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ORIGINAL ARTICLE

Enhancing grain size in durum wheat using RNAi to knockdown *GW2* genes

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Abstract

Key message **Knocking down GW2 enhances grain size by regulating genes encoding the synthesis of cytokinin, gibberellin, starch and cell wall.**

Abstract Raising crop yield is a priority task in the light of the continuing growth of the world's population and the inexorable loss of arable land to urbanization. Here, the RNAi approach was taken to reduce the abundance of *Grain Weight 2* (*GW2*) transcript in the durum wheat cultivar Svevo. The effect of the knockdown was to increase the grains' starch content by 10–40%, their width by 4–13% and their surface area by 3–5%. Transcriptomic profiling, based on a quantitative real-time PCR platform, revealed that the transcript abundance of genes encoding both cytokinin dehydrogenase 1 and the large subunit of ADP-glucose pyrophosphorylase was markedly increased in the transgenic lines, whereas that of the genes encoding cytokinin dehydrogenase 2 and gibberellin 3-oxidase was reduced. A proteomic analysis of the non-storage fraction extracted from mature grains detected that eleven proteins were differentially represented in the transgenic compared to wild-type grain: some of these were involved, or at least potentially involved, in cell wall development, suggesting a role of GW2 in the regulation of cell division in the wheat grain.

Introduction

Durum wheat (*T. turgidum* ssp. *durum*) is an allotetraploid species used primarily for the preparation of pasta, couscous and bulgur. The crop is produced mainly in southern Europe, North Africa and North America, but significant quantities are also produced in the central Asia and India (Kadkol and Sissons 2016). Although the productivity of

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This paper is dedicated to the memory of our colleague and friend Prof. Renato D'Ovidio.

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durum wheat is below that of bread wheat, the demand for its grain has been rising from year to year.

Crop yield is both a genetically complex trait and one which is strongly influenced by environmental factors. The grain yield of wheat is conventionally expressed as the product of a number of sub-traits, namely the mean weight of each grain, the number of grains set per spike and the number of fertile spikes per unit area (Sreenivasulu and Schnurbusch 2012). Although various genetic analyses have mapped a number of loci associated with wheat grain size in durum wheat, the species' tetraploid nature tends to hinder attempts to isolate the genes underlying these effects (Bednarek et al. 2012; Hong et al. 2014; Simmonds et al. 2016). The situation is rather different in the diploid species rice, where a number of genes controlling grain size and shape have been mapped and/or isolated (Xing and Zhang 2010; Zhang et al. 2013). A prominent such gene is *Grain Weight 2* (*OsGW2*) which encodes a RING-type protein exhibiting E3 ubiquitin ligase activity and thought to be involved in the regulation of cell division (Song et al. 2007). In genotypes lacking a functional copy of *GW2*, grain fill is accelerated, leading to an increase in grain weight and width, while in *GW2* over-expressors, grain size is diminished (Song et al. 2007). The maize (a cryptic tetraploid) genome harbors two copies of *GW2*; sequence variation in the promoter region of one of these copies has been significantly associated with variation in both the width and weight of the kernels (Li et al. 2010). Meanwhile in the hexaploid bread wheat genome, *GW2* homologs have been mapped to the short arm of each of the homeologous group 6 chromosomes (Su et al. 2011). A negative relationship has been established between the abundance of the A genome homeolog (*TaGW2-A1*) and grain weight (Su et al. 2011; Zhang et al. 2013; Jaiswal et al. 2015; Simmonds et al. 2016), while sequence variants in *TaGW2-A1*'s promoter region have been associated with diversity both with respect to the gene's transcript abundance and grain width (Su et al.2011; Zhang et al. 2013; Jaiswal et al. 2015). Simmonds et al. (2016) have reported an induced null mutant for *TaGW2-A1*; its associated phenotype was a significant increase in the mean weight, width and length of the grain.

The RNA interference (RNAi) platform, in which synthetic RNA sequences are introduced into cells in order to selectively and robustly induce the suppression of a specific target gene, has twice been used to study the effect of knocking down all three bread wheat *TaGW2* homeologs, but the results obtained have been inconsistent; thus, while Bednarek et al. (2012) observed a reduction in grain size and cell number in the endosperm, Hong et al. (2014) reported a significant increase in both grain width and weight. Here, a similar approach was taken, this time at the durum wheat level. Care was taken in designing the transgene to include a grain-specific promoter, so that alterations in the expression of the *TaGW2* homeologs in non-grain tissue were avoided.

Materials and methods

Plant material and growing conditions

Seedlings of wild-type (WT) durum wheat cultivar (cv.) Svevo and three derived RNAi transgenics were vernalized by holding at 4 °C for 4 weeks, after which the plants were raised in a regime of 20–28 °C during the light period (16 h) and 16–24 °C during the dark period (8 h); the light intensity was 300 μE m^{-2} s^{-1}.

Isolation of *GW2* sequences from durum wheat and their phylogeny

GW2-A1 and *GW2-B1* sequences were isolated from NCBI database (GenBank accession KP49899.1) and the durum wheat EST database available at University of Davis. A phylogenetic analysis, based on their deduced polypeptide sequences, was carried out using the neighbor-joining method, as implemented in the MEGA v7 software package (www.megasoftware.net/), applying 1000 bootstrapping replications (Felsenstein 1985).

The RNAi cassette and the biolistic transformation of immature embryos

The segment of *TaGW2-B1* (GenBank accession KJ697755.1) lying between nucleotides 838 and 1259 was PCR amplified from a template of RNA extracted from cv. Svevo grains harvested at 21 days post-anthesis. Extraction of the necessary RNA and its conversion to ss cDNA followed protocols described by Sestili et al. (2015). The PCRs were based on the primer pair XbaI/SalI/BamHI-GW2F and XbaI/XhoI/KpnI-GW2R (Table S1) in a 50 μL reaction containing 2 μL cDNA, 25 μL GoTaq®Hot Start Colorless Master Mix (Promega, Madison, WI, USA) and 0.5 μM of each primer. The resulting amplicon was introduced in both its sense and antisense direction into the plasmid pRDPT (Tosi et al. 2004) using, respectively, the SalI/KpnI and XbaI/XhoI restriction sites. The result was a construct termed pRDPT-GW2(RNAi) (Fig. S1). The transgene was placed under the control of an endosperm-specific promoter (Sestili et al. 2010). About 3000 immature cv. Svevo embryos were co-bombarded with a 3:1 molar ratio of pRDPT-GW2(RNAi) and pAHC20 (Christensen and Quail 1996), as described by Sestili et al. (2010). The pAHC20 construct harbors *Bar*, the product of which confers resistance to the herbicide bialaphos, thereby providing a selectable marker for recognizing transgenic regenerants.

PCR-based validation of putative transgenic plants

Genomic DNA was extracted from young leaves of T_0 regenerants using a NucleoSpin® Plant II Mini Kit (Macherey–Nagel, Düren, Germany). The presence of the two transgenes was PCR validated, using as primer pairs both pRDPT-Fw/Rev and BarFw/Rev (Table S1). Each 20 µL reaction contained 10 µL Hot GoTaq® Green Master Mix (Promega, Madison, WI, USA), 50 ng genomic DNA and 0.5 µM of each primer and was subjected to a 95 °C/2 min denaturation, followed by 35 cycles of 95 °C/1 min, 60 °C/1 min, 72 °C/1 min, ending in a final extension step of 72 °C/5 min. The amplicons were electrophoretically resolved through 1.5% agarose gels and visualized by EtBr staining.

RNA extraction and transcription profiling

Total RNA was extracted from embryos formed in WT and RNAi transgenic grains harvested 21 days post-anthesis, using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). A 1 µg aliquot of RNA represented the template for the synthesis of ss cDNA, achieved using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative real-time PCRs (qRT-PCRs) were performed using a CFX 96 Real-Time PCR Detection System device (Bio-Rad, Hercules, CA, USA), following the procedure described by Camerlengo et al. (2017). Relative transcript abundances were estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The chosen reference sequence was β-actin. The relevant primer pairs are listed in Table S1. Each genotype was represented by three biological replicates, each of which in turn was associated with three technical replicates.

Grain and spike phenotype

The following traits were monitored in mature plants: the number of spikelets per spike (SS), the weight of each spike (SW), the total grain weight for plant (TGW), the number of spikes per plant (SP), the surface area (GA), perimeter (GP), length (L) and width (W) of each grain and the weight of 100 grains (HGW). The various grain traits were obtained from scanned images of a sample of 100 grains of both WT and each RNAi line, obtained using a Perfection V750 PRO scanner (Epson Italia S.p.A., Milano, Italy) in conjunction with SilverFast v.6.5.0r4e software (www.silverfast.com). The trait values were derived using SmartGrain software (Tanabata et al. 2012) (www.kazusa.or.jp/phenotyping/smartgrain/index.htmL). The starch content of single grains (TS) was obtained using a Total Starch Assay kit (AA/AMG) (Megazyme Pty Ltd., Wicklow, Ireland), following manufacturer's protocol. Each line was represented by three biological replicates.

Grain yield and grain size traits have been expressed in the form mean ± standard error. Significant differences between mean values were identified by applying a one-way analysis of variance, in conjunction with the post hoc Tukey's HSD test. Significant differences were confirmed using the Scheffé, Bonferroni and Holm multiple comparison tests. The significance threshold was set at 0.05.

Extraction of the metabolic fractions and in-solution digestion

All chemicals were of the highest purity commercially available and were used without further purification. Ammonium bicarbonate, NaCl, NaH_2PO_4, formic acid (FA), dithiothreitol (DTT), iodoacetamide (IAA), and chicken lysozyme were obtained from Sigma-Aldrich (Milan, Italy). Modified porcine trypsin was purchased from Promega (Milan, Italy). Water and acetonitrile (OPTIMA® LC/MS grade) for LC/MS analyses were provided from Fisher Scientific (Milan, Italy).

Three replicate 200 mg samples of flour milled from the grain of either WT or transgenic line IM17-33a were suspended in 2 mL 0.4 M NaCl, 0.067 M NaH_2PO_4 (pH 7.6). The suspensions were mixed for 15 min, centrifuged at 9.000*g* for 10 min at 4 °C and the supernatants were removed. The procedure was repeated two more times, and the three supernatants were pooled and the final volume made up to 10 mL. The concentration of protein in each pooled sample was determined using a fluorometer assay kit (Invitrogen QubitTM Protein Assay kit, ThermoFisher Scientific, Milan, Italy). An aliquot, containing 50 µg protein (typically around 20 µL), was lyophilized under vacuum and dissolved in 20 mM ammonium bicarbonate (pH 8.3) to give a concentration of 1 µg/µL; 0.4 µg of chicken lysozyme was added as internal standard. Disulfide bridges reduction was carried out by the addition of 38.9 µg DTT dissolved in 2.5 µL of the same buffer, followed by 3 h incubation in the dark at 25 °C. Alkylation was performed by the addition of IAA at the same molar ratio overtotal thiol groups, and thereaction was allowed to proceed for 1 h in the dark at 25 °C. The reduced and alkylated proteins were finally subjected to tryptic digestion by incubation with modified porcine trypsin in ammonium bicarbonate (pH 8.3) at an enzyme–substrate ratio of 1:50 at 37 °C for 4 h. The digests were made up to 2 mL with 5% aqueous FA and analyzed by nano UHPLC/High Resolution nano ESI–MS/MS (Cunsolo et al. 2004, 2012).

Liquid chromatography and tandem mass spectrometry

Mass spectrometry (MS) data were acquired using an Orbitrap Fusion Tribrid (Q-OT-qIT) mass spectrometer (ThermoFisher Scientific, Bremen, Germany) equipped with a ThermoFisher Scientific Dionex UltiMate 3000 RSLCnano system (Sunnyvale, CA). A 1 µL aliquot of the in-solution digestion was loaded onto an Acclaim®Nano Trap C18 column (100 µm i.d. × 2 cm, 5 µm particle size, 100 Å). After washing the trapping column with solvent A (H_2O + 0.1% FA) for 3 min at a flow rate of 7 µL/min, peptides were eluted from the trapping column onto a PepMap® RSLC C18 EASY-Spray, 75 µm × 50 cm, 2 µm, 100 Å column and were separated by elution at a flow rate of 0.25 µL/min at 40 °C, with a linear gradient of solvent B (CH_3CN + 0.1% FA) in A from 5 to 65% over 82 min, followed by 65–95% over 5 min, at 95% for 5 min and finally from 95 to 5% over 10 min. The eluted peptides were ionized by a nanospray (Easy-spray ion source, Thermo Scientific) using a spray voltage of 1.7 kV and introduced into the mass spectrometer through a heated ion transfer tube (275 °C). Survey scans of peptide precursors in the m/z range 400–1600 were performed at resolution of 120,000 (@ 200 m/z) with a AGC target for Orbitrap survey of 4.0×10^5 and a maximum injection time of 50 ms. Tandem MS was performed by isolation at 1.6 Th with the quadrupole, and high-energy collisional dissociation (HCD) was performed in the ion routing multipole (IRM), using a normalized collision energy of 35 and rapid scan MS analysis in the ion trap. Only precursors with charge state 2–4 and an intensity above the threshold of 5000 were sampled for MS2. The dynamic exclusion duration was set to 60 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. AGC target and maximum injection time (ms) for MS/MS spectra were 10,000 and 100, respectively. The instrument was run in top speed mode with 3 s cycles, meaning the instrument continuously performed MS2 events until the list of non-excluded precursors diminishes to zero or 3 s, whichever occurred soonest. MS/MS spectral quality was enhanced enabling the parallelizable time option (i.e., by using all parallelizable time during full scan detection for MS/MS precursor injection and detection). Each WT and transgenic line extracts was injected in triplicate, in order to assess the reproducibility of the MS data. This generated a total of 18 MS data sets. MS calibration was performed using the Pierce® LTQ Velos ESI Positive Ion Calibration Solution (Thermo Fisher Scientific). MS data acquisition was performed using the Xcalibur v. 3.0.63 software (Thermo Fisher Scientific).

Database search

LC–MS/MS data were processed by PEAKS software v. 8.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). The data were searched against the 881,439 entries "Wheat" UniProt database (SwissProt and trEMBL, release March 2018) to whom chicken lysozyme sequence was added. Tryptic peptides with a maximum of three missed cleavage sites were subjected to an in silico search. Cysteine carboxyamidomethylation was set as fixed modification, whereas oxidation of methionine and transformation of N-terminal glutamine and N-terminal glutamic acid residues in the form of pyroglutamic acid were included as variable modifications. The precursor mass tolerance threshold was 10 ppm, and the maximum fragment mass error was set to 0.6 Da. Peptide spectral matches (PSM) were validated using Target Decoy PSM Validator node based on *q*-values at a 0.1% False Discovery Rate (FDR). A protein was considered as identified if a minimum of two peptides matched and if its coverage was ≥ 5% in at least two biological replicates and in two technical replicates of either the WT or the transgenic line. Proteins containing the same peptides which could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Label-free quantification data were obtained using PEAKS Q software, which detected the reference sample and automatically aligned the sample runs. Proteins present in distinctly different concentration between the two genotypes were identified by a statistical analysis tool set with the following filters: protein fold change ≥ 2, protein significance ≥ 20 and unique peptides ≥ 1. The data have been displayed in a heat map format for ready visualization.

Results

The GW2 proteins formed by WT cv. Svevo

The *TaGW2-A1* and *TaGW2-B1* cDNA sequences (GenBank accessions AFU88754 and AFU88755, respectively) were used to identify the corresponding genomic regions in the cv. Kronos genome as mapping to the short arms of chromosomes 6A and 6B. The sequences of the two homeologs were closely related to one another both at the nucleotide (98.3% identity) and at the polypeptide (96.9% identity) levels (Fig. S2 and S3). The coding sequence length of both genes was 1275 nt; it was interrupted in both by seven introns, producing a predicted 424 residue products of molecular weight ~ 47 kDa (Fig. S3). The 21 nucleotide polymorphisms which distinguished the two sequences (Fig. S2) were predicted to generate 13 residue differences. Both products' N-termini harbored two highly conserved sequences, namely the NES motif LRKLILE and the 43 residue RING domain

identified by Song et al. (2007) (Fig. S3). The former is shared with GW homologs encoded by a number of grass species genomes, including those of barley, rice, maize, sorghum, *Brachypodium distachyon* and foxtail millet; the latter is present in each of barley, maize, sorghum, *B. distachyon* and foxtail millet, but in rice, the identity of the position 96 residue differs (Fig. S4). A phylogenetic analysis of the GW2 polypeptide sequences revealed that the wheat GW2 proteins were most closely related to that of barley (Fig. 1).

The production of GW2-RNAi transgenic lines

A total of 850 immature cv. Svevo embryos were bombarded with pRDPT-GW2(RNAi) and pAHC20, from which 25 putative transgenic plants were regenerated. A PCR-based assay confirmed the presence of both pRDPT-GW2(RNAi) and pAHC20 of 14 of these plants, while eight harbored only pAHC20 and three lacked both transgenes. After self-pollination to the T_2 generation, it was possible to identify transgene homozygotes using the same PCR assays (Table S2). The three independent homozygous transgenic lines IM17-15a, IM17-33aII and IM17-81 were carried forward for the subsequent experiments.

The abundance of *GW2* transcript in the transgenic lines

The abundance of *GW2* transcript in the three GW2-RNAi lines was estimated by a qRT-PCR assay based on three sets of primer pairs, two of which were homeolog specific and one of which recognized both homeologs. Immature grains, harvested 21 days post-anthesis, were sampled from three independent plants per each line. Transcription from both homeologs was equally affected. The abundance of *GW2* transcript was reduced by > 75% in all three GW2-RNAi lines, with some variation seen in the extent of the knock-down between the lines: the reduction was 76% in IM17-81, 81% in IM17-15a and 87% in IM17-33aII (Fig. 2).

The effect of GW2 down-regulation on grain phenotype

The effect of *GW2* down-regulation on the set of grain and spike traits (HGW, SW, TGW, SP, SS, TS, GA, GP, GL and GW) was assessed by comparing the performance of the three transgenic lines with that of WT plants. Significant differences were observed for several of the traits. In IM17-33aII, HGW was raised by 18%, SW by 20%, TGW by 22%, GA by 13%, GP by + 7%, GL by 7% and GW by 5% (Tables 1, 2; Fig. 3). GW and GA were increased by, respectively, 4–13% and 3–5% across the three transgenic lines, whereas HGW and GL were enhanced only in IM17-33aII. As anticipated (since the transgene promoter was endosperm specific), neither SP nor SS was altered. With respect to SW, the increase experienced by IM17-33aII was accompanied by a fall of ~ 25% in each of the other two transgenic lines. TS measured from flour samples was not significantly affected by the presence of the transgene, but

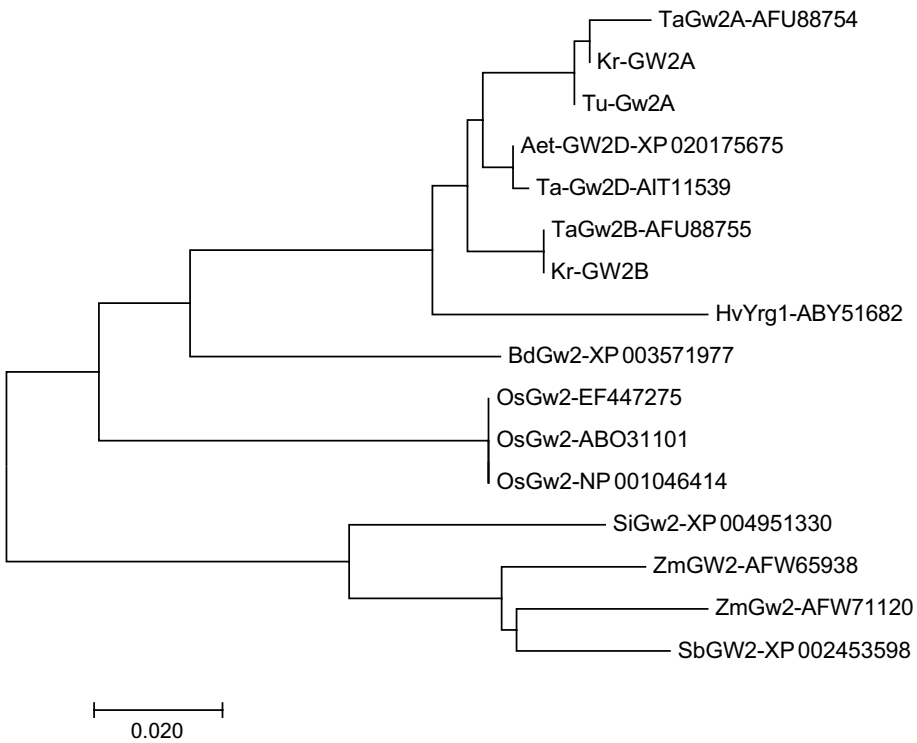

Fig. 1 Phylogenetic analysis of the GW2 protein family. Bootstrap values relating to each node are shown. Ta: *T. aestivum* (GenBank accessions AFU88754, AIT11539, AFU88755); Td: durum wheat cv. Kronos (TdGW2A GenBank accession KP49899.1; TdGW2B UCW_Tt-k55_contig_11353); Tu: *T. urartu*; Aet: *Ae. tauschii* (GenBank accession XP_020175675); Hv: barley (GenBank accession ABY51682); Bd: *B. distachyon* (GenBank accession XP_003571977); Os: rice (GenBank accessions EF447275, AB031101, NP_001046414); Si: foxtail millet (GenBank accession XP_004951330); Zm: maize (GenBank accessions AFW65938, AFW71120); Sb: sorghum (GenBank accession XP_002453598)

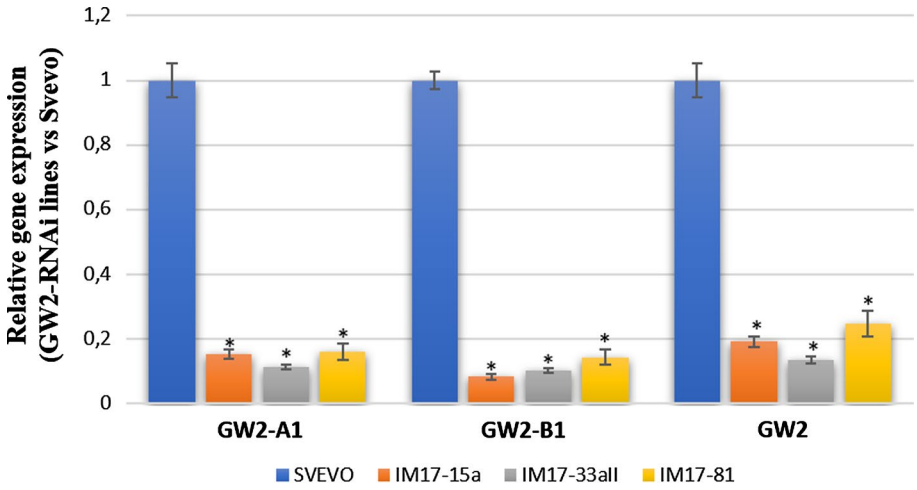

Fig. 2 Abundance of *GW2* transcript in grain harvested 21 days post-anthesis, as measured by qRT-PCR. Data expressed in the form of fold differences between the abundance in the grain set by WT and each of the three independent GW2-RNAi lines IM17-15a, IM17-33aII and IM17-81 plants. Three sets of primer pairs were deployed, two of which each targeted one homeolog, while the third recognized both. Data shown in the form mean ± standard error (SE) ($n=3$). *Means differ from one another significantly ($P \leq 0.05$)

Table 1 Variation between the RNAi transgenic line and WT plants with respect to the expression of the weight of 100 grains (HGW), the weight of each spike (SW), total grain weight for plant (TGW), total grain starch content (TS), the number of spikes per plant (SP) and the number of spikelets per spike (SS)

Lines	HGW (g)	SW (g)	TGW (g)	TS (mg/seed)	SP	SS
Svevo	5.42 ± 0.12^{a} (100)	2.58 ± 0.15^{a} (100)	5.58 ± 2.26^{a} (100)	37.85 ± 1.51^{a} (100)	5.72 ± 0.38	12.53 ± 0.43
IM17-15a	5.50 ± 0.11^{a} (101)	1.90 ± 0.12^{b} (74)	6.80 ± 1.97^{b} (122)	53.18 ± 2.91^{b} (140)	7.44 ± 0.69	11.78 ± 0.32
IM17-33aII	6.38 ± 0.14^{b} (118)	3.09 ± 0.12^{c} (120)	6.93 ± 1.90^{b} (124)	49.57 ± 2.50^{bc} (131)	6.00 ± 0.60	13.44 ± 0.27
IM17-81	5.49 ± 0.12^{a} (100)	1.98 ± 0.11^{b} (77)	5.32 ± 1.54^{a} (95)	41.62 ± 2.13^{ac} (110)	6.70 ± 0.42	11.79 ± 0.26

Values followed by different letters differ significantly ($P \leq 0.01$) from one another

To facilitate comparisons, all values are also reported (in parentheses) in the form of a percentage of the corresponding WT value

Table 2 Variation between the RNAi transgenic line and WT plants with respect to the expression of grain surface area (GA), perimeter (GP), length (GL) and width (GW)

Lines	Area (GA) (mm^2)	Perimeter (GP) (mm)	Lenght (GL) (mm)	Width (GW) (mm)
Svevo	18.13 ± 0.17^{a} (100)	18.90 ± 0.11^{a} (100)	7.78 ± 0.04^{a} (100)	3.10 ± 0.02^{a} (100)
IM17-15a	19.64 ± 0.20^{b} (108)	19.39 ± 0.11^{b} (102)	7.93 ± 0.05^{a} (102)	3.25 ± 0.02^{b} (105)
IM17-33aII	20.57 ± 0.16^{c} (113)	20.19 ± 0.09^{c} (107)	8.30 ± 0.04^{b} (107)	3.26 ± 0.02^{b} (105)
IM17-81	18.95 ± 0.19^{b} (104)	19.10 ± 0.11^{ab} (101)	7.79 ± 0.04^{a} (100)	3.19 ± 0.02^{b} (103)

Values followed by different letters differ significantly ($P \leq 0.01$) from one another

To facilitate comparisons, all values are also reported (in parentheses) in the form of a percentage of the corresponding WT value

when assessed on a single grain basis, its level proved to be significantly higher in both IM17-15a (by 40%) and IM17-33aII (by 31%).

Differentially expressed proteins (DEPs) in the metabolic fraction of the mature grain proteome

An exploration of the proteomic effect of *GW2* knockdown was investigated using the contrast between WT and line IM17-33aII. The RP-nUHPLC/nESI-MS/MS analyses and subsequent database search against the "Wheat" UniProt database identified a set of 2613 proteins in Svevo and 2672 in the transgenic line IM17-33aII, among which was a considerable number of uncharacterized proteins. Based on a threshold of an at least twofold difference in abundance, eleven were classed as DEPs (Fig. 4). One of these was present at below the level of detection in IM17-33aII grain, while the other ten were more abundant in the transgenic grain (Figs. 4, S5). The former is identified by a group of seven peptides found in two different proteins, one of which is uncharacterized, while the other has been identified as a xylanase inhibitor. These two proteins have a sequence similarity of 92.8% (Table S3, Fig. S6a). Among the ten proteins which were more abundant in the transgenic grain, five were identified as Endoglucanase

Fig. 3 Variation with respect to grain length and width between the *GW2* knockdown line IM17-33aII and WT cv. Svevo

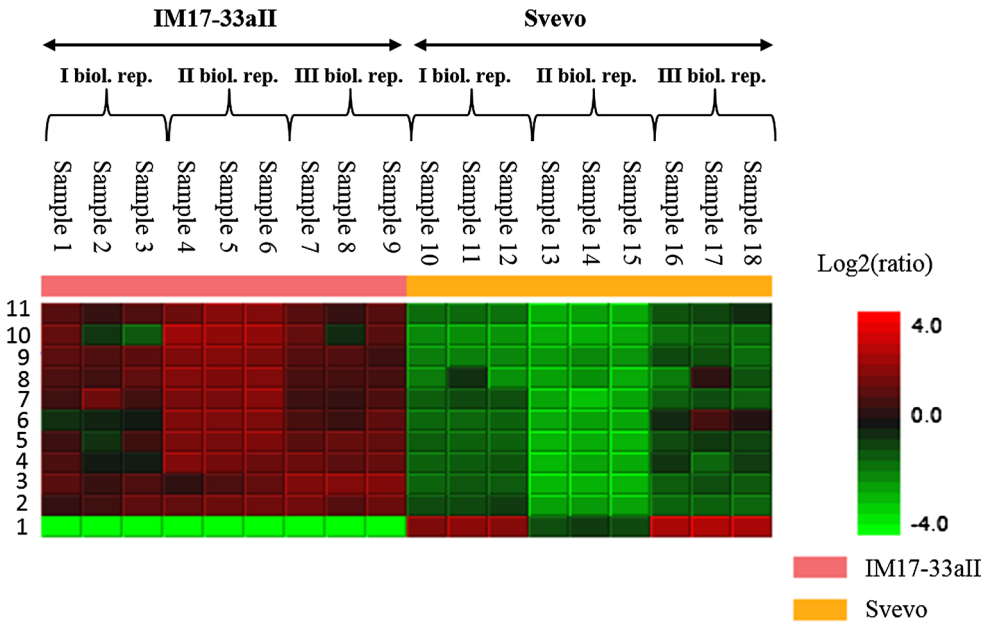

Fig. 4 Proteins differentially abundant in the grain of WT cv. Svevo and that of transgenic line IM17-33aII. (1) Xylanase inhibitor XIP-III OS = *Triticum aestivum* (UniProt accession Q4W6G2), (2) Globulin OS = *Triticum urartu* (UniProt accession H9XH65), (3) 12S seed storage globulin 1 OS = *Triticum urartu* (UniProt accession M7ZK46), (4) Farinin protein OS = *Brachypodium distachyon* (UniProt accession W8QN15), (5) Type 2 non-specific lipid-transfer protein OS = *Triticum aestivum* (UniProt accession Q2PCC3), (6) Putative non-specific lipid-transfer protein OS = *Aegilops tauschii* (UniProt accession M8BVH7), (7) Endoglucanase OS = *Triticum aestivum* (UniProt accession A0A1D6ADY9), (8) 60S ribosomal protein L23a OS = *Triticum urartu* (UniProt accession M8A553), (9) Trypsin/alpha-amylase inhibitor CMX1/CMX3 OS = *Triticum urartu* (UniProt accession M8A1S2), (10) Trypsin/alpha-amylase inhibitor CMX1/CMX3 OS = *Triticum urartu* (UniProt accession M8A1S2), (11) Alpha-amylase/trypsin inhibitor CM16 OS = *Triticum aestivum* (UniProt accession P16159). A 0.4 µg aliquot of chicken lysozyme was added to each 50 µg sample as an internal standard. Three replicates of each of WT and the transgenic line were analyzed, with each replicate represented by three technical replicates

(UniProt acc. A0A1D6ADY9), Type 2 non-specific lipid-transfer protein (UniProt acc. Q2PCC3), Globulin (UniProt acc. H9XH65), Putative non-specific lipid-transfer protein (UniProt acc. M8BVH7), 12S seed storage globulin 1 (UniProt acc. M7ZK46) with a sequence coverage ranging from 20 to 75% (Tables S3). Within the other five, a group of 42 peptides identified two very highly similar proteins (differing by just one residue, see Fig. S6b): both were classified as a CM16 α-amylase/trypsin inhibitor. A group of seven peptides was shared by four proteins, two of which are uncharacterized, whereas the other two resembled the 60S ribosomal protein L23a either

present in the diploid wheat *T. urartu* (Fig. S6c, d) or in the wheat D genome donor species *Aegilops tauschii* (Fig. S6e). A BLAST search (blast.ncbi.nlm.nih.gov/Blast.cgi) detected eight peptides in one of the three uncharacterized proteins and 18 in a second which matched the sequence of a trypsin/α-amylase inhibitor harbored by *T. urartu* (Fig. S6f, g). The third uncharacterized protein featured a sequence similarity of 99.3% with a *B. distachyon* farinin protein (Fig. S6 h).

The transcriptional consequences of knocking down *GW2*

The transcriptional behavior in the transgenic lines of four genes documented as being responsive to the knocking down of *GW2-A1* in bread wheat (Geng et al. 2017; Li et al. 2017) was examined via qRT-PCR: the genes included two encoding a cytokinin dehydrogenase (*CKX1*, *CKX2*), one a gibberellin oxidase (*GA3-ox*) and one a large subunit of ADP-glucose pyrophosphorylase (*AGPL*). Both *CKX1* and *AGPL* proved to be up-regulated in all three transgenic lines, the former by 2.2–3.2-fold and the latter by 1.7–2.3-fold (Fig. 5a). *CKX2* and *GA3-ox* behaved very differently: both were down-regulated in IM17-33aII and IM17-81, but the abundance of their transcript was unaltered in IM17-15a. The qRT-PCR platform was further used to explore the transcription in immature grain samples of some of the genes responsible for the DEPs. The outcome of this analysis was consistent with the proteomic analysis with just one exception: *XIP-III* was down-regulated in IM17-33aII, whereas *CM3*, *CM16*, *EG11* and *nsLTP* were all up-regulated, by, respectively, 2.8-, 2.1-, 4.5- and 1.8-fold (Fig. 5b); the exception was a gene encoding farinin, which was not differentially transcribed in the immature grain.

Discussion

Grain weight is a key component of the economic yield of cereal crops. The impact of intensive selection for this trait has been illustrated recently by a demonstration of the extent of the decline in sequence polymorphism remaining at *GW2* in wheat since domestication (Qin et al. 2017). In cv. Svevo, the two *GW2* homeologs share a very high degree of homology, both at the nucleotide and the polypeptide levels. The function of GW2 is now well established in rice to be a negative regulator of cell division, since loss-of-function mutants form larger grains weight as a result of their higher grain filling rate (Song et al. 2007). In both bread and durum wheat, negative associations have been established between the abundance of *GW2-A1* transcript and grain weight (Su et al. 2011; Yang et al. 2012; Zhang et al. 2013; Hong et al. 2014; Jaiswal et al. 2015; Simmonds et al. 2016). Recently, a novel *GW2-A1* allele, lacking a 114 nt segment of the promoter sequence, has been shown to result in a reduction in the gene's transcription(Zhai et al. 2018); the same allele is present in the Chinese bread wheat cultivar Lankaodali (unpublished data), which produces particularly long grains. According to Hong et al. (2014), however, the abundance of both *TaGW2-B1* and *TaGW2-D1* transcript appears to be positively associated with grain width. An analysis of gene-editing derived knockout mutants involving either one, two or all three bread wheat *GW2* homeologs did not support the notion that the products of either the B or the D genome homeologs counteract the action of *GW2-A1* (Zhang et al. 2018); rather, the phenotype of these mutants demonstrates that both products likely participate in the negative

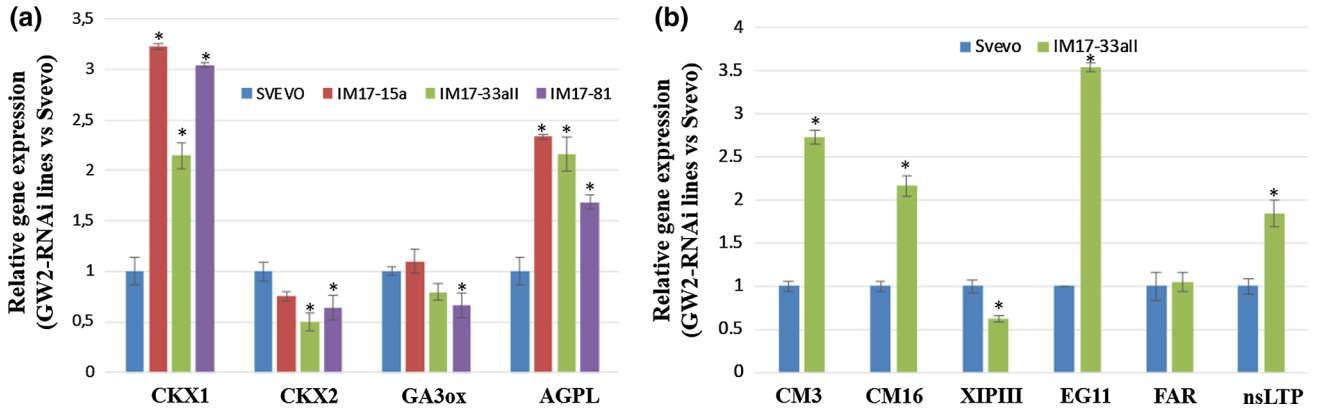

Fig. 5 Transcriptional behavior of **a** *CKX1*, *CKX2*, *GA3-ox* and *AGPL*, genes known to be responsive to *GW2* knockdown, and **b** of genes encoding the DEPs. The template represented cDNA prepared from grains harvested 21 days post-anthesis. Data expressed in as fold differences between the abundance in WT and in IM17-33aII grain. *Means differ significantly at $P \leq 0.05$

regulation of grain width, modulating cell number and length in the grain outer pericarp. Attempts to down-regulate the bread wheat *GW2* homeologs using RNAi technology, meanwhile, have given rise to conflicting results. While Bednarek et al. (2012) reported the effect to be a major drastic reduction in grain size, Hong et al. (2014) found the opposite to be the case. The discrepancy may be artifactual, since the use of the full length of the *GW2* sequence used by Bednarek et al. (2012) for the purpose of RNAi could have generated unexpected off-target effects; alternatively, the results may reflect a background effect, since the two studies did not use the same bread wheat cultivar. To date, the complete suppression of *GW2* has not been reported in durum wheat.

Here, the RNAi approach was used to simultaneously knock down both durum wheat *GW2* homeologs. Following the suggestion made by Hong et al. (2014), the RNAi cassette incorporated only part of the target sequence, and as an additional measure, the transgene was placed under the control of an endosperm-specific promoter to ensure that it was expressed only in the intended time and place. The resulting transgenics exhibited a major decrease in the abundance of *GW2* transcript (by 76–87%), a level of effectiveness which was higher than that achieved in bread wheat by both Hong et al. (2014) and Bednarek et al. (2012). The phenotypic effect of the knockdown was marked; although there was some variability between the independent transgenics for certain of the traits, all three lines produced grain which showed a pronounced increase in width, consistent with the outcome of silencing *GW2* homeologs at the hexaploid level (Hong et al. 2014; Zhang et al. 2018). In contrast to the experience of Zhang et al. (2018), which noticed that *GW2* genome-edited grains were morphologically wrinkled compared to the control, in our case, there was no evidence of any grain shriveling. This different behavior could be perhaps because, unlike the situation where the genes had been completely disrupted, here there still remained a low level of *GW2* transcript and hence presumably also some GW2 function.

An analysis carried out on the metabolic fraction of the mature grain proteome established that the abundance of eleven proteins varied significantly in the grain formed by WT and RNAi-GW2 transgenic line IM17-33aII plants. A much larger number of such proteins have been identified from a comparison between the immature grain proteome of the model bread wheat cultivar Chinese Spring and that of a *GW2-A1* knockout (Du et al. 2016). The likely most probable reason for such a different outcome is that here the analysis was performed on mature grains and the filters used were more restrictive. Of the DEPs, at least three (EG11, nsLTP2 and XIP-III) have some association with cell wall synthesis. Endo-1,4-β-D-glucanases are required for cell expansion, since they act to cleave the β-1,4-glycosidic bonds present in cellulose and xyloglucan (Lopez-Casado et al. 2008; Glass et al. 2015). The nsLTPs are small proteins which mediate phospholipid transfer, participate in plant defense against pests and act to enhance cell wall extension (Wang et al. 2012). According to Nieuwland et al. (2005), nsLTPs are associated with hydrophobic wall compounds, causing non-hydrolytic disruption of the cell wall and subsequently facilitating wall extension. XIP proteins (xylanase inhibitors) act to slow the spread of fungal pathogens (Dornez et al. 2010); in durum wheat, to date, only XIP-II has been characterized (Elliott et al. 2009), leaving the physiological function of XIP-III as yet unknown. It has been suggested that xylanase activity is required for remodeling cell wall during the growth and development of the cereal grain, so the possibility does exist that XIP inhibitors are used in a regulatory capacity during this process (Gebruers et al. 2002). In the grain formed by line IM17-33aII plants, both EG11 and nsLTP2 were more abundant than in WT grain, while XIP-III was not detectable in the former. The implication is that the knocking down of *GW2* in cv. Svevo could reduce the rigidity of the cell walls, making it easier for the cells to expand. Among the other DEPs present in higher abundance in the knockdown line's grain were proteins thought to act as α-amylase/trypsin inhibitors; their potential involvement in the process of cell wall development has not been reported to date.

As well as affecting the grain proteome, the knockdown of *GW2* also had a transcriptomic footprint, particularly involving genes encoding starch and phytohormone synthesis. In the transgenic lines, the gene encoding the large subunit of AGPase, an enzyme which catalyzes the conversion of glucose-1-phosphate to pyrophosphate plus ADP-glucose (Jeon et al. 2010), was strongly up-regulated. Consistent with an enhancement to AGPase activity, the starch content of the transgenic grain was higher than that of the WT grain. A similar up-regulation of genes encoding AGPase occurs in bread wheat lines silenced for *GW2-A1* (Geng et al. 2017). The cytokinins (CKs) and gibberellins (GAs) act as regulators for a wide range of processes, from cell growth to seed development (Huttly and Phillips 1995; Locascio et al. 2014; Zürcher and Müller 2016). In the grain, CKs are particularly prominent during periods of rapid cell division, but lose their importance as maturity approaches, when cell expansion takes over from cell division (Locascio et al. 2014). In contrast, GAs tend to accumulate both during the differentiation of the embryo and late during the grains' maturation phase (Locascio et al. 2014). In the GW2 knockdown lines' grains, the abundance of *CKX1* transcript (a gene which encodes a CK degrading enzyme) was higher than in the WT grains, while that of *CKX2* was lower. According to Geng et al. (2017), the absence of a functional *GW2-A1* results in a significant reduction in the abundance of at least three *CKX* genes (*CKX1*, *CKX2* and *CKX6*), an

observation taken to imply a heightened accumulation of CK; the conclusion was that *GW2-A1* in some way controls the expression of *CKX* genes. Once again, the most likely explanation for the lack of agreement with the present observations lies in the different physiological stages chosen to sample the transcriptomes, although it is also possible that the consequences of a complete abolition of *GW2* transcription differ from those caused by its less than complete abolition. *GW2* knockdown did not have significant effects on the transcription of *GA3-ox* in either IM17-15a or IM17-33aII grain, whereas it did have a marginal suppressive effect in IM17-81 grain. A rather different scenario has been reported by Li et al. (2017), who observed a significant increase in the abundance of *GA3-ox* transcript in grains harvested 20 days post-anthesis froma bread wheat line silenced for *GW2-A1*. The gene's transcription, however, fluctuated during grain development, being greatly down-regulated in very young grains (12 DPA), but up-regulated in grains sampled at 15 DPA.

Here, the intention was to characterize the effect of knocking down both of the *GW2* homeologs present in durum wheat. A range of phenotypic, molecular, proteomic and biochemical data was used to confirm that the product of *GW2* acts as negative regulator of grain yield in durum wheat grain. Our results suggest that the suppression of *GW2* genes is a successful strategy to increase grain size in durum wheat. Although the approach, which we used, is transgenic, the finding represents an important proof-of-concept to realize novel durum wheat genotypes with improved yield using alternative non-transgenic techniques, such as the exploration of either natural or induced mutants of *GW2*.

Author contribution statement FS prepared the RNAi construct, performed the phylogenetic analysis, coordinated the experiments, analyzed the data and drafted the manuscript in conjunction with DL. IM, ST and SM were responsible for the plant transformation. EB identified homozygous transgenic lines. AP performed qRT-PCR analysis on *GW2* genes. RP collected the phenotypic data and performed the qRT-PCR analysis of other genes. AZ, RS and SF performed the proteomic experiments and interpreted the resulting data. DL conceived the research. All of the authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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