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Allelic Variants of Glutamine Synthetase and Glutamate Synthase Genes in a Collection of Durum Wheat and Association with Grain Protein Content

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Abstract: Wheat is one of the most important crops grown worldwide. Despite the fact that it accounts for only 5% of the global wheat production, durum wheat (*Triticum turgidum* L. subsp. *durum*) is a commercially important tetraploid wheat species, which originated and diversified in the Mediterranean basin. In this work, the candidate gene approach has been applied in a collection of durum wheat genotypes; allelic variants of genes glutamine synthetase (*GS2*) and glutamate synthase (*GOGAT*) were screened and correlated with grain protein content (GPC). Natural populations and collections of germplasms are quite suitable for this approach, as molecular polymorphisms close to a locus with evident phenotypic effects may be closely associated with their character, providing a better physical resolution than genetic mapping using ad hoc constituted populations. A number of allelic variants were detected both for *GS2* and *GOGAT* genes, and regression analysis demonstrated that some variations are positively and significantly related to the GPC effect. Additionally, these genes map into homoeologous chromosome groups 2 and 3, where several authors have localized important quantitative trait loci (QTLs) for GPC. The information outlined in this work could be useful in breeding and marker-assisted selection programs.

Keywords: durum wheat; genetic diversity; grain protein content; glutamine synthetase; glutamate synthase

1. Introduction

Wheat, together with rice and maize, is one the most important cereal crops grown worldwide. Most of the cultivated cultivars and varieties belong to the hexaploid *Triticum aestivum* L. (genomes AABBDD, bread wheat) or to the tetraploid *T. turgidum* L. var. *durum* (genomes AABB, durum wheat), which are different in genome size (bread wheat also having a D genome), grain composition, and food end-use quality attributes. Several agronomic traits and composition aspects determine the final quality of grains.

Among them, grain protein content (GPC) contributes to the nutritional value and the baking properties of common wheat and to the pasta-making technology characteristics of durum wheat. GPC is a quantitative trait influenced by a complex genetic system and affected by environmental factors as well as by management practices. Indeed, one of the most pursued goals of breeders

in the last decades has been the improving of grain protein concentration. However, because of the negative correlation between grain yield and GPC, simultaneous increases of both traits have been difficult to achieve [1,2]. Nitrogen fertilizers are today extensively used to increase both crop yield and protein content. The current agricultural system requires growers to optimize the use of nitrogen fertilizers to avoid pollution, while maintaining reasonable profit margins. Numerous studies allowed the identification of candidate genes that encode enzymes involved in nitrogen assimilation and recycling [3], and many of them co-localized with agronomic and physiological traits related to nitrogen metabolism [4,5]. Two genes resulted to be particularly important in the first step of ammonia assimilation: glutamine synthetase and glutamate synthase. These two enzymes work synergistically in a cycle known as GS-GOGAT shunt, involved in the first step of N metabolism and glutamate synthesis.

The glutamine synthetase (GS) enzyme has an essential role in the assimilation and re-assimilation of inorganic N. GS genes represent a gene family with three to five isoforms, depending on the species [6]. On the bases of phylogenetic studies and mapping data in wheat, 10 GS cDNA sequences were classified into four sub-families denominated *GS1* (a, b and c), *GSr* (1 and 2), *GSe* (1 and 2), active in cytosol, and *GS2* (a, b and c), localized in plastids [7,8]. Several studies have been carried out on various isoforms as candidates for improving nitrogen use efficiency (NUE) in wheat, and their relationships with GPC have been investigated [8–12]. Once glutamine is synthetized, another important enzyme is involved in the second step of the reaction to the synthesis of glutamate: the glutamate synthase (Glutamine-2-oxoglutarate amidotransferase). This enzyme is responsible for the transfer of the amide group of glutamine to 2-oxoglutarate, with the result of two yielded glutamate molecules, one of which is then available for aminoacid synthesis and the other of which returns to the GS-GOGAT cycle [13]., Gene regulation, as well asenzymes structure, have been reported in previous works [4,14]. Based on the electron donors, GOGAT exists in plants in two different isoforms: A ferredoxin (Fd)-dependent (EC 1.4.7.1) form and an NADH-dependent (EC 1.4.1.14) form, both of which are located in plastids but in two different types of tissues. The Fd-GOGAT enzyme is usually present in photosynthesizing tissues, while the NADH-GOGAT enzyme is the predominant form in non-photosynthesizing cells. These differences in tissue location and enzyme roles have been well studied in both rice and conifers [15,16]. Additionally, mutagenesis studies showed that mutated and/or silenced *GOGAT* not only reduced enzyme activity but also seemed to be involved in changes in aminoacid metabolism [17–20].

Only a few studies have reported gene isolation and sequencing of *GOGAT* genes in plants, probably due to their length and structural complexity. GOGAT genes genomic sequences have been reported for maize [21], tobacco [22], Arabidopsis [23] and barley [24] and partial sequences were also reported in bread wheat [25]. Recently, NADH and Fd-GOGAT gene structures, genomic sequences, gene localization, and involvement in GPC control have been reported in durum wheat [26,27].

Considering the importance and central role of these two genes in nitrogen metabolism, the main objectives of this study were to investigate the presence of allelic variants of both the plastidic *GS2* and the *GOGAT* genes in a collection of tetraploid wheat genotypes and to validate the relationships between variants and GPC.

2. Materials and Methods

2.1. Plant Material

A collection of 236 tetraploid wheat genotypes (*Triticum turgidum*), including wild and cultivated accessions of seven subspecies (*durum*, *turanicum*, *polonicum*, *turgidum*, *carthlicum*, *dicoccum* and *dicoccoides*), was screened for *GS2* and *GOGAT* allelic variants (List of accessions is reported in Table S3). The collection was bred in an experimental field of the University of Bari at Valenzano (Bari, Italy) in 2009 and 2010 and in Foggia (Italy) in 2009 in a randomized complete block design study with three replications. Each plot consisted of 1 m rows, 30 cm apart, and the seeding rate was 50 seeds per plot.

Genomic DNA was isolated from fresh leaves and subsequently purified using a method previously described [28] and subsequently purified via phenol-chloroform extraction.

2.2. PCR Condition and Sequencing

PCR reactions were performed in final volumes of 20 µL in BIORAD thermo cyclers. The reaction mixture contained each deoxynucleotide in 200 µM concentrations, each primer in 0.5 µM concentrations, 1× buffer, 0.02 U/µL Taq polymerase (Phusion High-Fidelity DNA Polymerase, Thermo Fisher Scientific, Waltham, MA, USA), and 50 ng of template DNA. Using Oligo Explorer software, a set of genome specific primer pairs were designed for *GS2* and *GOGAT* genes, as reported in Tables S1 and S2. PCR fragments were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) cloned into the pCR4-TOPO vector (Invitrogen, Cloning Kit, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions, and subsequently sequenced (3500 Genetic Analyzer, Applied Biosystem, Foster City, CA, USA). Sequences alignments were carried out using ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and CodonCode Aligner software (CodonCode Corporation, Centerville, MA, USA).

2.3. Digestion with CEL I and Revelation Fragments

In order to discover mutations within *GOGAT* gene sequences in the durum wheat collection previously described, single nucleotide polymorphisms (SNPs) were detected using the Surveyor nuclease kit (Transgenomic, Omaha, NE, USA). Heteroduplex formation, *CelI* digestion and gel analysis were performed following a procedure previously reported [27].

2.4. Protein Content Quantification and Regression Analysis

Grain protein content (GPC) and yield components were evaluated in the durum wheat collection previously described grown in three different environments. GPC was assessed on 3 g of whole meal flour using a dual beam near infrared reflectance spectrophotometer (Zeutec Spectra Alyzer Premium, Zeutec Büchi, Rendsburg, Germany). Linear regression analysis between each allelic variant of both *GS* and *GOGAT* genes and GPC were carried out using MSTAT-C software developed by Freed et al. [29], Michigan State University.

3. Results and Discussion

3.1. Phenotypic Characterization for the Protein Content of a Collection of Tetraploid Wheat Genotypes

A collection of 236 tetraploid wheats genotypes, including durum cultivars, landraces, and wild accessions, has been characterized in terms of genetic diversity and population structure [30] and used for genome-wide association mapping of loci controlling some qualitative important traits, such as β-glucan content [31], carotenoid content [32] and phenolic acids [33]. GPC was determined in three replicated field experiments. Table 1 shows the summary data of protein content expressed as grams of protein on 100 g of dry weight of wholegrain. Variance analysis revealed significant differences between genotypes ($p < 0.001$).

Table 1. Mean, standard deviation (SD), ranges, and coefficient of variation (CV %) of grain protein content (GPC) (µg/g dry matter) in a tetraploid wheat collection evaluated in three different environments.

Environment	Foggia 2009	Valenzano 2009	Valenzano 2010
Mean	14.1	16.2	15.5
SD	1.55	2.01	2.21
Min	10.8	11.8	12.5
Max	20.4	22.9	23.5
CV (%)	10.9	12.4	14.3

The highest GPC mean value (16.2%) was found for the trial of Valenzano 2009; however, looking at the three trials, minimum and maximum values ranged from 10.8 and 23.5, respectively. Additionally, the coefficient of variation showed different values among the trials, ranging from 10.9 to 14.3. Variance analysis revealed significant differences in $p < 0.001$ between genotypes.

3.2. GS2 Allelic Variants and Relationship with GPC

Based on the genomic sequences of the two homoeologous genes *GS2-2A* and *GS2-2B* previous isolated [10], several pairs of specific genomic primers reported in Table S1 were drawn. These were amplified and screened for genomic variations in the entire collection of tetraploid wheat (see Materials and Methods).

Three major polymorphisms were found in the *GS2* gene. An insertion/deletion of a 239 bp MITE (Miniature Inverted-Repeat Transposable Element), located in the second intron of the 2A homoelogous chromosome, identifies the only allelic variant for the *GS2-2A* gene. Out of the analyzed 236 wheat genotypes, the intronic Miniature Inverted Transposable Element was found in 96 genotypes. On the other hand, two different polymorphisms were identified on the 2B homoelogous chromosome: a repeated 5 bp microsatellite (GATTA) and a 33 bp indel, respectively, located in the first and second introns of the *GS2-2B* gene. Figure 1 shows peaks corresponding to MITE deletion in the second intron of the *GS2-2A* gene (peak at 480 bp, and the 33 bp deletion/insertion of the *GS2-2B* gene (473 and 507 bp, respectively) [10].

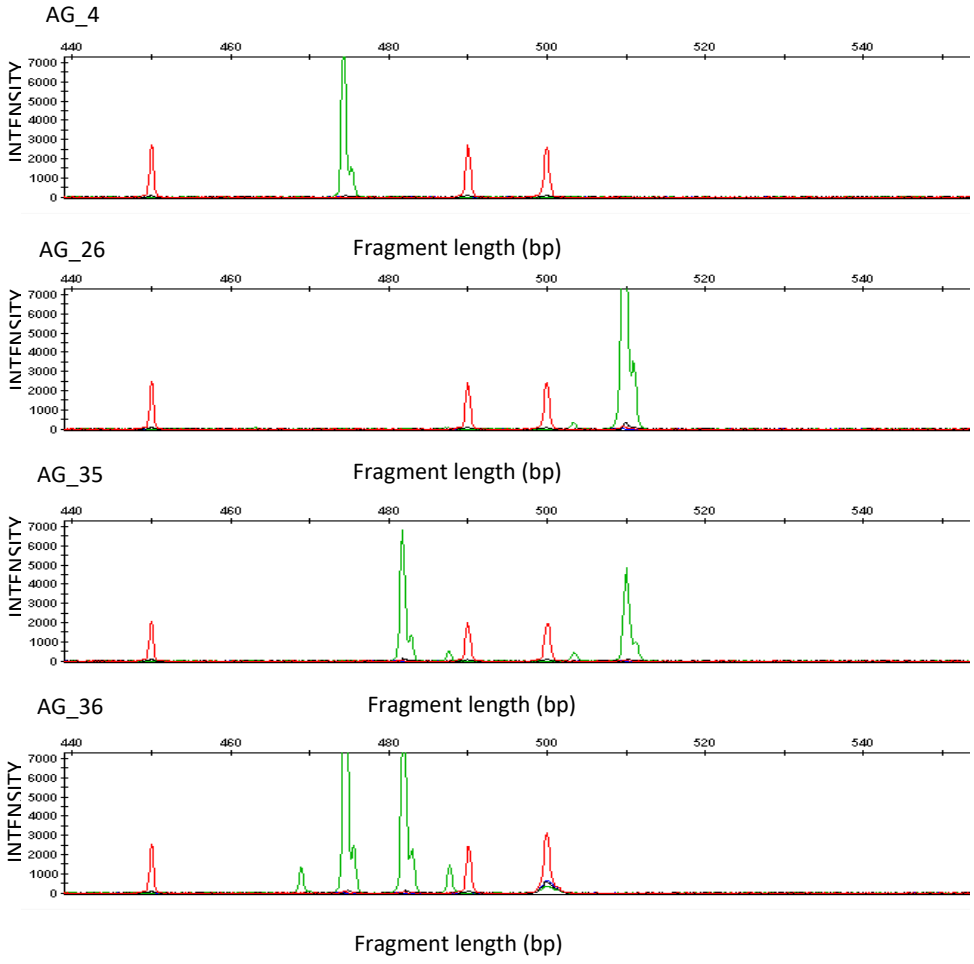

Figure 1. Polymorphisms detected in the *GS2* genes in genotypes of the durum collection. Peaks corresponding to MITE deletion in the second intron of the *GS2-2A* gene (peak at 480 bp) and the 33 bp deletion/insertion of the *GS2-2B* gene (473 and 507 bp, respectively) are shown.

We ran a regression analysis between the *GS2* allelic variants and the GPC in the described tetraploid wheat collection evaluated in three replicated field experiments. The regression analysis with the *GS2-A2* alleles showed that the amplicon of 480 bp absent of MITE is positively correlated with GPC in all three environments (Table 2). The stronger correlation was found in the Foggia 2009 environment ($p \geq 0.001$), while the lowest one, albeit still significant at $p \geq 0.05$, was found in Valenzano 2009. The phenotypic variation ranged from 4.1 to 9.2%. Although significant results were previously reported [10], no significant correlation was instead outlined by the regression analysis between GPC and the 33 bp indel of the *GS2-B2* gene, likely due to the fact that an unequal distribution of alleles occur in the collection of genotypes.

Table 2. Regression analysis between *GS2* allelic variants and GPC (% DW^{-1}) in a tetraploid wheat collection evaluated in three replicated field experiments.

Gene	Amplicons (bp)	Frequency	Environments								
			Foggia 2009			Valenzano 2009			Valenzano 2010		
			$\log_{10}(p)$	Effect	R^2	$\log_{10}(p)$	Effect	R^2	$-\log_{10}(p)$	Effect	R^2
GS2-A2	480/719	131–90	5.3 ***	0.92	9.2	2.6 *	0.83	4.1	3.7 **	1.11	6.1
GS2-B2	473/507	184–37	0.4	0.24	0.4	0.4	−0.33	0.4	0.1	0.13	0.0

*, **, and *** = significant at $p \geq 0.05$, $p \geq 0.01$, and $p \geq 0.001$, respectively, using the Bonferroni threshold ($p/12$) to control for multiple testing. R^2 = Phenotypic variation (%).

Glutamine synthetase plays a key role in the use of absorbed nitrogen, process of primary importance for plant growth. The absorption and utilization of nitrogen are closely related both to production and to the accumulation of grain proteins in various cereal species. Haplotype analysis of *GS2* gene and their association with NUE and yield-related traits was performed in bread wheat [34]. MITE was also found in Chinese bread wheat genotypes, and an interesting association with yield-related traits was also found.

Recently, a genome-wide association analysis with high-density SNP markers was conducted in order to identify genomic regions that may be associated with NUE traits in Great Plains hard winter wheat germplasm [35]. Interestingly, it was found that SNP markers on the long arm of a 2D chromosome were associated with NUE traits, in the same distal position where *GS2* genes map.

Other authors reported a colocalization between GS genes and GPC quantitative trait loci (QTLs) in a DHL population in three different environments [36]. A Meta-QTL for GY and GPC detection was carried out using three inter-connected doubled haploid populations grown in a large multi-environment trial network, identifying several genomic regions having GY and GPC [37]. Among them, they found 2A and 2D chromosomes carrying important QTLs, suggesting that genomic regions close to *GS2* genes are involved in GPC control.

3.3. GOGAT Allelic Variants and Relationship with GPC

Along with glutamine synthetase, glutamate synthase (GOGAT) forms an enzymatic complex considered to be one of the bottlenecks of the early stages of nitrogen metabolism, in particular as regards the absorption, assimilation, and amelioration of ammonia nitrogen in the plant. As previously reported [26,27], *GOGAT* genes have a complex intronic-exon structure: *Fd-GOGAT* is comprised of 33 exons and has a size of about 15 kb, while *NADH-GOGAT* is comprised of 22 exons separated by 21 introns and has a size of about 10 kb. In order to find polymorphisms more easily in such long sequences, *Fd-GOGAT* and *NADH-GOGAT* genes were screened via an EcoTILLING approach. The different combinations of specific genomic primers (A and B) reported in Table S2 were amplified in the genotypes of the collection and analyzed for the presence of polymorphisms.

In this work, each combination was amplified as reported in the Materials and Methods section, and SNPs and indels polymorphisms were determined by duplex ether formation and digestion with

the *CelI* enzyme. The duplex was obtained from each single genotype in the collection and the cv Svevo was used as control.

Digestion probes of different primer combinations for *Fd-GOGAT* genes allowed the identification of six SNPs polymorphism. Out of the six SNPs found, three were located in intronic regions (Introns 5, 10 and 31), and three were located in the exon region (Exons 6, 31 and 32). None of them determined a change in aminoacidic predicted sequences.

With the aim of determining the association between SNP markers found in *Fd-GOGAT* genes and the protein content of the caryopsis, regression analysis was conducted between the SNPs markers and the percentages of grain protein of the trials previously described. As reported in Table 3, the six SNPs identified three allelic variants for both *Fd-GOGAT-A2* and *Fd-GOGAT-B2* genes, named as a, b, and c allelic variants, respectively. Out of the three identified variants in the *Fd-GOGAT-A2* gene, the *Fd-GOGAT-A2a* allelic variant showed a highly significant correlation with GPC. This polymorphism was a C/T transition, significant at $p \geq 0.001$ in all three environments. Phenotypic variation ranged from 6.9 to 14.6% in Valenzano 2010, suggesting its potential effect on GPC. On the other hand, none of the three allelic variants identified for *Fd-GOGAT-B2* showed any correlation with GPC.

Table 3. Regression analysis between Fd-GOGAT allelic variants and GPC (% DW^{-1}) in a tetraploid wheat collection evaluated in three different environments.

Gene	Allele	Frequency	Environments								
			Foggia 2009			Valenzano 2009			Valenzano 2010		
			$-\log_{10}(p)$	Effect	R^2	$-\log_{10}(p)$	Effect	R^2	$-\log_{10}(p)$	Effect	R^2
Fd-GOGAT-A2a	C/T	204–17	4.1 ***	−1.47	6.9	6.7 ***	−2.48	11.7	8.4 ***	−3.05	14.6
Fd-GOGAT-A2b	C/T	115–99	0.2	−0.09	0.1	0.0	−0.02	0.0	0.2	−0.14	0.1
Fd-GOGAT-A2c	A/G	100–117	0.3	0.12	0.2	0.0	0.02	0.0	0.2	0.13	0.1
Fd-GOGAT-B2a	A/G	181–38	0.9	−0.41	1.1	2.3	−0.98	3.5	2.7 *	−1.19	4.3
Fd-GOGAT-B2b	A/G	37–181	1.1	0.47	1.4	2.6 *	1.06	4.0	3.0 *	1.28	4.9
Fd-GOGAT-B2c	A/G	117–101	0.3	−0.15	0.3	0.0	0.00	0.0	0.2	−0.14	0.1

* and *** = significant at $p \geq 0.05$ and $p \geq 0.001$, respectively, using the Bonferroni threshold (P/18) to control for multiple testing. R^2 = Phenotypic variation (%).

The same approach was followed in order to find out polymorphisms in *NADH-GOGAT* genes. A very similar scenario was detected for this gene: six different SNPs were detected in the screened genotypes, and specifically three SNPs for each genome resulting in three allelic variants for each homoeologous gene, named as a, b and c allelic variants, respectively (Table 4). The results of regression analysis showed that, out of the three allelic variants for *NADH-GOGAT-A3*, only the T/G transversion, identified as the *NADH-GOGAT-A3b* allelic variant, showed a very high and significant association at $p \geq 0.001$ with GPC in the three considered environments. Phenotypic variation ranged from 7.0 to 11.1% in Valenzano 2010. *NADH-GOGAT-A3a*, a C/T transition, was found to be significantly associated in one environment only, Foggia 2009, at $p \geq 0.05$ with a phenotypic variation of 4.4%.

As found in the *NADH-GOGAT-A3* gene, the 3B homoeologous gene showed three allelic variants, identified as a, b, and c and corresponding to a G/T transversion, and C/T and A/G transitions, respectively. None of them showed a strong correlation nor a phenotypic effect as the *NADH-GOGAT-A3b* allelic variant, but two of them, *NADH-GOGAT-B3a* and *NADH-GOGAT-B3c*, had a significant association at $p \geq 0.05$ with GPC in the Foggia 2009 environment only, with an R^2 of 4.6% and 4.2%, respectively.

Gene expression and post-transcriptional modification have a great effect on enzyme activity. However, mutations located both in exonic and intronic regions can affect gene expression levels. Insertion, deletions, and point mutations (single nucleotide polymorphisms) in introns can, for instance, introduce novel splice sites, activate novel promoters, or introduce/eliminate enhancer activity.

A recent work of Zeng et al. [38] showed how a single nucleotide polymorphism, leading to an aminoacid substitution in rice *Fd-GOGAT* genes, resulted in an increased GPC, confirming its important role as a potential candidate in NUE improvement.

Table 4. Regression analysis between NADH-GOGAT allelic variants and GPC (% DW^{-1}) in a tetraploid wheat collection evaluated in three different environments.

Gene	Allele	Frequency	Environments								
			Foggia 2009			Valenzano 2009			Valenzano 2010		
			$-\log_{10}(p)$	Effect	R^2	$-\log_{10}(p)$	Effect	R^2	$-\log_{10}(p)$	Effect	R^2
NADH-GOGAT-A3a	C/T	145–70	2.7 *	0.68	4.4	1.3	0.58	1.9	1.6	0.73	2.4
NADH-GOGAT-A3b	G/T	42–176	6.1 ***	−1.24	10.6	4.1 ***	−1.36	7.0	6.4 ***	−1.87	11.1
NADH-GOGAT-A3c	A/G	111–103	0.1	−0.04	0.0	0.7	−0.36	0.8	1.3	−0.60	1.9
NADH-GOGAT-B3a	G/T	71–147	2.8 *	−0.68	4.6	1.5	−0.61	2.1	1.8	−0.76	2.6
NADH-GOGAT-B3b	C/T	115–103	0.1	−0.06	0.0	0.9	−0.43	1.1	1.6	−0.68	2.3
NADH-GOGAT-B3c	A/G	146–72	2.6 *	0.65	4.2	1.2	0.54	1.6	1.5	0.68	2.1

* and *** = significant at $p \geq 0.05$ and $p \geq 0.001$, respectively, using the Bonferroni threshold (P/18) to control for multiple testing. R^2 = Phenotypic variation (%).

The *NADH-GOGAT* gene was also identified as a major candidate gene for cereal NUE by a cross-genome ortho-meta QTL study of NUE [39]. QTL for GPC has also been reported on chromosome 3AL in in the homoeologous position of the *NADH-GOGAT-3B* gene [40] A proof of the central role of *GOGAT* genes was obtained in rice; the suppression of both GOGAT genes reduced yield per plant and thousand kernel weight, phenotypic indications of nitrogen starvation [41].

4. Conclusions

GPC is one of the most important wheat agronomic trait, in relation to both nutritional and technological properties. GPC is a typical quantitative trait, controlled by several genes and influenced by environmental factors. Different physiological processes also influence GPC, such as nitrogen uptake, assimilation, and remobilization to the grain. Several studies, carried out on different genetic materials, have reported the influence of the homoeologous chromosome groups 2 and 3 on GPC control. In 1990, important QTLs for GPC on group 2 chromosomes were firstly reported on durum wheat [42]. QTLs for GPC were also found on the short arms of homoeologous group 2 chromosomes in both bread and durum wheat [40,43]. Stable QTLs for GPC were also identified on 2A and 2B chromosomes in Canadian durum wheat populations [44]. Several authors have focused on deciphering GPC and NUE quantitative traits, and genetic diversity at candidate genes have lately been considered for this purpose. Both *GS2* and *Fd-GOGAT* genes have been mapped in homoeologous group 2 chromosomes and have been found to co-localize with important QTLs for GPC in durum wheat [10,27]. Homoeologous group 3 was also found to be important for GPC control. An otho-Meta QTLs analysis for NUE identified *NADH-GOGAT* as one of the major effectors of NUE in wheat, rice, sorghum, and maize [39]. A QTL for GPC was also reported in the homoeologous position of chromosome 3AL [40]. Another important aspect to be considered in exploiting GPC and NUE is the importance of genotypic variation in gene and QTL expressions. Nigro et al. [12] showed how the genotype plays an important role in *GS* expression, especially at different N regimes. Previously, other authors have reported the importance of genotypic variation in NUE and final grain nitrogen content, determining that the genotype was one of the causes of variation in analyzed traits, after the N-rate and the growth stage [45].

The above data suggest that the genomic region surrounding *GS2* and *GOGAT* genes are involved in grain protein accumulation, and the identification of new useful alleles for marker-assisted selection is valuable for breeding wheat varieties with improved agronomic performance and N-use efficiency.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/9/4/52/s1, Table S1: Genome specific primer combination for GS2 genes, Table S2: Genome specific primer combination for GOGAT genes, Table S3: List of tetraploid accession used in the study.

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Author Contributions: Domenica Nigro, Agata Gadaleta and Antonio Blanco conceived and designed the experiments; Domenica Nigro, Stefania Fortunato and Stefania Lucia Giove performed the experiments; Giacomo Mangini, Ines Yacoubi and Antonio Blanco analyzed the data; Domenica Nigro, Rosanna Simeone and Agata Gadaleta wrote the paper.

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