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***Storage protein activator* controls grain protein accumulation in bread wheat in a nitrogen dependent manner**

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Abstract

The expression of cereal grain storage protein (GSP) genes is controlled by a complex network of transcription factors (TFs). Storage protein activator (SPA) is a major TF acting in this network but its specific function in wheat (*Triticum aestivum* L.) remains to be determined. Here we generated an RNAi line in which expression of the three *SPA* homoeologs was reduced. In this line and its null segregant we analyzed GSP accumulation and expression of GSP and regulatory TF genes under two regimes of nitrogen availability. We show that down regulation of *SPA* decreases grain protein concentration at maturity under low but not high nitrogen supply. Under low nitrogen supply, the decrease in *SPA* expression also caused a reduction in the total quantity of GSP per grain and in the ratio of GSP to albumin-globulins, without significantly affecting GSP composition. The slight reduction in GSP gene expression measured in the *SPA* RNAi line under low nitrogen supply did not entirely account for the more significant decrease in GSP accumulation, suggesting that SPA regulates additional levels of GSP synthesis. Our results demonstrate a clear role of *SPA* in the regulation of grain nitrogen metabolism when nitrogen is a limiting resource.

Keywords

Nitrogen nutrition, storage proteins, Storage protein activator, *Triticum aestivum*, wheat quality

34 **Introduction**

35

36 Bread wheat (*Triticum aestivum* L.*)* is the most important cereal crop in the world in terms of area
37 harvested and commercial exchange. It provides on average 20% of the total protein in the human diet .
38 Wheat grain proteins have unique properties making them suitable for use in a considerable number of
39 food and non-food products [3]. Both the total grain protein concentration (GPC) and the relative
40 composition of the storage protein fraction govern the cohesiveness and viscoelasticity of gluten, the
41 network formed by wheat grain storage proteins (GSPs) when mixed with water [4]. Breeding for high-
42 yielding genotypes has decreased GPC, while modern uses of end-products require higher GPC than
43 traditional products, limiting the potential usefulness of some varieties[5,6]. Most wheat GSPs belong to the
44 glutenin and gliadin prolamin families. Glutenins usually account for 35 to 45% of total grain protein and
45 are composed of high-molecular-weight (HMW-GS) and low-molecular-weight (LMW-GS) sub-units,
46 which together form very large macropolymers during grain desiccation [7]. Gliadins are monomeric
47 proteins classed as ω5-, ω1,2-, α/β- or γ-gliadins [8] and make up between 18% and 35% of total grain
48 protein.

49 Transcriptional control of GSP genes plays an important role in the endosperm specific synthesis of
50 GSPs during cereal grain development[9] through a network of interacting transcription factors (TFs). At
51 least twelve TFs involved in the regulation of GSP genes have been identified in different cereal species,
52 along with the *cis*-elements they bind to. The wheat GCN4-like motif (GLM) is bound by SPA, a basic
53 leucine zipper TF of the Opaque2 (O2) subfamily [10,11], with an ortholog in barley (*Hordeum vulgare* L.)
54 named BLZ2 that binds to the same motif [12], as does another member of the family in barley, BLZ1 [13]; the
55 ortholog of BLZ1 in wheat is SPA Heterodimerizing Protein (SHP) and is a negative regulator of GSPs [14].
56 Two non-homologous DNA-binding with one finger (DOF) TFs: prolamin box binding factor (PBF) and
57 scutellum and aleurone-expressed DOF (SAD), interact with the prolamin box [15,16]; in bread wheat, PBF
58 and SAD promote the transcription of glutenin genes by binding to the prolamin box and this activity is
59 additive to the induction of the expression of glutenin genes by SPA [17]. In barley, an AACA motif is
60 recognized by GAMYB (gibberellic acid-dependent of the MYB superfamily of transcriptional activators,
61 Diaz et al. 2002). In barley, two R1MYB family TFs, Myb-related CAB promoter-binding protein
62 (MCB1) and MYBS3, can bind a GA response complex motif [19,20] and FUSCA3, a B3-type TF, interacts
63 with an RY box [21]. The DOF proteins mediate the formation of several binary or ternary TF complexes
64 [16,18,19] in addition to the interactions between FUSCA3 and BLZ2 [21] and between BLZ1 and BLZ2 [12]. The
65 interaction of FUSCA3 with the RY box of a glutenin gene and with SPA has been demonstrated in wheat
66 [22]. New transcription factors regulating the expression of prolamin genes have been discovered more
67 recently in wheat: three negative regulators of GSPs, SPR and ODORANT1 [23–25], for which the exact

68 location where they bind on prolamin promoters is still unclear, and one positive regulator, NAC109 that
69 interacts with GAMYB [26].
70 SPA/O2/BLZ2 is one of the most studied TFs in this regulatory network of grain storage proteins. In
71 wheat, a study of different *SPA* haplotypes has shown that this gene affects the amount of nitrogen
72 allocated to the gliadin fraction [27]. In maize, *O2* is associated with grain lysine content [28] and an *o2* mutant
73 shows reduced expression of some GSP genes and corresponding proteins [29]. The effect of modifying
74 expression levels of RISBZ1, a *SPA* homolog in rice, has been tested [30,31]. Transient overexpression of
75 *RISBZ1* in protoplasts induced trans-activation of several GSP gene promoters, which was synergistically
76 enhanced by the simultaneous overexpression with the rice homolog of *PBF* [31]. The knockdown of
77 *RISBZ1 in planta* caused only slight changes in GSP accumulation, but when both *RISBZ1* and *RPBF*
78 were knocked down, GSP accumulation and gene expression were significantly reduced [30]. In wheat, the
79 overexpression of the copy of *SPA* located on the B genome led to lower accumulation of glutenin and ω-
80 gliadin and lower expression of *PBF* [32].
81 Nutrient availability has a major effect on GSP quantity and composition [33]. Higher nitrogen input
82 increases the amount of GSP that accumulates in the grain [34]. Differences in GSP composition related to
83 nitrogen availability have been found to follow allometric scaling laws [35–37], which may be a consequence
84 of the complex transcriptional regulation network controlling GSP gene expression [27]. One particular *cis*-
85 element on GSP gene promoters, the GLM, which is bound by SPA, plays an important role in the
86 transcriptional response to nitrogen. It was found to be essential for the activation of GSP gene
87 transcription in response to amino acids and ammonium, but this activation is only optimal in synergistic
88 interaction with the endosperm box (EB), the motif formed by the GLM and the prolamin box in tandem
89 [38], and other *cis*-elements [39]. Interestingly, when nitrogen supply is low, the GLM may act as a negative
90 regulatory motif for GSP gene transcription.
91 SPA orthologs thus have a prominent role in controlling GSP synthesis in cereals, particularly in
92 response to changes in nitrogen availability. To confirm that SPA is a positive regulator of GSP gene
93 expression in bread wheat and determine its role in the response of GSP accumulation and composition to
94 nitrogen availability, plants with reduced expression of *SPA* were grown with high or low amounts of
95 nitrogen. We analyzed the relative accumulation of the different GSP families and sub-groups and
96 measured the expression of GSP and their regulatory TF genes throughout grain development. We show
97 that a decrease in the expression of *SPA* caused a reduction in GPC, which was more significant under low
98 nitrogen availability. GSP accounted for most of the decrease in GPC compared to other protein classes,
99 with reductions in the amounts of all GSP families. We also describe the changes in regulatory TF
100 expression that contribute to the response to nitrogen with notably *PBF, MYBS3* and *SHP* being

upregulated by high nitrogen. Our results suggest that transcriptional regulation is not the sole mechanism determining the response of GSP synthesis to nitrogen availability.

Methods

All methods were performed in accordance with the relevant guidelines and regulations.

Plant material and growth conditions

Immature seeds of the spring wheat (*Triticum aestivum L.*) line NB1 (a non-commercial Spring wheat variety obtained from Limagrain Europe, Saint-Beauzire, France) were transformed by *in planta* inoculation using *Agrobacterium tumefaciens* and transgenic lines were regenerated [40]. The *SPA-A* full length coding sequence was previously amplified by PCR from a cDNA library from immature seeds of the bread wheat cultivar Récital. The binary vector pSCV was used to produce transgenic plants expressing both sense and antisense *SPA-A* cDNA separated by the first intron of rice tubulin to generate a hairpin RNA (Fig. 1). ~~The RNAi cassette is controlled by the promoter of the allele encoding the Dx5 subunit of *Glu-D1-1* (a HMW-GS)~~ The transgene was under the control of the promoter of the subunit Dx5 of the *Glu-D1-1* HMW-GS gene –and the *Nos* terminator. The plasmid includes a kanamycin resistance cassette for selection, *NptII*, controlled by the actin promoter and *Nos* terminator. For each transformation event, the number of T-DNA insertions was evaluated by qPCR performed on genomic DNA and the integrity of the transgene was verified by PCR. Transformants with several copies of the transgene were discarded. T0 plants were self-pollinated to generate the T1 generation composed of 25% homozygotes, 50% hemizygotes and 25% null segregant plants. The segregation ratio was established by cultivating 30 plants on a medium with kanamycin allowing the identification of homozygotes, hemizygotes and null segregants (Table S1). The zygosity of progenies from self-pollinated homozygotes and respective null segregants was verified by quantitative real-time PCR. Selfing of confirmed homozygotes and null segregant T2 plants gave rise to the T3 generation, i.e. the RNAi line and its null segregant to be used as a control. ~~The plasmid includes a kanamycin resistance cassette for selection, *nptII*, controlled by the actin promoter and Nos terminator. For each transformation event, the number of T-DNA insertions was evaluated by Southern blot and the integrity of the transgene was verified by PCR. Transformants with several copies of the transgene were discarded. T0 plants were self-pollinated to generate the T1 generation composed of 25% homozygotes, 50% hemizygotes and 25% null segregant plants. The zygosity of progenies from self-pollinated homozygotes and respective null segregants was checked by quantitative real-time PCR. Selfing of confirmed homozygotes and null segregant T2 plants gave rise to the T3 generation, i.e. the RNAi line and its null segregant to be used as control.~~

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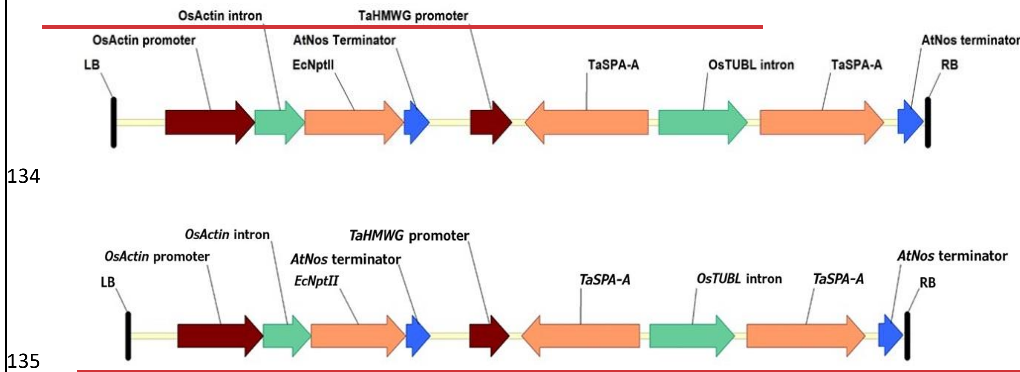

Figure 1 *Storage protein activator* RNA interference construct used for wheat transformation. LB, left border; RB, Right border.

T4 seeds were germinated for two to three days at room temperature on wet filter paper in Petri dishes. Germinated seeds were then transferred to soil in 50-mL PVC columns (inner diameter 7.5 cm, length 50 cm, 2 plants per column) and arranged in a greenhouse in a strip-plot design with the genotypes as rows and the N treatments (see below) as columns with four replicated blocks to form a homogeneous stand with a plant density of 261 plants m^{-2}. Temperature was controlled at 22°C during the day and 18°C during the night. Day length was 16 h, maintained with artificial light when needed. Plants received 68 mL $column^{-1}$ day^{-1} of water or nutrient solution. ~~For four weeks plants received a 3 mmol N L^{-1} nutrient solution (N3) containing 1 mM KH_2PO_4, 1 mM $Ca(NO_3)_2$, 0.5 mM NH_4NO_3, 2 mM $MgSO_4$, 3 mM $CaCl_2$, 5 mM KCl, 10 µM H_3BO_3, 0.7 µM $ZnCl_2$, 0.4 µM $CuCl_2$, 4.5 µM $MnCl_2$, 0.22 µM MoO_3, and 50 µM EDFS Fe. Then until anthesis plants received a 15 mmol N L^{-1} nutrient solution (N15), which was the same as the N3 solution except it contained 5 mM KNO_3, 4 mM $Ca(NO_3)_2$, 1 mM NH_4NO_3, no $CaCl_2$ and no KCl. At anthesis, continuous water irrigation was used to remove any excess of the nutrient solution in the soil; afterwards irrigation was maintained to its previous level with water. At 300°Cdays after anthesis, the columns were rinsed again, then they received either the N15 nutrient solution or a nutrient solution containing no nitrogen (N3 solution in which $Ca(NO_3)_2$ and NH_4NO_3 were replaced with 4 mM $CaCl_2$) until grain ripeness.~~ Three nutrient solutions were used to feed the plants, N0, N3 and N15. N0 contained 1 mM KH2PO4, 0.5 mM NH4NO3, 2 mM MgSO4, 7 mM CaCl2, 5 mM KCl, 10 µM H3BO3, 0.7 µM ZnCl2, 0.4 µM CuCl2, 4.5 µM MnCl2, 0.22 µM MoO3, and 50 µM EDFS-Fe; N3 contained 1 mM KH2PO4, 1 mM Ca(NO3)2, 0.5 mM NH4NO3, 2 mM MgSO4, 3 mM CaCl2, 5 mM KCl, 10 µM H3BO3, 0.7 µM ZnCl2, 0.4 µM CuCl2, 4.5 µM MnCl2, 0.22 µM MoO3, and 50 µM EDFS-Fe; N15 contained 1 mM KH2PO4, 5 mM KNO3, 4 mM Ca(NO3)2, 1 mM NH4NO3, 2 mM MgSO4, 10 µM H3BO3, 0.7 µM

160 ZnCl2, 0.4 µM CuCl2, 4.5 µM MnCl2, 0.22 µM MoO3, and 50 µM EDFS-Fe. All plants received N3 for
161 four weeks, then N15 until anthesis. At anthesis, continuous water irrigation was used to remove any
162 excess of the nutrient solution in the soil; afterwards irrigation was maintained to its previous level with
163 water. Differences in treatment between N- and N+ started at 300°Cdays after anthesis, when the columns
164 were rinsed again and lasted until grain ripeness: N+ plants received the N15 nutrient solution while N-
165 plants received N0. Main stems were tagged when the anthers of the central florets appeared. Degree-days
166 were calculated as the sum of the average daily temperatures after anthesis with a base temperature of
167 0°C.
168

169 **Determination of grain dry mass and protein concentration**

170 Grains from four ears (except at 200°Cdays after anthesis, where five ears were used) were sampled from
171 each replicate every 100°Cdays from 200°Cdays after anthesis to maturity (grain ripeness, 900°Cdays
172 after anthesis) and again at 1050°Cdays after anthesis. Four grains per ear were sampled between
173 200°Cdays and 700°Cdays after anthesis for RNA analysis and were immediately frozen in liquid nitrogen
174 and stored at -80°C. The remaining grains of the ear were also frozen and stored at -80°C until they were
175 freeze-dried. We measured the dry mass and nitrogen concentration of a sub-sample of grains (ca. 65%).
176 The remaining grains were oven-dried at 80°C for 48 h to calculate the percentage of remaining water. At
177 maturity, grain yield per ear was calculated using all grains of each ear harvested.
178 Grains were milled for 2 min using a custom ball mill. Flour (5 mg) was weighed in tin capsules and the
179 total N concentration was determined with the Dumas combustion method (Association of Analytical
180 Communities International approved method no. 992.23) using a FlashEA 1112 N/Protein Analyzer
181 (Thermo Electron Corp, Waltham, MA). Grain protein concentration (GPC) was calculated by multiplying
182 grain N concentration by 5.62 [41].
183

184 **Sequential extraction, separation and quantification of grain protein fractions**

185 Non-prolamin, gliadin and glutenin protein fractions were sequentially extracted from 60 mg of freeze-
186 dried wholemeal flour as described by Triboi et al. [37] and modified by Plessis et al. [42]. Each 2 ml tube
187 contained one stainless steel bead (5 mm diameter) and samples were stirred by placing the tubes on a
188 rotating wheel (40 rpm) during each extraction and washing step. The non-prolamin protein fraction was
189 extracted for 30 min at 4 °C from 100 mg wholemeal flour with 1.5 ml of 50 mM phosphate buffer (pH
190 7.8) containing 0.1 M NaCl. After centrifugation for 10 min (18 000 g) at 4 °C, the supernatant was
191 collected and the pellet was washed twice for 10 min each time with 1.5 ml of the same buffer. After
192 centrifugation in the same conditions, all supernatants were pooled. The same steps were used to extract
193 the gliadin protein fraction from the previous pellet with 70% (v/v) ethanol. Finally, the glutenin protein

fraction was extracted in 50 mM borate buffer (pH 8.5) containing 2% SDS (w/v) and 1% dithiothreitol (w/v). The supernatants (80 µl) of each protein fraction were oven dried overnight at 60 °C in tin capsules and their total N concentration was determined with the Dumas combustion method as described above. Protein fractions from samples of the same flour from cultivar Récital were extracted, analysed as a control in each of the 21 sets of extractions and used to determine the coefficient of variation for each of the protein fractions, which were 3.48, 5.10, 2.19, 2.61, and 1.96% for the non-prolamin, gliadin, and glutenin protein fractions, storage proteins, and total proteins, respectively.

Gliadin classes (ω1,2-, α/β-, and γ-gliadins) and glutenin sub-units (HMW-GS and LMW-GS) were separated and quantified by HPLC (Figs S1 and S2) using an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, http://www.agilent.com) as described in Triboi at al. [35]. The total N concentration of each protein fraction was determined by the Dumas combustion method, as described above. The gliadin extracts used were those obtained by sequential extraction, but glutenins were extracted from flour independently with a protocol adapted from Fu and Kovacs [43]. The gliadin and glutenin extracts were filtered through regenerated cellulose syringe filters (0.45–lm pore diameter, UptiDisc; Interchim, http://www.interchim.com), and 4 ll (gliadin) or 2 ll (glutenin) of protein extract was injected into a C8 reversed-phase Zorbax 300 StableBound column (2.1 9 100 mm, 3.5 lm, 300 A; Agilent Technologies) maintained at 50°C. The eluents used were ultra-pure water (solvent A) and acetonitrile (solvent B), each containing 0.1% trifluoroacetic acid. The flow rate was 1 ml min^{-1} ~~-1~~. Proteins were separated by using a linear gradient, from 24 to 50% solvent B over 13 min for gliadin, and from 23 to 42% solvent B over 25 min for glutenin. Proteins were detected by UV absorbance at 214 nm. After the gradient, the column was washed with 80% solvent B for 2 min and then equilibrated at 24% (for gliadins) or 23% (for glutenins) solvent B for 2 min at the same flow rate. Chromatograms were processed with CHEMSTATION 10.1 software (Agilent Technologies). The signal obtained from a blank injection was subtracted from the chromatograms before integrating the data. The HPLC peaks corresponding to each of the four gliadin classes were identified following the observations of Wieser et al. [44]. The quantity of each gliadin class or glutenin subunit as a percentage of total gliadin or total glutenin, respectively, was calculated by dividing the areas under each HPLC peak by the total area under the chromatogram trace. The quantity of each gliadin class (or glutenin subunit) per grain was calculated by multiplying the proportion of each gliadin class (or glutenin subunit) in total gliadin (or total glutenin) by the total quantity of gliadin (or glutenin) per grain, as quantified by Dumas analysis. By subtracting the quantity of all GSPs from N_{tot}, we calculated the amount of the remaining protein fraction, mainly constituted of albumin-globulins.

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RNA extraction and measurement of gene expression

Four grains per ear (same ears as for protein analysis) were sampled at 200, 300, 400, 500, 600 and 700°Cdays, the embryos were cut out and the rest of the grain immediately frozen in liquid nitrogen and kept at -80°C. RNA was extracted from 75 mg of grain powder in 750 µL of extraction buffer (200 mM Tris–HCl pH 9, 400 mM KCl, 200 mM sucrose, 35 mM $MgCl_2$, 25 mM EDTA) and 600 µL phenol/chloroform (pH 8). The suspension was homogenized by vortexing for 30 s and then centrifuged for 10 min at 15,000 × *g*. The supernatant was collected. The pellet was resuspended in 600 µL of phenol/chloroform, centrifuged using the same conditions and the supernatant collected, and the whole step repeated. Supernatants were pooled. RNA was precipitated by adding 1 M acetic acid (1/10 volume) and ethanol (2.5 volumes). The RNA pellet was washed with 3 M Na acetate (pH 6) and resuspended in water. A second acetic acid/ethanol precipitation was performed before resuspending the pellet in 50 µL RNase free water. RNA was treated with RNase-free DNase according to the instructions of the supplier (AMBION). The RNA in solution was quantified by measuring the absorbance at 260 nm in a spectrophotometer. Approximately 2 µg of total RNA were reverse transcribed using oligo(dT)20 and reverse transcriptase (Bio-rad iScriptTM Select cDNA Synthesis kit) in a final volume of 40 µl. Transcript levels of four housekeeping genes and the storage protein and transcription factor (TFs) genes were quantified by real-time q-PCR using Lightcycler 480 SYBR Green I Master (Roche) in 15 µl with 5 µl of cDNA diluted 10 times. Relative expression (RE) was calculated as: $RE = \varepsilon^{\Delta Cp}$, where ε is the efficiency of the primers for the measured gene and ΔCp is the normalized crossing point (Cp); $\Delta Cp = (Cp_1 \times Cp_2 \times Cp_3 \times Cp_4)^{0.25} - Cp_g$, where Cp_g is the Cp for the measured gene and Cp_1, Cp_2, Cp_3 and Cp_4 are the Cp values of the four housekeeping genes (Pfaffl *et al*., 2004). The primer sequences are given in Table S2.

Soluble protein extraction and western blot analysis

Wheat flour (50 mg) from grains collected at 500°Cdays after anthesis was dissolved in extraction buffer (10 mM sodium phosphate, 10 mM NaCl, pH 7.8 at 4°C) supplemented with a protease inhibitor cocktail (P9599, Sigma, St. Louis, MO, USA). Proteins were precipitated from the extract supernatant with ice-cold acetone overnight at -20°C. The dried protein pellet was dissolved in SDS-PAGE buffer containing 80 mM Tris-HCl pH 8, 2% (w/w) SDS, 40% glycerol (v/v), 0.002% bromophenol blue (w/w), supplemented with 2% (v/v) DTT and 2.5% iodoacetamide (w/w). The protein concentration was determined using the Bradford protein assay (B6916, Sigma, St. Louis, MO, USA). In order to quantify the SPA protein, different quantities of total soluble protein extracts (10, 20, 30, and 40 µg) were separated on SDS-polyacrylamide gel (T = 10.3%, C = 1.3%).

After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Hybond, ECL, GE Healthcare) using a Criterion blotter (Biorad). The membrane was incubated for 1 h at room temperature in a blocking buffer containing 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.01% (v/v) Tween 20, and 5%

(w/w) skimmed milk. The membrane was then incubated for 1 h at room temperature and overnight at 4°C with a 1:1000 dilution of anti-SPA antibody (Eurogentec S.A., Belgium). Antirabbit IgG conjugated with horseradish peroxidase (GE Healthcare) was used as the secondary antibody (diluted 1:50000). The s~~S~~ignal was detected using an Immobilon™ Western Chemiluminescent HRP Substrate (ECL Millipore) following the manufacturer's protocol. Anti-SPA signals were quantified by image analysis using Image J software (http://imagej.nih.gov/ij). The fold-change in SPA protein abundance was calculated as the ratio of the slope of the relationship between protein amount and anti-SPA signal for the SPA RNAi and NS lines [45].

Data analysis

All statistical analyses were done in R-4.2.3~~3.2.1~~ for Windows [46] (code provided in the Supplementary Information). An ANOVA model with two factors (genotype and block) ~~procedure for a strip-plot design was used~~was used- to analyze the results ~~at maturity (R package 'Agricolae' [47])~~. Genotype and~~,~~ block~~, and N treatments were~~ were regarded as fixed effects. Variance homogeneity was tested using the Bartlett test and the normality of the residuals with the Shapiro-Wilk test. The block effect was never statistically significant. Differences between NS line and *SPA* RNAi line were tested using the post-hoc Dunnett test, with the NS line used as control. Statistical differences were judged at the 5% level. Differences in SPA protein abundance between NS line and *SPA* RNAi line were tested by comparing the slopes of the standard major axis regression between the normalized volume of anti-SPA signal and the total protein mass using the 'smatr' package [48].

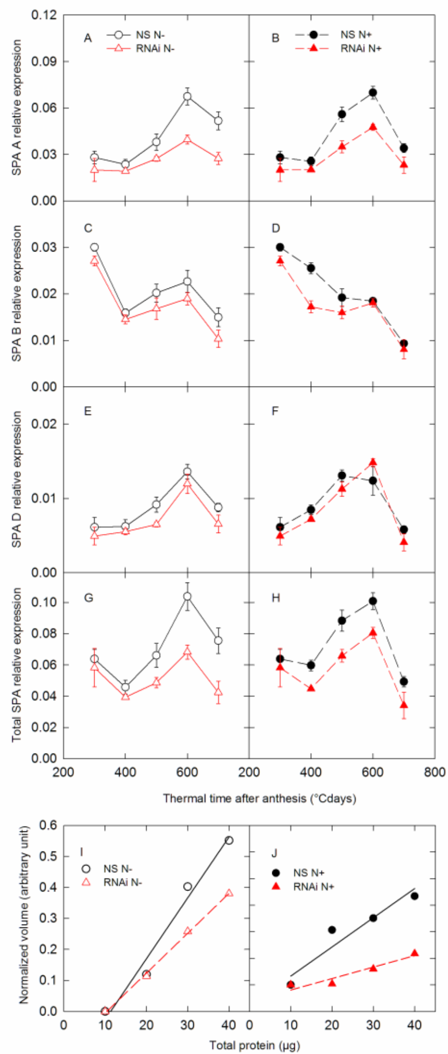
Results

SPA RNA and protein quantities are reduced in the *SPA* RNAi line

To investigate the role of SPA in regulating storage protein accumulation in bread wheat grain we generated RNAi transgenic lines to down regulate this gene. The transgene was under the control of the promoter of a HMW-GS gene. More precisely, the promoter of the allele encoding the Dx5 subunit of *Glu-D1-1* was used. This promoter is grain specific and highly induced during grain development [49] (Fig. 1). We grew *SPA* RNAi plants and their null~~on~~-segregant siblings (NS) in the greenhouse. Grain developmental stage was measured in degree-days (°Cdays) after anthesis to take into account the effect of temperature on development. At 300°Cdays after anthesis the plants were either supplied with 15 mM nitrogen (N+ treatment) or no nitrogen (N- treatment) until the end of grain filling about 800°Cdays after anthesis. We sampled grains every 100°Cdays from 200°Cdays to 800°Cdays after anthesis and then at maturity, 900°Cdays and 1050°Cdays after anthesis.

We demonstrated RNA interference in one of the transgenic lines generated by measuring the expression of the three *SPA* homoeologs during grain development using q-PCR (Fig. 2). In the RNAi line fewer *SPA-A* transcripts were detected throughout development under both nitrogen treatments compared to NS. As *SPA-A* is the most highly expressed of the three homoeologs, the decrease in its expression was largely responsible for the overall decrease in the relative abundance of *SPA* transcripts (Fig. 2Gg and Hh). For example, relative expression of total *SPA* transcripts was > 20% less in the RNAi line than in NS 500°Cdays after anthesis for the N- treatment and 400°Cdays after anthesis for the N+ treatment. Interestingly, SPA-B showed a different time-course of expression from SPA-A and SPA-D in both the NS and RNAi line. This difference has already been observed in different genetic backgrounds [27] and suggests distinct regulation of the different homoeologs of SPA.

We also checked the effect of RNAi on SPA protein levels. We carried out a western blot analysis on grains collected 500°Cdays after anthesis (Fig. 2Ii and Jj). The quantity of SPA protein was reduced by 34% (P = 0.071) for the N- treatment (Fig. 2Ii) and 61% (P = 0.057) for the N+ treatment (Fig. 2Jj and Fig. S3). Therefore less SPA protein accumulated when expression of *SPA* was down regulated by RNAi.



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312 **Figure 2** Down-regulation of *SPA* in the RNAi line. *SPA* null segregant (NS, circles) and RNAi (RNAi,
313 triangles) lines of bread wheat were grown in the greenhouse with low (N-, open symbols) and high (N+,
314 closed symbols) nitrogen supply. (~~a~~A) to (F~~f~~) Relative expression of *SPA* homoeologs. (G~~g~~) and (H~~h~~)
315 Relative expression of the sum of the three SPA homoeologs. (I~~i~~) and (J~~j~~) Quantification of the anti-SPA
316 signal from western blots with different quantities of total protein extract at 500°Cdays after anthesis. (A~~a~~)
317 to (H~~h~~) Data are means ~~± 1 s.e.~~ for $n = 4$ independent replicates.

Nitrogen accumulation is modified in the *SPA* RNAi line under low nitrogen availability

For plants subjected to the N- treatment, the total quantity of nitrogen per grain (N_{tot}) and grain protein content (GPC) at maturity were reduced by 6% (P = 0.043) and 9% (P < 0.001) respectively in the *SPA* RNAi line compared with the NS. In the case of GPC, the decrease in the RNAi line compared to the NS was already significant (P < 0.05) at 400 and 700°Cdays after anthesis (Fig. 3C~~c~~). Under these conditions of low nitrogen availability, the greatest difference in GPC between *SPA* RNAi line and the NS was at maturity, which can be attributed to a late increase in single grain dry mass and a decrease in N_{tot} (Fig. 3~~a~~A, B~~b~~ and C~~c~~).

In contrast, for plants subjected to the N+ treatment, N_{tot} was not significantly different (P = 0.30) between the *SPA* RNAi and NS lines (Fig. 3B~~b~~) and GPC was only significantly reduced (-5%) in the RNAi line at 400 and 500°Cdays after anthesis (Fig. 3C~~c~~). Grain yield per ear was increased by 9% while it was not significantly altered in the N- treatment.

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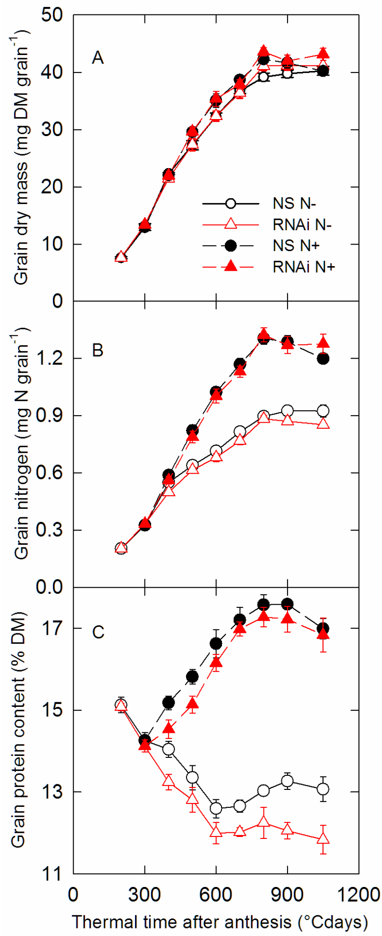
Single grain dry mass at maturity was not significantly different between the *SPA* RNAi and NS lines for either of the nitrogen availability treatments (Table 1).

Table 1 Single grain dry mass, grain yield per ear, total quantity of N per grain (Ntot), grain N yield per ear, and grain protein concentration at maturity (900 and 1050°Cdays) for the null segregant (NS) and *SPA* RNAi (RNAi) lines under low (N-) and high (N+) nitrogen availability. Data are means ± 1 s.e. for n = 8 independent replicates. Within a single N treatment, * (P < 0.05), ** (P < 0.01) and *** (P < 0.001) indicate significant differences between values for NS and RNAi lines from an ANOVA followed by the Dunnett post-hoc test.

Variable	Treatment			
	N-		N+	
	NS	RNAi	NS	RNAi
Single grain dry mass (mg DM grain^{-1})	40.0 ± 0.5	41.2 ± 0.8	40.9 ± 0.6	42.6 ± 0.7
Grain yield per ear (g DM ear^{-1})	0.96 ± 0.03	1.05 ± 0.05	1.04 ± 0.02	1.14 ± 0.04*
Grain N (mg N grain^{-1})	0.92 ± 0.02	0.86 ± 0.01**	1.24 ± 0.02	1.27 ± 0.03
Grain N yield per ear (mg N ear^{-1})	22.4 ± 0.5	22.3 ± 0.8	32.0 ± 0.8	35 ± 1.3
Grain protein concentration (% of DM)	13.2 ± 0.2	11.9 ± 0.2***	17.3 ± 0.2	17.0 ± 0.3



333

334 **Figure 3** Changes in grain dry mass, total quantity of nitrogen (N_{tot}) in the grain and grain protein
335 concentration during grain development. *SPA* null segregant (NS, circles) and RNAi (RNAi, triangles)
336 lines of bread wheat were grown in the greenhouse with low (N-, open symbols) and high (N+, closed
337 symbols) nitrogen supply. DM, dry mass. Data are means ± 1 s.e. for n = 4 independent replicates.

338

339 **Storage protein accumulation but not composition is affected in the *SPA* RNAi line under low**
340 **nitrogen availability**

341 We determined grain protein composition throughout grain development in the NS and *SPA* RNAi lines.
342 Under N- treatment, decreases in all GSP fractions were measured from around 400°Cdays and onwards

343 in the *SPA* RNAi line compared to NS, while a slight decrease in the albumin-globulin (AG) fraction was
344 only detected at maturity (Fig. 4). Decreases in GSPs must have contributed more to the overall decrease
345 in GPC under low nitrogen availability as the GSP to AG ratio was lower in the *SPA* RNAi line than in NS
346 (Table 2). In the N+ treatment, the glutenin subunits, gliadin classes and AG mostly accumulated at
347 similar rates in the *SPA* RNAi and NS lines (Fig. 4) and at maturity the GSP to AG ratio was the same
348 (Table 2). At maturity, for both N treatments, the gliadin to glutenin ratio tended to be lower (-6%) in the
349 *SPA* RNAi line than NS but the difference was not statistically significant ($P = 0.5$).
350 For N- conditions, the relative proportions of GSP fractions in total GSP were the same in the NS and
351 *SPA* RNAi lines (Table 2), showing that all GSP fractions contributed to the same relative extent to the
352 decrease in GSP quantity when *SPA* was downregulated. An exception was ω1,2-gliadin, which was
353 reduced by 18% ($P < 0.001$) in the *SPA* RNAi line compared with NS. However ω1,2-gliadin makes up
354 less than 4% of the total amount of GSP.
355

Table 2 Grain storage protein (GSP) to albumin-globulin (AG) ratio under low (N-) and high (N+) nitrogen availability and GSP composition at maturity (900 and 1050°Cdays) under low nitrogen availability, shown as the percentage of high (HMW-GS) and low (LMW-GS) molecular weight glutenin subunit, ω1,2-gliadin, α/β-gliadin and γ-gliadin fractions in total GSP. NS, null segregant line. RNAi, *SPA* RNAi line. Data are means ± 1 s.e. for $n = 8$ independent replicates. Within a single N treatment, * ($P < 0.05$) and *** ($P < 0.001$) indicate significant differences between NS and RNAi lines from an ANOVA followed by the Dunnett post-hoc test.

Variable	Treatment			
	N-		N+	
	NS	RNAi	NS	RNAi
GSP to AG ratio	1.59 ± 0.04~~1.98 ± 0.029~~	1.59 ± 0.04~~1.90 ± 0.03*~~	1.59 ± 0.04 1.59 ± 0.04~~2.2 ± 0.05~~	1.59 ± 0.04~~2.3 ± 0.04~~
HMW-GS (% GSP)	20.4 ± 0.24	21.0 ± 0.34	19.0 ± 0.52	18.5 ± 0.18
LMW-GS (% GSP)	42.4 ± 0.50	42.6 ± 0.52	40.6 ± 0.62	40.0 ± 0.28

ω1,2-gliadins (% GSP)	2.8 ± 0.08	2.4 ± 0.12***	5.3 ± 0.28	5.1 ± 0.10
α/β-gliadins (% GSP)	17.4 ± 0.31	17.0 ± 0.37	18.9 ± 0.54	19.7 ± 0.19
γ-gliadins (% GSP)	15.8 ± 0.28	15.7 ± 0.37	15.1 ± 0.35	15.6 ± 0.19

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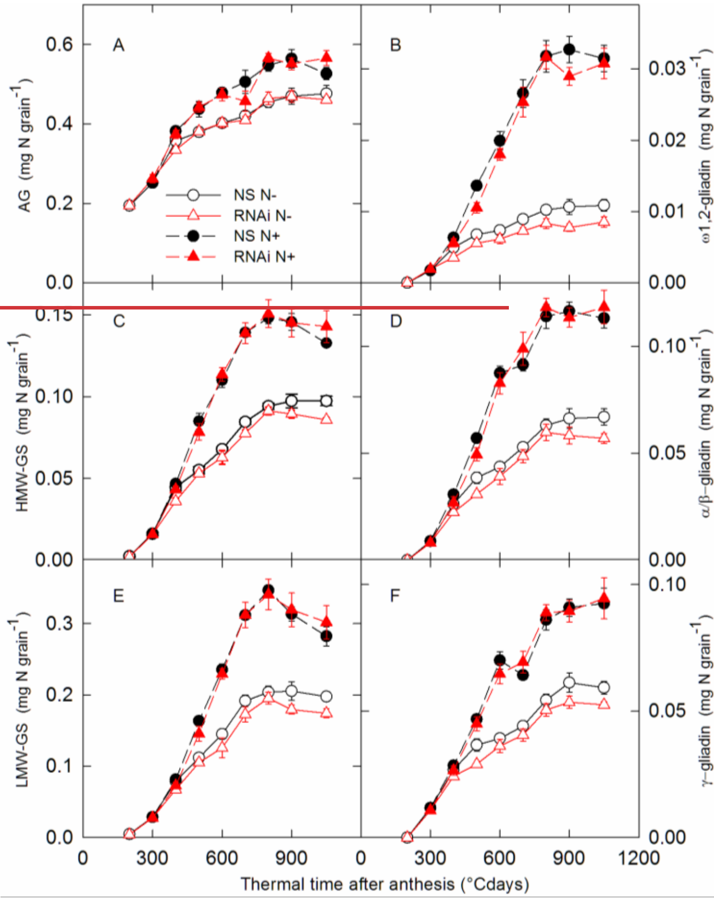
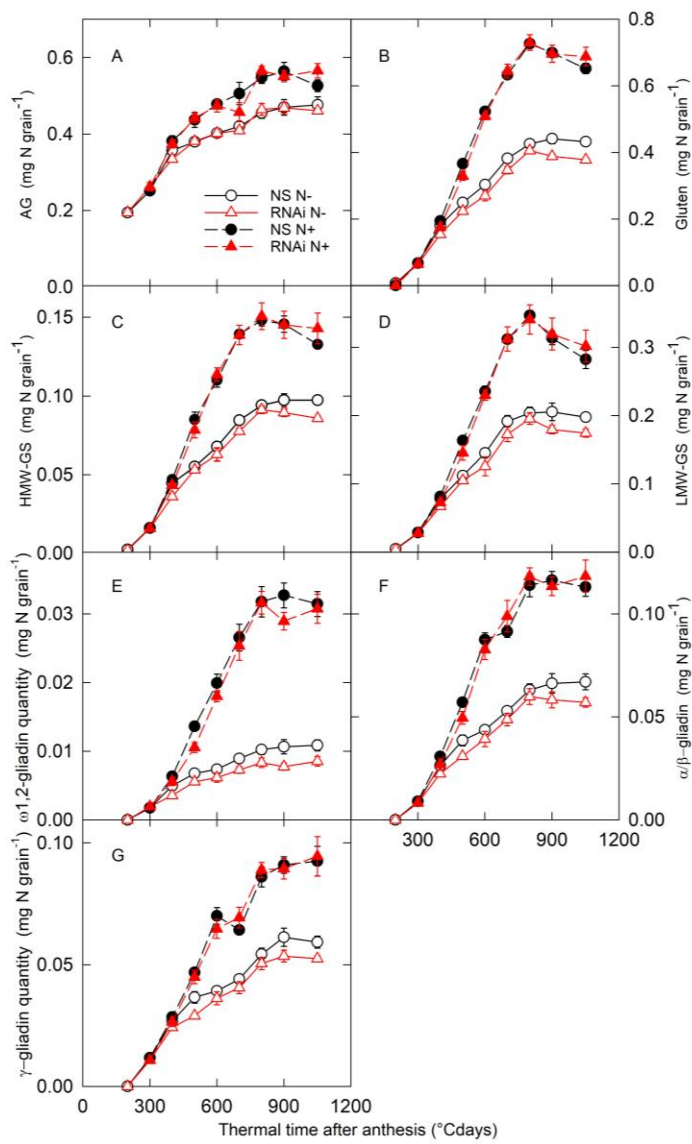




358

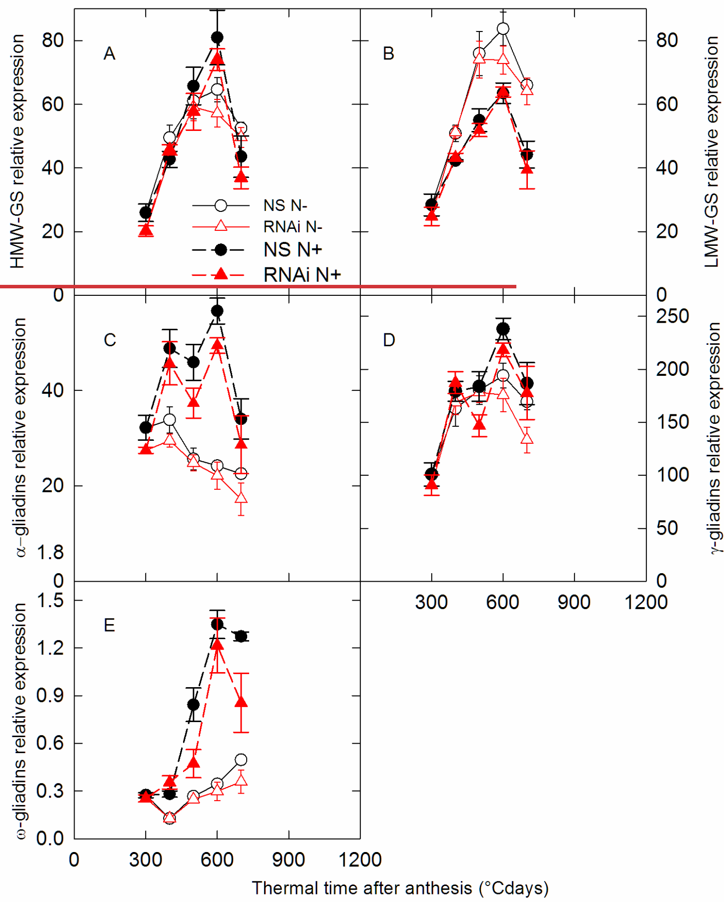
359 **Figure 4** Changes in the quantity per grain of protein fractions during grain development. *SPA* null
360 segregant (NS, circles) and RNAi (RNAi, triangles) lines of bread wheat were grown in the greenhouse
361 with low (N-, open symbols) and high (N+, closed symbols) nitrogen supply. AG, albumin-globulin;
362 HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits.
363 Data are means ± 1 s.e. for n = 4 independent replicates.

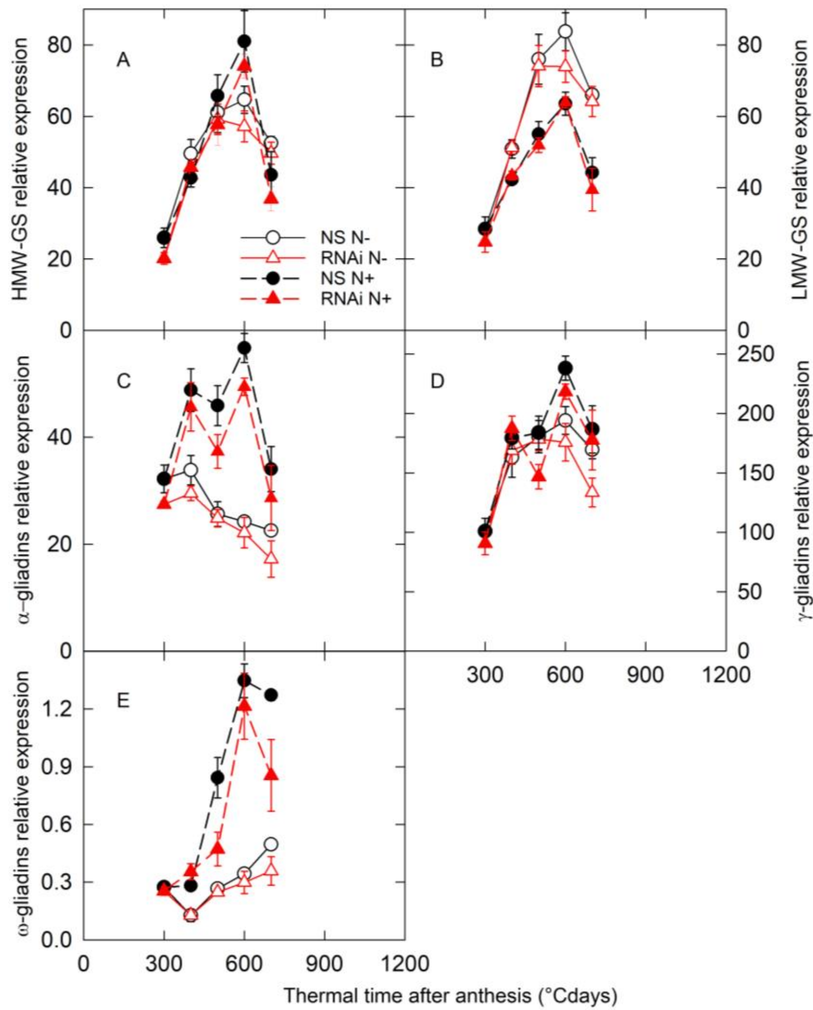
364

365 ***SPA* under-expression decreases gliadin gene expression more than glutenin gene expression~~mainly~~**
366 **~~decreases gliadin gene expression~~**

367 As in bread wheat SPA is a transcriptional regulator of GSP genes [14], we measured the expression of
368 genes belonging to the different GSP families in the wheat *SPA* RNAi line (Fig. 5). For most families we
369 used generic q-PCR primers to amplify transcripts of all the genes of a given family. For HMW-GS we
370 amplified transcripts of the four HMW-GS genes expressed in the line used for transformation separately
371 then summed the result. Our results show that all gene families were down regulated in the *SPA* RNAi line
372 compared to NS for at least one time point of either of the treatments. In N+ conditions, the expression of
373 some GSP genes was affected in the *SPA* RNAi line, mainly at the later time points (500°Cdays after
374 anthesis and onwards). The time points coincide with the largest differences in *SPA* expression in the
375 RNAi line (Fig. 2). In the N+ treatment, glutenin genes were the least affected in the *SPA* RNAi line with
376 no change in expression detected for LMW-GS, while gliadin genes showed the most striking decrease in
377 expression (Fig. 5). Similar results were observed for the N- treatment but the differences between the
378 *SPA* RNAi line and the NS line were smaller than for the N+ treatment.

379





380

381 **Figure 5** Changes in gene expression of grain storage proteins during grain development. *SPA* null
382 segregant (NS, circles) and RNAi (RNAi, triangles) lines of bread wheat were grown in the greenhouse
383 with low (N-, open symbols) and high (N+, closed symbols) nitrogen supply. HMW-GS, high molecular
384 weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits. Data are means ± 1 s.e. for *n*
385 = 4 independent replicates.

386

387 Expression of TFs known to be part of cereal GSP regulatory network was also quantified during the
388 linear grain filling period (Fig. 6). The expression of *PBF*, *MYBS3*, *GAMYB* and *MCB1* was lower in N+
389 than in N- conditions in both *SPA* RNAi and NS lines. The expression of *SAD* and *SHP* was upregulated
390 in the N+ treatment compared with the N- treatment in NS for at least two time points, but in the *SPA*
391 RNAi line *SHP* did not respond to nitrogen supply. *PBF* and *GAMYB* expression was downregulated for
392 one or more time points in the *SPA* RNAi line compared with NS under N- conditions, while *SHP* and
393 *GAMYB* were downregulated in the *SPA* RNAi line compared with NS under N+ conditions for at least
394 one time point.
395

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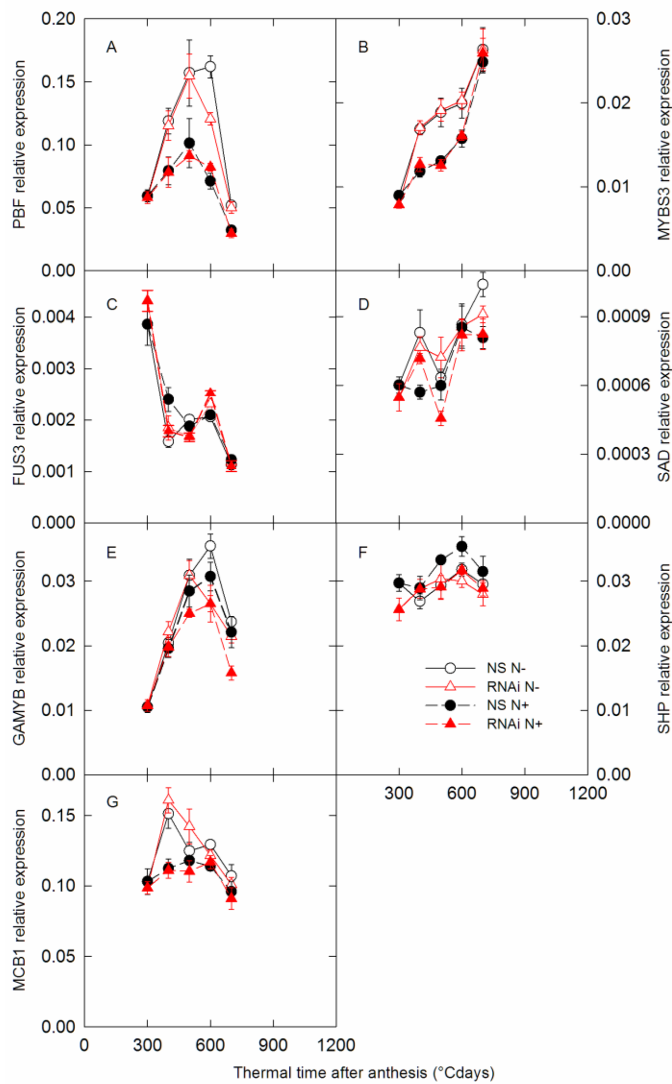

397 **Figure 6** Changes in gene expression of transcription factors of the grain storage protein transcriptional
398 regulation network during grain development. *SPA* null segregant (NS, circles) and RNAi (RNAi,
399 triangles) lines of bread wheat were grown in the greenhouse with low (N-, open symbols) and high (N+,
400 closed symbols) nitrogen supply. Full details of gene names are provided in the text. Data are means ± 1
401 s.e. for n = 4 independent replicates.

Discussion

In this study, we show that the under-expression of *SPA* in bread wheat can result in reduced expression of GSP genes and when nitrogen is limiting a decrease in GPC and the GSP to AG ratio of grain. These results confirm previous indications that SPA has a role in the transcriptional regulation of GSP genes [14] like its orthologs in other cereals [12,29,31]. However, down-regulating *SPA* had a fairly limited effect on GSP gene expression. This may have been because the decrease in *SPA* gene expression was not large enough to induce a stronger effect or because of functional redundancy like in rice where the under-expression of both *RISBZ1*, the *SPA* ortholog, and *RPBF* resulted in a much more significant decrease in GSP gene expression than in the *RISBZ1* knock-down line [30].

Our results show that *SPA* under-expression has a stronger effect on the expression of gliadin than glutenin genes. This is consistent with a study of natural genetic variation in wheat where two haplotypes of *SPA*-A were identified. Different levels of *SPA*-A expression from each haplotype led to the allocation of different quantities of total grain nitrogen to the gliadin fraction, but equal amounts to the glutenin fraction [27].

SPA activates the expression of LMW-GS and HMW-GS by binding the GLM [10,11,50], so we expected to observe a lower level of glutenin expression in the *SPA* RNAi line. However, HMW-GS and LMW-GS gene expression was barely affected by *SPA* under-expression (Fig. 5A and 5B). For both HMW-GS and LMW-GS, the level of gene expression we measured here was the combined expression of several members of the gene family. Nucleotide diversity in promoters of LMW-GS gene family members [51] could however result in differences in expression patterns. As we were not able to monitor the expression of each individual LMW-GS gene, particularly the one used in previous studies [10,50], the known effect of SPA regulation might have been masked by different responses from the rest of the gene family. The GLM motifs identified in the promoters of HMW-GS genes were shown to be functional as they are activated after SPA binding [11]; the same applies to the G-like box with bZIP proteins [49]. As these boxes belong to a common regulatory framework shared by all the HMW-GS promoters [11], it is expected that the entirety of HMW-GS genes respond in the same way.

Changes in GSP gene expression alone did not account for all of the modifications in GSP accumulation observed in the *SPA* RNAi line under low nitrogen supply. In addition differences in gene expression did not always lead to differences in protein accumulation. For example, gene expression of gliadins was lower in the *SPA* RNAi line than in the WT under high nitrogen availability at some time points, but this did not result in any detectable change in grain protein accumulation or composition. This indicates that mechanisms other than transcription regulate GSP synthesis compensating for the deregulation of GSP expression. Discrepancies between gene expression and protein accumulation in wheat have already been

436 observed for γ-gliadins in a study of the effect of nitrogen and sulfur availability on the regulation of
437 wheat GSP synthesis [35] and for gliadins as part of the characterization of *nac019* triple mutants [26].
438 Moreover, an association study of wheat grain protein composition found that several nitrogen
439 assimilation and metabolism genes were associated with GPC and N_{tot}, further evidence of levels of non-
440 transcriptional regulation of grain protein accumulation [42]. Field studies have shown that N_{tot} is mainly
441 determined by the supply of N to grains and its accumulation is therefore mainly source driven [36,52,53].

442 The effect of under-expressing *SPA* was dependent on nitrogen availability as we only observed changes
443 in grain protein content and composition under the low nitrogen treatment. This does not seem to be
444 related to *SPA* being more highly expressed in the RNAi line when nitrogen was available, as on the
445 contrary, there was still an obvious decrease in SPA protein concentration compared to the control. GLM
446 binding by SPA has already been shown to have a role in integrating the effects of nitrogen availability at
447 the transcriptional level on GSP synthesis in barley grain [39]. Here we saw in wheat that this occurs mostly
448 at the protein synthesis level. In *o2*, a maize mutant for the *SPA* ortholog *O2*, genes involved in amino
449 acid metabolism are differentially expressed [29]. Under varying levels of nitrogen and sulfur availability,
450 amino acid transport and metabolism are modulated to adjust wheat GSP synthesis and composition [35].
451 Thus SPA probably modulates the nitrogen response at different levels, both directly by regulating GSP
452 gene expression and indirectly by controlling the expression of other genes involved in regulating GSPs
453 either transcriptionally, as suggested by our results, or translationally [54].

454 We attempted to generate plants over-expressing *SPA* but none of the transformed lines showed the
455 expected increase in *SPA* gene expression. This could mean that over-expression of *SPA* is detrimental to
456 the development of the embryo or to germination. Our use of an HMW-GS gene promoter for the *SPA*
457 transgene may have resulted in a lethal dose of SPA as it contains an activation domain for *SPA* itself that
458 might have generated a feed-forward regulatory loop. Another study was more successful in producing
459 SPA over-expressors in bread wheat, achieving ten to 20-fold increases in the expression of *TaSPA-B* [32];
460 while they used an HMW-GS promoter like us, it came from a different allele and it is unclear which part
461 of the promoter they used, therefore it is possible the absence of some boxes led to lower, and thus non-
462 lethal, levels of *SPA* expression than in our transformants. Unexpectedly in regards to our results and
463 previous studies [14], Guo et al. [32] found that the over-expression of *SPA* did not lead to higher accumulation
464 of GSP, and on the contrary diminished the quantity of glutenin and ω-gliadin. This could be at least
465 partly due to the indirect effect of SPA over-expression reducing the expression of the glutenin activator
466 *PBF* [17] and increasing the expression of the glutenin repressor *SHP* [14]. The study by Guo et al. [32]was done
467 at a single level of nitrogen supply and our results, along with previous work [39], show that SPA regulation
468 of GSP accumulation and the expression of other transcription factors in the regulatory network is
469 dependent on nitrogen availability. It is thus possible that different consequences of increasing the

470 expression of *SPA* would have been obtained varying nitrogen supply and that using plants with modified
471 expression of SPA might improve GPC under certain conditions of fertilization. More generally, any
472 attempt at improving GPC in cereals should involve testing in a wide range of nutritional conditions.
473
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476
477 **Competing interests**
478 The authors have no relevant financial or non-financial interests to disclose.
479
480 **Author contributions**
481 TR and FT created and selected the transgenic lines. PM, CR and AP designed the experiment. AP
482 supervised the cultivation of the plants and harvested grain samples. AP and ND analysed grain
483 composition. AP and MD carried the RNA extraction and measured gene expression. MM extracted grain
484 proteins, performed and analysed the western blot. AP and PM analysed the data. AP, PM and CR wrote
485 the manuscript. All authors commented on the manuscript.
486
487 **Data availability**
488 The datasets generated during the current study are available from the corresponding author on reasonable
489 request.
490
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619 **Supplemental information**

620 **Fig. S1** HPLC chromatogram of glutenin from whole meal flour of the *Triticum aestivum* line NB1.

621 **Fig. S2** HPLC chromatogram of gliadin from whole meal flour of the *Triticum aestivum* line NB1.

622 **Table S1** Sequences of primers used for q-PCR

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