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Increasing the Grain Yield and Grain Protein Content of Common Wheat (*Triticum aestivum*) by Introducing Missense Mutations in the Q Gene

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Abstract: Grain yield (GY) and grain protein content (GPC) are important traits for wheat breeding and production; however, they are usually negatively correlated. The Q gene is the most important domestication gene in cultivated wheat because it influences many traits, including GY and GPC. Allelic variations in the Q gene may positively affect both GY and GPC. Accordingly, we characterized two new Q alleles (Q2 and Q1-N8) obtained through ethyl methanesulfonate-induced mutagenesis. Compared with the wild-type Q allele, Q2 contains a missense mutation in the sequence encoding the first AP2 domain, whereas Q1-N8 has two missense mutations: one in the sequence encoding the second AP2 domain and the other in the microRNA172-binding site. The Q1 allele did not significantly affect GPC or other processing quality parameters, but it adversely affected GY by decreasing the thousand kernel weight and grain number per spike. In contrast, Q2-N8 positively affected GPC and GY by increasing the thousand kernel weight and grain number per spike. Thus, we generated novel germplasm relevant for wheat breeding. A specific molecular marker was developed to facilitate the use of the Q1-N8 allele in breeding. Furthermore, our findings provide useful new information for enhancing cereal crops via non-transgenic approaches.

Keywords: wheat quality; agronomic trait; mutation; breeding

1. Introduction

Common wheat (*Triticum aestivum*) is a major food crop that serves as the primary protein source in the human diet. Wheat provides approximately 18% of the calories and 20% of the proteins consumed by humans worldwide [1]. Therefore, grain yield (GY) and grain protein content (GPC) are critical traits to be considered for wheat breeding and production. Because of increased demand driven by population growth and improvements in living conditions, there is an urgent need for wheat varieties with increased GY and GPC.

Nitrogen applications during wheat production are vital for increasing GY and GPC [2-4]. In order to produce wheat with a high GY and GPC, farmers tend to apply large amounts of nitrogen fertilizer to wheat fields, which increases cultivation costs and environmental pollution. Breeding to increase the wheat GY and GPC remains a considerable challenge because of the confirmed negative relationship between the two parameters [5-8].

Wheat flour has unique processing properties that enable it to be used to make diverse end-products. The end-use quality of wheat is significantly influenced by GPC. The unique processing quality of wheat flour depends on the seed storage proteins, especially gliadins and glutenins, which account for 60–80% of the total GPC [9,10]. Gliadins are monomeric...
compounds that contribute to dough extensibility [11], whereas glutenins, which are polymeric compounds linked by intermolecular disulfide bonds, affect dough elasticity. Glutenins consist of high and low molecular weight glutenin subunits [12,13].

Because the Q gene (TraesCS5A02G473800) influences many important traits, including GY, GPC, grain threshability, grain size, spike morphology, rachis fragility, plant height, and flowering time, it plays a major role in wheat domestication, de-domestication and breeding [14–18]. This gene is located on the long arm of chromosome 5A and encodes a member of the APETALA2 (AP2) transcription factor family [15,19]. The AP2 transcription factors have diverse functions affecting plant development [20]. The Q allele originated from a spontaneous mutation in the microRNA172-binding region of the q allele [15]. Similarly, the introduction of another point mutation in the microRNA172-binding site of the Q allele resulted in the Qc1 allele [17]. The q, Q, and Qc1 transcription levels are correlated with the number of point mutations in the microRNA172-binding site [15,17]. Compared with the effects of the Q allele, Qc1 increases GPC by approximately 60 g kg$^{-1}$, reflecting the value of Qc1 for wheat breeding. However, Qc1 decreases the longitudinal cell size of rachises, resulting in compact spikes and decreases in GY [17]. Missense mutations in the Q sequence encoding the AP2 domain can lead to decreased spike density [15,21]. These challenges underline the need to identify or generate new Q alleles that positively affect GY and GPC.

In this study, we characterized two new Q alleles, namely Qs1 and Qc1-N8, which have a single missense mutation in the sequences encoding the first and second AP2 domains, respectively. They were obtained via the chemical treatment of common wheat lines carrying the Q and Qc1 alleles, respectively. The effects of Qs1 and Qc1-N8 on the wheat GY and GPC were investigated.

2. Results

2.1. Phenotype of the Mutant ss1 Carrying the Qs1 Allele

In order to elevate the effect of missense mutations in the sequence encoding the AP2 domain of the Q protein, a mutant carrying the Qs1 allele (ss1; sparse spike 1) was isolated from the M2 population of the common wheat cultivar ‘Shumai482’ (Figure 1). The ss1 plants produced a speltoid-like spike (Figure 2a). In contrast to the Q allele (GenBank No. KX580301.2), Qs1 has a missense mutation (GenBank No. OK041024) in the sequence encoding the first AP2 domain (Figure 3 and Figure S1). Compared with the WT control (Figure 1), ss1 plants were taller (Figure 2b, Table 1) and had a longer main spike (Figure 2a, Table 1) but a lower spike density (Table 1). Regarding the examined yield-related traits, the spikelet number per the main spike, grain number per the main spike, thousand kernel weight (Table 1), and grain width (Figure 2d,e) were lower for ss1 than for the WT control. In contrast, the grain length (Figure 2c,f) was greater for ss1 than for the WT control. Notably, there was no significant difference in the productive tiller number between the ss1 and WT plants (Table 1). Therefore, GY was significantly lower ($p < 0.01$) for ss1 (0.46 kg m$^{-2}$) than for the WT control (0.58 kg m$^{-2}$) in the 2018–2019 growing season. Under our experimental conditions, the Qs1 allele decreased GY by 20.6% by decreasing the thousand kernel weight and the grain number per spike.

An analysis of the processing quality traits revealed a lack of a significant difference between the ss1 and WT plants in terms of GPC, wet gluten content, gluten index, Zeleny sedimentation value, water absorption, development time, stability time, and loaf volume (Table 2; Figure 4).
Grain number per main spike

2018–2019

2019–2020

58.81 ± 5.83 *
44.08 ± 1.71 *
50.43 ± 4.14 *
65.13 ± 8.23 *

Standard deviation. ‘+’ and ‘−’ indicate more and less than the WT control, respectively. ‘+ 0.2′ in panel (c) indicates the grain was 0.2 mm longer than the WT grain (on average).

Phenotype of the mutant

Figure 2. Phenotype of the mutant ss1. (a) Spikes of the wild-type (WT) (left) and ss1 (right) plants. Scale bar, 2 cm. (b) WT (left) and ss1 (right) plants. Scale bar, 10 cm. Comparisons of grain length (c) and grain width (d). **, p < 0.01; *, p < 0.05. Data are presented as the mean ± standard deviation. ‘+’ and ‘−’ indicate more and less than the WT control, respectively. ‘+0.2′ in panel (c) indicates the ss1 grain was 0.2 mm longer than the WT grain (on average). ‘+2.6%’ in panel (c) indicates Q^2 increased the grain length by 2.6% (on average). (e) Kernel width of the WT (upper) and ss1 (lower) samples. Scale bar, 2 cm. (f) Kernel length of the WT (upper) and ss1 (lower) samples. Scale bar, 2 cm.

Figure 1. Outline of the generation of the mutants ss1 and NS8 in the T. aestivum cv. ‘Shumai 482′ genetic background.
Table 1. Comparison of the agronomic traits of the mutant ss1 and the wild-type (WT) control.

| Traits                        | Growing Season | ss1           | WT             | E              | G              | E × G     |
|-------------------------------|----------------|---------------|----------------|----------------|----------------|-----------|
| Plant height (cm)             | 2018–2019      | 83.41 ± 4.05 ** | 79.44 ± 3.09  | 205.087 **     | 24.459 **      | 0.534     |
|                               | 2019–2020      | 71.82 ± 2.30 ** | 67.11 ± 2.00  | 123.331 **     | 15.408 **      | 2.680     |
| Spike length (cm)             | 2018–2019      | 14.65 ± 0.68 * | 14.06 ± 0.75  |                |                |           |
|                               | 2019–2020      | 12.88 ± 0.80 ** | 11.87 ± 0.46  |                |                |           |
| Spikelet number per main spike| 2018–2019      | 21.00 ± 1.15 ** | 22.94 ± 1.44  | 15.038 **      | 24.858 **      | 0.110     |
|                               | 2019–2020      | 19.93 ± 0.70 ** | 21.43 ± 1.45  |                |                |           |
| Spike density                 | 2018–2019      | 1.43 ± 0.07 ** | 1.63 ± 0.09   | 45.434 **      | 84.944 **      | 13.78     |
|                               | 2019–2020      | 1.55 ± 0.10 ** | 1.80 ± 0.08   |                |                |           |
| Grain number per main spike   | 2018–2019      | 58.81 ± 5.83 * | 65.13 ± 8.23  | 43.376 **      | 8.860 **       | 0.044     |
|                               | 2019–2020      | 50.43 ± 4.14 * | 54.64 ± 4.25  |                |                |           |
| Thousand kernel weight (g)    | 2018–2019      | 44.08 ± 1.71 * | 46.13 ± 0.43  | 50.427 **      | 13.780 **      | 0.216     |
|                               | 2019–2020      | 48.76 ± 1.25 * | 51.90 ± 1.67  |                |                |           |
| Tiller number                 | 2018–2019      | 5.30 ± 1.03   | 5.25 ± 1.33   | 27.478 **      | 0.730          | 1.207     |
|                               | 2019–2020      | 3.85 ± 0.74   | 4.25 ± 0.79   |                |                |           |

**p < 0.01; *p < 0.05; E, environment; G, genotype; E × G, interaction between the environment and genotype. Data are presented as the mean ± standard deviation.
Table 2. Comparison of the processing quality parameters of the mutant ss1 and the wild-type (WT) control.

| Trails                        | Growing Season | ss1          | WT           | E       | G      | E × G |
|-------------------------------|----------------|--------------|--------------|---------|--------|-------|
| Grain protein content (%)     | 2018–2019      | 13.12 ± 0.43 | 13.45 ± 0.36 | 30.313  | 2.562  | 0.323 |
|                               | 2019–2020      | 14.53 ± 0.60 | 15.16 ± 0.76 | 29.114  | 0.301  | 4.315 |
| Zeleny sedimentation value (mL) | 2018–2019      | 32.65 ± 3.64 | 27.73 ± 4.20 | 21.54 ± 2.51 | 23.88 ± 1.98 | 14.645 ** | 0.570  | 2.996 |
| Wet gluten content (%)        | 2018–2019      | 24.72 ± 1.60 | 24.98 ± 3.06 | 27.21 ± 1.49 | 29.66 ± 2.05 | 65.599 ** | 0.004  | 0.315 |
| Glucose index (%)             | 2018–2019      | 92.87 ± 2.59 | 87.32 ± 8.94 | 63.37 ± 9.02 | 65.04 ± 6.69 | 428.875 ** | 0.783  | 0.077 |
| Water absorption (%)          | 2018–2019      | 50.36 ± 0.71 | 50.88 ± 1.67 | 58.74 ± 1.12 | 58.28 ± 0.45 | 7.440 * | 0.365  | 2.875 |
| Development time (s)          | 2018–2019      | 78.00 ± 13.22| 77.67 ± 9.14 | 61.50 ± 10.71 | 68.40 ± 10.46 | 7.440 * | 0.365  | 2.875 |
| Stability time (s)            | 2018–2019      | 260.86 ± 121.40 | 203.83 ± 66.50 | 193.20 ± 56.34 | 230.40 ± 54.54 | 0.394  | 0.125  | 1.560 |

***, p < 0.01; *, p < 0.05; E, environment; G, genotype; E × G, interaction between the environment and genotype. Data are presented as the mean ± standard deviation.

Figure 4. Q8 has no effect on the bread loaf volume. (a) Comparison of the intact ss1 (left) and wild-type (WT) (right) leaves. Scale bar, 2 cm. (b) Comparison of the ss1 and WT loaf volumes. ns, not significant.

2.2. Characterization of the Mutant NS8 Carrying the Q8-N8 Allele

Because a missense mutation in the sequence encoding the AP2 domain can decrease the spike density without altering processing quality parameters, the normal-spikes mutant NS8 containing the Q8-N8 allele was isolated from the M2 population of the mutant S-Cp1-1. The NS8 plants had a normal spike, which was similar to the ‘Shumai482’ spike (Figures 5a and S2a). Compared with the Q allele sequence, Q8-N8 contains two missense mutations (GenBank No. OK041023), with one in the sequence encoding the second AP2 domain and the other in the microRNA172-binding region (Figures 3 and S1). When compared with the WT control (plants with only the Q allele; Figure 1), the mutant NS8 plants were shorter (Figures 5b, 6b, and S2b), but had a similar spike length (Figures 5a, 6d, and S2a) and spike density (Figures 6f and S2n).
The CAPS primer pair (N8-CAPS-F1 + N8-CAPS-R1; Table 3) amplified a 300 bp fragment from the genomic DNA of NS8 and WT. The 300 bp fragment of WT could be digested into 210 bp and 90 bp bands. In contrast, the amplicons of NS8 remained undigested.

Figure 5. Phenotype of the mutant NS8 during the 2020–2021 growing season. (a) Spikes of the wild-type (WT) (left) and NS8 (right) plants. Scale bar, 2 cm. (b) WT (left) and NS8 (right) plants. Scale bar, 10 cm. Comparisons of the grain length (c), grain width (d), thousand kernel weight (e), and grain protein contents (f) of the WT and NS8 samples. **, p < 0.01; *, p < 0.05. Data are presented as the mean ± standard deviation. ‘+’ indicates more than the WT control. ‘+4.7%’ in panel (c) indicates the NS8 grain was 0.3 mm longer than the WT grain (on average). ‘+4.7%’ in panel (e) indicates that Qf1-N8 increased the grain length by 4.7% (on average). (g) Kernel width of the WT (upper) and NS8 (lower) samples. Scale bar, 2 cm. (h) Kernel length of the WT (upper) and NS8 (lower) samples. Scale bar, 2 cm.

Of the yield-related traits, the grain number per spike (Figure 6a and Figure S2i), thousand kernel weight (Figure 5e and Figure S2e), grain width (Figure 5d,g and Figure S2c,h), and grain length (Figure 5c,h and Figure S2c,h), were greater for the mutant NS8 than for the WT control. However, there were no significant differences in the spikelet number per the main spike (Figure 6e and Figure S2m) and productive tiller number (Figure 6c and Figure S2k) between the NS8 and WT plants. As expected, the Qf1-N8 allele positively affected GPC (Figure 5f and Figure S2f) and MY by increasing the thousand kernel weight and grain number per spike.

In order to facilitate the use of the Qf1-N8 allele in breeding, the missense mutation in the sequence encoding the second AP2 domain was converted to a CAPS marker (Figure 7). The CAPS primer pair (N8-CAPS-F1 + N8-CAPS-R1; Table 3) amplified a 300 bp fragment from the genomic DNA of NS8 and WT. The 300 bp fragment of WT could be digested into 210 bp and 90 bp bands. In contrast, the amplicons of NS8 remained undigested.
CR products amplified by using the h2Fig-0.3′ in panel (Q).

**Table 3. Primers used in this study**

| mRNA | Name |
|------|------|
| N8   | AP2startF |
| N8   | AP2.2 |
| N8   | AP45.1R |
| N8   | AP2.16F |
| N8   | AP2.8R |
| N8   | -CAPS |
| N8   | QF7 |
| N8   | -Q- |

**Figure 6.** Effect of Q^I^-N8 on agronomic traits during the 2020–2021 growing season. The subfigures (a–f) are the comparisons of the grain number per main spike, plant height, tiller number, main spike length, spikelet number per main spike, and spike density of the WT and NS8 samples, respectively. The data in the bar graphs are presented as the mean ± standard deviation. *, p < 0.05; ns, not significant. ‘+’ and ‘−’ indicate more and less than the wild-type (WT) control, respectively. ‘+7.1’ in panel (a) indicates NS8 plants had 7.1 more grains per main spike than the WT plants (on average). ‘+13.2%’ in panel (a) indicates that Q^I^-N8 increased the grain number per main spike by 13.2% (on average).

**Figure 7.** Gel separation of digested and undigested PCR products amplified by using the primer pair N8-CAPS-F1 + N8-CAPS-R1. Lanes 1 and 2 are the digested results of the Q allele. Lanes 3 and 4 are the digested results of the Q/Q^I^-N8 alleles. Lanes 5 and 6 are the undigested results of the Q^I^-N8 allele. Lane 7 is the DNA marker.
Table 3. Primers used in this study.

| Primers Name       | Sequences (5'-3')                                      | Reference | Objective                        |
|--------------------|--------------------------------------------------------|-----------|----------------------------------|
| AP2startF          | ATGGTGCTGGATCTAATGTGAGTGCAGGGCAGGGCA                  | [15]      |                                  |
| AP2.8R             | CGCGGCAATACGGGCGAATCCAAAGGAAATCTCAGGA                 |           |                                  |
| AP2.2-1F           | ATCTTAGCTGTATGGGCTGTG                                  | This study|                                  |
| AP2.2-1R           | TCAACGAGATAGGGGTGTCG                                   | This study| Cloning of the genomic DNA        |
| AP2.2-2F           | AGGCTCCACATAAGTATATGATCAGTC                           | This study| DNA sequence of Q gene            |
| AP2.2-2R           | CTGATTTCAAGGAAACGTTTATGCG                             | This study|                                  |
| AP2.16F            | CTGCTTGGTGCGTCCACAGGGTACGAAA                         | [15]      |                                  |
| AP45.1R            | CAGAAGGCGCAACGTTAAGCCGCAACAAATGCG                    | [15]      |                                  |
| Q-mRNA-F2-123      | TCGGAGATGGTGCGAT                                        | This study| Cloning the full open             |
| Q-mRNA-R1-1479     | GCCAGCTTCAGTGTGCCG                                      | This study| reading frame of Q gene           |
| QF7                | GACCAGCCAGTAGTCACCC                                     | This study| Genotyping of Q<sup>-1</sup>-N8   |
| QR7                | TCTTGCAGTTCCATCAGGCC                                   | This study| Q<sup>-1</sup>-N8 allele         |
| N8-CAPS-F1         | ACTAGAGTGAGTGAGGAAGATT                                 | This study| CAPS marker for Q<sup>-1</sup>-N8 |
| N8-CAPS-R1         | AGCAACTGTAGGCGTCCAAA                                    | This study|                                  |

3. Discussion

Because GY and GPC determine the profitability of wheat production, they are the primary traits upon which wheat breeders and growers focus. More specifically, GY is critical for ensuring food security, especially in many developing countries, while GPC is a crucial index for assessing the nutritional and unique processing quality of wheat [22]. Increasing both GY and GPC is important to improving the availability of high-quality food and, by extension, the living standards of humans. As an essential macronutrient for plants, nitrogen is crucial for establishing a balance between GY and GPC during wheat production [3,4,23]. Accordingly, the most frequent cultivation practice used by farmers to increase GY and GPC is the application of nitrogen fertilizers [4,24], but this leads to increased cultivation costs, decreased nitrogen use efficiency, and increased environmental pollution. Moreover, after incremental additions of nitrogen fertilizer, GPC reaches a maximum and then remains constant [24]. In this study, we created a new Q allele (i.e., Q<sup>-1</sup>-N8), which breaks the negative relationship between GY and GPC and can synchronously increase the wheat GY and GPC. Thus, Q<sup>-1</sup>-N8 may be useful for breeding more profitable wheat varieties.

‘Shumai482’ is an elite commercial wheat cultivar with a relatively high GY and GPC. The Q<sup>-1</sup>-N8 allele in the ‘Shumai482’ genetic background can increase both GY and GPC. Notably, Q<sup>-1</sup>-N8 decreased the plant height (Figure 5b, Figure 6b and Figure S2b,j), thereby enhancing lodging resistance. Lodging is still a major factor limiting global wheat production, especially in regions with heavy rain and strong winds, because it leads to serious decreases in GY. We are currently assessing the breeding value of Q<sup>-1</sup>-N8 in multiple environments, in diverse genetic backgrounds, and in field plot experiments.

As important parts of the Q protein, the AP<sub>2</sub> domains are critical for DNA binding and for physical interaction with other proteins. Modifying the amino acids in the AP<sub>2</sub> domains of the Q protein may reverse the unfavorable agronomic traits of the mutant S-Cp<sub>1</sub>-1 carrying the Q<sup>-1</sup> allele [17] by affecting the expression of downstream genes and the interaction between Q and other proteins. For example, the transcription factor TaLAX1 physically interacts with Q to antagonistically regulate grain threshability and spike morphology [25]. However, most downstream genes of Q for regulating agronomic traits remain unknown. The mutation in the Q<sup>-1</sup> allele results in a single amino acid change in the first AP<sub>2</sub> domain (Figure 3 and Figure S1), which negatively affects the thousand kernel weight (Table 1). The overexpressed Q<sup>-1</sup> allele has a missense mutation in the mircoRNA172-binding site (Figure 3 and Figure S1) that increases the thousand kernel weight [17]. It is likely that at least some missense mutations in the sequences encoding the AP<sub>2</sub> domains and those in the microRNA172-binding site have the opposite effect on the thousand kernel weight. The Q<sup>-1</sup>-N8 allele has two missense mutations, with one in the sequence encoding the AP<sub>2</sub> do-
main and the other in the microRNA172-binding site (Figure 3 and Figure S1); this allele is associated with an increase in the thousand kernel weight (Figure 5e and Figure S2e). Therefore, the opposite effects of the two-point mutations in the $Q^{1-1}-N8$ allele are relatively well balanced to increase the thousand kernel weight. However, further increases in GY require the creation of new alleles with mutation(s) beyond the AP$_2$ domain-encoding sequences; four previously reported $Q^i$ alleles (i.e., $Q^{i1}$–$Q^{i4}$) [17] might be useful for generating new alleles.

Common wheat is a hexaploid species (AABBDD; 2n = 6x = 42) that contains three homologous genomes (i.e., A, B, and D). An earlier study revealed the dosage effect of the Q gene in wheat [26]. To date, only the Q gene copy in the A genome has been optimized. To further improve GY and GPC, the stepwise optimization of the Q copies in the B and D genomes of common wheat and related species is ongoing.

Durum wheat (Triticum turgidum ssp. durum) is a tetraploid species (AABB; 2n = 4x = 28) and is the main and preferred raw material for pasta production [27]. The GPC is a determining factor influencing durum wheat quality, and grains with a high GPC tend to produce good cooking quality pasta [27–29]. The $Q^{1-1}$-N8 allele, which is located in the A genome, may be useful for durum wheat breeding.

In addition to the Q gene, many other plant genes include microRNA-binding sites, including some genes encoding a conserved AP$_2$ domain. The directed evolution of these genes via the introduction of point mutations in their microRNA-binding sites and other domain-encoding sequences may be an efficient and effective way to ensure global food security.

Increases in GY and GPC are also required for other major cereal crops, such as rice, maize, barley, sorghum, and foxtail millet, all of which carry Q gene orthologs and homologs [20,30–34]. Moreover, these orthologous and homologous genes seem to have conserved functions among cereals [31,33,34]. Similar to the allele development in this study (i.e., Q allele to $Q^{i1}$ and then to $Q^{i1}$-N8), elite alleles for the Q gene orthologs and homologs can be created by the stepwise optimization of their expression (e.g., by introducing point mutations in the microRNA172-binding site or in other elements) and by enhancement of the activities of the encoded proteins (e.g., by introducing point mutations in the sequences encoding the AP$_2$ domains or other domains), affecting specific downstream gene(s) and interacting protein(s). As they are applied in breeding programs involving non-transgenic methods, these attributes illustrate the utility of creating a set of elite alleles of the Q gene orthologs and homologs to increase the GY and GPC of cereal crops.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

The seeds of common wheat cultivar ‘Shumai482’ ($Q$ allele) and its compact-spike mutant S-Cp1-1 ($Q^{c2}$ allele) [17] were treated with 0.8% and 0.4% ethyl methanesulfonate (Catalog number: M0880-100G; Sigma-Aldrich, St Louis, MO, USA), respectively. Seeds from the leading spikes of the M$_1$ plants were harvested and sown to generate the M$_2$ population. The mutant ss1 (sparse spike 1) was obtained from the M$_2$ population of ‘Shumai482’. The mutant NS8 (normal spike 8) was isolated from the M$_2$ population of S-Cp1-1. The Q genes of ss1 ($Q^{c1}$ allele) and NS8 ($Q^{c2}$-N8 allele) were sequenced.

The mutants were backcrossed with ‘Shumai482’ to assess the effects of $Q^{c2}$ and $Q^{1-1}$-N8 on agronomic traits and processing quality parameters. Ten BC$_2$F$_3$ homozygous lines (five with the Q allele and five with the $Q^{c2}$ allele) and 10 BC$_2$F$_4$ homozygous lines (five with the Q allele and five with the $Q^{c1}$ allele) (Figure 1) were grown at the experimental farm of Sichuan Agricultural University in Wenjiang (30°43′16″ N, 103°52′15″ E) during the 2018–2019 and 2019–2020 wheat growing seasons, respectively. Field trials were performed using a randomized block design. Each line was cultivated in a 2 m × 3 m area, with a row spacing of 20 cm × 5 cm. The BC$_1$F$_2$ and BC$_2$F$_2$ plants carrying Q or $Q^{1-1}$-N8 (Figure 1) were grown with a row spacing of 20 cm × 10 cm in Wenjiang during the 2020–2021 and
The growing seasons, respectively. A nitrogen:phosphorous:potassium (15:15:15) compound fertilizer was applied before sowing (450 kg per hectare).

At the GS87 growth stage [35], agronomic traits, including plant height (cm), spike length (cm), spikelet number per spike, grain number per spike, and productive tiller number, were recorded. Spike density was calculated as the ratio of the spike length to the spikelet number per spike. For the BC$_2$F$_3$ and BC$_2$F$_4$ homozygous lines with the Q or Q$^{s1}$ allele, 20 representative plants of each line were examined. For the homozygous BC$_1$F$_2$ and BC$_2$F$_2$ plants carrying the Q or Q$^{s1}$-N8 allele, the agronomic traits of 20–30 plants were also evaluated.

After harvesting samples and drying them under the sun at approximately 35 °C to a constant weight, the thousand kernel weight (g), grain length (mm), and grain width (mm) were determined. For each BC$_2$F$_3$ and BC$_2$F$_4$ homozygous line with the Q or Q$^{s1}$ allele, the thousand kernel weight was measured by randomly selecting 1000 seeds. For the BC$_1$F$_2$ and BC$_2$F$_2$ plants carrying the Q or Q$^{s1}$-N8 allele, the thousand kernel weight was measured on the basis of 200 randomly selected mature seeds. In order to measure the grain length and width, 100 randomly selected seeds were scanned using the Epson Eu-88 A3 Transparency Unit (Seiko Epson, Nagano, Japan). The resulting images were analyzed using the WinSEEDLE Analysis System (Regent Instruments, QC, Canada).

4.2. Gene Cloning

Young leaves collected from individual plants at the GS13 growth stage [35] were ground to a fine powder in liquid nitrogen. Genomic DNA and total RNA were extracted from the ground materials using Plant DNA/RNA extraction kits, respectively (Catalog numbers: DN32-100 and RN33050; Biofit, Chengdu, China). First-strand cDNA was synthesized using the Prime Script™ 1st Strand cDNA Synthesis Kit (Catalog number: 6110A; Takara, Dalian, China). All kits were used as recommended by the manufacturers.

The Q cDNA and genomic DNA sequences of the mutants ss1 and NS8 were cloned and sequenced. The PCR amplifications were completed in a 50 µL volume consisting of genomic DNA or cDNA, 200 µM dNTPs, 10 µM each primer, 1 U Phanta Max Super-Fidelity DNA Polymerase (Catalog number: P505-d1/d2/d3; Vazyme, Nanjing, China), and 25 µL 2 × supplied buffer (with Mg$^{2+}$). The PCR was performed using the Mastercycler Pro thermal cycler (Eppendorf, Hamburg, Germany) with the following program: 95 °C for 5 min; 35 cycles of 95 °C for 45 s, 60–68 °C for 30 s, and 72 °C for 2 min; 10 min at 72 °C. The PCR products were separated on a 1.5% agarose gel (Catalog number: 5260; Takara). The target fragments were purified using the FastPure Gel DNA Extraction Mini Kit (Catalog number: DC301-01; Vazyme) and then inserted into the pCE2 TA/Blunt-Zero vector using the 5 min TA/Blunt-Zero Cloning Kit (Catalog number: C602-01; Vazyme). Positive colonies were sequenced by Sangon Biotech (Chengdu, China). The cloning and sequencing experiments were repeated at least three times. Sequences were analyzed using DNAMAN (version 8) (Lynnon Biosoft, San Ramon, CA, USA). The primers used are listed in Table 3.

4.3. Genotyping for Q$^{s1}$-N8

Genomic DNA extracted from individual plants in the BC$_1$F$_2$ and BC$_2$F$_2$ population of the mutant NS8 was used as the PCR template. The QF7 + QR7 primer pair (Table 3) flanking the microRNA172-binding site was used. The PCR amplifications were performed as described above. The PCR products were sequenced to determine the presence/absence of Q$^{s1}$-N8.

4.4. Development of CAPS Marker for Q$^{s1}$-N8

The point mutation in the sequence encoding the second AP$_2$ domain of the Q$^{s1}$-N8 allele was converted to the CAPS (Cleaved Amplified Polymorphic Sequence) marker by using DNAMAN (version 8). This CAPS marker (N8-CAPS-F1 + N8-CAPS-R1; Table 3) was tested in the BC$_1$F$_2$ and BC$_2$F$_2$ populations of the mutant NS8. The PCR amplifications
were performed as described above. The PCR products (about 5 µg) were digested with five units of the restriction enzyme BbvI (Catalog number: R0173S; New England Biolabs, Ipswich, MA, USA) along with given buffer at 37 °C for 120 min. The digested fragments were separated on a 1.5% agarose gel.

4.5. Processing Quality Analysis

Mature grains were dried under the sun, cleaned, and stored at room temperature for 2 months. The GPC (dry weight) was measured as described by [36]. In order to assess the effect of Q^{s1} on processing quality, the moisture content of the ss1 (Figure 1) and wild-type (WT) grains were adjusted to 16.5% before the samples were milled using the CD1 Laboratory Mill (CHOPIN Technologies, Villeneuve-la-Garenne Cedex, France). The Zeleny sedimentation value, wet gluten content, gluten index, and dough rheological properties were determined as previously described [36]. A farinograph (Brabender GmbH & Co., Duisburg, Germany) was used to determine the rheological properties.

A baking test was performed according to a slightly modified version of AACC method 10.09-01 [37]. Specifically, a standard rapid mix test involving 50 g flour (14% moisture content) was conducted. There were two loaves of bread per flour sample. The loaf volume was determined using the BVM6630 volume meter (Pertern, Stockholm, Sweden), as described by the manufacturer.

4.6. Statistical Analysis

All data were calculated using Excel 2010 (Microsoft, Redmond, WA, USA). The significance of the differences in the mean values for the agronomic traits and processing quality parameters between the WT and mutant samples was determined according to Student’s t-test implemented in the Data Processing System (DPS) software (version 18.10) (Zhejiang University, Hangzhou, China) [38]. The DPS software was also used to perform an analysis of variance.

5. Conclusions

In this study, we characterized two new Q alleles (Q^{s1} and Q^{c1}-N8) and demonstrated that the negative correlation between GY and GPC of wheat could be broken by Q^{c1}-N8 via a non-transgenic approach. Q^{c1}-N8 synchronously increases the wheat GY and GPC, and a specific molecular marker was developed to facilitate its use in breeding. It is possible to further increase both GY and GPC by progressively optimizing the Q gene in the three genomes of wheat.

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