

# Functional characterization of *GPC-1* genes in hexaploid wheat

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**Abstract** In wheat, monocarpic senescence is a tightly regulated process during which nitrogen (N) and micronutrients stored pre-anthesis are remobilized from vegetative tissues to the developing grains. Recently, a close connection between senescence and remobilization was shown through the map-based cloning of the *GPC* (*grain protein content*) gene in wheat. *GPC-B1* encodes a NAC transcription factor associated with earlier senescence and increased grain protein, iron and zinc content, and is deleted or non-functional in most commercial wheat varieties. In the current research, we identified ‘loss of function’ ethyl methanesulfonate mutants for the two *GPC-B1* homoeologous

genes; *GPC-A1* and *GPC-D1*, in a hexaploid wheat mutant population. The single *gpc-a1* and *gpc-d1* mutants, the double *gpc-1* mutant and control lines were grown under field conditions at four locations and were characterized for senescence, GPC, micronutrients and yield parameters. Our results show a significant delay in senescence in both the *gpc-a1* and *gpc-d1* single mutants and an even stronger effect in the *gpc-1* double mutant in all the environments tested in this study. The accumulation of total N in the developing grains showed a similar increase in the control and *gpc-1* plants until 25 days after anthesis (DAA) but at 41 and 60 DAA the control plants had higher grain N content than the *gpc-1* mutants. At maturity, GPC in all mutants was significantly lower than in control plants while grain weight was unaffected. These results demonstrate that the *GPC-A1* and *GPC-D1* genes have a redundant function and play a major role in the regulation of monocarpic senescence and nutrient remobilization in wheat.

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## Abbreviations

DAA	Days after anthesis
DIC	<i>Triticum turgidum</i> ssp. <i>dicoccoides</i> (Körn.) Thell
DM	Dry matter
EMS	Ethyl methanesulfonate
GPC	Grain protein content
NAC	NAM; ATAF1,2; CUC2
NY	Newe Ya’ar Regional Research Center, Israel
TAU	Tel Aviv University, Israel
TILLING	Targeting Induced Lesions IN Genoms
TKW	Thousand kernel weight
UCD	University of California, Davis

## Introduction

Wheat accounts for approximately 25 % of worldwide protein supply (FAOSTAT 2009) and is also an important source for micronutrients such as Zn and Fe. Therefore, increasing grain protein content (GPC) and micronutrient concentration is an important research and breeding objective for programs aiming to increase the nutritional value of wheat. In addition, high levels of GPC are also desirable for their positive effect on bread and pasta quality.

Increasing the rate of N remobilization to developing grains utilizes stored N more efficiently and increases GPC (Kade et al. 2005). While most of the carbohydrates found in the wheat grain are the result of assimilation during the grain filling period (Austin et al. 1977a; Blake et al. 2007), grain protein is predominantly derived from the remobilization of N from degraded leaf proteins, which are stored during wheat vegetative growth (Dalling et al. 1976; Austin et al. 1977b; Simpson et al. 1983; Kichey et al. 2007).

*GPC-B1* is the first identified wheat gene responsible for variation in GPC. The allele for high GPC was identified in a wild emmer accession (*Triticum turgidum* ssp. *dicoccoides* (Körn) Thell, henceforth referred to as DIC) collected in Israel (Avivi 1978). This accession was crossed with the durum wheat variety Langdon [*Triticum turgidum* subsp. *durum* (Desf)] and used to create a full set of chromosome substitution lines each containing a different DIC chromosome in the modern durum wheat background (Joppa and Cantrell 1990). These lines were used to discover a major QTL for GPC on the short arm of chromosome 6B (Joppa et al. 1991, 1997) that was later shown to also confer higher Fe and Zn grain concentrations (Cakmak et al. 2004; Distelfeld et al. 2007) and accelerated senescence (Uauy et al. 2006a).

The map-based cloning of *GPC-B1* (also called *NAM-B1*) revealed that this gene encodes a NAC-domain (NAM; ATAF1,2; CUC2) transcription factor that has a functional copy in DIC and a non-functional copy in most modern wheat varieties, due either to a frame shift mutation or to its complete deletion (Uauy et al. 2006b; Hagenblad et al. 2012). In addition to *GPC-B1*, hexaploid wheat has two other functional homoeologous genes, on chromosome 6A (*GPC-A1*) and 6D (*GPC-D1*) and paralogous copies (*GPC-2*) on chromosome 2 (Uauy et al. 2006b). Silencing of all homoeologous *GPC-I* and *GPC-2* genes using RNA interference (RNAi) results in a 24–30 day delay in senescence and a reduction in grain protein, zinc and iron content of more than 30 % in comparison to the wild-type control plants (Uauy et al. 2006b). An RNAseq experiment was carried out to compare the transcriptomes of the *GPC*-RNAi and control lines at an early stage of senescence and identified 691 differentially expressed genes, which included transporters, hormone-regulated genes and

transcription factors, many of which were up-regulated during senescence (Cantu et al. 2011). These results suggest that the *GPC* genes are early regulators of senescence and that senescence is an active process that involves the precise regulation of hundreds of genes. Similar results have been observed in previous transcriptome analyses in other plant species in which senescence-associated gene networks have been shown to be regulated by numerous transcription factors, particularly members of the NAC and WRKY families (Miao et al. 2004; Gregersen and Holm 2007; Jukanti et al. 2008; Breeze et al. 2011).

To better understand the function of the individual homoeologous *GPC-I* genes in common wheat, we isolated knockout mutants for the *GPC-A1* and *GPC-D1* genes from a hexaploid wheat Targeting Induced Local Lesions IN Genomes (TILLING) population (Slade et al. 2005; Uauy et al. 2009). Here we report the effects of single and combined *GPC-I* mutations on senescence, GPC, micronutrient content and yield components under field conditions. In separate greenhouse experiments, we also analyzed the effects of these mutations on the expression of genes that were previously found to be differentially regulated in the *GPC*-RNAi transgenic lines.

## Materials and methods

### Wheat TILLING mutant screen

An EMS (ethyl methanesulfonate)-treated (1 %) population of mutants of the hexaploid wheat (*Triticum aestivum* L. cv. Express, PI 573003; seeds from Western Plant Breeders Inc., <http://www.westbred.com>) was screened for mutations in the *GPC-A1* (2,688 plants) and *GPC-D1* genes (2,880 plants) using the two primer sets described below and procedures described previously (Slade et al. 2005).

### *GPC-A1*

A-genome specific primers TAGCTTGCTAGGGGGAACG AAGAAGATCC and CGTCCAACCTGATGAGACGACG TACAGAA (the underlined G is an intentional mismatch introduced to achieve homoeolog-specific amplification) were designed to amplify the first 953-bp of the *GPC-A1* gene including the first two exons and a part of the third exon.

### *GPC-D1*

D-genome specific primers CGCTCGATCTGGTGGGAT CATCCG and AGACGCGTCGGTGAGCGGTATTCA were designed to amplify a 790-bp fragment of the *GPC-D1* gene, starting 75-bp before the ATG start codon and including all of the first exon and most of the second exon.

## Marker-assisted selection of wheat *GPC-I* mutants

One truncation mutation was selected for each gene and cleaved amplified polymorphism sequence (CAPS) markers were designed to select the mutations during the backcross and intercross process as described below.

### *GPC-A1*

Primers TCAAAACCAACTGGATCATGCA and GGA GAGGATTCATAAATGCGGA were used to amplify a 736-bp fragment of the *GPC-A1* gene that was then digested with one unit of the restriction enzyme *BsrI*. The selected *GPC-A1* mutation eliminates one *BsrI* restriction site and, therefore, the wild-type (four fragments of 15, 159, 226 and 336-bp) and the mutant (three fragments of 15, 159 and 562-bp) alleles can be differentiated by their divergent restriction digest pattern.

### *GPC-D1*

A 790-bp region including the *GPC-D1* mutation was amplified using the D-genome-specific primers described above and was then digested with one unit of the restriction enzyme *BsrI*. The selected *GPC-D1* mutation eliminates one *BsrI* restriction site and, therefore, the wild-type allele shows three restriction fragments (32, 132, and 626-bp) while the mutant allele shows two fragments (32 and 758-bp).

RNA was extracted from *gpc-a1* and *gpc-d1* mutants and control plants from both greenhouse and field experiment (UCD 2012), and cDNAs were sequenced to identify and validate the TILLING mutations detected in the genomic DNA.

Selected M<sub>3</sub> plants from the EMS-treated population were backcrossed to non-mutagenized cv. Express to reduce the mutation load. The BC<sub>1</sub>F<sub>1</sub> plants were self-pollinated and plants homozygous for the mutations were selected from the BC<sub>1</sub>F<sub>2</sub> plants using the relevant CAPS markers. The selected homozygous plants from each progeny were crossed to combine mutations in *GPC-A1* and *GPC-D1*. The different mutant combinations were backcrossed two additional times to cv. Express to further reduce the mutation load. BC<sub>3</sub>F<sub>2</sub> plants were used to select homozygous single mutants (*gpc-a1* and *gpc-d1*), double mutant (*gpc-I*) and control plants (sister lines with no *GPC-I* mutations). The selected plants were self-pollinated (BC<sub>3</sub>F<sub>3</sub>) to increase seed numbers for field evaluations.

## Field experiments

Phenotypic evaluations of *GPC* mutant lines were conducted for two consecutive years. During the 2010–11 growing season, experiments were carried out at three locations

in Israel; Newe Ya'ar Regional Research Center (NY 2011), Tel Aviv University (TAU 2011) and Kokhav Michael (KM 2011). During the 2011–2012 growing season, experiments were conducted at two locations in Israel (TAU 2012 and NY 2012) and one location in the USA at the University of California, Davis (UCD 2012). All experimental plots were arranged in a randomized complete block design.

In addition, a greenhouse experiment was performed in UCD during the winter of 2011 and a net-house experiment was performed in TAU during the winter of 2013 (TAU 2013). In both experiments, plants were grown in five liter pots and anthesis date was recorded for each spike.

## Field locations and description

### 2010–2011

In Newe Ya'ar Regional Research Center (NY 2011, 32°42'N 35°10'E, 600 mm annual precipitation) planting occurred on December 24th 2010, on heavy soil (inorganic matter 18.7 %; sand, 22.6 %; silt, 57.0 %; clay and organic matter, 1.7 %).

In Tel Aviv University (TAU 2011, 32°6'N 34°48'E, 546 mm annual precipitation) planting occurred on January 6th 2011 on hamra soil (90 % sand and 10 % clay, soil N 8.2 mg kg<sup>-1</sup>).

In Kokhav Michael (KM 2011, 31°37'N 34°40'E, 446 mm annual precipitation) planting occurred on January 2nd 2011 on dark brown soil.

### 2011–2012

TAU 2012 and NY 2012 were located at the same locations as TAU 2011 and NY 2011, respectively. TAU 2012 planting occurred on December 11th 2011. NY 2012 planting occurred on December 7th 2011.

In Israel, all experiments (2011 and 2012) consisted of 5 randomized complete blocks (except for NY 2011 which had 3 blocks). Seeds were germinated in germination paper and seedlings were planted 4 days later. Seedlings were hand-sown in 80 cm rows with 8 plants per row, 30 cm between rows and 4 rows per genotype totaling 32 plants (genotype/block). Fertilizer application for all experiments in Israel (excluding NY 2011, which did not receive any fertilizer) consisted of a basal N fertilization of 100 kg ha<sup>-1</sup>.

The UCD 2012 experiment at the UC Experimental Field Station in Davis, CA (UCD 2012, 38°32'N, 121°46'W), consisted of 7 m<sup>2</sup> plots with five randomized complete blocks. Sowing occurred in early November (fall planting) in a Yolo loam soil (NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub><sup>-</sup>, 8.9/35.5 ppm) and the fertilization consisted of basal N fertilization 89 kg ha<sup>-1</sup> and a top dressing of 112 kg ha<sup>-1</sup> (in February 2011) at tillering.

## Senescence evaluations

### *Chlorophyll content*

Relative chlorophyll levels were measured in flag leaves of the main spike of ten plants from each genotype at each time point where each chlorophyll measurement is an average of five reads per leaf. Plants were tagged at anthesis and the date of each chlorophyll measurement relative to anthesis was expressed as days after anthesis (DAA). For chlorophyll measurements hand-held chlorophyll meters were used; CCM-200 (Opti-Science) was used in the Israel experiments and a SPAD-502 (Minolta, Milton Keynes, UK) in the US experiment. All CCM-200 results were normalized to SPAD values (Richardson et al. 2002).

### *Spike water content*

Spike weight (g) was measured immediately after harvesting and later after complete drying. Spike water content (g) was calculated as the difference between the two measurements. This parameter was determined for the TAU 2011 experiment only. Five spikes of each genotype were harvested at 42, 49, 57 and 63 DAA.

### *Photosynthesis measurements*

The rate of photosynthesis ( $A_{\max}$ ,  $\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) in control and mutant plants was measured in the TAU 2011 experiment using an infrared gas analyzer (LI-6400 portable photosynthesis system, LI-COR). Flag leaf measurements were taken 3 days before anthesis and at 22, 45 and 59 DAA.

## Remobilization of N

### *Accumulation of N in flag leaf and peduncle*

N percent (%) was measured using a CHNS-O Elemental Analyzer (FLASH EA 1112 series, Thermo Finnegan, Italy). 30 plants were collected at harvest time from each of the *gpc-1* and control plants in NY 2012 experiment, and from each plant flag leaf and peduncle samples were analyzed. Another 15 flag leaves were collected at 50 DAA from the *gpc-1* and control plants at TAU 2013 net-house pot experiment.

### *Accumulation of N during grain development*

Total grain N ( $\text{mg spike}^{-1}$ ) content was determined from grain samples collected at NY 2012, at four time points; 10, 25, 41 and 60 DAA. Six spikes from two blocks of each genotype were analyzed using a CHNS-O Elemental Analyzer (FLASH EA 1112 series, Thermo Finnegan) at each time point. The total grain weight of each spike sample was

multiplied by the N % and the results are presented in milligrams of N per spike total grain weight ( $\text{mg spike}^{-1}$ ).

## Grain evaluations

### *Grain protein content at maturity*

GPC ( $\text{g kg}^{-1}$ ) was determined using near-infrared (NIR) spectrometry. In the Israel experiments, GPC was measured by NIR using NIR Systems 6500 (Foss NIRSystems, Hillerød, Denmark) and in UCD experiments using a grain analyzer-Perten IM9200 (Perten Instruments AB, H.Q. Stockholm, Sweden).

### *Grain Fe and Zn content*

Grain Fe and Zn content (ppm) was measured for each genotype from TAU 2012 and UCD 2012 experiments using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies, Santa Clara, CA, USA) at the University of California, Davis.

### *Spike weight during grain development*

During the grain filling period, five spike samples of each genotype from each block, a total of 25 spikes per genotype, were collected in the NY 2012 experiment at four time points; 10, 25, 41 and 60 DAA. Whole spikes [including grains, glumes and rachis—total spike dry matter (DM)] were completely dried and then weighed.

### *Thousand kernel weight*

Grain samples collected from every plot from all the experiments (2011 and 2012) were counted and weighed to determine thousand kernel weight (TKW).

### *Yield parameters*

All above-ground tissues from five plants from each of the experimental blocks and all the genotypes were collected at harvest time from the TAU 2012 and NY 2012 experiments. The following parameters were measured: plant biomass, spike number per plant, total grain weight per plant, harvest index (HI; total grain weight/plant biomass) and grain weight per spike.

## qRT-PCR

The transcription profiles of eight *GPC*-regulated genes identified in a previous study (Cantu et al. 2011) were compared in *gpc-1* double mutant and control plants from the UCD 2011 greenhouse pot experiment. RNA was extracted

from the flag leaves of ten biological replicates of each genotype harvested at four time points; heading date, 2, 12 and 22 DAA. All sample preparation and qRT-PCR reactions were performed as described by Cantu et al. (2011). Transcript levels are expressed as fold-*ACTIN* levels and are the average of ten biological replicates  $\pm$  SE. Each qRT-PCR plate included two genotypes (*gpc-1* mutant and control), four time points and eight genes plus the endogenous control ( $2 \times 4 \times 9 = 72$ ). Ten qRT-PCR plates were used for the ten biological replicates.

#### Statistical analysis

Statistical analysis was performed using R 2.15.1 (R Development Core Team 2012). The Tukey-HSD test was used for multiple comparisons of the means. In all experiments performed in Israel two wild-type control lines (two independent sister lines carrying no *GPC-1* mutations) were used. Since there were no significant differences between the results for both controls, the data from these two lines were combined and will be referred to as one control.

## Results

### Wheat *GPC-1* knockout mutants

#### *GPC-A1*

We screened the hexaploid wheat, *Triticum aestivum* cv. Express TILLING population using specific primers for *GPC-A1* and identified 32 independent mutations. Among these mutations, we selected a substitution of guanine to adenine in position 919 from the start codon (G919A) that results in a premature stop codon at position 196 of the *GPC-A1* protein (W196\*). This protein truncation will be referred to hereafter as *gpc-a1*. Since the premature stop codon results in the elimination of more than half of the protein (209 of 405 amino acids) including part of the fifth conserved region of the NAC domain, it is most likely a knockout mutation.

#### *GPC-D1*

A similar screen of the TILLING population using primers specific to the *GPC-D1* gene revealed 50 independent mutations. We selected a substitution of guanine to adenine at position 550 from the start codon (G550A) which results in the introduction of a premature stop codon at position 114 of the *GPC-D1* protein (W114\*). This protein truncation will be referred to hereafter as *gpc-d1*. This premature stop codon results in the elimination of 294 of 408 amino acids including the complete fourth and fifth and half of the

third conserved regions of the NAC domain, strongly suggesting a non-functional protein.

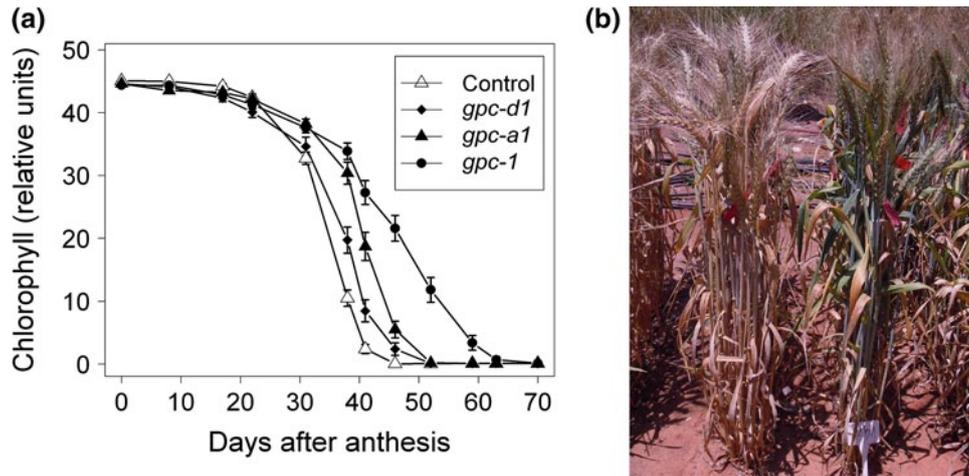
The sequencing of cDNA from *gpc-1* and control plants confirmed the presence of the predicted stop codons in the transcripts of both *gpc-a1* (G919A) and *gpc-d1* (G550A).

Since the hexaploid wheat, *T. aestivum* cv. Express carries a deletion of the *GPC-B1* gene (Uauy et al. 2006b), combining the *GPC-A1* and *GPC-D1* mutations results in a full *GPC-1* knockout plant. To achieve this, the *GPC-A1* and *GPC-D1* mutations were backcrossed and combined as described in Materials and methods, resulting in BC<sub>3</sub>F<sub>3</sub> seeds of sister lines homozygous for the four possible mutant combinations: single mutants (*gpc-a1* and *gpc-d1*), double mutants (*gpc-1*) and wild-type control plants (no induced *GPC-1* mutations except for the natural deletion of *GPC-B1*).

### Mutations in *GPC-1* delay monocarpic senescence in hexaploid wheat

Before anthesis, the *GPC-1* mutants and control plants exhibited identical patterns of development. We detected no effect of the *GPC-1* mutations on growth rate, plant size, flowering time and spike morphology (shape and number of spikelets per spike) that might have been caused by the EMS mutagenesis. However, differences between the genotypes appeared at a later stage, between 30 and 40 DAA, when the control plants senesced at a normal rate while the single and double *GPC-1* knockout mutants senesced later and at a slower rate in all six environments (location/year combinations). These differences were quantified by measuring the chlorophyll content in the flag leaves (Fig. 1a). Chlorophyll levels of control plants were significantly lower than those of the single mutants (*gpc-a1* and *gpc-d1*) and double mutant (*gpc-1*) at several time points from 30 to 40 DAA (Table S1). In all of the tested environments the flag leaves in the control plants had senesced completely by 40 DAA, whereas in both single mutants, this did not occur until around 45–50 DAA. In the double mutant, senescence was even further delayed, with flag leaves being fully senesced only around 60–65 DAA, a delay of 20–30 days compared to the control lines (Fig. 1a; Table S1).

Photosynthesis rates were measured in plants at the TAU 2011 experiment to determine whether the leaves of the *GPC-1* mutants, which remained green longer than the control plants, also retained photosynthetic activity. We found no differences between any of the *GPC-1* mutants and control plants before anthesis or at 22 DAA, but differences in photosynthesis rates were significant ( $P < 0.001$ ) at 45 and 59 DAA (Fig. S1). At 45 DAA, *gpc-a1* and *gpc-1* mutants maintained a positive photosynthetic rate ~16 and ~25 % of their pre-anthesis level, respectively, while *gpc-d1* mutants had negligible photosynthetic activity



**Fig. 1** Differences in senescence between *GPC-1* mutants and control plants. **a** Flag leaf chlorophyll content in the TAU 2012 field experiment; control (open triangle), *gpc-d1* mutant (filled diamond), *gpc-a1* mutant (filled triangle), *gpc-1* double mutant (filled circle). The experiment consisted of five completely randomized blocks, for

each genotype in each block ten plants were tagged at anthesis and measured. Data are mean value ( $n = 5 \pm \text{SE}$ ). **b** Difference in senescence at TAU 2012 between control plants (left) and *gpc-1* double mutant plants (right) at 53 days after anthesis (DAA)

and control plants had zero activity. At 59 DAA only *gpc-1* plants remained photosynthetically active although at ~20 % of their level before anthesis. These results are associated with the pattern of chlorophyll degradation, which showed that the control plants and both the single mutants had undergone complete leaf senescence by 50 DAA, whereas the double *gpc-1* mutant plants remained green (Fig. 1b) and showed higher chlorophyll levels (Fig. 1a).

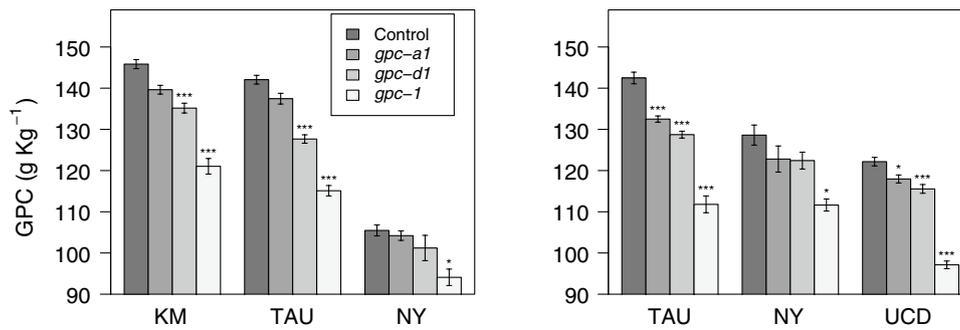
In addition to the flag leaves, differences between the genotypes were also visible in the spikes. In the TAU 2011 experiment, at 35 DAA, control spikes began to dry and yellow while spikes from mutant plants remained green. To evaluate the effect of *GPC-1* on senescence in the sink tissues we measured the difference in spike water content between the mutant and control plants during the grain filling period. In the control plants, complete spike senescence occurred at ~50 DAA (Fig. S2), which is approximately 10–15 days later than the complete senescence of the flag leaves (~35 to 40 DAA) (Fig. 1a). At 42 DAA, spike water content in control plants was lower than in the *GPC-1* mutants (*gpc-a1*;  $P < 0.02$ , *gpc-d1*;  $P < 0.001$  and *gpc-1*;  $P < 0.001$ ). At 49 DAA, the single mutants showed an intermediate water content between the *gpc-1* mutant and the control ( $P < 0.02$ , compared to control). The *gpc-1* mutants retained their spike water content longer than control plants at all time points ( $P < 0.001$ ) and from the single mutants at 57 ( $P < 0.01$ ) and 63 DAA ( $P < 0.01$ ). At 63 DAA, the spikes of the *gpc-1* mutants were still not completely dry, with water content equal to a fifth of the harvested spike average weight (Fig. S2).

#### Grain protein concentration

In experiments carried out in 2011, the GPC of control plants averaged  $105 \text{ g kg}^{-1}$  in NY,  $142 \text{ g kg}^{-1}$  in TAU and  $146 \text{ g kg}^{-1}$  in KM while in the 2012 experiments the control plants averaged  $122 \text{ g kg}^{-1}$  in UCD,  $129 \text{ g kg}^{-1}$  in NY and  $142 \text{ g kg}^{-1}$  in TAU (Table S2). The *gpc-d1* plants exhibited 4–10 % lower GPC in 2011 and 2012 compared to control plants, a reduction which was significant in TAU (2011 and 2012) ( $P < 0.001$ ), KM 2011 ( $P < 0.001$ ) and UCD 2012 ( $P < 0.001$ ) but not in NY (2011 and 2012). The *gpc-a1* mutant had a 1–7 % reduction in GPC compared to control plants in all locations, although this reduction was significant only in the TAU 2012 ( $P < 0.001$ ) and UCD 2012 ( $P < 0.02$ ) experiments. The *gpc-a1* mutants showed slightly higher GPC levels than the *gpc-d1* mutant but the differences between the two mutants were significant only in TAU 2011 ( $P < 0.001$ ) and KM 2011 ( $P < 0.01$ ). The *gpc-1* double mutant showed the greatest 10–21 % reduction in GPC, which was significantly lower than both single mutants and wild-type control plants in all experiments (Fig. 2).

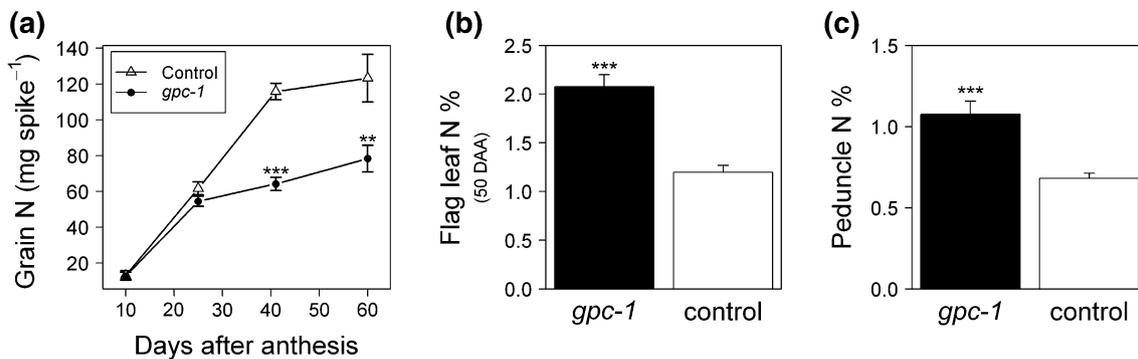
#### Remobilization of N

To investigate the effect of the *GPC-1* genes on the time course of GPC accumulation during grain development, we measured the N content in developing grains of the *gpc-1* mutant and control genotypes from the NY 2012 experiment. The N accumulation profile was similar in both genotypes until ~25 DAA. After this time point, N accumulation in the control plants continued to rise until it reached a



**Fig. 2** Grain protein content ( $\text{g kg}^{-1}$ ) of *GPC-1* mutant and control plants grown in 2011 (left) and 2012 (right) winter growing season in three locations in Israel (TAU, KM and NY) and in one location in

the USA (UCD), data are mean value ( $n = 5 \pm \text{SE}$ ). Asterisks indicate significance compared to control using Tukey-HSD test for multiple comparisons.  $*P < 0.05$ ,  $***P < 0.001$



**Fig. 3** N accumulation in different tissues and time points. **a** Total N accumulation ( $\text{mg spike}^{-1}$ ) during grain development at 10, 25, 41 and 60 DAA, of *gpc-1* (filled circle) and control (open triangle) collected at NY 2012 experiment. N levels (%) in *gpc-1* and control plants were measured in flag leaves (**b**) at 50 DAA from a pot experi-

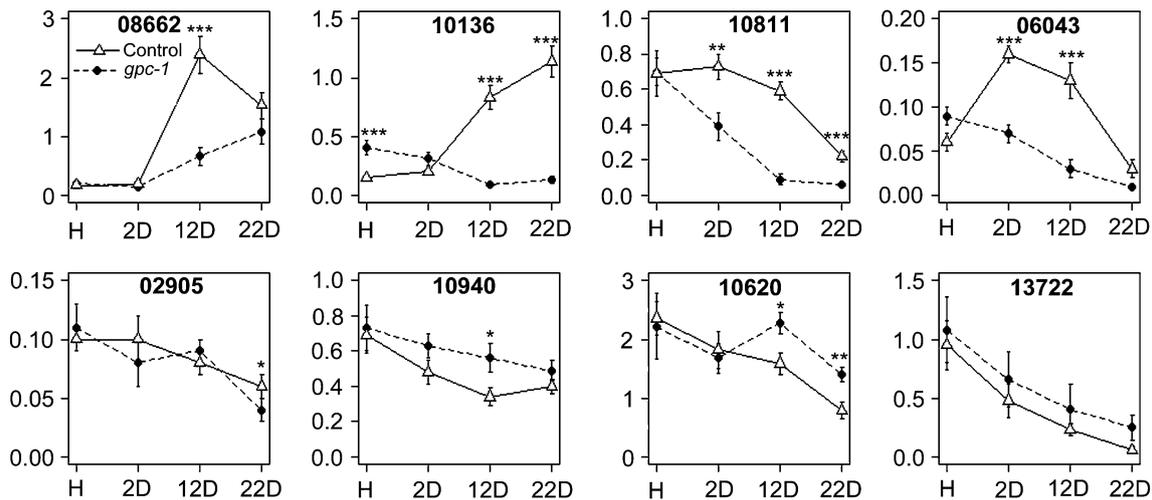
ment at TAU 2013 and peduncles (**c**) collected at harvest time (completely yellow) at NY 2012 experiment. Data are mean value  $\pm \text{SE}$  and asterisks indicate significance using a *t* test.  $**P < 0.01$ ,  $***P < 0.001$

plateau at the next measurement point (41 DAA). This was in contrast to the *gpc-1* mutants where the N levels were lower at 41 DAA compared to control plants ( $P < 0.001$ ) and peaked at a lower level compared to control plants at 60 DAA ( $P = 0.01$ ) (Fig. 3a), indicating that this process is much slower and less efficient in the absence of the *GPC-1* genes.

We further examined the allocation of N from the source tissues by measuring the N in flag leaves at 50 DAA, and in flag leaves and peduncles after harvest. At 50 DAA, the leaves of the *gpc-1* mutants had higher N (2.07 %) than the control plants (1.19 %,  $P < 0.001$ , Fig. 3b). In this experiment, the differences were not significant when the leaves were completely dry at full maturity (data not shown). In addition, N levels in the peduncles were significantly higher ( $P < 0.001$ ) in the *gpc-1* mutants (1.07 %) than in control plants at full maturity (0.68 %) (Fig. 3c).

### Micronutrient analysis

To determine the effect of the individual *GPC-1* homologs on Fe and Zn accumulation, we measured the concentrations of these micronutrients in mature grains from the TAU 2012 and UCD 2012 experiments using ICP-MS. We found a decrease in Fe and Zn content in the *GPC-1* mutants relative to the control, which was largest in the *gpc-1* double mutants (Fig. S3). For the single mutants, a reduction in grain Fe was observed both in the TAU experiment (*gpc-a1* = 8 % and *gpc-d1* = 11.7 % reduction) and in the UCD experiment (*gpc-a1* = 25 % and *gpc-d1* = 19 %) but these differences were significant only in the UCD experiment (*gpc-a1*;  $P < 0.001$ , *gpc-d1*;  $P = 0.002$ ). For the *gpc-1* double mutants, the decrease in Fe in the grain was significant in both experiments (TAU = 31.5 % and UCD = 28.6 %,  $P < 0.001$  for both environments) compared to the wild-type control. Zn



**Fig. 4** Validation of putative *GPC*-regulated genes. The relative expression levels of eight isogroups taken from Cantu et al. (2011) were compared in control (open triangle) and *gpc-1* double mutants (filled circle) across a senescing time course by qRT-PCR. Y-axis

values represent the fold-*ACTIN* level of expression of each isogroup  $\pm$  SE based on ten biological replicates. Asterisks indicate significance using a *t* test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*  $P < 0.001$ . H heading date, D days after anthesis

concentration was also reduced in the *GPC-1* mutants. The *gpc-1* and *gpc-1* mutants had intermediate Zn values that were non-significant compared to the control. The *gpc-1* mutant showed reductions of 4.7 and 2.9 % in TAU and UCD, respectively, whereas the *gpc-1* mutant showed reductions of 7.6 and 5.3 % in the same locations. Reductions were greater in the *gpc-1* double mutant (TAU = 12.1 % and UCD = 8.9 %) compared to control plants but were significant only in the UCD 2012 ( $P = 0.02$ ) experiment.

#### Yield components

Despite clear differences in senescence, photosynthesis, GPC and micronutrient content between control and mutant plants, we found no consistent and significant differences in grain weight between the genotypes in any location (Fig. S4). Furthermore, dry spike weight (total spike DM) measurements at 10, 25, 41 and 60 DAA at NY 2012 showed that the control and mutant plants exhibited a similar accumulation of spike weight, with no significant differences at any time point (Fig. S5).

To evaluate the effect of the *GPC-1* mutations on other yield components, several parameters were measured (Table S3) in the TAU 2012 and NY 2012 experiments. No difference was found between *GPC-1* mutants and control plants in any parameter in the NY 2012 experiment. In the TAU 2012 experiment the *gpc-1* double mutant plants had similar biomass and number of spikes per plant but lower grain yield ( $P < 0.05$ ) HI ( $P < 0.001$ ) and grain yield per spike ( $P < 0.01$ ) compared to control plants.

#### Validation of *GPC*-regulated genes

A previous RNAseq experiment identified 691 differentially expressed genes between control and *GPC*-RNAi plants at 12 DAA (Cantu et al. 2011). A small subset of these genes were validated by qRT-PCR and confirmed to be differentially regulated in *GPC*-RNAi plants in a time course ranging from heading date to 22 DAA (Cantu et al. 2011). In the current study, we compared the expression of eight of these genes between the control and *gpc-1* double mutants at the same time points. Seven of the eight target genes which were differentially regulated in *GPC*-RNAi plants at either 12 DAA or 22 DAA showed significant differences between *gpc-1* double mutant and control plants in at least one of the studied time points (Fig. 4).

#### Discussion

GPC is an important trait in wheat breeding programs because of its impact on the nutritional value and quality of the end product (Brevis et al. 2010). Environmental factors are known to have a major effect on GPC (Groos et al. 2003; Brevis and Dubcovsky 2010) and this was confirmed in our experiments, which revealed large differences in GPC between different locations. Despite this variation, the phenotypic effects (including GPC) of the different genotypes were consistent in all six tested environments and demonstrated the strong effect the *GPC-1* homoeologs have on senescence and nutrient remobilization in wheat.

Since the *GPC*-RNAi transgenic plants showed a simultaneous down-regulation of all homoeologous copies of

the *GPC-1* and *GPC-2* genes, it was not possible to determine the individual contributions of each individual gene or their homoeologs (Uauy et al. 2006a, b). However, the backcross replacement of the non-functional *GPC-B1* allele with the functional DIC allele was sufficient to accelerate senescence and improve N and micronutrient remobilization, a result which suggested that *GPC-1* plays a major role in these processes (Uauy et al. 2006a, b; Distelfeld et al. 2007). The *gpc-a1* and *gpc-d1* single mutants and the *gpc-1* double mutant generated in the current study allowed us to determine the relative contributions of each *GPC-1* homoeolog to the regulation of senescence and nutrient remobilization.

In addition, the *GPC* mutants have the advantage of producing complete knockouts of gene function compared with the partial reduction of transcript levels observed in the *GPC*-RNAi transgenic plants. In these transgenic plants, the transcript levels of all the *GPC-1* and *GPC-2* genes were reduced by roughly 50 % (Uauy et al. 2006b). In this study, we were able to identify individual mutants with premature stop codons in *GPC-A1* or *GPC-D1* genes. These mutations eliminated more than half of the GPC-1 proteins including parts of the conserved NAC domain and therefore, are almost certainly non-functional. Since the *GPC-B1* copy is deleted in cv. Express, the combination of the truncated *GPC-A1* and *GPC-D1* mutations in the *gpc-1* double mutant results in plants with no functional copies of *GPC-1*.

#### Functional characterization of *GPC-1* homoeologous genes

When grown under greenhouse conditions, the *GPC*-RNAi transgenic plants exhibited a 24–30 day delay in senescence relative to the non-transgenic control. This result is similar to the 20–30 day delay in senescence observed in the current study in field-grown *gpc-1* double mutants when compared to control plants. The *gpc-d1* and *gpc-a1* single mutants exhibited a milder delay in senescence (5–10 days) suggesting that the *GPC-A1* and *GPC-D1* genes may have overlapping functions in accelerating senescence that are partially additive. Therefore, the full effect of the *GPC-1* genes is observed only in the double mutant plants. The delay in senescence observed in the *gpc-1* double mutant suggests that a large part of the delay observed in the *GPC*-RNAi experiment was likely caused by the down-regulation of the *GPC-1* genes. However, additional factors may have affected this comparison. The *GPC*-RNAi and TILLING mutant experiments were performed in different environments (greenhouse vs. field conditions), involved different genetic backgrounds (bobwhite vs. express) and represent different levels of down-regulation of the genes (~50 % reduction vs. complete knockout).

Delayed senescence was also detected in the spikes of mutant plants. Spikes of single mutant plants were

completely dried 5–10 days later than control plants while the *gpc-1* mutants retained some water even 15 days after spikes from control plants were dry. A similar result was previously observed in isogenic lines for the *GPC-B1* gene, in which the lines carrying the non-functional allele showed a significant delay in grain maturity (1–2 days in common wheat and 3–5 days in durum wheat) relative to the lines with the functional *GPC-B1* allele (Brevis and Dubcovsky 2010). These results show that the *GPC-1* genes affect, directly or indirectly, spike maturity.

The delay in senescence of the *GPC-1* mutants was associated with a decrease in GPC but not with any consistent differences in TKW or any other yield parameters indicating that the lower GPC was not caused by an indirect dilution effect as a result of differences in total grain weight. The average decrease in GPC for all environments in the *gpc-a1* (4 % reduction) and *gpc-d1* (7 % reduction) single mutants was smaller than in the *gpc-1* double mutant (17 % reduction), which again illustrates the partially additive and overlapping function of the two *GPC-1* homoeologs. The delay in senescence of the *GPC-1* mutants was also associated with a decrease in grain Fe and Zn levels. The reduction in grain Fe content in the *gpc-1* double mutants is similar to that previously observed in the *GPC*-RNAi transgenic lines (38 % reduction, Uauy et al. 2006b) suggesting that *GPC-1* genes play a major role in the regulation of Fe remobilization in hexaploid wheat. The reductions in grain Zn levels in the single and double *GPC-1* mutants were smaller than the reductions observed for Fe levels in the same experiments. Although Zn levels in the grain were reduced in all mutant lines relative to the control plants, the statistical analyses of individual experiments showed significant differences only for the double mutants in the UCD experiment ( $P = 0.02$ ). It would be interesting to investigate if the greater reduction in grain Zn previously reported for the *GPC*-RNAi transgenic plants (36 % decrease) relative to those reported here for the *gpc-1* double mutants (average 11 %) is due to the different experimental conditions or to a more predominant role of *GPC-2* in the remobilization of Zn during senescence in hexaploid wheat.

#### N accumulation during grain development

Our measurements in the NY experiment also revealed differences in the pattern of N accumulation during grain development. In the control plants, the increase in grain N was constant until 41 DAA. However, while the *gpc-1* double mutant showed a similar increase in grain N until 25 DAA, at 41 DAA the grain N levels were lower compared to the control ( $P < 0.001$ ). Similar results were obtained in greenhouse experiments using the transgenic *GPC*-RNAi plants and their corresponding controls, which showed differential grain N accumulation. Control and *GPC*-RNAi

plants showed similar grain N accumulation until ~35 DAA, but whereas the control plants continued to accumulate N beyond this time point, the *GPC*-RNAi plants accumulated a small amount of additional grain N (Waters et al. 2009). The differences in grain N accumulation during the second half of the grain filling period (between 25 and 41 DAA) correspond with the period when the delayed leaf senescence phenotype observed in the *gpc-1* double mutant plants was most apparent (30–40 DAA).

The vegetative tissues of these plants also exhibited significant differences in their nitrogen content, but with an opposite trend to GPC levels. Nitrogen levels in the *gpc-1* mutants were significantly higher than in the control plants both in the flag leaves (50 DAA) and in the peduncles at post-harvest. Similar results have been reported before for the *GPC*-RNAi transgenic plants (Uauy et al. 2006a, b; Waters et al. 2009) and for tetraploid and hexaploid wheat isogenic lines for the *GPC-B1* functional and non-functional alleles (Brevis and Dubcovsky 2010). Taken together, these results suggest that the *GPC-1* genes accelerate the remobilization of N from vegetative tissues to the grain. In the absence of efficient remobilization to the grain, N accumulates in the vegetative tissues of the mutant plants.

It is important to note that the change in the rate of total N accumulation was not a result of differential changes in grain weight as was indicated from the similar spike weight gains among genotypes during the grain filling period (Fig. S5). The reduced GPC observed in the *gpc-1* double mutants may be associated with the reduced ability of these mutants to hydrolyze leaf proteins (e.g., RubisCo). This hypothesis is supported by previous studies in tetraploid wheat, which showed that lines with the non-functional *GPC-B1* allele had significantly lower concentrations of soluble amino acids in the flag leaf than their corresponding isogenic lines carrying the functional *GPC-B1* allele (Kade et al. 2005). The concentration of soluble amino acids is important because a substantial percentage of the N in the wheat grain is supplied by amino acids remobilized from vegetative tissues (Barneix 2007; Gregersen et al. 2008; Waters et al. 2009). Further support for this hypothesis comes from studies in barley; where near isogenic lines carrying a *GPC-H1* allele associated with accelerated senescence and higher GPC showed an up-regulation of genes coding for both plastidial and extraplastidial proteases later during senescence than sister lines carrying the *GPC-H1* allele associated with delayed senescence and low GPC (Jukanti et al. 2008).

Another factor that could contribute to the improved N, Fe and Zn remobilization in the lines with more functional *GPC-1* alleles, is the effect of *GPC-1* in the up-regulation of numerous genes involved in transport processes (e.g., NRAMP and ZIP metal transporters, Cantu et al. 2011). However, we currently do not know if any of the

up-regulated transporters are limiting factors for the remobilization of N, Fe and Zn to the grain.

#### Stay-green phenotype and its potential effects on yield components

‘Stay-green’ is a phenotype characterized by delayed senescence compared to wild-type plants (Thomas and Howarth 2000). A functional stay-green trait enables the plant to synthesize and store greater levels of assimilates, which can lead to increased crop yields under certain environments (Richards 2000; Christopher et al. 2008; Zhou et al. 2011; Derkx et al. 2012). Our photosynthesis measurements showed that the *GPC-1* mutants maintain photosynthetic activity for longer than the control lines; hence we can classify the *GPC-1* mutants as functional stay-green mutants. However, despite having a longer period of photo-period activity in the leaves, the *GPC-1* knockout mutants did not show any significant increase in grain weight or other yield components in our particular environments. We currently do not know if this was a result of unfavorable environmental conditions during the extended stay-green period or a finite capacity of the grains of the tested variety to increase grain weight beyond a certain limit. Common wheat plants containing the wild emmer *GPC-B1* segment showed higher GPC with no yield penalties (Mesfin et al. 1999; Brevis and Dubcovsky 2010; Kumar et al. 2011; Carter et al. 2012; Tabbita et al. 2013) but under certain conditions a negative effect has been shown on TKW (Brevis and Dubcovsky 2010; Carter et al. 2012; Tabbita et al. 2013). Kernel weight is also under tight genetic control, with large variability among wheat varieties (Gegas et al. 2010). Therefore, it is also possible that these genetic differences may affect the ability of different wheat varieties to benefit from a stay-green phenotype. For example, the greenhouse experiments comparing the *GPC*-RNAi transgenic lines with their non-transgenic control, showed no differences in grain weight with the non-transgenic control lines despite the very large differences in senescence (~1 month) and the continuous supply of water and nutrients under favorable environmental conditions (Waters et al. 2009). These results suggest that increases in wheat grain yield will require the integration of increases in C remobilization from the source tissues with a larger genetic plasticity in grain size or number of grains per spike to accommodate the increase in transported carbohydrates.

#### Validation of *GPC-1* regulated genes

Finally, we used the *gpc-1* double mutants to validate a set of genes previously found to be differentially expressed between transgenic *GPC*-RNAi and control lines (Cantu et al. 2011). Of the eight target genes which

were differentially regulated at 12 DAA or 22 DAA in the *GPC*-RNAi plants, seven were also differentially regulated between the control and *gpc-1* double mutant. This result confirms that these targets are regulated by the *GPC-1* homoeologous genes and therefore supports a critical role for the *GPC-1* genes in the regulation of senescence and the transport of micronutrients to the developing grain.

In summary, our results establish that the knockout of the *GPC-1* homoeologous genes alone is sufficient to generate large effects on senescence and nutrient remobilization and to modify the transcription profiles of putative *GPC*-regulated genes. In addition, this study demonstrates that the *GPC-A1* and *GPC-D1* homoeologs have overlapping functions in senescence and nutrient remobilization processes, which is not surprising given the relatively recent origin of hexaploid wheat (Dubcovsky and Dvorak 2007).

Our results show that the knockout of all *GPC-1* homoeologs results in the reduction in the rate of total N accumulation in the grains during the second half of the grain filling period and suggest that this reduction might be related to the lower efficiency of the *GPC-1* mutants to disassemble leaf proteins and transport them to the grains before they reach full capacity. Our results also show that in the background and environments tested, the *GPC-1* genes do not have a significant effect on carbohydrate accumulation in wheat grains. Therefore, the possibility of using the stay-green phenotype of these mutants to increase yield should be considered with caution and probably will require simultaneous improvement of the seed capacity to accumulate additional carbohydrates.

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