Use of mutagenesis to induce novel allelic variation for genes involved in starch biosynthesis

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INTRODUCTION

Starch constitutes 65-75% of wheat grain dry weight and is the main carbohydrate reserve. The relative amounts of the two glucose polymers amylose and amylopectin are responsible for starch physical and chemical properties with strong influences on the functional properties of flour and on its specific uses in the food and manufacturing industries[1;2]. Enzymes involved in starch synthesis and, particularly, the role of five isoforms of starch synthases have been identified[3]. Four of these (SS) are involved in amylopectin synthesis, along with branching and debranching enzymes; whereas the granule bound starch synthases (GBSSI or waxy proteins) are responsible for amylose synthesis in storage tissues.

In bread wheat three different waxy isoforms are present which are encoded by three genes designated as *Wx-A1*, *Wx-B1* and *Wx-D1* located on chromosome arms 7AS, 4AL and 7DS[4,5,6]; similarly, three homoeologous *SSII* genes, located on chromosome arm 7AS, 7BS and 7DS, encode the SGP-1 proteins (SGP-A1, SGP-B1 and SGP-D1) involved in amylopectin synthesis[7].

Given the important role starch plays in food and non food uses, several efforts are being addressed to the manipulation of its composition through modification of the amylose/amylopectin ratio in different crops in general and in wheat in particular. Approaches used to achieve this goal are being pursued through the manipulation of the genes involved in the starch biosynthetic pathway using natural or induced mutations and transgenic methods. The use of mutagenesis to produce novel allelic variation represents a powerful tool to increase genetic diversity and this approach seems particularly suitable for starch synthase genes for which limited variation exist. Recently, taking advantage of gene sequence information, a non-transgenic approach has been developed to investigate the function of specific genes but also to identify genetic variation in genes influencing useful traits. The approach termed TILLING (Targeting Induced Local Lesions In Genomes) is based on the identification of single base pair changes in specific genes within a population of plants produced by treating seeds or pollen with a chemical mutagen.

In this work the amylose/amylopectin ratio has been altered by mutagenesis through the identification of EMS-induced mutants, containing loss-of-function alleles of *Sgp-1* and *Waxy* genes.

MATERIALS AND METHODS

Plant material

TILLING analysis was performed on genomic DNAs extracted from leaves of an M3 population of the bread wheat cv Cadenza mutagenised with EMS.

SDS-PAGE analysis

The preparation of starch granules from half seeds and the separation of granule-bound starch proteins by SDS-PAGE followed the method reported by Zhao and Sharp[8] with some modifications, as in Mohammadkhani *et al.*[9]. Protein bands were visualized by silver staining.

Tilling analysis

Sgp-1 gene-specific primers were designed for TILLING analysis. Forward-strand primers were end-labeled with IRDye 700 dye and reverse-strand primers with IRDye 800 dye. The standard high throughput TILLING protocol was followed for mutation discovery[10]. PCR products were denatured and annealed to form heteroduplexes between complementary strands. Heteroduplexes were then cleaved using nuclease CelI and the products were separated on denaturing polyacrylamide gels using a Li-Cor 4300 DNA analyzer. Cleaved heteroduplexes produced two smaller molecular weight products, one labeled with IRDye 700 and the other with IRDye 800. The sum of the length of the two bands is equal to the size of the expected amplicon.

RESULTS

Screening and characterization of the bread wheat Cadenza treated with EMS

In order to identify new mutations in genes involved in starch synthesis, an M_4 population (500 plants) of the bread wheat cultivar Cadenza, obtained after EMS treatment in Rothamsted and grown in Martonvasar, was analysed. Results of SDS-PAGE of granule bound starch proteins led to the identification of different lines not expressing SGP-A1 (3 lines), SGP-B1 (1 line), SGP-D1 (7 lines), Wx-A1 (1 line), Wx-B1 (2 lines) and Wx-D1 (2 lines) proteins (Fig. 1). Molecular characterization of identified single null mutants were performed using gene-genome specific primer pairs for *Sgp-1* and *Wx* genes (Tab. 1). The primer pairs used for the *Waxy* genes are those reported in Slade *et al.*[11]. In particular, one mutant in each of three loci *Wx* and *Sgp-1* was analysed. By sequencing the amplified PCR fragments the different mutations responsible of gene silencing

were identified. In two mutant lines, lacking the Wx-A1 and Wx-D1 proteins, nucleotide substitutions (C with T and G with A), in positions 1978 and 1497 produce premature stop codons (TAG and TGA, respectively). In a line lacking the Wx-B1 protein, the substitution (G with A) falls on the splice site (GT) between the IV exon and IV intron. Similarly, in a line lacking the Sgp-B1 protein, the substitution is located on a splice site. In two lines, respectively, Sgp-A1 and Sgp-D1 null a nucleotide substitution is responsible for the formation of a premature stop codon.

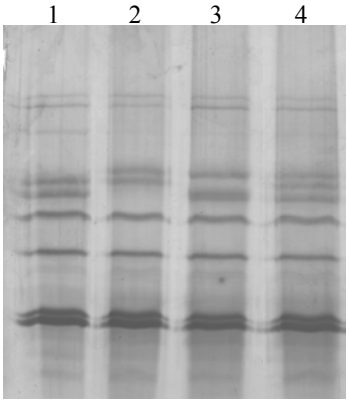

Figure 1. SDS-PAGE separation of starch granule proteins extracted from Sgp-1 mutants. 1-) Sgp-A1^{-}; 2-) Sgp-B1^{-}; 3-) Sgp-D1^{-}; 4-) Cadenza (wt).

Identification of induced mutants and new allelic variants in *Sgp-1* genes by TILLING approach

Twelve Sgp-1 gene- and genome- specific primer pairs were developed and tested by PCR on genomic DNAs of ditelosomic lines lacking, respectively, the short arm of chromosome 7A, 7B and 7D (Tab. 1).

Table 1. Sgp-1 gene-genome specific primer pairs.

Primer	Nucleotide sequence	Amplicon	Reference
Sgp-A1 F1	CCT TCG GAC AAG AAG TTG	711 bp	
Sgp-A1 R1	GGA GTC CAG CGT GCT CAG		
Sgp-A1 F2	ATG TTC TCT TCA CCG GCG C	825 bp	
Sgp-A1 R2	CCA CAC ACA GAC ACA CACATA C		
Sgp-A1 F3	GCG TTT ACC CCA CAG AGC	451 bp	Shimbata *et al.*[12]
Sgp-A1 R3	ACG CGC CAT ACA GCA AGT CAT A		Shimbata *et al.*[12]
Sgp-A1 F4	ACC AAC TTC TCC CTG AGC AC	800 bp	Chibbar *et al.*[13]
Sgp-A1 R4	GGA CCA GAT CGA GAT CGG A		Chibbar *et al.*[13]
Sgp-B1 F1	GGC TCA AAT TTC GTG CCC	715 bp	
Sgp-B1 R1	GCG TGG TTA TCA GCG TTC		
Sgp-B1 F2	ATT TCT TCG GTA CAC CAT TGG CTA	671 bp	Shimbata *et al.*[12]
Sgp-B1 R2	TGC CGC AGC ATG CC		Shimbata *et al.*[12]
Sgp-B1 F3	CAT CGT ATC ACG ATC ACC CAC	805 bp	
Sgp-B1 R3	GGA AGC AGA AGC CGA GGG CAC		
Sgp-B1 F4	CTG GGG ACG CTG GAC TC	780 bp	Chibbar *et al.*[13]
Sgp-B1 R4	GCT ACG GAC CAG ATC GGA A		Chibbar *et al.*[13]
Sgp-D1 F1	GGG AGC TGA AAT TTT ATT GCT TAT TG	558 bp	Shimbata *et al.*[12]
Sgp-D1 R1	TCG CGG TGA AGA GAA CAT GG		Shimbata *et al.*[12]
Sgp-D1 F2	CCG CGA ACC GTA CCA TCT C	798 bp	
Sgp-D1 R2	GAG CAG AGG CCG AGG ACT C		
Sgp-D1 F3	TTT CGA GTC CTC GGC CTC TG	776 bp	
Sgp-D1 R3	TCC TTC TTT GTG AAA TCT GGC		
Sgp-D1 F4	CAC CAA CTT CTC CCT GAG GAC	700 bp	Chibbar *et al.*[13]
Sgp-D1 R4	GCG CAA TGC AGT TCC AT		Chibbar *et al.*[13]

Three of these pairs were used for TILLING analysis on DNA isolated from 1344 lines of EMS treated seeds of the cultivar Cadenza (M_3 generation). PCR was performed on pools of genomic DNA (each pool contained two individual DNAs) and resulted in the identification of 33 probable mutants. Mutations, visible as dark bands on acrylamide gel, are shorter than full-length PCR product (Fig. 2). We identified 5 missense, 1 truncation and 1 splice junction mutations. All mutations were transitions (G to A or C to T) as expected from alkylation by EMS treatment.

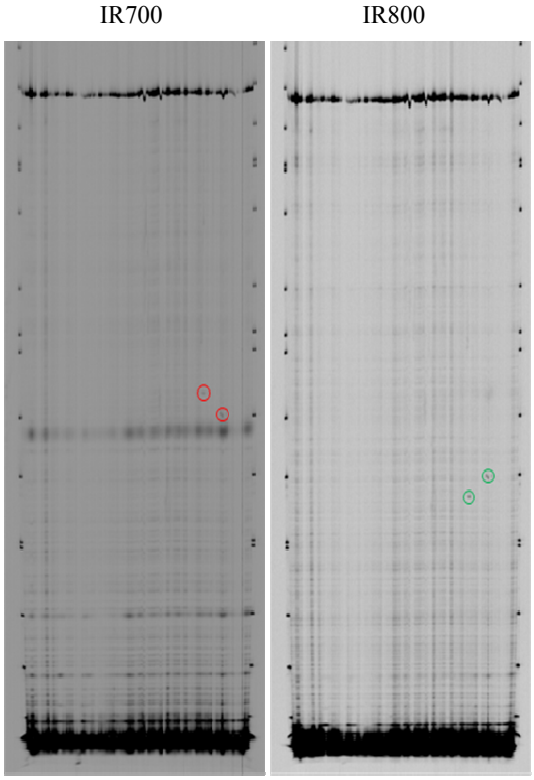

Figure 2. EMS-induced mutation identified by TILLING. The images of IR700 and IR800 channels are reported on the left and right side of the picture. CelI-cleaved products (circled in red and green) appear as dark bands shorter than expected full amplicon. The addition of the bands in same well corresponds to expected size of full-length product in the top of panel.

CONCLUSION

The possibility to manipulate starch composition in cereals, and particularly in wheat, is receiving an increased attention, due to the recognition of its important role in food and non food applications. Starch is used as a basic material for the production of non-food products in paper, plastic, adhesive, textile, medical and pharmaceutical industry. At present the research is focusing on high amylose starches because derived foods have an increased amount of resistant starch, which has been shown to have beneficial effects on human health. The nutritionists believe that the resistant

starch has a role similar to dietary fibre inside the intestine, protecting against diseases as colon cancer, type II diabetes, obesity and osteoporosis. Moreover, pasta produced with semolina containing higher amylose content shows good cooking resistance and firmness, satisfying consumer preferences.

Low amylose starches can be used for the production of higher quality noodles and frozen foods, additionally, they have positive impact on food shelf life.

Different approaches for increasing the amount of amylose content in cereals, as a means to increase resistant starch in foods, have recently been described[14]. Both transgenic and non transgenic tools are available, and the latter, along with novel technologies (e.g. TILLING), offers the possibility to manipulate starch composition and develop new wheat varieties without facing all the regulatory and consumer acceptance issues linked to transgenic crops and novel foods. In this respect we have identified induced mutants for *Sgp-1* and *Waxy* genes by combining traditional and reverse genetic approaches in bread wheat. These materials will be crossed to obtain double and triple null lines. Previous works have demonstrated that *Sgp-1* triple null lines have an increased amylose content and resistant starch[15,16], whereas complete *Waxy* genotypes have starches with very low amylose[17]. In contrast to natural mutants used previously to develop Sgp-1 and Waxy deficient lines, the induced mutants that we have identified result from single nucleotide polymorphisms rather than from deletions of part of the genes. These materials properly backcrossed with the parent cultivar will lead to the development of lines varying in the amount of amylose content without the problems associated with major gene deletions as it can occur in natural mutants.

ACKNOWLEDGEMENTS

This study is partially financially supported by the European Commission in the Communities 6th Framework Programme, Project HEALTHGRAIN (Food-CT-2005-514008). It reflects the authors' views and the Community is not liable for any use that may be made of the information contained in this publication.

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