Down-regulation of the TaGW2 gene by RNA interference results in decreased grain size and weight in wheat

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Abstract

For important food crops such as wheat and rice, grain yield depends on grain number and size. In rice (Oryza sativa), GW2 was isolated from a major quantitative trait locus for yield and encodes an E3 RING ligase that negatively regulates grain size. Wheat (Triticum aestivum) has TaGW2 homologues in the A, B, and D genomes, and polymorphisms in TaGW2-A were associated with grain width. Here, to investigate TaGW2 function, RNA interference (RNAi) was used to down-regulate TaGW2 transcript levels. Transgenic wheat lines showed significantly decreased grain size-related dimensions compared with controls. Furthermore, TaGW2 knockdown also caused a significant reduction in endosperm cell number. These results indicate that TaGW2 regulates grain size in wheat, possibly by controlling endosperm cell number. Wheat and rice GW2 genes thus seem to have divergent functions, with rice GW2 negatively regulating grain size and TaGW2 positively regulating grain size. Analysis of transcription of TaGW2 homoeologues in developing grains suggested that TaGW2-A and -D act in both the division and late grain-filling phases. Furthermore, biochemical and molecular analyses revealed that TaGW2-A is a functional E3 RING ubiquitin ligase with nucleocytoplasmic subcellular partitioning. A functional nuclear export sequence responsible for TaGW2-A export from the nucleus to the cytosol and retention in the nucleolus was identified. Therefore, these results show that TaGW2 acts in the regulation of grain size and may provide an important tool for enhancement of grain yield.

Key words: Divergent function, E3 RING ligase, grain size, orthologous, RNA interference, TaGW2, wheat.

Introduction

Wheat is one of the world’s major cereal crops and is of considerable importance to human nutrition, supplying one-fifth of the calories consumed by humans. Wheat is a hexaploid plant, having three homoeologous genomes, A, B, and D. The wheat grain is a caryopsis and consists of the embryo, the endosperm, and the teguments, with the endosperm being the primary storage site for starch and protein. Wheat grain development can generally be divided into five successive but overlapping stages based on endosperm development (Simmonds and O’Brien, 1981): fertilization [0 days post-anthesis (DPA)], coenocytic endosperm, cellularization, and cell division (these three processes occurring from 1 to 5 DPA), early grain filling (6–13 DPA), late grain filling (14–24 DPA), and grain desiccation (25–38 DPA).

In cereals, grain yield is a complex agronomic trait that is controlled by quantitative trait loci (QTLs) and affected by environmental factors. It can be broken down into a number of components that are also under QTL control, such as spike number per plant, grain weight per spike, spikelet number per spike, and 1000 grain weight (TGW). Grain weight is the most important component of grain yield and is largely determined by grain size, which is specified using its three dimensions (length, width, and thickness), and the degree of filling. In wheat, several QTLs for grain yield have been identified (Kato et al., 2000;
McCartney et al., 2005; Röder et al., 2008). However, no gene controlling a QTL for wheat grain size has yet been isolated and fully characterized. In contrast, in rice, several genes controlling QTLs for yield component traits have been cloned and functionally studied, such as GS3 (Fan et al., 2006), GW2 (Song et al., 2007), and, more recently, GW3 (Weng et al., 2008).

GW2 was isolated from a QTL controlling grain width and weight. GW2 was mapped on the short arm of chromosome 2 and the 1278 bp cDNA encodes a RING-type E3 ligase. E3 ligases (E3s) mediate specifically ubiquitination on protein substrates in the ubiquitin–26S proteasome system (Vierstra, 2009). The enzymatic reaction is carried out by a set of three enzymes, E1, E2, and E3, and the latter catalyses the transfer of ubiquitin from E2 to the targeted protein. The GW2 protein is a 425 residue polypeptide of ~47 kDa, with a CSHC2 RING domain in its N-terminal region (Song et al., 2007). Loss of GW2 function increases the number of cells in the spikelet hull, yielding wider spikelet hulls and consequently wider rice grains, and also accelerates the grain milk-filling rate, resulting in heavier grains. The GW2 protein therefore functions as a negative regulator of cell division. The substrate targeted by GW2 during cell division in the rice spikelet hull is still unknown and the mechanism by which GW2 alters the grain milk-filling rate also remains unclear. Recently, GW2 homologues were identified in maize and wheat (Li et al., 2010a; Su et al., 2011). In maize, ZmGW2-CHR4 is significantly associated with grain width and 100 grain weight (GHW). Moreover, ZmGW2-CHR4 was located in a HGW QTL (Li et al., 2010a). In bread wheat, TaGW2 is constitutively expressed, and TaGW2 homologues in the A, B, and D genomes are located on the homoeologous group 6 chromosomes (Su et al., 2011). TaGW2-A was mapped on the short arm of chromosome 6A, near the centromere. Association analyses in a Chinese common wheat core collection revealed that a marker in the promoter region of TaGW2-A was significantly associated with grain width and TGW. This marker was also found to be strongly associated with heading and maturation date (Su et al., 2011). More recently, Yang and colleagues (2012) identified a single T base insertion in the eighth exon of TaGW2-A leading to a premature stop codon. These authors found this single nucleotide polymorphism (SNP) genetically associated with grain width in an F2 segregating population (Lankaodali-Chinese Spring).

The aim of this study was to investigate the function of TaGW2 in wheat grain development. The hexaploid nature of the wheat genome makes finding and combining mutations in genes that are expressed by the three genomes a challenging proposition. In contrast, gene silencing through RNA interference (RNAi) can simultaneously suppress target gene expression from each locus. Here, RNAi was used to silence the three expressed copies of TaGW2, and the results showed that the down-regulation of TaGW2 expression resulted in a significant reduction in final grain weight and size. To gain further insights into TaGW2 function, biochemical and molecular analyses were performed. First, the results showed that TaGW2-A and -D are mainly expressed during the division and late grain-filling stages. In addition, the TaGW2-A protein is a functional E3 RING ligase, like its rice counterpart, but displays nucleocytoplasmic localization. An N-terminal LxxLxL-type nuclear export signal (NES) that is responsible for shuttling TaGW2-A between the nucleus and cytosol and retaining TaGW2-A in the nucleolus was identified.

Materials and methods

Plant material and growth conditions

Seeds from Triticum aestivum cv Récital and transgenic plants were grown as described in Capron et al. (2012) and in the Supplementary information SI available at JXB online.

For the determination of grain developmental stages, the first flowering spikelets of all tillers on each plant were tagged at anthesis and only grains from florets with the same anthesis date were harvested. The developmental stages of grain were evaluated in terms of growth degree days (°Cd) from anthesis; that is, the sum of daily mean temperatures accumulated over the period considered.

For transgenic experiments, immature seeds were harvested 12–14 DAP and sterilized, and immature embryos were isolated for bombardment.

Generation of RNAi lines

The RNAi construct (pTaGW2-RNAi) was made in the vector pSTARGATE supplied by CSIRO (http://www.pi.csiro.au/mai/vectors.htm). RNAi lines were produced using particle bombardment-mediated transformation of immature cv Récital embryos. The pTaGW2-RNAi plasmid was digested with NotI, and the fragment containing the RNAi cassette was co-bombarded with a phospho-mannose isomerase selection cassette with a 2:1 molar ratio. Regeneration and selection of the transformed plants were performed essentially as described by Wright et al. (2001) and Pellegrineschi et al. (2009).

Genotypyng of transgenic plants was performed by PCR of genomic DNA using PDK–intron primer pairs (Supplementary Table S1). In positive and negative transgenic lines, endogenous transcription levels of each TaGW2 were investigated by quantitative real-time PCR (qRT-PCR) as mentioned below, in RNA samples from leaves, using the RNAiTaGW2endo primer pairs (Supplementary Table S1 at JXB online).

Quantitative real-time PCR

Total RNA and first-strand cDNA were obtained as described by Capron et al. (2012) and in Supplementary information SI at JXB online. Absence of genomic DNA contamination was checked by PCR using the PDI–intron primer pair (Paolacci et al., 2009).

Specificity and PCR efficiency of primer pairs were determined as described in Supplementary information SI. Only pairs with efficiencies between 85% and 100% were used for quantification. qRT-PCR assays were performed on 4 µl of cDNA diluted 1:40. Three independent biological replicates were used for quantification analyses and three technical replicates were analysed per biological replicate. PCRs were cycled as described in Capron et al. (2012). Relative expression was determined using the ΔCt method corrected for primer efficiencies (Pfaffl, 2004). Data were normalized to the expression of the Ta2776 gene (Paolacci et al., 2009). To compare TaGW2 homoeologue expression, the relative expression values of TaGW2-A were calculated using the 2-ΔΔCt method, with the 40 °Cd stage as a reference sample for ΔΔCt, according to the method described by Schmittingen and Livak (2008).

In RNAi lines, two independent technical replicates were used for quantification analyses and three replicates were analysed per technical replicate. The Ta54227 gene was used as the internal control. Relative expression values were calculated using the 2-ΔΔCt method, with the mean of ΔCt values of the null segregants from the transgenic event as a calibrator.

Phenotyping of RNAi transgenic lines

Three independent transgenic lines were conducted and phenotyped, separately in time. Phenotyping of T3 progeny from T105 and T111 events
was conducted between June 2010 and January 2011 and between
November 2010 and May 2011, respectively.

For each transgenic event, spikes from all tillers of each plant were
tagged at anthesis when the first spikelets of the spike flowered, and the
date was recorded. For both transgenic events, spikes were harvested
at full maturity (1000 °C after anthesis). The numbers of spikelets per
spike, and the numbers of grain per spike, were then determined.

Measures of individual grain fresh mass, grain dry mass, volume, water
mass estimated as the difference between fresh and dry masses, length,
width, and thickness were carried out on the two basal grains of the two
central spikelets on each spike of the plant. Grain volume was estimated
using a water displacement method (pycnometry), and grain size traits
were determined using a scaled camera-assisted system. Grain fresh
mass, volume, length, width, and thickness were obtained from freshly
harvested caryopses and grain dry mass after oven drying of the grains at
80 °C for 48h. All remaining grains from a spike were counted, pooled,
and weighed, and ground to whole meal flour using a 6800 Cyclotec
mill. Total grain nitrogen content was then determined with the Dumas
combustion method (AOAC method no. 992.23) using a FlashEA 1112
N/Protein Analyzer (Thermo Electron). Each extract was analysed in
duplicate. Total grain protein content per grain was calculated by multi-
plying the grain nitrogen content by 5.7 (Sosulsik and Imafidon, 1990).

For the T105 transgenic event, basal grains of central spikelets of
spikes were also harvested at ~260 °C, and the number of cells in the
endosperm was then counted according to the method described by
Singh and Jenner (1982).

Results

Cloning and characterization of TaGW2
homoeologous cDNAs

To isolate the homoeologous cDNAs encoding TaGW2, prim-
ers (TaGW2 FL CDS primer pair, Supplementary Table S1 at
JXB online) were designed from the rice GW2 CDS sequences
(EF447275.1), and then cDNAs encoding TaGW2 from leaves of T. aestivum were amplified and cloned (cv Récital).

Sequence analysis revealed two different cDNAs. Two groups
of wheat expressed sequence tags (ESTs) were also found in
GenBank, one group corresponding to each of the cDNAs,
and a single EST (CK206178) with slight differences from the
other two groups. Each group of sequences was then assigned
to the short arm of chromosomes 6A, 6B, and 6D by PCR on
aneuploid lines of T. aestivum cv Chinese Spring (Sears, 1966),
using copy-specific primers (TaGW2-6A, -6B, and -6D primer
pairs, designed from an alignment of the cDNA sequences;
Supplementary Table S1 and Fig. S1). These results revealed
that the full-length sequences of TaGW2-A and -B (GenBank
accession numbers JN896622 and JN896623, respectively) were
thus obtained. The EST CK206178 corresponded to a 3′-partial
sequence of TaGW2-D (from 679 bp to 1275 bp of the TaGW2-A
and -B cDNAs, Supplementary Fig. S1).

Production of RNAi lines

To investigate the function of TaGW2 during wheat grain de-
velopment, RNAi was performed to reduce the transcript levels of all
TaGW2 homoeologues. The RNAi construct was made with a
1242 bp fragment of the TaGW2-A cDNA (nucleotides 34–1275).
The 1242 bp sequence was used to query the wheat genomic data-
bases available for sequences that could be potentially targeted by
the RNAi construct. Blastn searches returned 13 and eight hits
with stretches of identity of ≥20bp in CerealsDB (www.cereal-
sdb.uk.net) and URGI (http://urgi.versailles.inra.fr) databases,
respectively. Four hits with no homology with known genes were
found in URGI. One sequence on each of the chromosomes 7AL
and 3AL and two sequences on the 2AS chromosome were found
together to correspond to non-coding DNA or to a Gypsy-like
retrotransposon in the provisional wheat genome sequence. All
remaining hits were considered to be TaGW2 genes.

Two series of 354 and 246 immature embryos of winter hexa-
eploid wheat variety Récital were transformed by co-bombard-
ment with the RNAi construct and a selection cassette. Two
independent transgenic plants, T105 and T111, were identified.
Transgenic T0 plants were self-pollinated and the presence or
absence of the transgene in T1 progeny was determined by PCR
on genomic DNA. QRT-PCR analysis in T 1 progeny enabled
the identification of one positive T105 plant with a substantial
reduction of overall TaGW2 expression (75% reduction) and four
positive T111 plants with an average reduction in overall TaGW2
expression of 50%. The plants were self-pollinated and the
resulting T2 progeny were genotyped. QRT-PCR of T2 progeny
identified 21 and eight plants from T105 and T111 lines, respec-
tively, with an average reduction in overall TaGW2 expression
of 40% compared with null-segregant control lines (the overall
TaGW2 reduction level ranged from 20% to 76% and from 35%
to 64% in positive plants from T105 and T111 lines, respect-
ively, Fig. 1A). The expression levels of the TaGW2 genes in the
RNAi lines were analysed using homoeologue-specific primers
(TaGW2-A,-B, and -D reverse primers; Supplementary Table S1
at JXB online). In both transgenic lines, a significant reduction
in transcript levels of each TaGW2 homoeologue was observed
(Fig. 1A). The A and B copies of TaGW2 were reduced by almost
45% in both transgenic events compared with control lines, and
the D copy by 39% and 22% in T105 and T111 transgenic lines,
respectively.

Measurement of grain yield-related traits in RNAi lines

To determine whether reduction of TaGW2 affected grain yield,
T2 transgenic and null-segregant plants from T105 and T111 lines
were phenotyped at maturity for grain yield and size-related traits. Significant effects on grain size were observed in mature grains of both transgenic lines compared with the controls (Fig. 1B–D). Spikelet position on the spike impacts grain weight and nitrogen accumulation (Calderini and Ortiz-Monasterio, 2003; Andersson et al., 2004). Therefore, to compare traits accurately between transgenic and control lines, all measurements were performed on the two basal grains of the two central spikelets from the main spike of each plant. Individual grain fresh mass was reduced in transgenic plants compared with controls by 38.6% and 22.2%, grain dry mass was reduced by 37.3% and 21.9%, and grain water mass by 31% and 25%, in T105 and T111 lines, respectively (Fig. 1E). Transgenic plants also displayed a 34.6% and 20.4% reduction in grain volume, a 10% and 11.8% reduction in grain width, and a 13.3% and 9.1% reduction in thickness, in T105 and T111 lines, respectively. No significant differences were observed in either transgenic line in the number of grains per spike, the number of spikelets per spike, the anthesis date, or the nitrogen and protein contents per grain. Differences in grain length and number of spikes per plant in transgenic plants compared with the controls were only observed in the T111 line (Fig. 1E).

In T2 progeny from the T105 line, the number of cells in the endosperm of grains harvested at 260 °Cd (considered as the end of the division stage in cv Récital; Nadaud et al., 2010) was also phenotyped. Cell numbers in the endosperm were significantly reduced by ~25% in transgenic plants compared with control plants (Fig. 1E). Unfortunately, endosperm cell numbers of T2 progeny from the T111 line could not be counted because of the limited number of spikes yielded by these plants (transgenic and null-segregant control, Fig. 1E). Taken together, these results suggest that TaGW2 is a positive regulator of wheat grain size and grain weight, unlike GW2 in rice, and that TaGW2 is a positive regulator of cell number in the endosperm.

Expression of TaGW2 homoeologues during grain development

GW2 and its homologues are constitutively expressed in rice, maize, and wheat (Song et al., 2007; Li et al., 2010a; Su et al., 2011), but these studies only covered grain division stages, and the specific expression patterns of each TaGW2 homoeologue were not investigated. To analyse TaGW2 homoeologue expression during grain development, a qRT-PCR assay was performed using homoeologue-specific primer pairs (Supplementary Table S1 at *JXB* online), on eight grain developmental stages, at times measured in cumulative degree days (°Cd; see Materials and methods), in *T. aestivum* cv Récital from 40 °Cd to 800 °Cd. All transcripts were detected from 40 °Cd (Fig. 2). TaGW2-A and -D were mainly expressed during the beginning of the division stage (40–120 °Cd) and the late grain-filling stage (600 °Cd). A high level of TaGW2-D transcript was maintained during grain desiccation (800 °Cd). TaGW2-B transcript mainly accumulated during the late grain-filling stage (600 °Cd). Therefore, the expression patterns of TaGW2-A and -D strongly suggested their involvement in both the division and late grain-filling phases of wheat grain development. Furthermore, the high level of TaGW2-D at 800 °Cd suggested that it may have a role in grain maturation. TaGW2-B may only act in late grain filling. Based on these results, the A and D copies appeared to have major roles in the regulation of division and filling in wheat grain development. Likewise, Su and colleagues (2011) reported the A-copy to be associated with grain width. With this in mind, it would thus be interesting to characterize further the TaGW2-A and -D proteins. Unfortunately, in this study, the full-length cDNAs of TaGW2-A and -B and only a partial sequence of TaGW2-D were obtained. Biochemical analyses therefore focused on the functional characterization of the TaGW2-A protein.

TaGW2-A is a functional RING-type E3 ligase

GW2 was previously characterized as a functional E3 RING ligase, *in vitro*. To determine whether TaGW2-A is also a functional E3 ligase, an *in vitro* ubiquitination assay was thus performed. A 6×His-TaGW2-A fusion protein (52.2 kDa) was produced in *Escherichia coli* and purified using a histidine affinity column. TaGW2-A displayed self-ubiquitination in the presence of Ub, E1, and E2 in the reaction mixture, as shown by the high molecular weight bands of ubiquitinated protein detected by western blot analysis using an anti-ubiquitin antibody (Fig. 3, lane 5). The molecular weight of the bands matched the molecular weight of the 6×His-TaGW2-A fusion protein, with one Ub moiety (60.2 kDa) or more. The same pattern of bands was observed for rice GW2 tested under the same reaction conditions (data not shown). TaGW2-A autoubiquitination was abolished if the E1 or E2 enzymes were absent (Fig. 3, lanes 1–4). These results thus show that TaGW2-A displays E3 ubiquitin ligase activity *in vitro*, as does its rice counterpart.

TaGW2-A is a nucleocytoplasmic protein and is exported from the nucleus via a functional LxxLxL-type nuclear export signal

To gain insight into the cellular function of TaGW2-A, its subcellular localization was next examined. Confocal microscopy experiments were performed to assess the distribution of TaGW2-A fused to yellow fluorescent protein (YFP) (Fig. 4A). YFP–TaGW2 was transiently co-expressed by agroinfiltration in tobacco leaf epidermal cells, with cytosolic cyan fluorescent protein (cCFP) as a control. Both proteins were observed in the cytosol and the nucleus (Fig. 4B, merged image). YFP–TaGW2 was also detected in the nucleolus (Fig. 4B, merged image, arrow). The nuclear localization of cCFP results from passive diffusion through nuclear pore complexes because of its small size (22.3 kDa). As passive diffusion is generally limited to proteins less than ~40 kDa (Wagstaff and Jans, 2009), the size of YFP–TaGW2 (69.6 kDa) excludes its passive nuclear diffusion, indicating that TaGW2-A has a genuine nuclear localization. The fact that TaGW2-A was detected in both the cytoplasm and the nucleus prompted a search for TaGW2-A motifs that may govern its intracellular localization. It remains unclear how TaGW2-A enters the nucleus, as no nuclear localization signal has been yet identified in the primary sequence of TaGW2-A. However, a leucine-rich region (LxxLxLxL) was identified that might act as an NES. To determine whether this putative NES was functional, deletions and mutations of TaGW2-A

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Fig. 1. Molecular characterization and phenotyping for grain yield-related traits of T2 transgenic and null-segregant control plants from T105 and T111 events. (A) Relative transcript level of endogenous TaGW2 homoeologues in T2 transgenic (n=21 and n=8, in T105 and T111 lines, respectively, grey) and null-segregant control plants (n=5 and n=9, in T105 and T111 lines, respectively, black). Quantification
fused to YFP were generated (Fig. 4A). In the YFP–mNESTaGW2 substitution mutant, the three leucines were replaced by alanine (34AxxAxA39). Consistent with a disruption of NES function, YFP–mNESTaGW2 accumulated exclusively in the nucleus (Fig. 4C). This result indicates that the LxxLxL motif is essential for nuclear export of YFP–TaGW2, and that disruption of this motif led to nuclear retention of the chimeric protein. Subsequently, the first 39 amino acids of TaGW2-A, which include the putative LxxLxL-type NES, were investigated to be used to export cYFP from the nucleus [(1–39)TaGW2–YFP]. The cYFP protein alone, like cCFP, accumulated in the cytosol and diffused passively into the nucleus (Fig. 4D). (1–39)TaGW2–YFP was mainly located in the cytosol (Fig. 4E), although the size of this fusion protein (26.83 kDa) would still allow it to diffuse passively into the nucleus. Thus amino acids 1–39 of TaGW2-A were sufficient to export cYFP from the nucleus. However, amino acids 19–29 of TaGW2-A [(19–39)TaGW2–YFP] were not sufficient to export cYFP from the nucleus (Fig. 4F). This result shows that the LxxLxL motif alone in the N-terminal part of TaGW2-A was not sufficient to shuttle cYFP out of the nucleus and further suggests the probable importance of the three-dimensional conformation, which makes the LxxLxL motif accessible.

Interestingly, YFP–mNESTaGW2 was excluded from the nucleolus, which suggests that the NES might also act as a retention signal in the nucleolus. Indeed, the fusion of TaGW2-A amino acids 19–29 to YFP clearly resulted in an accumulation of the fusion protein in the nucleolus (Fig. 4F, indicated by the asterisks). This indicates that the LxxLxL motif alone in the N-terminal part of TaGW2-A was not sufficient to shuttle cYFP out of the nucleus and further suggests the probable importance of the three-dimensional conformation, which makes the LxxLxL motif accessible.

Fig. 2. Relative TaGW2 homoeologue mRNA levels at different grain development stages. Time is measured in cumulative growth degree days (°Cd). Quantification was performed by qRT-PCR. Relative expression values were calculated using the 2-ΔΔCt method, using Ta2776 as an internal control, and the 40 °Cd stage as a calibrator. All data are given as the mean of three independent biological replicates. Comparisons of transcript accumulation between grain developmental stages were performed independently for each copy using Scheffe’s multiple comparison test. Asterisks indicate that means are significantly different at P < 0.01. (This figure is available in colour at JXB online.)

Fig. 3. E3 ubiquitin ligase activity of TaGW2-A. 6×His-TaGW2-A was assayed for E3 activity in the presence or absence of human E1 (A1S9), E2 [glutathione S-transferase (GST)-UbcH5b], and Ub (GST-Ub). Ubiquitin and ubiquitinated proteins were detected by western blotting using anti-ubiquitin antibody (P4D1, Santa Cruz). An asterisk indicates monoUb-E2 intermediates; in the absence of E3, ubiquitin cannot be transferred to E3 and remains bound to the E2 enzyme. Double asterisks indicate monoUb-TaGW2-A.
Discussions

Silencing of TaGW2 impacts wheat grain size

To summarize, RNAi-mediated down-regulation of TaGW2 in wheat resulted in significant decreases in final grain fresh, dry and water masses, and grain volume, width, and thickness. No significant difference was observed in the number of grains per spike. A negative correlation between grain number per spike and grain size is frequently observed; the reduced grain dry mass in transgenic plants thus does not result from a larger number of grains per spike (Fisher et al., 1977). Furthermore, no significant differences in the number of spikelets per spike, the number of spikes per plant (in the T105 line), or the anthesis date were observed in transgenic plants compared with the controls, suggesting that the transgene exerted no effect on vegetative traits of the plants and that a direct intrinsic effect of the transgene on grain development was therefore likely.

Two studies previously investigated the genetic association between TaGW2-A and wheat yield components (Su et al., 2011; Yang et al., 2012). Although both studies suggest TaGW2-A as a negative regulator of grain width, like GW2 in rice, they diverge as regards the nature of the causal mechanism. By comparing the three homoeologous sequences of TaGW2 between small and large grain varieties, Su et al. (2011) did not find any variation in the coding regions. Instead, a base substitution in the promoter region of TaGW2-A was found to be associated with grain width and TGW. Moreover, the authors established a negative correlation between the expression level of TaGW2 and grain width, in immature grains at 10 d post-flowering. Yang et al. (2012) did not report any variation for the coding sequences from chromosomes 6B and 6D but identified a single T base insertion in the eighth exon of TaGW2-A of a large grain variety, Lankaodali. This insertion leads to a non-functioning protein, which is similar to the deletion in rice GW2. This marker was found associated with grain width and TGW in an F2 population derived from a cross between Lankaodali and Chinese Spring. The present study shows that overall down-regulation of TaGW2 copies results in smaller wheat kernels, thus suggesting that TaGW2 is a positive regulator of grain size-related traits. The results are notably consistent with those of Su et al. (2011) as both imply a modulation of the expression level. However, there are discrepancies between the present results and the results reported by Su et al.
Su et al. (2011) and Yang et al. (2012). Three possible hypotheses may explain these discrepancies. One is that the previous studies focused on the A copy of TaGW2 and, to date, nothing is known as regards the other two copies of the gene. Here, RNAi enabled silencing of the three copies of TaGW2 at a time. Thus a potential cumulative effect of silencing the three TaGW2 homoeologues during the whole developmental cycle of the plant may have been observed. A second explanation is that the previous studies dealt with genetic associations between polymorphic markers within the A copy of TaGW2 and yield components. However, both studies indicated that TaGW2-A is located in a genomic region with a major yield QTL, close to the 6A centromere (Snape et al., 2007). Therefore, the observed genetic association could be explained by linkage disequilibrium between these markers and a gene not yet identified with a large effect on yield components. Interestingly, Li et al. (2010a) conducted similar studies in maize and identified the favourable alleles (i.e. the relative contribution of each parental allele) using either an association analysis or a QTL mapping approach. The two approaches provided conflicting results and the authors suggested that the polymorphic marker they used could be in linkage disequilibrium with the actual functional polymorphism (Li et al., 2010a). A third possibility is off-target effects caused by the long RNAi hairpin used in this study to down-regulate TaGW2 genes. Although a search in URG1 and CerealsDB wheat genomic databases did not reveal any 20 nucleotide identity stretches in coding sequences with known function, it cannot be excluded that the RNAi hairpin silences additional genes which positively impact grain size-related traits.

Although similar results were obtained in both transgenic lines, interestingly the effect of TaGW2 down-regulation on grain yield-related traits was always less substantial in T111 transgenic plants than in T105. In both transgenic lines, the A and B copies of TaGW2 were silenced to similar levels. However, TaGW2-D transcript accumulation was reduced by almost twice as much in the T105 line as in T111. This difference in TaGW2-D silencing could thus explain the different magnitudes of the observed effects in both transgenic lines. Moreover, this result suggests that the different homoeologous genes contribute to this quantitative trait, and that any modulation of the expression level of one copy could quantitatively impact the yield components.

Various approaches have been attempted to determine grain weight potential in wheat and other crops. Final grain dry weight, which results from an accumulation of dry matter, is mainly governed by the number of endosperm cells and their expansion (Hoshikawa, 1962; Brocklehurst, 1977; Vilhar et al., 2002). Water uptake is a major determinant of grain size during the early phase of wheat grain development (Schnyder and Baum, 1992; Nadaud et al., 2010). Indeed, the initial growth of grains is mostly due to a rapid net deposition of water in the grain (Schnyder and Baum, 1992). Schnyder and Baum (1992) proposed that water content largely determines volumetric growth of the grain during endosperm cell division. Other studies support the hypothesis that grain weight determination is driven by early growth of the pericarp, which restricts grain expansion and consequently grain volume (Calderini et al., 1999a; b; Calderini and Reynolds, 2000; Garcia et al., 2005; Ugarte et al., 2007). During the grain-filling stage, grain dry weight increases, with starch accumulating between 13 and 34 DPA and storage proteins between 10 and 36 DPA (Schnyder and Baum, 1992; Dupont and Altenbach, 2003). Moreover, during this stage, water and dry matter dynamics are closely related (Schnyder and Baum, 1992; Calderini and Reynolds, 2000), and the end of water accumulation in the endosperm coincides with the end of dry matter accumulation (Schnyder and Baum, 1992). Finally, during grain desiccation, the endosperm tissue undergoes programmed cell death and the kernel rapidly loses all but 10–15% of its water content.

The expression patterns of TaGW2-A and -D described in this study were consistent with a role for TaGW2 in regulating endosperm cell number. Based on the RNAi results and on current knowledge of the determination of grain weight potential in wheat, a model for the role of TaGW2 in the regulation of grain size and grain weight in wheat can be proposed (Fig. 5). For the time being, it is difficult to identify clearly which homoeologous copy acts in these processes, so the overall TaGW2 will...
TaGW2 in wheat and GW2 in rice have divergent functions in grain development

Interestingly, the grain traits (grain weight, width, and thickness) affected by the RNAi of TaGW2 were the same as those altered in the GW2 antisense lines and in the WY3 rice variety, which encodes a loss-of-function variant of GW2 (Song et al., 2007). However, TaGW2 acts positively on these traits, opposite to rice GW2, which acts negatively. In addition, no significant differences in grain number per spike and in the number of spikes per plant in wheat were observed, but GW2 loss-of-function in rice reduced the grain number per spike by 29.9% and increased the panicle number per plant by ~27%. It is also noteworthy that in rice, GW2 regulates the number of cells in the spikelet hull but has no effect on the number of cells in the endosperm; in contrast, TaGW2 regulates endosperm cell number, at least in the T105 line. These two homologous genes thus might function differently during grain development in wheat and rice. This discrepancy of function between two orthologous genes was unexpected as orthologous genes often have conserved functions (Fan et al., 2006; Li et al., 2010b). Interestingly, Distelfeld et al. (2012) reported that, despite their orthologous relationship, the wheat GPC genes and the rice Os07g37920 gene have divergent functions. The wheat GPC genes control senescence, but the rice orthologous Os07g37920 gene controls anther dehiscence. Furthermore, divergence of function between several orthologues has also been reported between human and mouse. Further examination of TaGW2 function, including identification of its interacting proteins, will both shed light on the differences compared with rice GW2 and provide useful insights on the mechanisms of regulation of grain size and thus yield in important crop species.

GenBank accession numbers

The GenBank accession numbers of TaGW2-A and -B are JN896622 and JN896623, respectively.

Supplementary data

Supplementary data are available at JXB online. Supplementary information SI. Detailed Materials and methods.

Figure S1. Nucleotide and putative amino acid alignments of the TaGW2-A, -B, and -D CDS.
Figure S2. Amino acid alignment of GW2 and TaGW2-A.
Table S1. List of the primers used in this study.

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