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Allelic variation for *GS* and *GOGAT* genes in a tetraploid wheat collection

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Abstract. Nitrogen is one of the major limiting nutrients in most plant species and is mostly assimilated as reduced form of ammonium. Ammonium is assimilated into amino acids through the synergic activity of two enzymes: glutamine synthetase (*GS*) and glutamate synthase (*GOGAT*). While Glutamine synthetase genes are a gene family whose enzymes are located in both cytoplasm (*GS1*, *GSe* and *GSr*) and plastids (*GS2*), glutamate synthase exists in two different isoform depending on the electron donor used as cofactor: NADH-dependent and Fd- dependent *GOGAT*, both active in plastids. *GS* catalyses the incorporation of ammonium into glutamate, producing glutamine. *GOGAT* catalyses the transfer of the amide group of glutamine to 2-oxoglutarate, resulting in the formation of two molecules of glutamate. This assimilation requires cofactors, reducing equivalents and other compounds generated during photosynthesis. Glutamine and glutamate serve as nitrogen donors for the biosynthesis of many other molecules, mainly for amino acid, directly involved in protein biosynthesis and ultimately in grain protein content. The aim of the present work was to assess the correlation between grain protein content and *GS* genes through identification of new allelic variations in a collection of durum wheat genotypes. For this purpose a collection of 240 tetraploid wheat genotypes (*Triticum turgidum* L.), was analyzed allowing the identification of 5 different haplotypes for the genes *GS2-A2* and *GS2-B2* of which the "a" allele of *GS2-A2* was found significantly correlated with grain protein content.

Keywords. *GS* – Wheat – Functional markers – Association mapping.

Variation allélique des gènes *GS* et *GOGAT* dans une collection de blé tétraploïde

Résumé. L'azote est l'un des principaux nutriments limitant pour la plupart des espèces de plantes et est le plus souvent assimilé comme une forme réduite de l'ammonium. L'ammonium est assimilé aux acides aminés grâce à l'activité synergique de deux enzymes: la glutamine synthétase (*GS*) et la glutamate synthase (*GOGAT*). Alors que les gènes de la glutamine synthétase sont une famille de gènes dont les enzymes sont situées à la fois dans le cytoplasme (*GS1*, *GSe* et *GSr*) et les plastes (*GS2*), la glutamate synthase existe sous deux isoformes différents selon le donneur d'électrons utilisé comme cofacteur: *GOGAT* NADH dépendante et FD-dépendant, tous les deux actifs dans les plastes. *GS* catalyse l'incorporation de l'ammonium dans le glutamate, produisant la glutamine. *GOGAT* catalyse le transfert du groupe amide de la glutamine à 2-oxoglutarate, conduisant à la formation de deux molécules de glutamate. Cette assimilation nécessite des cofacteurs, des équivalents réducteurs et d'autres composés générés lors de la photosynthèse. La glutamine et le glutamate servent de donneurs d'azote pour la biosynthèse de nombreuses autres molécules, principalement pour les acides aminés, directement impliqués dans la biosynthèse des protéines et en fin de compte dans la teneur en protéines du grain. L'objectif de ce travail a été d'évaluer la corrélation entre la teneur en protéines du grain et les gènes *GS* grâce à l'identification de nouvelles variations alléliques dans une collection de génotypes de blé dur. A cet effet, une collection de 240 génotypes de blé tétraploïdes (*Triticum turgidum* L.) a été analysée permettant l'identification de 5 haplotypes différents pour les gènes *GS2-A2* et *GS2-B2* dont l'allèle "a" de *GS2-A2* a été trouvé significativement corrélé avec la teneur en protéines du grain.

Mots-clés. *GS* – Blé – Marqueurs fonctionnels – Cartographie d'association.

I – Introduction

Wheat, together with rice and maize, is one of the most important cereal crops grown worldwide and provides most of the proteins in human diet. As reviewed by Chatzav *et al.* (2010), the demand for cereal continues to grow as a consequence of a constantly increasing world population (for wheat, approx. 2% per year; Skovmand *et al.*, 2001). So far, increasing yield has been one of the main objectives in plant breeding programs. But another important concern of wheat breeding programs is the nutritional value of staple food crops (Cakmak, 2008; Cakmak *et al.*, 2010; Chatzav *et al.*, 2010). Genetic diversity existing in crop plants is important for their eventual use in breeding programs for enhanced food production. As a result of the intense breeding techniques carried in the last decades, modern variety show a high uniformity with a reduction of genetic variation. Genetic diversity could be the result of geographical impact through evolution and hence traits could be considered as a function of variety (Benadeki, 1992). Choosing the appropriate parents is essential in crossing programs aimed to enhance the genetic recombination for potential yield increase (Islam, 2004). Among the most efficient tools for parental selection in wheat hybridization programs there are the estimation of genetic distance and the evaluation of the level and structure of genetic diversity (Khodadadi *et al.*, 2011). In this context, landraces, wild forms (*Triticum* spp.), and other related wild species can have crucial roles in breeding programs (Peleg *et al.*, 2008).

Nitrogen uptake is an essential element in crop improvement, either directly for grain protein content or indirectly for photosynthetic production. One factor determining nutritional value in cereal is grain protein content (GPC), also strictly related to the baking properties of common wheat (*Triticum aestivum* L. ssp. *aestivum*) as well as the pasta-making characteristics of durum wheat (*Triticum turgidum* L. ssp. *durum*) (Blanco *et al.*, 2012). Domestication and the intense wheat management practices used in the last decades determined a serious erosion of genetic diversity resulting in genetic uniformity of modern varieties (Tanksley and McCouch, 1997; Ladinzinsky, 1998). GPC is a typical quantitative trait controlled by a complex genetic system and influenced by environmental factors and management practices, as well as nitrogen and water availability, temperature and light intensity. This character was found influenced by two major enzymes responsible for cyclic assimilation of ammonium into amino acids in the biochemical pathway of NH₄⁺ assimilation; i.e., glutamine synthetase: (GS) and glutamine-2-oxoglutarate amidotransferase: (GOGAT) (Nigro *et al.*, 2013; Gadaleta *et al.*, 2011; Gadaleta *et al.*, 2014). These two enzymes are involved in assimilation and recycling of mineral N catalyzing ATP-dependent conversion of glutamine into glutamate using ammonia as substrate (Cren and Hirel, 1999; Ireland and Lea, 1999). Glutamine synthetase gene encodes for an enzyme responsible of the first step of ammonium assimilation and transformation into glutamine, essential compounds in aminoacid-biosynthetic pathway. On the bases of phylogenetic studies and mapping data in wheat, ten GS cDNA sequences were classified into four sub-families denominate GS1 (a, b, and c), GS2 (a, b, and c), GSr (1 and 2) and GSe (1 and 2) (Bernard *et al.* 2009). Genetic studies in rice (Obara *et al.* 2004) and maize (Hirel *et al.* 2001, 2007; Galais and Hirel 2004) demonstrated co-localisations of QTLs for GS protein or activity with QTLs relating to grain parameters at the mapped GS genes. The aim of the present work was to assess the genetic variation of GS2 genes in a collection of durum wheat genotypes.

II – Material and methods

A collection of 229 tetraploid wheat genotypes (*Triticum turgidum* L.), including old and modern cultivars of durum wheat (*T. turgidum* L. ssp. *durum*) and wild relatives was used for genetic studies. The collection, including 128 old and modern cultivars of durum wheat (*T. turgidum* L. var. *durum*) and 103 wild and domesticated tetraploid wheats, was grown in the experimental field of the University of Bari at Valenzano (Bari, Italy) in 2009 using a randomized complete block design with three replications and plots consisting of 1-m rows, 30 cm apart, with 50 germinating seeds per plot. Genomic DNA was isolated from fresh leaves using the method described by Sharp *et al.*

(1988) and subsequently purified by phenol-chloroform extraction. DNA quality and concentration was determined by spectrophotometer analysis at 260 and 280 nm (A260/A280 ratio = 1.6-1.8) and by agarose gel electrophoresis. Functional markers were designed by using Primer3 and OligoExplorer software for GS2 genes based on sequences reported by Gadaleta et al. (2011).

DNA amplifications for fragment sequencing were carried out in 25 µl reaction mixtures, each containing 25 ng template DNA, 2 µM of each primer, 200 µM of each dNTP, 2.5 mM MgCl₂, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 10 mM KCl) and 0.5 unit of Taq DNA-polymerase. The following PCR profile in a Bio-Rad DNA Thermal Cycler was used: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C/65°C (depending on the tested primer combination) for 2 min, 72°C for 1 min with a final extension at 72°C for 15 min. PCR products were detected by agarose gel electrophoresis 1.5%. Single PCR fragments were directly purified with EuroGold Cycle Pure Kit and sequenced in both strands by BMR Genomics service. Sequence assembly was obtained with “Codone Code Aligner” and “Geneious” assembly programs. Gaps and uncertain sequence were resolved by primer walking. Regions of less coverage or ambiguous reads were rechecked with primers designed to cover those regions.

III – Results and discussion

Genetic diversity in wheat refers to both genetic and phenotypic variance. For example, plants could show different seed size, height, flowering time, flavor, but also they could be different for other characteristics such as resistance to biotic and abiotic stresses, as diseases and pests or heat/cold response, respectively. That means that variations exist in almost every trait, also complex ones such as nutritional quality and taste. But, when a trait of interest cannot be found in the modern crops due to their genetic uniformity, valuable alleles could be identified in the wild ancestors of crop plants (Aaronsohn, 1910; Tanksley and McCouch, 1997). For this purpose, association mapping analysis was applied for studying nitrogen metabolism in wheat. In higher plants inorganic nitrogen, in the form of ammonia, is assimilated via the glutamate synthase cycle or GS-Gogat pathway.

In the present work candidate gene approach has been applied to the study of grain protein content in durum wheat, focusing the study on the glutamine synthetase genes as potential candidates for determining grain protein content (GPC) (Gadaleta *et al.*, 2011). The aim of the present work was to assess the correlation between grain protein content and GS2 genes through a study of association mapping in a collection of durum wheat genotypes. For this purpose a collection of 240 tetraploid wheat genotypes (*Triticum turgidum* L.), including old and modern cultivars of durum wheat (*T. turgidum* L. ssp. *durum*) and wild relatives were evaluated for grain protein content in replicated trials and under different environmental conditions. The analysis of variance revealed highly significant differences at $P < 0.001$ among genotypes. The mean GPC of the 234 tetraploid accessions was 46.2% with a range of 11.8% to 25.2%; the heritability was of 0.64. Two functional markers were designed for the two homoeologous genes GS2-A2 and GS2-B2 genes and analyzed in the whole collection. Different haplotypes were identified for both genes. The analysis allowed the identification of 5 different haplotypes for both genes confirmed by sequences analysis of the obtained fragments. Functional markers were amplified in the wheat collection and electrophoretic pattern of GS-A2 gene is reported in figure 1. In particular, we considered two alleles for the gene GS2-A2 (named “a” and “b”, corresponding to the presence/absence of a fragment of 480 bp physically mapped on 2A chromosome. The regression analysis carried out between the functional markers and grain protein content trait showed a positive significant correlation. The “a” allele of GS2-A2 was found significantly correlated with grain protein content, with a probability of $P > 0.001$. The GS2-A2 gene co-localized with a major QTL for GPC identified by Gadaleta *et al.* (2011) and Blanco *et al.*, (2012).

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