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TILLING mutants of durum wheat result in a high amylose phenotype and provide information on alternative splicing mechanisms

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ABSTRACT

The amylose/amylopectin ratio has a major influence over the properties of starch and determines its optimal end use. Here, high amylose durum wheat has been bred by combining knock down alleles at the two homoelogous genes encoding starch branching enzyme IIa (*SBEIIa-A* and *SBEIIa-B*). The complete silencing of these genes had a number of pleiotropic effects on starch synthesis: it affected the transcriptional activity of *SBEIIb*, *ISA1* (starch debranching enzyme) and all of the genes encoding starch synthases (*SSI*, *SSIIa*, *SSIII* and *GBSSI*). The starch produced by grain of the double *SBEIIa* mutants was high in amylose (up to ~1.95 fold that of the wild type) and contained up to about eight fold more resistant starch. A single nucleotide polymorphism adjacent to the splice site at the end of exon 10 of the G364E mutant copies of both *SBEIIa-A* and *SBEIIa-B* resulted in the loss of a conserved exonic splicing silencer element. Its starch was similar to that of the *SBEIIa* double mutant. G364E *SBEIIa* pre-mRNA was incorrectly processed, resulting in the formation of alternative, but non-functional splicing products.

1. Introduction

Grain wheat starch comprises a combination of the two glucan polymers amylose and amylopectin. Amylopectin is produced by the concerted action of several classes of starch synthase (SSI, SSII and SSIII), branching (SBEI, SBEIIa, and SBEIIb) and debranching enzymes (isoamylases ISA1, ISA2, ISA3 and limit dextrinase [LD]); in contrast, the sole enzyme involved in amylose synthesis is the granule-bound starch synthase I (GBSSI) [1]. Durum wheat (*Triticum turgidum* subsp. *durum*) is a widely cultivated crop species used for the manufacture of pasta and couscous (along with other similar products in Western Asia and North Africa) and forms a major ingredient in both leavened and unleavened bread throughout the Mediterranean Basin.

The amylose to amylopectin ratio has a major effect on pasta quality [2,3]. High amylose starch has a higher resistant starch (RS) content; like dietary fibre, its consumption can be beneficial for human health [4–7]. About 25% of wheat starch comprises amylose, but this proportion can be increased by suppressing one or more of the genes – particularly *SSII* and *SBEIIa* – involved in amylopectin synthesis [8–16].

There has been a recent resurgence of interest in mutagenesis, both as a genetic and a breeding tool [17–19]. The TILLING (Targeting Induced Local Lesions IN Genomes) approach combines chemical mutagenesis with DNA-based screening to identify mutations to a specific gene target [20]. First developed in the model species *Arabidopsis thaliana* [20], it has been applied very widely among crop species [21]. Several wheat TILLING projects have been initiated, targeting either agronomic or quality traits [11,13–15,22–28]. The study and characterization of loss-of-function and missense mutations can contribute to understand molecular processes not well-established, as the pre-mRNA processing or the catalytic and regulatory mechanisms of target enzymes. Regard to pre-mRNA processing, the role of splice

Abbreviations: AC, amylose content; DP, Aday post anthesis; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; GBSSI, granule bound starch synthase; HGW, hundred grain weight; HRM, high resolution melting; ISA1, 2, 3, isoamylase 1, 2, 3; LD, limit dextrinase; qRT-PCR, quantitative real time-PCR; RS, resistant starch; RT-PCR, reverse transcription-PCR; SBEIIa, IIb, starch branching enzyme IIa, IIb; SSI, II, III, starch synthase I, II, III; TILLING, targeting induced local lesions in genomes; TS, total starch.

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junction sites has been widely studied in humans, animal and plant kingdom. However, the correct pre-mRNA splicing not only requires the presence of splicing sites, but also additional intronic and exonic regulatory sequences, involved in the recognition of nearby splice sites [29].

The role of some *cis*-regulatory elements, such as exonic splicing enhancers (ESE) and exonic splicing silencers (ESS), has been investigated in humans, while it remains still poorly understood in plant. In humans it was observed that the mutations located in ESS and ESE elements may modify consensus 5′- or 3′-splice sites, thereby causing aberrant messengers [30,31]. Although the alternative splicing in plant is underexplored, compared to the human genome, it has been suggested that genome-wide computational analysis can contribute to provide new knowledge [32].

Here, the production and characterization of high amylose durum wheat has been described, obtained by pyramiding SBEIIa mutations, previously identified [26]. The molecular characterization of a missense mutation responsible for an incorrect processing of *SBEIIa* pre-mRNA is also given.

2. Materials and methods

2.1. Plant material

The durum wheat cv. Svevo and three *SBEIIa* mutants (*SBEIIa-A*$^{-}$, *SBEIIa-B*$^{-}$ and the mutant selection G364E) previously identified using a TILLING [26] were vernalized at 4 °C for three weeks. The plants were grown at 20/24 °C with a 16 h light period and light intensity of 300 μE m^{-2} s^{-1}. M_5 generation progeny of G364E and F_3 progeny of the cross *SBEIIa-A*$^{-}$ × *SBEIIa-B*$^{-}$ were grown in the field. The G364E mutant harbours a missense mutation responsible for a residue alteration in the α-amylase catalytic domain.

2.2. High resolution melting (HRM) genotyping

F_2 progeny bred from the cross *SBEIIa-A*$^{-}$ × *SBEIIa-B*$^{-}$ which lacked fully functional *SBEIIa* alleles at both loci were identified using an HRM-based assay. A nested PCR strategy was used, in which the first reaction generated the allele-specific amplicons, as described in Botticella et al. [13]. The second reaction, made up to a volume of 10 μL, included as template a 1 μL aliquot of a 1:60 dilution of the first reaction, 5 μL GoTaq® Hot Start Colorless Master Mix (Promega, Madison, USA), 1 μL LCGreen Plus (Idaho Technology Inc., Salt Lake City, USA) and 1.5 μL of each primer to give a final primer concentration of 0.5 μM. The sequence of all of the nested PCR primers used is given in Supplementary Table 1. The PCR program comprised a 95 °C/5 min initial denaturation, followed by 39 cycles of 95 °C/30 s, 60 °C/20 s, 72 °C/20 s. At the end of the final extension step, the reaction was held at 95 °C for 30 s, then at 25 °C for 60 s. The second PCR was run in 96 well Frame-Star plates (4titude Ltd, Surrey, UK) and the "Amplicon genotyping" routine included with the LightScanner instrument (Idaho Technology Inc.) was used to analyze the melting curves.

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.009.

2.3. Reverse transcription PCR (RT-PCR)

Transcripts of *SBEIIa-A* and *SBEIIa-B* genes of the G364E mutant were assayed in 50 μL RT-PCRs containing 25 μL Hot GoTaq® Green Master Mix (Promega), 2 μL cDNA and 0.5 μM of each of the G364E-F and -R primers (Supplementary Table 1). The reactions were subjected to an initial denaturation (95 °C/15 min), followed by 39 cycles of 95 °C/30 s, 60 °C/30 s, 72 °C/60 s, and a final extension of 72 °C/5 min. The amplicons were electrophoresed through a 2% agarose/TBE gel and individual fragments were recovered using a NucleoSpin® Gel and a PCR Clean-up kit (Macherey-Nagel, Düren, Germany). DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany).

2.4. Quantitative real time (qRT-PCR)

Total RNA was extracted from snap-frozen immature (21 days post anthesis [DPA]) grains of greenhouse-grown plants, following Laudencia-Chingcuanco et al. [33], as modified by Sestili et al. [12]. The RNA formed the template for the synthesis of cDNA, based on a QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Subsequent 20 μL qRT-PCRs were performed using an iCycler iQ instrument (Bio-Rad Laboratories, Hercules, CA, USA); each reaction contained 10 μL iQ™ SYBR Green Supermix 2× (Bio-Rad Laboratories), 1 μL cDNA and 0.5 μM of each primer. The primer pairs used to assay the transcription of *SSI*, *SSII*, *SSIII*, *SBEI*, *SBEIIb*, *ISA1*, *LD* and *GBSSI* have been reported elsewhere [12]. The *SBEIIa-A* and *SBEIIa-B* primer pair sequences are given in Supplementary Table 1. *Ta2526* was used as the reference sequence, as recommended by Nemeth et al. [34]. The $2^{-\Delta\Delta CT}$ method was used to estimate relative levels of transcript abundance [35]. Each qRT-PCR data point represented the mean of three technical replicates for each biological sample. Mean relative transcript abundances were compared using the Student's *t* test.

2.5. Determination of amylose (AC), total starch (TS) and RS content

The starch formed by field-grown F_3 grains from the cross *SBEIIa-A*$^{-}$ × *SBEIIa-B*$^{-}$ was characterized from two independent samples per genotype, based on at least six technical replicates per sample. For the double *SBEIIa* mutant, the analyses were performed on two sister lines. AC was determined from starch isolated following Zhao and Sharp [36], using an iodometric assay developed by Chrastil [37]. A standard curve was prepared using a mixture of potato amylose (Fluka, Neu-Ulm, Germany) and amylopectin isolated from waxy durum wheat. TS and RS content were measured from wholemeal flour using kits provided by Megazyme Pty Ltd. (Wicklow, Ireland). In critical lines, RS was additionally estimated from purified starch. Statistical analysis was performed using Tukey's test.

2.6. Starch granule morphology

Starch was extracted as described above, critical-point dried in a Balzer's apparatus equipped with a liquid CO_2 inlet and metal-shadowed in a gold sputtering unit equipped with an argon inlet. The preparations were examined using a Cambridge Stereoscan 240 scanning electron microscope.

2.7. Isolation and sequencing of the SBEIIa-B allele

Genomic DNA was extracted from cv. Svevo and the G364E mutant using a NucleoSpin® Plant II kit (Macherey-Nagel). The entire coding region, split into 22 exons, was amplified using five primer pairs (Supplementary Table 2) in 50 μL reactions comprising 100 ng genomic DNA, 1× GoTaq® Hot Start Colorless Master Mix (Promega) and 0.5 μM of each primer. The amplification regime was as described above for the RT-PCR, except that the extension time was extended to 1.5–3 min. The resulting amplicons were sequenced by Eurofins Genomics (Ebersberg, Germany).

Supplementary Table 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.009.