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ORIGINAL ARTICLE

TaGW2-6A allelic variation contributes to grain size possibly by regulating the expression of cytokinins and starch-related genes in wheat

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Abstract

Main conclusion **Functional allelic variants of *TaGW2-6A* produce large grains, possibly via changes in endosperm cells and dry matter by regulating the expression of cytokinins and starch-related genes via the ubiquitin–proteasome system.**

In wheat, *TaGW2-6A* coding region allelic variants are closely related to the grain width and weight, but how this region affects grain development has not been fully elucidated; thus, we explored its influence on grain development based mainly on histological and grain filling analyses. We found that the insertion type (NIL31) *TaGW2-6A* allelic variants exhibited increases in cell numbers and cell size, thereby resulting in a larger (wider) grain size with an accelerated grain milk filling rate, and increases in grain width and weight. We also found that cytokinin (CK) synthesis genes and key starch biosynthesis enzyme AGPase genes were significantly upregulated in the *TaGW2-6A* allelic variants, while CK degradation genes and starch biosynthesis-negative regulators were downregulated in the *TaGW2-6A* allelic variants, which was consistent with the changes in cells and grain filling. Thus, we speculate that *TaGW2-6A* allelic variants are linked with CK signaling, but they also influence the accumulation of starch by regulating the expression of related genes via the ubiquitin–proteasome system to control the grain size and grain weight.

Keywords Cytological analysis · Gene expression · Grain development · Grain filling · *TaGW2-6A*

Abbreviations

AGPase	ADP glucose pyrophosphorylase
CK	Cytokinin
CKX	Cytokinin oxidase gene family
CS	Wheat line Chinese Spring
DAA	Days after anthesis
IPT	Isopentenyl transferase gene family
NIL31	Near-isogenic line 31
TKW	Thousand-kernel weight
UPS	Ubiquitin–proteasome system
Z	Zeatin
ZR	Zeatin riboside

Introduction

Due to rapid increases in the global population and losses of arable land, increasing crop yields is important for addressing food shortages throughout the world. In particular, the grain weight is the most important component of the grain yield and it is largely determined by the size and composition of the endosperm (Wan et al. 2008; Bednarek et al. 2012).

The endosperm is the major component of the wheat caryopsis, where it comprises most of the volume of the mature grain. Thus, the grain size and weight are determined mainly by the degree of endosperm growth (Reddy and

Juan Geng and Liqun Li contributed equally to this study.

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Daynard 1983; Chojecki et al. 1986; Liu et al. 2009). Song et al. (2007) found that a larger cell size in the endosperm and heavier grains were caused by a faster rate of dry matter accumulation, which were determined by the grain milk filling rate. During endosperm development, the grain-filling period may be related to cell division and enlargement (Xu et al. 2007). Furthermore, the development of the wheat caryopsis is related to starch synthesis and accumulation, which contribute directly to the yield and quality of wheat (Zhao et al. 2003).

CKs are important hormones that regulate cell division and grain filling, and they are significantly correlated with seed development (Yang et al. 2002). Liu et al. (2013) indicated that the Z + ZR (types of CKs) levels in kernels were positively correlated with the maximum kernel weight as well as the maximum and average grain milk filling rates. In addition, high levels of CKs were generally found in the developing wheat endosperm, where they may be required for cell division during the early stage of seed setting (Liu et al. 2013). Isopentenyl transferases (*IPTs*) and CK oxidases (*CKXs*) are important gene families for maintaining CK homeostasis (O'Keefe et al. 2011). Higher endogenous CK levels can be achieved by upregulating *IPT* genes, *TaIPT2*, *TaIPT5*, and *TaIPT8*, or by downregulating *CKX* genes, *TaCKX1* and *TaCKX2* (Song et al. 2012).

In fact, grain filling is due to starch accumulation. Thus, as the most important component of the wheat endosperm, starch comprises most of the dry weight of the wheat caryopsis (Becraft 2001; Uhlmann and Beckles 2010; Wei et al. 2010). ADP-glucose pyrophosphorylase (AGPase) plays an important role in starch synthesis, and improving the AGP activity can enhance the sink strength of developing seeds (Liang et al. 2001; Smidansky et al. 2002). AGPase, which comprises two large subunits (AGPase LS) and two small subunits (AGPase SS) (Huang et al. 2014), catalyzes the rate-limiting reaction in starch biosynthesis in plants where it uses the substrates glucose 1-phosphate and ATP to produce ADP-glucose and pyrophosphate. ADP-glucose is the glucose donor for starch synthases (Smidansky et al. 2002). Moreover, as shown in previous studies, the wheat homologues of *OsRSR1* (*TaRSR1*) and *OsbZIP58* (SPA) are negative regulators of most starch metabolic genes (Fu and Xue 2010; Kang et al. 2013; Wang et al. 2013), and thus they are candidate genes for improving wheat with high amylase starch.

The ubiquitin–proteasome system (UPS) is important for determining the seed size and stress tolerance in plants (Santner and Estelle 2010; Capron et al. 2012). Capron et al. (2012) showed that the crosstalk between phytohormones and UPS might play a key role in wheat grain development. Some E3 ligase and hormone-related genes seem to be up- or downregulated during the early and late stages of the grain development. After clarifying these hormonal signaling pathways, it has become clear that UPS plays an important role in hormone perception and response (Dharmasiri and Estelle 2004; Santner and Estelle 2009, 2010). The close relationships between the UPS and hormones were first described based on the identification of an F-box protein called TIR1 (Transport Inhibitor Response 1), which acts as an auxin receptor (Gray et al. 1999). Subsequently, RING ligases were found to promote normal ABA signaling by regulating the abundance of ABA responsive transcription factors (Zhang et al. 2005; Stone et al. 2006).

As a homologue of *OSGW2* in rice, *TaGW2* is a weight-related gene that encodes a functional E3 RING-type ubiquitin ligase (Su et al. 2011). In recent years, several studies have demonstrated the effect of *TaGW2* in wheat on the grain size parameters, and several single nucleotide polymorphisms have been found in its promoter region (Su et al. 2011; Zhang et al. 2013; Jaiswal et al. 2015). Further analysis has shown that *TaGW2-6A* negatively regulates the kernel width and kernel weight (Hong et al. 2014; Jaiswal et al. 2015). Interestingly, a single T base insertion in *TaGW2-6A* in the eighth exon causes premature termination in the large-kernel variety (Lankaodali), thereby leading to increases in the grain width and weight (Yang et al. 2012).

The functional effect of *TaGW2-6A* on the weight of the wheat caryopsis has been studied widely. However, little is known about how *TaGW2-6A* allelic variations affect wheat caryopsis development. Thus, in the present study, we investigated the cytological and grain filling characteristics of *TaGW2-6A* allelic variants using a near-isogenic line (NIL). We also determined the changes in the expression levels of CK and starch-related genes to understand the influence of *TaGW2-6A* allelic variations. Our results showed that *TaGW2-6A* allelic variation may regulate the expression of related genes via the UPS to control the grain size and weight. These findings provide new insights into the influence of *TaGW2-6A* allelic variations on the grain size and kernel weight in bread wheat.

Materials and methods

Plant materials and growth

Two wheat lines were selected for this study: Chinese Spring (CS) and NIL31. NIL31 was derived from a cross between the Chinese winter wheat cultivar Lankaodali (TKW = 57.49 ± 0.88 g, with the insertion of a T base at the 977-bp position in the eighth exon of the *TaGW2* allele compared with Chinese Spring) and CS (TKW = 27.75 ± 0.62 g), where recurrent backcrossing with the parent CS was performed for six generations to obtain the BC6F2 population, which was accompanied by marker-assisted selection with SNPs (Yang et al. 2012). One BC6F2

plant was self-pollinated for four generations to obtain NIL31 based on its larger kernel size, higher grain weight phenotype, and the T base insertion in the *TaGW2* coding sequence genotype. A previous mapping assay located the *TaGW2* mutation allele on chromosome 6A (Yang et al. 2012). The specific recipient genomic compositions of the NIL31 are shown in Supplemental Fig. S1. The basic characteristics of the materials are provided in Supplemental Table S1.

NIL31 and CS were planted in stress-free soil conditions at Northwest Agriculture and Forestry University (108°4′E, 34°16′N) in China during the cropping seasons of 2014–2015 and 2015–2016. Thirty seeds per row were manually planted individually with eight lines at 25 cm apart per 2 m row, with a line spacing of 15 cm, and the field plots were managed according to the same methods employed locally for commercial production. We performed three biological replicates for each treatment. When the anthers first appeared in the upper part of the floret spikelets, the ears of the main stem were marked with different colored tags in the morning. Labeled spikelets in NIL31 and CS were sampled at seven different dates after anthesis, i.e., 3, 6, 9, 12, 15, 20, and 25 days after anthesis (DAA) (two further sampling dates were added to test grain filling, i.e., 30 and 35 DAA). About one-third of the sampled grains were frozen in liquid nitrogen for 1 min and then stored at −80 °C for gene expression and endogenous hormone analyses. The other two-thirds of the sampled grains were used for grain milk filling and cytological studies.

Gene expression analysis

Total RNA was extracted from the NIL31 and CS seeds sampled on different dates using TRIzol reagent (Takara, Dalian, Liaoning, China), according to the manufacturer's instructions. First-strand cDNA was synthesized with purified RNA, avian myeloblastosis virus (AMV) reverse transcriptase, and oligo (dT15) primers according to the manufacturer's instructions (Takara). cDNA was diluted to 100 ng μL^{-1} with Tris–EDTA buffer and some of the diluted cDNA was used for PCR amplification. Quantitative real-time PCR was performed in 96-well blocks using a LightCycler®96 detection system (Roche, Basel, Switzerland). All of the quantitative real-time PCR primer sequences are listed in Supplemental Table S2. Primers were designed using the NCBI website and the specificity was confirmed by running BLAST. The genes studied in this experiment by quantitative real-time PCR are shown in Supplemental Table S3. The reaction mixtures contained 10 µL of 2× Fast-start Essential DNA Green Master (Roche), 0.6 µL of each primer (10 mM), 100 ng of cDNA template, and double-distilled H_2O to make up a final volume of 20 µL. The same thermal profile was used for all of the PCR reactions, i.e., 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 57–64 °C for 15 s, and 72 °C for 15 s, and then at 72 °C for 10 min. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), where the bread wheat 18S rRNA was used as an internal reference. Transcript abundance data were normalized against the average transcript abundance of 18S rRNA. Three biological replicates were performed for each allelic variant and three technical replicates were analyzed for each biological replicate, where the 15 DAA stage of CS was used as the reference sample for ΔΔCT.

Endogenous hormone analysis

Extraction and purification of the CKs (Z and ZR) was conducted according to Agar et al. (2006) with some modifications. The frozen samples (1 g) were powdered in liquid N_2 and 10 mL of cold methanol containing 1 mM butylated hydroxytoluene as an antioxidant was added to the fine powder, before storing at 4 °C for 24 h in the dark. The samples were filtered through 0.45-µm polytetrafluoroethylene (Sartorius) filters. The extract was redissolved in 0.1 M KH_2PO_4 buffer (pH 8.0) and centrifuged at 8000 *g* for 10 min at 4 °C. The supernatant was transferred into a vial containing 1 g of polyvinylpolypyrrolidone (MW = 30,000, Sigma Chemical). The samples were filtered through a 0.45-µm Millipore filter and injected into high-performance liquid chromatography system to detect Z and ZR.

An isocratic system was used for high-performance liquid chromatography analysis. The extracts in the vials were injected into high-performance liquid chromatography system equipped with a Waters 2695, ultraviolet detector (Unicam Analytical Systems, Cambridge, UK) and a symmetric RC18 column (250 mm × 4.6 mm, particle size, 5 µm). The mobile phases used for Z and ZR were prepared with methanol and ultrapure water at 2:3, and the flow rate and wavelength were 0.8 mL/min and 254 nm, respectively. The injection volume was 10 µL and the samples were injected three times to reduce the error. Under the chromatographic conditions mentioned above, Z and ZR standards (analytical grade, Sigma-Aldrich, St Louis, MO, USA) were prepared separately in the corresponding mobile phase and analyzed by high-performance liquid chromatography to determine the retention time with a run time of 40 min/sample. The Z and ZR standards were detected at 4.007 and 5.420 min, respectively. Standard curves were constructed based on five concentrations. Statistical analysis of the hormonal data was based on a completely randomized design with three replicates.

Dry weight and grain filling rate

For each variety, we collected ten ears in triplicate at each sampling time point. In total, nine sets of samples were obtained to measure the dry weights of the spikelets. The first and second flowers on each spikelet were removed from each spike to collect 20 grains and a total of 200 kernels were dried rapidly at 105 °C for 30 min, before drying at 80 °C until a constant weight was reached. The dry weights were determined based on three biological replicates. The grain filling rates at different time points were determined based on the dry grain weight using the following equation:

$$Y = K/(1 + ae^{-bt}). \quad (1)$$

The grain filling rate (V) was calculated as the derivative of Eq. (1):

$$V = Kabe^{-bt}/(1 + ae^{-bt})^2, \quad (2)$$

where Y is the average weight per grain (g), t is the number of days after flowering, K, and a and b are coefficients determined from the regression.

Histological analysis

The caryopses were collected at different DAAs, where they were cut into 2-mm-thick sections from the center of each caryopsis with a clean razor blade and then immersed immediately in 4% glutaraldehyde phosphate buffer fixative at 4 °C. The samples were rinsed four or five times (10 min each) with 0.1 mol L^{-1} phosphate-buffered saline (PBS, pH 6.8) and dehydrated using an ethanol series comprising 30, 50, 70, 80, and 90% (10 min each), and then 100% (three times, 20 min each). After rinsing, each sample was fixed using 0.1 mol L^{-1} osmic acid for 1.5–2.5 h and then rinsed five times (10 min each) with 0.1 mol L^{-1} PBS (pH 6.8). Next, LR White resin was used to infiltrate and embed the material. The samples were then polymerized at 70 °C for 12 h in an oven. Finally, the samples were cut into 1-μm slices using a histotome (RM2265, Leica), stained with 0.03 mol L^{-1} toluidine blue for 15 s, and observed and photographed under a confocal microscope equipped with a digital camera (Olympus DP80). The cell numbers in the endosperm were measured as described by Singh and Jenner (1982).

Measurements of grain traits

After natural maturation, the thousand-kernel weight (TKW), kernel width, kernel length, and kernel thickness were measured. After harvest, 30 grains were randomly selected from each cultivar and lined up lengthwise along a ruler to measure the average grain length, and then arranged breadth-wise to measure the grain width. Three biological replicates were performed for each of the two lines. The middle parts of 20 grains were measured with vernier calipers to establish the average grain thickness. Three independent samples of 250 grains were weighed and the means were converted to the TKW.

Data analyses

Statistical analyses were performed with SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences were detected using one-way ANOVA and the mean values were tested at 5% probability based on the least significant difference test. Figures were prepared using Sigmaplot 12.5.

Results

Effects of *TaGW2-6A* allelic variations on the grain size and grain weight

TaGW2-6A allele variations significantly increase the grain width and grain weight (Yang et al. 2012; Du et al. 2016). This conclusion was also verified by our experiments (Fig. 1). Compared with CS, we observed significant increases in the grain width (about +20%) in NIL31 (Fig. 1e), as well as the grain thickness (about +13.6%) (Fig. 1f) and grain length (about +12.9%) (Fig. 1d) to different extents. We also detected a significant increase (about +47.9%) in the TKW in NIL31 (Fig. 1c). These results demonstrate that the insertion type allelic variant of *TaGW2-6A* is closely related to the grain size and TKW.

Effects of *TaGW2-6A* allelic variations on endosperm development

To obtain insights into the functional effects of *TaGW2-6A* allelic variations on the endosperm, we compared cross sections and cell numbers in the endosperm of NIL31 and CS during grain development. As the endosperm developed, the length and width of the central endosperm cells increased gradually from 3 to 25 DAA in both CS and NIL31, where the cell size was larger in NIL31 than CS, especially from 9 to 15 DAA (Fig. 2a; Supplemental Table S4). The number of endosperm cells increased rapidly in the grains from 3 to 15 DAA, and reached a peak at 15 DAA in both lines. Subsequently, the number of endosperm cells decreased slightly, possibly due to programmed cell death starting at 14–20 DAA (Agarwal et al. 2011). However, the *TaGW2-6A* allelic variants had significantly higher cell proliferation rates than CS (Fig. 2c; Supplemental Table S5), which led to a higher sink strength. We also noted that the episperm gradually thickened in CS and NIL31, and the episperm cells

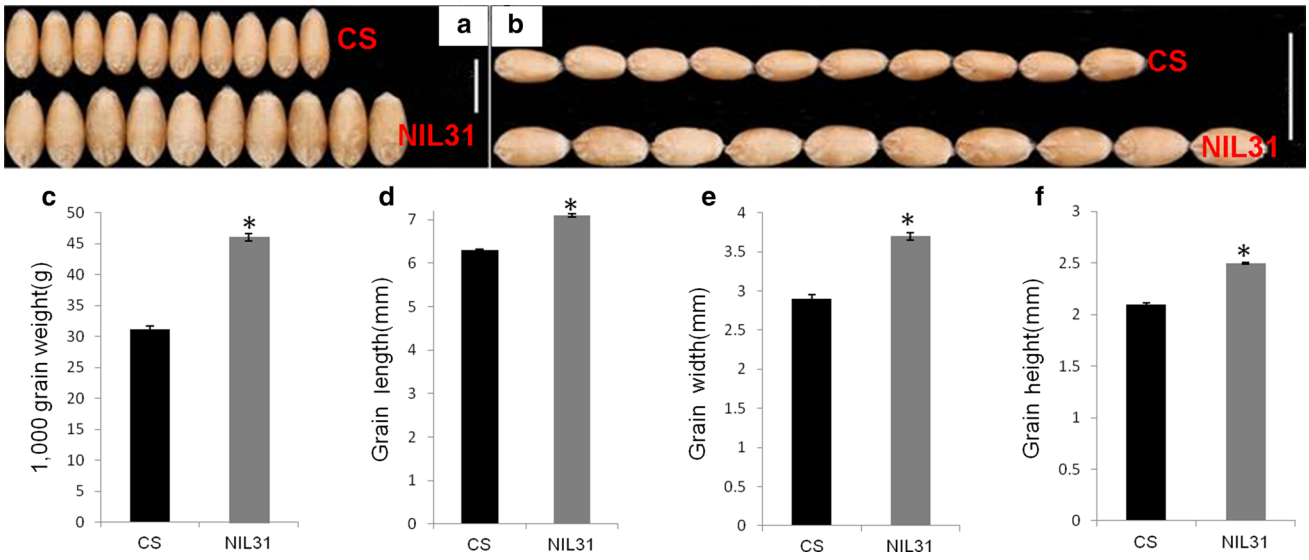

Fig. 1 The *TaGW2-6A* allele variation increases the grain size and weight in wheat. **a**, **b** Comparisons of the grain width and grain length, respectively, in CS and NIL31. **c** TKW in CS and NIL31. **d** Grain length in CS and NIL31. **e** Grain width in CS and NIL31. **f** Grain height in CS and NIL31. Data represent means ± standard errors based on three biological replicates (*$P < 0.05$). *Error bars* indicate the standard errors calculated from three independent experiments. *Scale bars* 5 μm (**a**) and 10 μm (**b**)

formed a compact seed coat after 9 DAA. Compared with CS, NIL31 had a relatively thinner episperm (Fig. 2a, b), which may have promoted the growth of endosperm cells, thereby increasing the setting strength.

Effects of *TaGW2-6A* allelic variations on the grain milk characteristics

The increases in the grain weight and grain filling rate for CS and NIL31 fitted by a logistic growth equation are shown in Fig. 3. Previous experiments have shown that a large kernel weight is closely related to a high filling rate (Egli 2006). NIL31 has a larger endosperm and heavier grains, so we investigated the grain milk filling characteristics of NIL31 and CS. At 3 DAA, the fresh weight of the endosperm was slightly higher in NIL31 than CS (Fig. 3a; Supplemental Table S5), although there was no difference in the dry weight (Fig. 3b; Supplemental Table S5). The fresh weight and dry weight of the endosperm were significantly higher in NIL31 than CS from 9 DAA, and these differences were maximized at around 35 DAA (Supplemental Table S5). The fresh weight and dry weight of the endosperm were 46.04 and 48.71% higher, respectively, in NIL31 compared with CS. NIL31 had a significantly higher filling rate during grain development, and the maximum grain filling rate was reached at 15 DAA in both (Fig. 3c; Supplemental Table S5). These results indicate that the larger endosperm (or the larger cell size in the endosperm) and heavier grain in NIL31 led to the more rapid accumulation of dry matter, which was promoted by the *TaGW2-6A* allele variations.

Effects of *TaGW2-6A* allelic variations on the transcription levels of CK-related genes

CKs are major phytohormones that regulate many physiological processes in plants, including cell division and enlargement, and grain filling. According to the endosperm development pattern, we selected samples from 6 DAA, 9 DAA, 12 DAA, and 15 DAA for gene expression analysis (Figs. 2, 3). We determined the transcript levels of CK synthesis genes, i.e., *TaIPT2*, *TaIPT3*, *TaIPT5*, and *TaIPT8*, and CK degradation genes, i.e., *TaCKX1*, *TaCKX2*, and *TaCKX6*. Quantitative real-time PCR showed that the transcript patterns of *TaIPT2*, *TaIPT3*, *TaIPT5*, and *TaIPT8* in NIL31 were similar to those in CS, whereas the transcript levels of these genes were significantly higher in NIL31 than CS (Fig. 4a). In contrast to the *TaIPTs*, the transcript levels of the *TaCKXs* were significantly higher in CS than NIL31 (Fig. 4b). Most of these genes were specifically expressed at 12 DAA, and thus they were closely related to the rapid changes in the numbers of cells and grain filling during this period (Figs. 2c, 3), which led to the increased grain size.

Effects of *TaGW2-6A* allelic variations on the level of endogenous CK

Figure 5 shows that the Z + ZR had similar patterns in CS and NIL31. The contents of Z + ZR in CS and NIL31 grains transiently increased during the early grain-filling stage and reached a maximum at 12 DAA before decreasing thereafter, which was consistent with the changes in CK-related gene

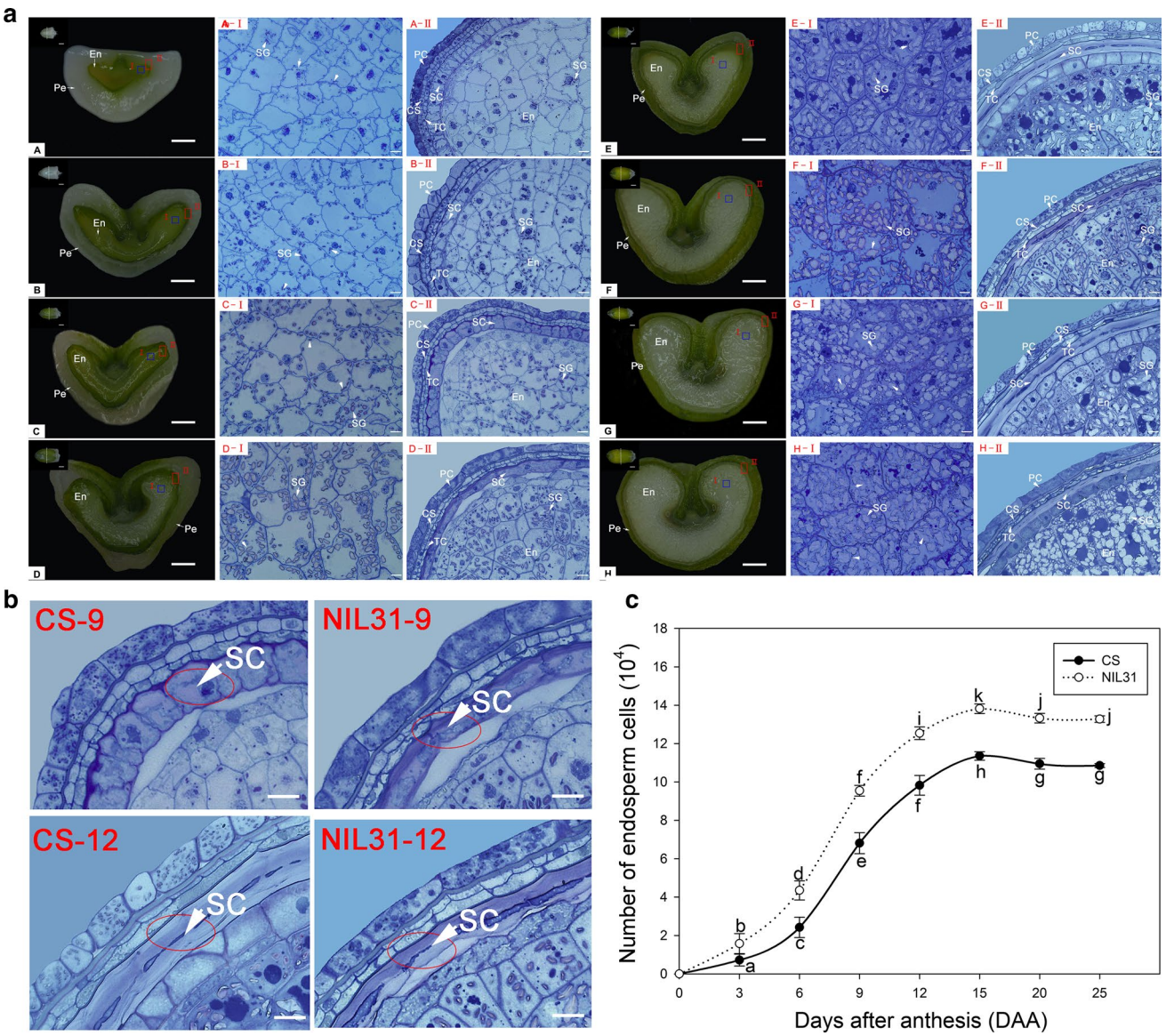

Fig. 2 Histological images of wheat caryopsis and cell proliferation during endosperm development. **a** Changes in endosperm cells during different developmental stages. *A–H* Transverse sections of the developing grains in CS (*A*, *C*, *E*, *G*) and NIL31 (*B*, *D*, *F*, *H*) at 6 DAA (*A*, *B*), 9 DAA (*C*, *D*), 12 DAA (*E*, *F*), and 15 DAA (*G*, *H*), where the *insets* show the intact grain in the corresponding period. I and II represent the central endosperm cells and pericarp in CS and NIL31 at 6–15 DAA, respectively. **b** Comparison of the seed coat in CS and NIL31 at 9 DAA and 12 DAA. The *red ovals* represent the episperm in CS and NIL31 at 9 DAA and 12 DAA, respectively. *Scale bars* 10 μm. **c** Proliferation of endosperm cells in NIL31 and CS during grain development. *PC* parenchyma cell, *CS* cross cell, *TC* tube cell, *SC* seed coat, *En* endosperm, *SG* starch granule. *Scale bars* 100 μm in *insets*, 50 μm (*A–H*) and 20 μm (*A–H-I* and *A–H-II*)

expression and the grain filling rate. However, the level of Z + ZR was higher in NIL31 grains than CS grains during 6–15 DAA, especially on 9 DAA and 12 DAA.

Effects of *TaGW2-6A* allelic variations on the transcript levels of starch-related genes

We also examined the expression of starch-related genes, including the genes for the key starch biosynthesis enzyme AGPase (*TaAGPL* and *TaAGPS*) and negative regulators (*SPA* and *TaRSR1*) (Fig. 4c). The expression levels of all four genes increased gradually and then decreased, where the peak was reached at 12 DAA in both lines. Moreover, the transcript levels of *TaAGPL* and *TaAGPS* were greatly elevated in NIL31, especially on 6 DAA (Fig. 4c), which was consistent with the rapid development of starch granules in NIL31 according to histological sectioning (Fig. 4d). In contrast to *TaAGPL*

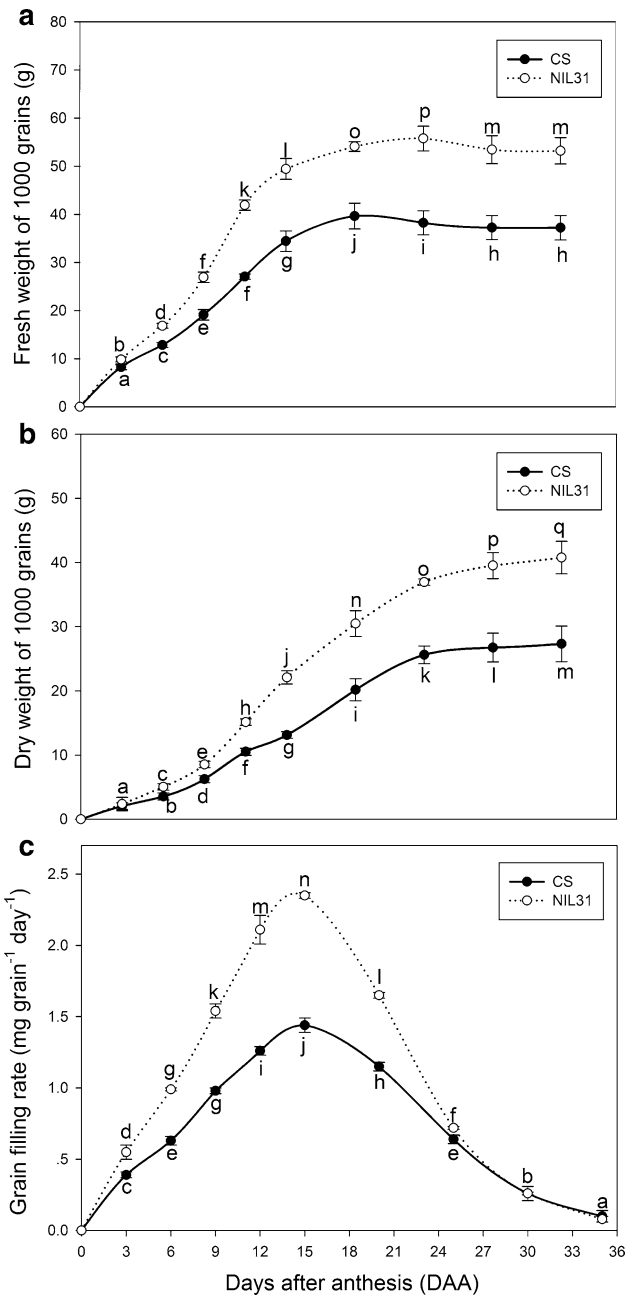

Fig. 3 Characterization of grain milk filling in NIL31 and CS. **a** Differences in the fresh weight. **b** Differences in the dry weight. **c** Differences in the grain filling rate

and *TaAGPS*, the expression levels of the negative regulators *SPA* and *TaRSR1* were higher in CS than NIL31 (Fig. 4c). Thus, our observations suggest that the earlier and more rapid starch synthesis and accumulation in NIL31 may be caused by changes in the expression levels of starch-related genes, which are affected by *TaGW2-6A* allelic variants.

Discussion

Large kernel size is an important evolutionary and agricultural trait, and it has been the main goal of selection during domestication and crop improvement. Thus, the genes associated with variations in kernel size may have been preferentially selected during the long-term domestication process. *TaGW2-6A* is a gene that is closely associated with grain development in wheat (Su et al. 2011; Bednarek et al. 2012; Yang et al. 2012). In recent years, several studies have explored the functional effects of the *TaGW2-6A* gene on grain size parameters and weight (Yang et al. 2012; Hong et al. 2014; Jaiswal et al. 2015). In the present study, we showed that a *TaGW2-6A* allelic variation (NIL31) could significantly increase the grain width and grain weight, thereby agreeing with the findings reported by Du et al. (2016).

The persistent endosperm forms the vast majority of the mature grain in rice, maize, and wheat. Previous studies have shown that there is a higher rate of dry matter accumulation and endosperm cell proliferation in large-grained varieties (Reddy and Daynard 1983; Chojecki et al. 1986). Thus, the grain size and weight are affected greatly by cell size via growth and expansion due to the vastly increased accumulation of storage material by endosperm cells. Similarly, in the current study, we found that the large-grained *TaGW2-6A* allelic variant (NIL31) had a wider (larger) grain due to the increased numbers of cells and the cells were expanded compared with those in the small-grained CS (Fig. 2a, c; Supplemental Table S4 and S5). Larger kernels allow greater endosperm growth and provide greater sink strength due to the accelerated rate of grain milk filling and starch accumulation (Figs. 2a, 3c). In addition, NIL31 had a thinner and looser episperm compared with CS, which may allow greater endosperm growth and provide a greater area in contact with the endosperm (Fig. 2b).

CKs are important hormones and they are directly responsible for endosperm growth and the final seed size (Li et al. 2013a, b). Several studies have shown that CKs can increase the seed size and grain yield by promoting grain filling and cell division (Yang et al. 2000, 2002; Rijavec et al. 2009). The homeostasis of CKs is determined by the balance between the de novo synthesis of *IPTs* and the irreversible degradation of *CKXs* (O'Keefe et al. 2011). Perturbing homeostasis by downregulating the expression of any one of *CKX1*, *CKX2*, or *CKX6* during seed development can result in the accumulation of CK (Ashikari et al. 2005). As shown by Song et al. (2012), we found that the increased expression of *TaIPT2*, *TaIPT3*, *TaIPT5*, and *TaIPT8*, and the decreased expression of *TaCKX1*, *TaCKX2*, and *TaCKX6* may lead to the accumulation of CK in NIL31. In addition, the Z + ZR content of NIL31 was higher than that of CS during 6–15 DAA, especially on 9 DAA and 12 DAA according to the

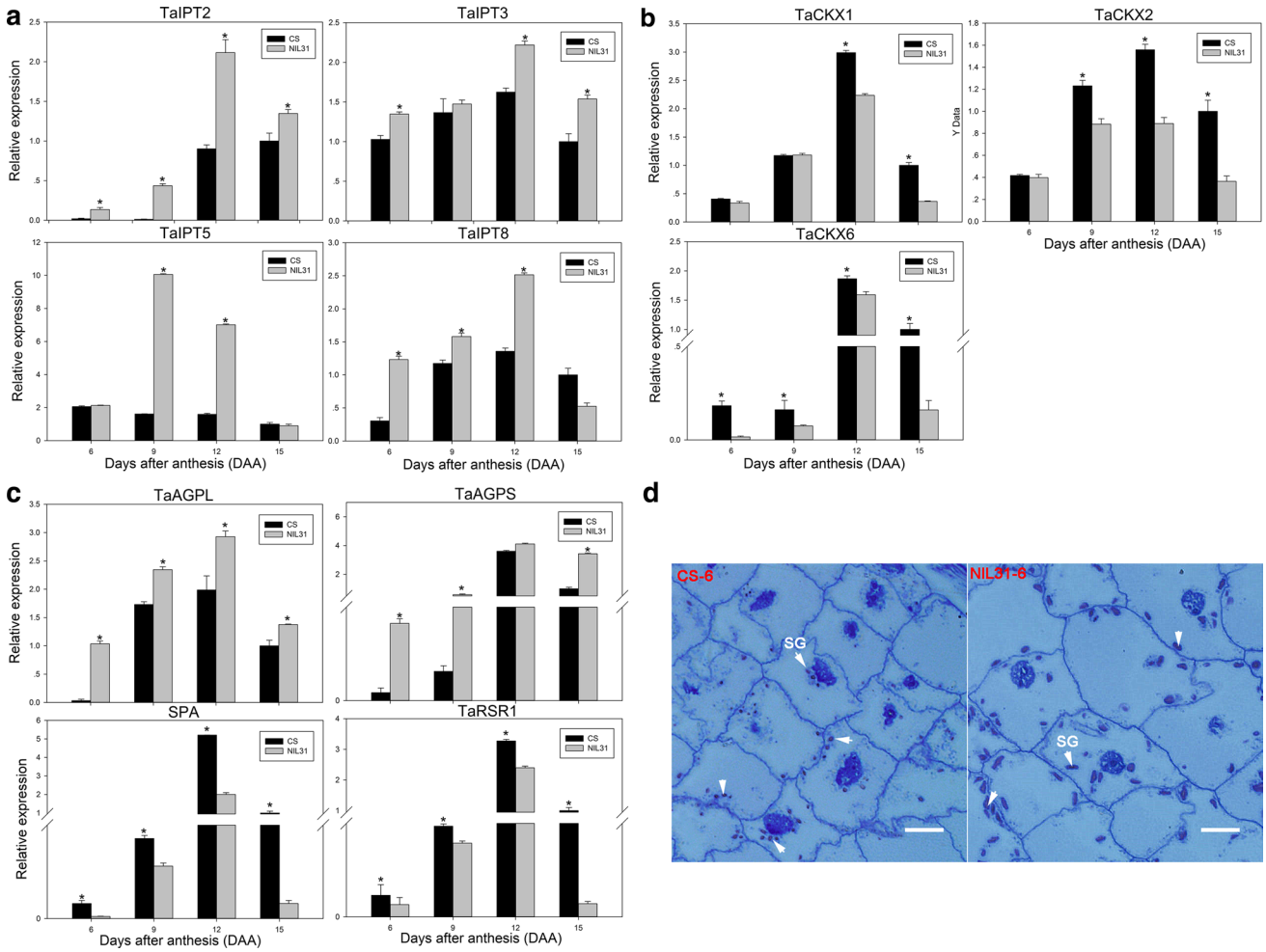

Fig. 4 Expression patterns of CKs and starch-related genes in CS and NIL31. **a** Expression patterns of *TaIPT2*, *TaIPT3*, *TaIPT5*, and *TaIPT8*. **b** Expression patterns of *TaCKX1*, *TaCKX2*, and *TaCKX6*. **c** Expression patterns of *TaAGPL*, *TaAGPS*, *SPA*, and *TaRSR1*. *Error bars* indicate the standard errors calculated based on three independent experiments (*$P < 0.05$). **d** Starch development in CS and NIL31 at 6 DAA. *Arrowheads* indicate starch granules; *SG* starch granules. *Scale bar* 10 μm

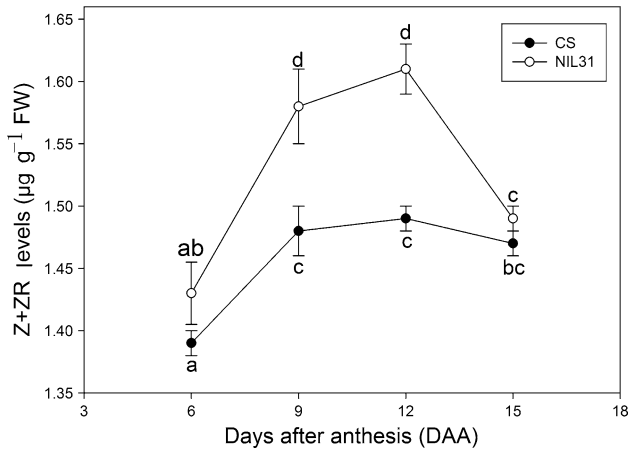

Fig. 5 Changes of endogenous cytokinin in CS and NIL31

determination of the CK content (Fig. 5). The higher levels of CK in the *TaGW2-6A* allelic variants may lead to more cells and larger grains.

A key role for AGPase in starch biosynthesis in the endosperm may determine endosperm filling, thereby enhancing the sink strength (Smidansky et al. 2003; Kato et al. 2007; Li et al. 2011). High transcription levels of AGPase genes are closely and positively related to starch synthesis (Ohdan et al. 2005; Li et al. 2011). In the present study, we speculated that the higher expression levels of *TaAGPL* and *TaAGPS* in NIL31 may lead to a greater rate of starch accumulation, which is consistent with the observations in Fig. 2. Reduced expression levels of *TaRSR1* and *SPA*, which can enhance starch biosynthesis and stimulate cell division (Singh et al. 2015; Liu et al. 2016), were also found in NIL31 in the present study (Fig. 4c). Thus, we consider that *TaGW2-6A* allelic variants may promote

the accumulation of starch in wheat grains by affecting the expression of starch-related genes. The enhanced accumulation of starch led to increases in the dry weight and fresh weight, which contributed to a higher seed yield and larger grain size (Li et al. 2011).

Evidence indicates that the expression of CK-related genes is tightly linked to the regulation of proteins (Jasinski et al. 2005; Yanai et al. 2005; Li et al. 2013a, b). Xu et al. (2011) found that the excessive accumulation of SARK reduced the accumulation and function of CKs by upregulating *CKXs* and downregulating *IPTs*. Completely the opposite changes in *IPTs* and *CKXs* were found in NIL31 in the present study. The *TaGW2-6A* allele variation encodes an E3 ubiquitin ligase and the 1-bp insertion could have led to a loss of function in the degradation of substrate proteins via UPS due to the truncation of 96 amino acids (Yang et al. 2012). These findings suggest that a protein substrate of E3 and its accumulation might lead to elevated CK levels by directly controlling the expression levels of *IPTs* and *CKXs* in NIL31. Furthermore, several specific proteins related to cell development have been reported in *TaGW2-6A* allele variants (Du et al. 2016) and we consider that the candidate protein may be one of these.

Starch is another major contributor to the grain yield and quality (Smidansky et al. 2002, 2003), where it is synthesized from ADP-glucose. In this study, we found that NIL31 had significantly enlarged and more abundant starch granules, as demonstrated by histological sectioning analysis, particularly at 6 DAA (Figs. 2a, 4d). We also detected the upregulated expression of the starch biosynthesis rate-limiting enzyme genes (*AGPL* and *AGPS*) as well as the downregulated expression of the negative regulatory genes *TaRSR1* and *SPA*, especially during 6–15 DAA (Fig. 4c). These findings strongly resemble those found in plants with enhanced sugar levels, where the expression levels of genes encoding starch biosynthesis enzymes are induced (Rook et al. 2001, 2006; Yin et al. 2010). Similarly, our previous study indicated that *TaGW2-6A* allelic variations may lead to greater starch accumulation in NIL31 due to higher sugar contents during the early stage of wheat development (Du et al. 2016). The involvement of the UPS in the sugar response has also been reported (Farrás et al. 2001). Thus, we hypothesize that sugar may be another substrate for RING E3 ubiquitin ligase encoded by the *TaGW2-6A* allele variants, and that the accumulation of sugar may stimulate the expression of starch-related genes, thereby promoting the accumulation of starch.

Therefore, we suggest that the enhanced endosperm size might be an indirect effect of *TaGW2-6A* allelic variation. Based on our results, we hypothesize that the *TaGW2-6A* allelic variant may utilize the UPS to change the expression levels of CK and starch-related genes to promote cell division and expansion as well as starch accumulation during grain filling, thereby allowing the formation of large grains. We present a model to explain the potential effect of *TaGW2-6A* allelic variation on the regulation of the grain width (size), weight, and yield according to our study (Fig. 6). It is important to understand the seed development process to improve the grain yield, but the functional effects of *TaGW2-6A* allelic variants during grain development on the final seed size and weight are still unclear in wheat. Our findings provide insights into the effects of *TaGW2-6A* allelic variants on seed development in wheat via the UPS. The *TaGW2-6A* gene is one of the key regulators of grain (seed) size and our results may facilitate breeding efforts to improve the grain yield in wheat.

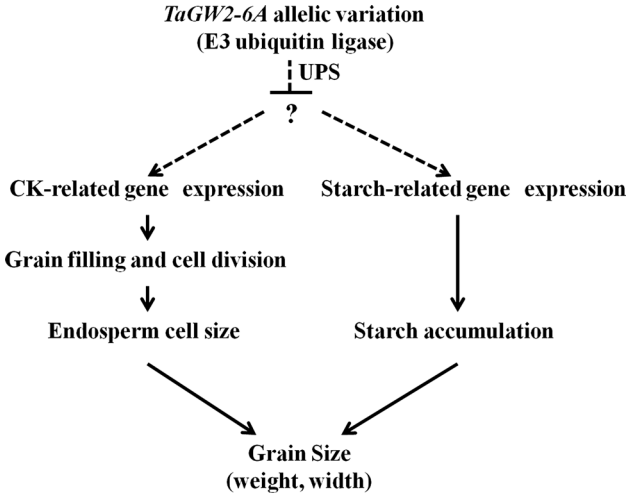

Fig. 6 Proposed model of the role of the *TaGW2-6A* allelic variant in the regulation of grain size and weight. The *TaGW2-6A* allelic variant prevents the degradation of the targeted substrate via the UPS, as well as regulating the expression of CK and starch-related genes, thereby indirectly influencing the grain milk filling rate, starch accumulation rate, endosperm cell size, endosperm size, and ultimately the grain size and grain weight

Author contribution statement JG conducted the experiments and wrote the manuscript. XL conceived and designed the research. LL contributed reagents or analytical tools. XL and QL contributed to the interpretation of results and provided a critical analysis of the manuscript. YZ, YL, and LZ contributed to the completion of this experiment. All of the authors read and approved the manuscript.

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