and degradation of starch in chloroplasts from spinach during the light period (Stitt and Heldt, 1981). Furthermore, *Arabidopsis* mutants deficient in starch degradation showed higher levels of starch under constant light, suggesting that under these conditions the polysaccharide levels are also determined by simultaneous synthesis and degradation (Baslam et al., 2017; Caspar et al., 1991). It was also described that *Arabidopsis* plants subjected to osmotic stress increase starch degradation during the light period (Thalmann et al., 2016). The remobilization of fixed carbon from starch during the day allow that the available light energy would be used for the

biosynthesis of different molecules such as amino acids, lipids and other structural precursors required for growth (Fernandez et al., 2017). This is in agreement with the observed increase in shikimate and other amino acids as well as the increase of fatty acids observed in the metabolomic experiments.

In addition, the overexpression of OSttaSBEIII also produced a higher phosphate content. Starch phosphorylation occurs mainly in the longer branches of amylopectin, with only a small proportion in the short chains of amylopectin, and is essentially absent in amylose (Blennow et al., 2002). When PWD and GWD have phosphorylated the starch granule, this structure becomes more accessible to the polysaccharide degradation machinery, and it is proposed that SEX4 acts immediately after eliminating the phosphate fraction (Zeeman et al., 2010). It was reported that the starch phosphate content strongly influenced starch granule morphology, amylose content and starch fine structure (Mahlow et al., 2016). The increase in phosphate content, as well as the increase in glucose levels are also in accordance with the increase in the expression and activity of starch degradative enzymes. Thus, the final appearance of the starch granules observed in OEOsttaSBE plants would be a complex mechanism that depends on various factors, not only by the activity of OsttaSBEIII, but also by the balance between the activities of starch synthesis enzymes and the increased activity of degradative enzymes during the light period, as well as by an alteration in the remobilization of carbon in OEOsttaSBE plants.

Although it was reported that in *Arabidopsis* leaves the average size of the starch granules is 1.8 μm, more than 50 % of the granules have a diameter between 2 and 3 μm (Crumpton-Taylor et al., 2012).The overexpression of OsttaSBEIII produced a higher proportion of small starch granules, between 85 and 92 % of granules of<2 μm. This type of small starch granules is generally found in microalgae. About the 80 % of the starch granules accumulated in these organisms have a narrow size distribution, between 0.5 and 2 μm (Tanadul et al., 2014).Granule size, size distribution and shape are among the most important morphologically distinguishing factors of starches from different origins (Lindeboom et al., 2004). In recent years there is a growing interest in obtaining small starch granules due to their high surface area and surface pores that enhance water uptake (Tanadul et al., 2014). The larger superficial area of small granules produces an increase in viscosity, provides more sites for crosslinking reactions for starch modification and provides a better surface for adhesion and also increase their degradability (Niu et al., 2019). Our results showed a higher degradability of the starch granules produced in OEOsttaSBE lines. It was reported that small starch granules degraded faster than larger ones *in vitro* and during storage conditions (Niu et al., 2019). The use of small granules is of particular interest in applications where starch is used as filler, such as the paper industry, where these types of granules provide a lower gelatinization temperature and give a smoother paste texture (Santelia and Zeeman, 2011). In addition, small granules are also used in food industries for free-fat food formulation, as additive material in pharmaceutics, cosmetics and plastics and biofilm or biofuel production (Lindeboom et al., 2004; Tanadul et al., 2014).

In conclusion, we presented a known strategy but with a new protagonist for restructuring starch granules in-planta by the overexpression of a SBE from *O. tauri,* which is structurally and functionally different from plant SBEs. The transgenic plants showed no penalty in development, an increased starch content with a high proportion of small size granules, which led to a higher degradability. Thus, the overexpression of OsttaSBE would have an important impact on the final structure of amylopectin. It would be interesting to know the characteristics of the starch produced and the branching points in plants of agronomic interest such as wheat, maize or barley. Due to the special interest to develop new approaches to obtain plants that synthesize and accumulate a higher quantity of starch with novel characteristics, our findings are particularly desirable for numerous biotechnological applications in food and non-food industries.

## Author's contributions

NH, JB, MIZ, HP, DFGC, MVB conceived, designed and analyzed the experiments. NH, JB performed the experiments. NH, JB, MIZ analyzed the results. JB, HP, DFGC and MVB wrote the manuscript. All authors read and approved the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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NH was doctoral fellow from CONICET. JB, DFGC, MIZ and MVB are research scientists from CONICET. This work was supported by ANPCyT (to MVB – HRP, PICT 2018 01440).

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2023.100131.

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