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ORIGINAL PAPER

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Partial sequences of nitrogen metabolism genes in hexaploid wheat

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Abstract Our objective was to partially sequence genes controlling nitrogen metabolism in wheat species in order to find sequence polymorphism that would enable their mapping. Primers were designed for nitrate reductase, nitrite reductase, glutamate dehydrogenase and glutamate synthase (GOGAT), and gene fragments were amplified on *Triticum aestivum*, *T. durum*, *T. monococcum*, *T. speltoides* and *T. tauschii*. We obtained more than 8 kb of gene sequences, mainly as coding regions (60%). Polymorphism was quantified by comparing two-by-two the three genomes of the hexaploid cultivar Arche and genomes of diploid wheat species. On average, the polymorphism rate was higher for non-coding regions, where it ranged from 1/60 to 1/23, than for coding regions (range: 1/110–1/40) except when the hexaploid D genome was compared to that of *T. tauschii* (1/800 and 1/816, respectively). Genome-specific primers were devised for the ferredoxin-dependent (Fd)-GOGAT gene, and they enabled the mapping of this gene on homoeologous chromosomes of group 2 using Chinese Spring deletion lines. A single nucleotide polymorphism (SNP) detected between the two hexaploid wheat cultivars Arche and Récital was used to genetically map Fd-GOGAT on chromosome 2D using a population of dihaploid lines. Fd-GOGAT-specific primers were used to estimate the SNP rate on a set of 11 hexaploid and nine Durum wheat genotypes leading to the estimate of 1 SNP/515 bp. We demonstrate that polymorphism detection enables heterologous, homeologous and even paralogous copies to be assigned, even if the elaboration of specific primer pairs is time-consuming and expensive because of the sequencing.

Keywords Glutamate dehydrogenase · Glutamate synthase · Homoeologous · Nitrate reductase · Nitrite reductase · SNP

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Introduction

Due to economic and ecological factors, there is a trend in Europe to limit chemical inputs for wheat, especially nitrogen (N). Varieties which have both a good grain yield and end-use quality at a low nitrogen level are therefore needed. Field experiments on cereals have shown that there are genetic differences at low available N levels with respect to N absorption and utilization efficiency (see Dhugga and Waines 1989; Le Gouis et al. 2000). The recent development of molecular markers has enabled quantitative trait loci (QTLs) to be detected, and these may help in gaining an understanding this variability, as has been done in maize (Bertin and Gallais 2001) and *Arabidopsis* (Loudet et al. 2003a, b). For this latter species, the complete genome sequence has been elucidated, and it is therefore easier to associate candidate genes to the QTLs. For example, Loudet et al. (2003b) identified an amino acid transporter, a cytosolic glutamine synthetase (GS) and a putative high-affinity nitrate transporter within the confidence interval of two of their QTLs. The identification of candidate genes is, however, more difficult for species such as wheat or maize with large genomes not yet sequenced. Consequently, information coming from model species like rice may also be used on the basis of gene colinearity even if the substantial evolutionary divergence between the species increases the possibility of finding independent duplications or non-homologous multigene families (Lagudah et al. 2001).

The early steps of nitrate and ammonia assimilation or reassimilation involve three gene families, those coding for nitrate reductase (NAR), nitrite reductase (NIR), glutamate synthase (GOGAT). Although its exact physiological function remains unclear (Dubois et al. 2003), glutamate dehydrogenase (GDH) has also been included in the set of genes related to inorganic nitrogen assimilation. Genes encoding NADH-NAR (EC 1.7.1.1) have been sequenced in several plants, including *Arabidopsis* (two genes; Wilkinson and Crawford 1993; Cheng et al. 1988), maize (two genes; Gowri and Campbell 1989) and barley (one gene; Schnorr et al. 1991). A gene encoding for a bi-specific NAD(P)H-NAR (EC 1.7.1.2) has been characterized in maize (Long et al. 1992) and barley (Miyazaki et al. 1991). In wheat, although no sequence data are available in the databases, two NADH-NAR genes located on linkage groups (LGs) 6 and 7 and one bi-specific NAR gene on LG 6 (Kilian et al. 1992) have been reported. Ferredoxin-dependent (Fd)-NIR (EC 1.7.7.1) catalyses the reduction of nitrite to ammonium in the second step of the nitrate assimilation cycle. This enzyme has been less studied than NAR, but sequences have been published for different plants, such as maize (Lahners et al. 1988) and rice (Tereda et al. 1995). GOGAT works conjointly with GS in the first step of ammonia assimilation. A unique gene for Fd-dependent GOGAT (EC 1.4.7.1) has been characterized in maize (Sakakibara 1991) and barley (Avila 1993), while two genes have been identified in *Arabidopsis* (Suzuki and Rothstein 1997; Coschigano 1998) and *Nicotiana plumbaginifolia* (Ficarelli et al. 1999). The NADH-dependent GOGAT (EC 1.4.1.14) gene has been characterized in *Medicago sativa* (Gregerson et al. 1993) and rice (Goto et al. 1998). The genes encoding the GDH isozymes, which can in vitro either assimilate ammonia (NADH-dependent reaction) or deaminate glutamate [NAD-dependent reaction (EC 1.4.1.3)], have been sequenced in several plant species, such as *Arabidopsis* (Melo-Oliveira et al. 1996), maize (Sakakibara et al. 1995) and *N. plumbaginifolia* (Ficarelli et al. 1999). Although NADP(H)-dependent GDH activity has in some cases been detected in plants (Dubois et al. 2003), the nucleotide sequence of the corresponding gene is only available from the green algae *Chlorella sorokinia* (Cock et al. 1991) and from several bacteria and yeasts.

The objective of the investigation reported here was to partially sequence the genes just mentioned in different polyploid and diploid wheat species in order to look for sequence polymorphism, which will enable these genes to be mapped using either deletions lines or a segregating population.

Materials and methods

Plant material and DNA extractions

DNA samples were prepared from leaves of *Triticum monococcum* (Tm; Am genome), *T. speltoïdes* (Ts; S genome), *T. tauschii* var. *meyere* (Tt; D genome) ,

Table 1 Gene fragment names, primer sequences, PCR conditions and size of the PCR product for each of the genes studied

Gene name[a]	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)	PCR conditions[b]	Fragment length (kb)
Fd-NIR	GGTGTGACGACGAGCGAGCAG	GCATGTACGCCAGGTCGTTGA	30× (20 s 94°C, 30 s 65°C, 1 min 72°C)	0.5
Fd-NIR	CGGAGGACCTGATCGACAAGA	GACGATCTTGCCGCTGCTGTT	30× (20 s 94°C, 30 s 60°C, 1 min 72°C)	0.5
NADH-NAR1	GAATTCCAAGCAGTTCACCATGTCCGA	GAATTCGATCTCGTCGACCATGCTCGT	30× (20 s 94°C, 30 s 58°C, 1 min 72°C)	0.5
NADH-NAR2	GGAGATCGAGGTGCTGGACC	CATGCACGTACATACGGTTCTC	35× (40 s 94°C, 40 s 58°C, 1 min30 s 72°C)	1.5
Fd-GOGAT	CCTGGTGATCTTGTGAACTAC	CCTTGCAGCCTAGCGTCAGC	35× (45 s 94°C, 1 min 55°C, 2 min 72°C)	2
NADH-GOGAT	ACCAGAATAGATGGCGTGAAGC	ACTGTACATTATTCAGGCTAC	35× (45 s 94°C, 1 min 55°C, 2 min30 72°C)	1.7
NAD-GDH	GAAGGGTGGAATCAGATACC	CTGTGTTTCACTCTCAGCCAC	40× (45 s 94°C, 1 min 55°C, 2 min30 72°C)	2.1
NADP-GDH	GATGAGTGGGCGGACACATAC	AAATCGCTCCCTCCAGCAGC	40× (45 s 94°C, 1 min 60°C, 2 min30 72°C)	1.7
Fd-GOGAT/A	GTTGGGTTATGAGAAG	GTACACAAATCTGCTC	40× (30 s 94°C, 30 s 52°C, 30 s 72°C)	0.35
Fd-GOGAT/B	GGCAGCACGAAAGGT	CCTTGCAGCCTAGCGTCAGC	40× (30 s 94°C, 30 s 57°C, 30 s 72°C)	0.4
Fd-GOGAT/D	GGCGTCACTCGCATC	GATTGTTCATCAAGG	40× (30 s 94°C, 30 s 54°C, 30 s 72°C)	0.45
Fd-GOGAT/D/Rec	GAACGCTTTCCCTCCA	GCATATCTGCCACTG	35× (30 s 94°C, 30 s 53°C, 30 s 72°C)	0.18

[a]See Introduction for complete name of each gene [b] Each PCR starts with 3 min at 94°C and ends with 10 min at 72°C in addition to the optimal conditions for each primer couple presented here

T. durum Excalibur (Ttu; AB genomes), *T. aestivum* Arche, Récital and Chinese Spring (TaArc, TaRec, TaCs, respectively; ABD genomes) and 147 doubled haploid lines derived from a cross between Arche and Récital. Physical mapping was performed on 19 nullitetrasomic lines (NT), 36 ditelosomic lines (Dt) and 85 deletion lines of cv. Chinese Spring (Sears 1954; Endo and Gill 1996). Finally, a single nucleotide polymorphism (SNP) detection using Fd-GOGAT genome-specific primers was carried out on a set of 19 French hexaploid and Durum wheat genotypes. The 11 hexaploid wheat genotypes included landraces (Barbu du Finistère, Blé de Redon, Mars de Suède Rouge Barbu, Ralet) and varieties registered between 1974 and 1998 (Apache, Arche, Courtot, Ornicar, Récital, Renan, Taldor). The eight Durum wheat genotypes were registered between1974 and 1991 (Armet, Brumaire, Durental, Ixos, Lloyd, Neodur, Primadur, Villemur).

PCR conditions, cloning and sequencing

PCR reactions were performed in final volumes of 25 µl in Eppendorf thermocyclers. The reaction mixture contained 0.2 m *M* of each deoxynucleotide, 200 n *M* of each primer, 1× buffer (1.5 m *M* $MgCl_2$, Tris-Cl, KCl, $(NH_4)_2SO_4$, pH 8.7), 0.75 U *Taq* polymerase (QIAGEN, Valencia, Calif.) and 50 ng of template DNA. Primers were synthesized by Invitrogen (Invitrogen, Paisley, UK) and the PCR conditions were optimized for each couple (Table 1). For the primers Fd-GOGAT/A and Fd-GOGAT/B, 1× of the Q-Solution (Qiagen) was added.

The PCR products were separated on a 1% agarose gel and visualized under UV light following ethidium bromide staining. Fragments were extracted from the gel using the GeneClean II kit (Q-BIOgene, Carlsbad, Calif.) and cloned into pCR2.1 TOPO T/A vector according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). The clones were analysed following isolation of the plasmid (miniprep kit, Qiagen) and *Eco*RI digestion. Samples of 3 and 14 colonies per gene fragment were sequenced for the hexaploid and diploid species, respectively (Genome Express, Meylan, France).

Sequences and linkage analysis

Existing cereal sequences of the genes of interest were extracted from the data bases and aligned, and primers were designed in the conserved regions. All of the sequence analyses were carried out under the Génoplante-Info interface (Samson et al. 2003). Dendrograms were constructed with the UPGMA cluster analysis using a similarity score calculated with the algorithm from Wilbur and Lipman (1983).

Linkage analysis was performed with MAPMAKER 3.0 (Lander et al. 1987) using a LOD threshold of 3.0, a maximum recombination fraction of 0.5 and the Kosambi mapping function (Kosambi 1944) and the ERROR DETECTION ON option to detect typing errors. Confidence intervals for distances between the two closest markers and the Fd-GOGAT gene were calculated by a bootstrap strategy with 1,000 random bootstrap samples of the same population size drawn with replacement (Liu 1998).

Results

Gene amplifications

Using gene-specific primers, we obtained eight fragments, each ranging in length from 486 bp to 2,195 bp (Table 1), that were totally or partially sequenced (Table 2). Overall, more than 8 kb of gene sequences was obtained from hexaploid wheat and related diploid species, of which approximately 40% consisted of non-coding regions (Table 2), mainly in the form of introns; the one exception was for NAR2 where 159 bp of the 3′ untranslated region (UTR) was sequenced.

Sequences analyses

For each gene fragment, sequences from hexaploid and diploid wheats were analyzed simultaneously (Fig. 1). In most cases, each sequence from hexaploid wheat was unambiguously related to a diploid sequence (Fig. 1b, d–g). The A and, in particular, the D genomes were easily identified, while the B genome exhibited a larger variation which made it more difficult to assign, mainly for NIR and NAR2 (Fig. 1a, c). Hypothetical B sequences from Arche and Récital clustered in the same group but were not related to *T. speltoides* sequences. In this situation, we aligned the sequences to refine the comparison and to attribute genome relationships. If identification remained ambiguous—i.e. there were not enough SNPs shared by the hexaploid sequence and those of related diploid species—designing specific primers became difficult. Additional sequence data of the gene would then be necessary to possibly find more SNPs.

No sequence was obtained for the Fd-GOGAT B-genome copy of Récital either because the number of sequenced colonies was not sufficient or because the primers we designed were not able to amplify the sequence because of a mutation or a deletion.

Polymorphism quantification

Sequence polymorphisms were quantified by comparing two-by-two the three genomes of the hexaploid variety Arche and the genomes of the diploid wheat species. The polymorphism rates ranged from 0 to 1/14 for

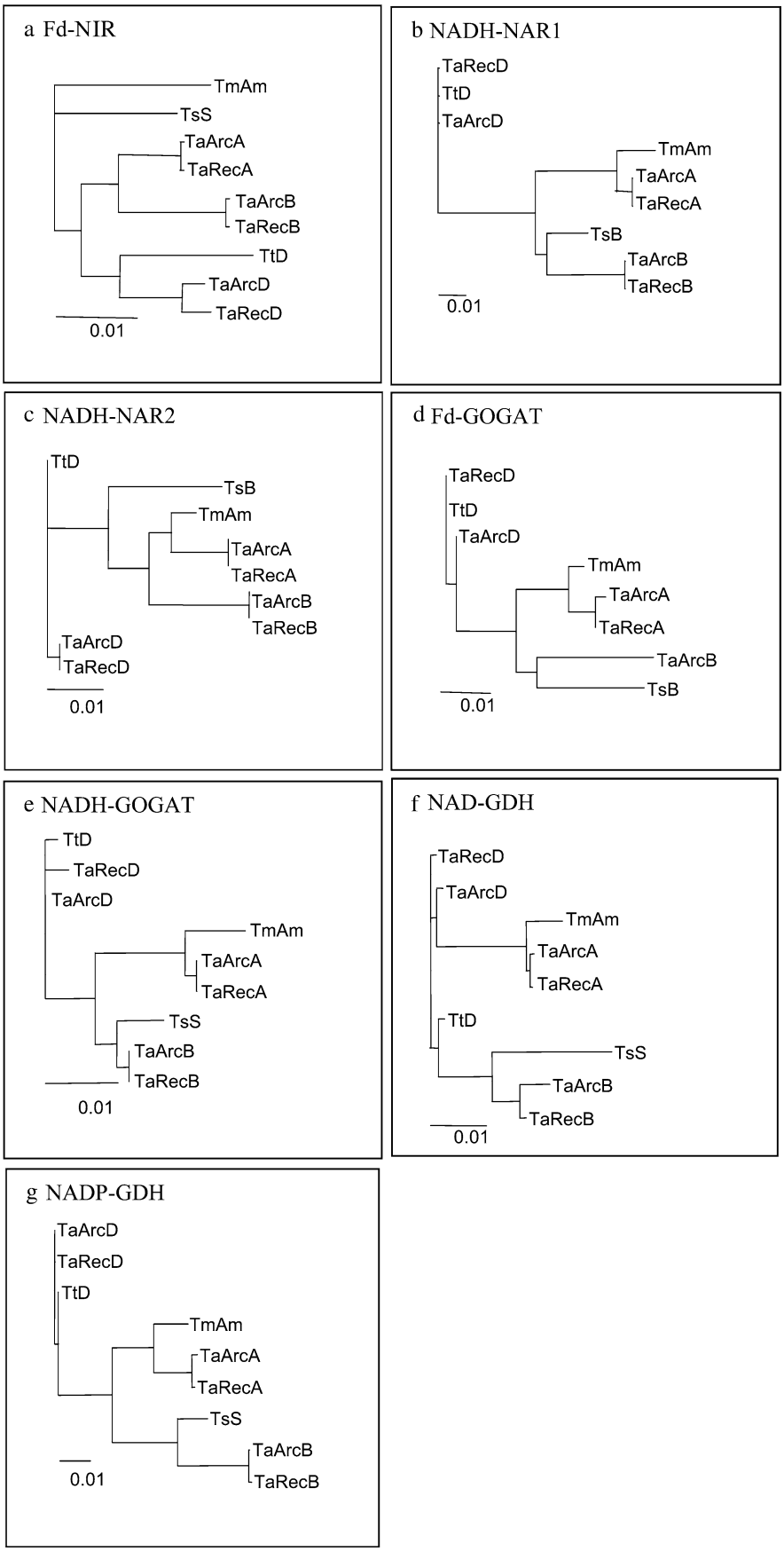

Fig. 1 Results of clustering on similarity scores between sequences obtained from diploid species (*Tm Triticum monococcum*, *Ts T. speltoides*, *Tt T. tauschii*) and two varieties of hexaploid wheat (*Ta T. aestivum*, *Arc Arche*, *Rec Récital*). **a** Nitrite reductase (*NIR*), **b** nitrate reductase 1 (*NAR1*), **c** nitrate reductase 2 (*NAR2*), **d** Fd-GOGAT, **e** NADH-GOGAT, **f** NAD-GDH, **g** NADP-GHD

Table 2 Number (first line) and frequency (second line) of single nucleotide polymorphisms (SNPs) and insertions/deletions (indels, in brackets) between the three homoeologous and diploid wheat genomes (*ND* not determined)[a]

Gene sequences	Sequence length (bp)	ArcA/ArcB	Tm/Ts	ArcA/ArcD	Tm/Tt	ArcB/ArcD	Ts/Tt	ArcA/Tm	ArcB/Ts	ArcD/Tt
Fd-NIR										
Coding	860	10	12	10	15	21	13	4	20	2
		1/49	1/72	1/49	1/57	1/41	1/66	1/124	1/43	1/430
Non-coding	110	ND	7(2)	ND	2(1)	6(3)	3(1)	ND	3(1)	0
		ND	1/12	ND	1/55	1/18	1/36	ND	1/36	0
NADH- NAR1										
Coding	497	34	25	33	31	31	25	6	22	0
		1/14	1/20	1/15	1/16	1/16	1/20	1/83	1/23	0
NADH- NAR2										
Coding	930	19	15	15	15	26	26	12	33	4
		1/49	1/62	1/62	1/62	1/36	1/36	1/77	1/28	1/232
Non-coding	159	13(3)	14(2)	8(4)	8(3)	8(2)	8(1)	2	11(1)	2
		1/12	1/11	1/20	1/20	1/20	1/20	1/80	1/14	1/80
Fd-GOGAT										
Coding	447	17	14	15	12	9	14	2	15	0
		1/26	1/32	1/30	1/37	1/50	1/32	1/223	1/30	0
Non-coding	496	18(1)	14(2)	12	13(1)	28(1)	20(1)	2(1)	20(2)	0
		1/28	1/35	1/41	1/38	1/17	1/25	1/246	1/25	0
NADH-GOGAT										
Coding	826	12	13	13	17	5	5	2	3	0
		1/69	1/64	1/64	1/48	1/165	1/165	1/413	1/275	0
Non-coding	485	9(5)	10(2)	11(4)	11(2)	8(2)	5(1)	4(2)	5(2)	0
		1/51	1/46	1/42	1/42	1/57	1/92	1/115	1/92	0
NAD-GDH										
Coding	683	14	17	9	14	20	12	4	5	0
		1/49	1/40	1/76	1/49	1/34	1/57	1/170	1/136	0
Non-coding	874	63(10)	59(13)	36(8)	40(10)	60(14)	57(14)	44(8)	46(7)	2
		1/14	1/15	1/24	1/22	1/15	1/15	1/20	1/19	1/437
NADP- GDH										
Coding	653	6	6(5)	4	4(3)	6	4(2)	11(2)	3(2)	0(1)
		1/108	1/108	1/163	1/163	1/108	1/163	1/59	1/326	0
Non-coding	1,065	27(5)	27(6)	20(4)	24(6)	14(3)	14(4)	1(4)	12(1)	0(1)
		1/39	1/39	1/53	1/44	1/76	1/76	1/1,065	1/88	0
Total										
Coding	4,896	1/40	1/48	1/46	1/45	1/42	1/49	1/110	1/48	1/816
Non-coding	3,199	1/23	1/24	1/35	1/33	1/26	1/30	1/60	1/33	1/800

[a]ArcA, -B, -D, *Triticum aestivum* Arche carrying the A, B or D genome copies, respectively; Tm, *T. monococcum*; Ts, *T. speltoides*; Tt, *T. tauschii*

coding regions (Table 2). On average, when we compared the three genome copies of hexaploid wheat or the three diploid genome copies two-by-two, we obtained almost the same results : 1/40, 1/46 and 1/42 for ArcA/ArcB, ArcA/ArcD and ArcB/ArcD, respectively, compared to 1/48, 1/45 and 1/49 for Tm/Ts, Tm/Tt and Ts/Tt, respectively. Very few sequence variants were found between the D genome of hexaploid wheat and *T. tauschii* (1/816). More polymorphism was found between the copy of the hexaploid B genome and that of *T. speltoides* (1/48) than between the hexaploid A genome and *T. monococcum* (1/110).

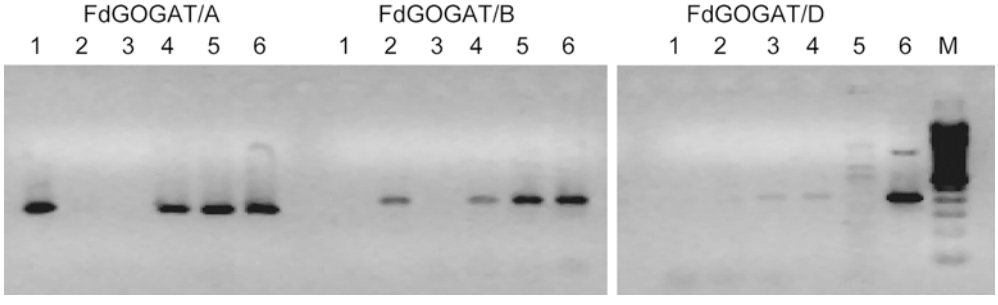

Fig. 2 Amplification of a Fd-GOGAT fragment using genome-specific primers (Fd-GOGAT/A, Fd-GOGAT/B and Fd-GOGAT/D) designed according to the presence of SNPs in the A, B and D genomes. *Lanes*: *1 Triticum monococcum*, *2 T. speltoides*, *3 T. tauschii*, *4 T. aestivum* Arche, *5 T. durum*, *6 T. aestivum* cv. Chinese Spring, *M* 100-bp marker

On average, the polymorphism rate was higher for the non-coding regions (Table 2), with values ranging from 1/60 to 1/23, than for the coding regions (1/110–1/40), with the exception of the hexaploid D genome and *T. tauschii* (1/800 and 1/816, respectively). Moreover, only on NADP-GDH were a few insertions/deletions (indels) found in the coding regions, while 43 were observed in non-coding regions. Interestingly, we also found a variable AG microsatellite motif in the NADH-GOGAT 3′ UTR region showing six, eight and ten repetitions in the A/Am, D and B/S genomes, respectively.

Differences between genes fragment sequences were also observed, with NADH-GOGAT or NADP-GDH being less polymorphic than NADH-NAR1 and Fd-GOGAT.

Unfortunately very few polymorphisms between the two hexaploid varieties Arche and Récital were detected. Of the 7,598 bp sequenced, only three SNPs were detected—two for the Fd-GOGAT fragment and one for the Fd-NIR fragment, and both in coding regions of the D genome.

Mapping

The mapping work concentrated on Fd-GOGAT since both deletion and genetic mapping could be carried out. The homeologous sequence variant (Somers et al. 2003) enabled the design of genome-specific primers. The three genome-specific primer pairs for Fd-GOGAT (Table 1) were first tested on *T. monococcum*, *T. speltoides*, *T. tauschii*, *T. aestivum* Arche and Chinese Spring and *T. durum*. As expected, specific amplifications were obtained for each primer (Fig. 2). For example, Fd-GOGAT/A primers led to an amplification with *T. monococcum*, *T. durum* and *T. aestivum* but not with *T. speltoides* and *T. tauschii*. Non-specific bands were obtained when the Fd-GOGAT/D primers were tested on *T. durum* and cv. Chinese Spring, but these were not considered further as the sizes of these bands did not correspond to the expected size. The genome-specific primers were then used on the Chinese Spring deletion lines (Endo and Gill 1996). Using Fd-GOGAT/D primers, we were able to assign the gene for Fd-GOGAT to chromosome 2D as it was not amplified on the nulli-2D line; more precisely, we could map it to the 2DS arm as it was not amplified on the ditelosomic DT-2DL line (Fig. 3). Its position was then refined as the gene was not amplified on the 2DS-1/2 and 2DS-5/1 deletion lines, consequently assigning it to the 2DS5-0.47-1.00 bin. In the same manner, Fd-GOGAT was also assigned to the 2AS5-0.78-1.00 and 2BS1-0.53-0.75 bins, which was in accordance to the assignation of the Fd-GOGAT EST (BE497494) by the National Science Foundation wheat EST mapping project (http://wheat.pw.usda.gov/west-sql/).

A specific primer was devised to amplify the Récital allele of the Fd-GOGAT genome D gene with the SNP in the 3′ position (Récital = A, Arche = G). The Fd-GOGAT/D/Rec primer (Table 1) was tested on DH lines issuing from the cross between Arche and Récital (Fig. 4). The gene locus that we named *Fdgo-D1* was then genetically mapped (Fig. 5) on chromosome 2D, 4.1 ± 4.6 cM and 21.1 ± 10.2 cM from the Xgpw4085 and the Xgwm102 microsatellite loci, respectively.

SNP quantification

Fd-GOGAT genome-specific primers were then used to assess the SNP rate on a set of 19 Durum and hexaploid wheat varieties. Only good-quality sequences were analysed to avoid false SNPs being identified. With the Fd-GOGAT/A primers, 286 bp were analysed without ambiguity (coding: 153 bp; non-coding: 133 bp), and these were sequenced on nine hexaploid and eight tetraploid French wheat genotypes as no sequences were obtained on Apache and Blé de Redon. We found no SNP on this material. The Fd-GOGAT/B primers

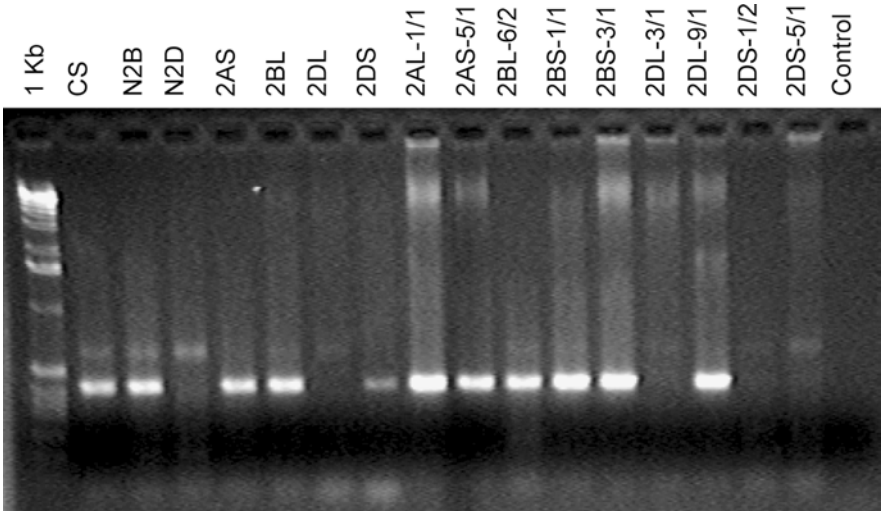

Fig. 3 Physical mapping of the D copy of Fd-GOGAT by amplifications using Fd-GOGAT/D primers on nullitetrasomic, ditellosomic and deletion lines from cv.Chinese Spring. *Lanes*: *1 kb* 1-kb marker, *CS* Chinese Spring, *N2BT2A*, *N2DT2A* nullitetrasomic lines, *2AS*, *2BL*, *2DL*, *2DS* ditellosomic lines, *2AL-1/1*, *2AS-5/1*, *2BL-6/2*, *2BS-1/1*, *2BS-3/1*, *2DL-3/1*, *2DL-9/1*, *2DS-1/2*, *2DS-5/1* deletion lines, *control* no DNA

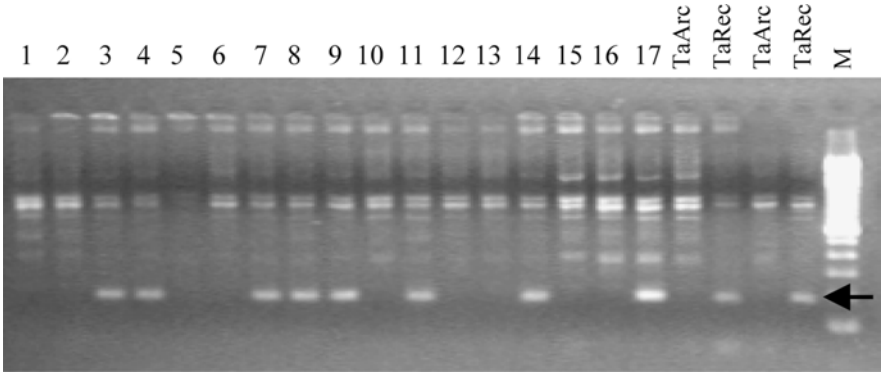

Fig. 4 Amplification of a 176-bp fragment from the D-copy of Fd-GOGAT on *Triticum aestivum* Arche (*TaArc*) and Récital (*TaRec*) and on 17 doubled haploid (DH) lines (*1–17*) issuing from their cross using primers Fd-GOGAT/D/Rec to reveal a SNP. *M* 100-bp marker

amplified sequences on all of the genotypes except Barbu du Finistère, Renan, Ralet, Ornicar, Blé de Redon, Neodur and Lloyd. Two SNPs on 351 bp were detected with these primers in the 3′ UTR region (243 bp); both were detected in Durum wheat while only one was detected in hexaploid wheat. No SNP was detected with the Fd-GODAT/D primer set that amplified 423 bp (coding: 233 bp; non-coding: 190 bp) on five wheat genotypes (Arche, Récital, Renan, Ralet, Taldor). The region amplified by this specific primers did not include the region with the SNP used for mapping. All in all, for the three genome-specific primers, this lead to a rate of two SNPs on 1,030 bp or one SNP per 515 bp.

Discussion

Bread wheat is an hexaploid species with three homologous genomes: A, B and D. The presence of these three highly similar genomes makes it difficult to conduct a direct PCR-based mapping and, consequently, genome-specific primers have to be developed for this purpose. If such specific primers are not developed, as a result of the polyploidy, a single PCR product would generally represent a mixture of sequences from the A, B, and D genomes. To identify the genome-specific markers we chose to clone and sequence the gene both in wheat and in related diploid species. It was always possible to assign each of the three wheat sequences to one of the diploids, although it was more difficult to do this for the B genome. This suggests that the actual A- and D-genome diploid species are closer to those that contributed to the creation of the bread wheat. Conversely, the fluctuation in the results observed for the B genome confirms the uncertainty of its origin. In addition, for the regions we analysed, very few variants were detected between the hexaploid D genome and *T. tauschii*, while many were observed between the hexaploid B genome and *T. speltoides*. These results are also in accordance with the known phyletic origin of hexaploid wheat from a recent cross between a tetraploid ancestor and *T. tauschii* and the doubt that exists concerning the origin of the B genome (Kimber and Sears 1987).

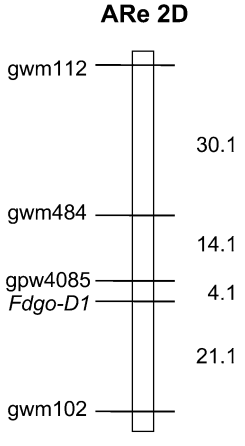

Fig. 5 Partial genetic linkage map of chromosome 2D obtained on 147 DH lines from the Arche × Récital population including four microsatellite loci and the *Fdgo-D1* locus. Genetic distances between loci are given on the *right* of the chromosome in Kosambi centiMorgans

We detected only three SNPs between the two hexaploid wheat varieties Arche and Récital (1/1466 bp). Although we observed that much more polymorphism was detected in non-coding regions and despite the fact that the D genome was less polymorphic, these three SNPs were located in the coding regions of the D genome. One of these SNPs was successfully used to genetically map the gene on the 2D chromosome. Using specific Fd-GOGAT genome primers on a set of 19 hexaploid and Durum wheat genotypes, we obtained a 1/515 SNP rate. Bundock et al. (2003) detected 1 SNP/131 bp on 11 barley varieties using cytochrome P450 public expressed sequence tags (ESTs). Kanazin et al. (2002) found 1 SNP/189 bp amplifying 54 loci on five cultivars using previously published sequence-tagged site (STS) primers. Somers et al (2003) detected 1 SNP/540 bp on 16 wheat varieties by comparing sequences issued from public ESTs and choosing contigs with 11–60 members. They were able to develop diagnostic SNPs for 89% of the contig and for 28% of what they supposed to be homeologous EST clusters. The approach we developed here has the advantage that it enables the detection of SNPs in both the coding and non-coding regions of selected genes. However, although introns were also considered here, the result we report is comparable to that from Somers et al. (2003). We estimated SNP rates using either sequencing after cloning or

sequencing after PCR with genome-specific primers. *Taq*-generated errors may alter the SNP rate found after cloning, but it should be limited in the present investigation as 14 clones were sequenced for hexaploid wheat and three clones for diploid wheat and the consensus sequences compared. The use of genome-specific primers enabled sequencing without cloning, thereby limiting the risk of errors.

In the present investigation we performed physical mapping on a set of deletion lines with genome-specific primers. Primers of 15–17 mers were designed with the specific SNP in the 3′ position. As we chose specific SNPs both in the hexaploid and in the corresponding diploid sequences, we were able to physically assign each of the copy in an homeologous position. In our case, the genes we selected gave a simple pattern—i.e. three copies—one on each homoeologous chromosome. However, some of the genes belong to multigenic families and mapping them is sometimes difficult. The SNPs will allow the assignation of both homeologous, heterologous and paralogous copies, even if the elaboration of specific primer pairs is time-consuming and sometimes expensive because of the quantity of amplification products to be sequenced. The primers we developed here were specific to two cultivars. It will then be interesting to test them on a larger set of varieties in order to determine (1) the frequency of distribution of our two alleles, (2) the possible existence of new alleles elsewhere in the vicinity and (3) if association studies can confirm the gene to be a good candidate gene involved in the expression of a quantitative trait—in the present investigation, protein content—even if a mutation alone will certainly not be sufficient and must be supplemented by additional evidence like genetic transformation.

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