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The down-regulation of the genes encoding Isoamylase 1 alters the starch composition of the durum wheat grain

Francesco Sestili[a], Francesca Sparla[b], Ermelinda Botticella[a], Michela Janni[a,c,1], Renato D'Ovidio[a], Giuseppe Falini[d], Lucia Marri[e], Jose A. Cuesta-Seijo[e], Stefano Moscatello[f], Alberto Battistelli[f], Paolo Trost[b], Domenico Lafiandra[a,*]

[a] Department of Agricultural and Forestry Sciences DAFNE, University of Tuscia, Via S. Camillo de Lellis, SNC, 01100 Viterbo, Italy
[b] Department of Pharmacy and Biotechnology FABIT, University of Bologna, Via Irnerio 42, 40126 Bologna, Italy
[c] National Research Council CNR-Istituto di Bioscienze e Biorisorse, Via G. Amendola, 165, 70126 Bari, Italy
[d] Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, 40126 Bologna, Italy
[e] Carlsberg Research Laboratory, Gamle Carlsberg Vej 10, Copenhagen, V DK-1799, Denmark
[f] National Research Council CNR-Istituto di Biologia Agroambientale e Forestale, Viale Marconi 2, 05010 Porano, TR, Italy

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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 partial down-regulation of *Isa1* and revealed a pleiotropic effect on the level of transcription of genes encoding other isoamylases, pullulanase and sucrose synthase. The polysaccharide content of the transgenic endosperms was different from that of the wild type in a number of ways, including a reduction in the content of starch and a moderate enhancement 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 after a treatment with hydrochloric acid, 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.

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-

Abbreviations: CSLF6, cellulose synthase like F6; DP, degree polymerization; HPAEC-PAD, High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection; ISA1, Isoamylase 1; ISA2, Isoamylase 2; ISA3, Isoamylase 3; PUL, pullulanase; qRT-PCR, quantitative real time PCR; RNAi, RNA interference; SEM, scanning electron microscopy; SUSY, sucrose synthase.

* Corresponding author.
E-mail addresses: francescosestili@unitus.it (F. Sestili), francesca.sparla@unibo.it (F. Sparla), e.botticella@unitus.it (E. Botticella), michela.janni@ibbr.cnr.it (M. Janni), dovidio@unitus.it (R. D'Ovidio), giuseppe.falini@unibo.it (G. Falini), lucia.marri@carlsberglab.dk (L. Marri), JoseA.Cuesta.Seijo@carlsberglab.dk (J.A. Cuesta-Seijo), stefano.moscatello@ibaf.cnr.it (S. Moscatello), alberto.battistelli@ibaf.cnr.it (A. Battistelli), paolo.trost@unibo.it (P. Trost), lafiandr@unitus.it (D. Lafiandra).
[1] Present address: CNR- Istituto Materiali Speciali per l'Elettronica e Magnetismo, Parco Area delle Scienze 37 A, 43124 Parma, Italy.

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1,6 linkages where at least three α-1,4-linkages lie adjacent to the ramification [10]. Three classes of isoamylases have been recognized, designated ISA1, ISA2 and ISA3 [11,12]. Two different models have been proposed to explain the role of ISA enzymes in amylopectin biosynthesis. The trimming model proposes that ISAs remove the improperly positioned branched chains [13–15]. The second model hypothesizes that ISA enzymes have an indirect role in the synthesis of amylopectin, cleaving the water-soluble polysaccharide (WPS) in the amylopasts and permitting to the branching enzymes to use as substrate only the pre-amylopectin [16]. Whichever the model, a further study reported that ISA1 and ISA2 are involved in amylopectin synthesis; whereas ISA3 seems to play a role, together with pullulanes, in starch degradation [11]. The absence of ISA1 activity in potato tubers and grains of maize, rice and barley produces the so-called *sugary* phenotype, in which starch granule morphology is radically altered, along with a substantial increase in phytoglycogen content [4,9,17–21]. ISA1 complexes with ISA2, forming a structure widely considered to be important for the starch granule initiation [7,16,18,20–22]. Utsumi et al. [23] demonstrated that in rice, endosperms lacking the ISA1 homo-oligomer are both deficient in starch and have an overabundance of water-soluble malto-dextrins. According to both Kubo et al. [4] and Utsumi et al. [23], the absence of ISA2 is not associated with an increase in phytoglycogen content in rice, but others have confirmed that both ISA1 and ISA2 activity is required to produce normal starch [7,18,20,24]. In wheat the genes encoding ISA1 have been successfully isolated [8,25], but as yet neither loss-of-function mutants nor transgenically derived *Isa1* knockouts have been identified, so the effect of 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 and screening of transgenic plants

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. [26]. The *Isa1* sequence from base 584–1071 (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 [27], using, respectively, the *SalI*/*KpnI* and *XbaI*/*PstI* restriction sites, to produce the construct pRDPT-ISA1(RNAi) (Fig. S1). An endosperm-specific promoter [28,29] 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 [30] in a 3:1 molar ratio. The bombardment target was immature cv. Svevo embryos harvested at 15 day 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. [31]. 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 [28].

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. [28].

The primer pairs used for the detection of *Isa1*, *Isa2*, *Isa3* and *Pul* transcripts have been reported by Kang et al. [32]; 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. [33].

2.4. Measurement of non structural carbohydrates

Samples for each genotype were obtained grinding four caryopses to a fine powder in a mortar (four replicates). The ground powder was used for carbohydrate analysis. For soluble sugar determination 10 mg of powder were extracted in screw cap plastic tubes with 1 mL of 80% ethanol at 80 °C for 1 h with vigorous shaking in a block heater. After centrifugation (13000*g* for 5 min) the supernatant was dried in the Concentrator Plus (Eppendorf Italia s.r.l.), the dry sample was re-suspended in 1 mL of water and analysed.

Soluble carbohydrates were analysed as in Santi et al. [34] with minor modifications. The analytical column used was the Dionex™ CarboPac™ PA210-Fast-4 μm Columns (Sunnyvale, CA U.S.A.). Elution was obtained at 30 °C with an isocratic flux at 0.8 mL min^{-1} of 12 mm NaOH. Sugars were identified by comparison of the retention time with appropriate standards (Sigma Aldrich, St. Louis, USA)

For phytoglycogen and water soluble α-glucans determinations, seed powder (30 mg) was extracted for one h in 0.8 mL of water at 4 °C with continuous shaking. Phytoglycogen was purified as in Powell et al. [9]; shortly, phytoglycogen was precipitated by adding 4 vol of pure ethanol to the water extracted supernatant and recovered as the pellet after centrifugation at 4000*g* for 10 min. After washing with pure cold ethanol, the pellet was re-suspended in water, autoclaved at 120 °C for 20 min and hydrolysed with 60 units of amyloglucosidase and 4 units of α-amylase (Sigma Aldrich, St. Louis, USA) for one h at 50 °C. Free glucose originated by phytoglycogen enzymatic hydrolysis was quantified with a spectrophotometric coupled enzymatic assay as in Famiani et al. [35]. The supernatant remaining after phytoglycogen precipitation was dried and re-suspended in 1.5 mL of 50 mM sodium acetate buffer (pH 4.6). After addition of 60 units of amyloglucosidase and 4 units of α-amylase (Sigma Aldrich, St. Louis, USA) and incubation for one h at 50 °C released glucose was analysed as described for phytoglycogen [35].