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Production of novel allelic variation for genes involved in starch biosynthesis through mutagenesis

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Abstract Given the important role that starch plays in food and non-food uses of many crops, particularly wheat, efforts are being made to manipulate its composition through modification of the amylose/amylopectin ratio. Approaches used to achieve this goal include 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 appropriate for starch synthase genes for which limited variation exists. In this work, an EMS-mutagenised population of bread wheat cv. Cadenza has been screened by combining SDS–PAGE analysis of granule bound starch proteins with a TILLING (Targeting Induced Local Lesions IN Genomes) approach at the gene level. In particular we have focused on two groups of synthase genes, those encoding the starch synthase II (*Sgp-1*) and those corresponding to the waxy proteins (*Wx*). SDS–PAGE analysis of granule bound proteins allowed the identification of single null genotypes associated with each of the three homoeologous loci. Molecular characterization of induced mutants has been performed using genome specific primer pairs for *Sgp-1* and *Wx* genes. Additional novel allelic variation has also been detected at the different *Sgp-1* homoeoloci by using a reverse genetic approach (TILLING). In particular single nucleotide substitutions, introducing a premature stop codon and creating amino acid substitutions, have been identified.

Keywords Wheat · Mutagenesis · TILLING · Starch synthases

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Introduction

The use of mutagenesis in crop breeding has resulted in major advances and the release of many cultivars with improved economically important traits. In bread wheat more than 150 mutant varieties have so far been released using this approach (Maluszynski et al. 2001). Recently, taking advantage of gene sequence information, a non-transgenic reverse genetics approach has been developed to investigate the function of specific genes and also to identify genetic variation in genes influencing useful traits that can be used in crop breeding. The approach, termed TILLING (Targeting Induced Local Lesions In Genomes), is based on the

identification, by mismatch cleavage of polymerase chain reaction (PCR) products, of single base nucleotide polymorphisms (SNPs) in specific genes within a population of plants, produced by treating seeds or pollen with a chemical mutagen (McCallum et al. 2000), or in diversity collections (Comai et al. 2004). A major advantage of TILLING is that chemical mutagenesis, using mutagens such as EMS (ethylmethane sulphonate), sodium azide and MNU (*N*-methyl-*N*-nitrosourea) is easily applicable to many plant species, resulting in its wide application in model organisms as well as in crops of economic value (Slade and Knauf 2005; Parry et al. 2009). Moreover this approach is particularly suitable for polyploid species with complex genomes such as wheat in which even null alleles may have no phenotype, due to complementation by homoeologous genes; Slade et al. (2005) demonstrated use of the TILLING technology to generate and identify new *waxy* alleles in durum and bread wheat through the TILLING technology, and combined alleles in homoeologous loci by crossing to generate a *waxy* phenotype.

Starch constitutes 65–75% of the wheat grain dry weight and is the main component of a vast array of foods which are a regular part of the human diet, including bread, pasta, noodles, biscuits, rolls, breakfast cereals, cookies and cakes. The relative amounts of the two glucose polymers amylose and amylopectin are responsible for the physical and chemical properties of starch and therefore have major effects on the functional properties of flour and on its specific uses in the food and manufacturing industries (Yoo and Jane 2002; Van Hung et al. 2007). Both low and high amylose wheat are useful for the different effects they have on flour or semolina functionalities. For example, low amylose starches are used for the production of higher quality noodles and frozen and baked foods; additionally, they have positive impact on food shelf life, retarding the staling process (Ellis et al. 1998; Bhattacharya et al. 2002).

At present researchers are also focusing on high amylose starches because foods derived from these have an increased amount of resistant starch, which has been shown to have beneficial effects on human health. The resistant starch has a role similar to dietary fibre inside the intestine, protecting against diseases such as colon cancer, type II diabetes, obesity and osteoporosis (Topping 2007). Moreover, pasta produced with durum wheat semolina containing higher amylose levels shows good cooking resistance and firmness, satisfying consumer preferences (Soh et al. 2006).

A number of enzymes are involved in starch synthesis and, particularly, the roles of five isoforms of starch synthase (SS) have been identified (Rahman et al. 2005). Four of these are involved in amylopectin synthesis, along with branching and debranching enzymes, while 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 (Chao et al. 1989; Yamamori et al. 1994); 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 (Yamamori and Endo 1996). The complete genomic sequences of the waxy genes and of the *Sgp-1* genes of wheat have been produced by Murai et al. (1999) and Shimbata et al. (2005).

Given the important role starch plays in food and non food uses, a great deal of effort is being applied to the manipulation of its composition through modification of the amylose/amylopectin ratio. Waxy mutations occur spontaneously in cereals such as maize, rice and barley (Eriksson 1970). In wheat, detection of waxy mutants has been hindered by polyploidy and only in the past decades, thanks to the extensive electrophoretic surveys carried out on established cultivar and genetic resources, the identification of partial waxy mutant lines, characterized by the lack of one or two waxy proteins has been possible. Crossing of these lines has permitted the construction of both completely null waxy lines of hexaploid and tetraploid wheat, and also partial nulls with different combinations of alleles (Nakamura et al. 1995; Urbano et al. 2002), allowing the contributions of the different homoeologous genes to be assessed.

Similarly, by crossing natural mutants lacking each of the three different Sgp-1 proteins, Yamamori et al. (2000) have produced a bread wheat line lacking all three Sgp-1 proteins. This line had an apparent high amylose, deformed starch granules and altered amylopectin structure. In other plant species, as pea (Craig et al. 1998), maize (Zhang et al. 2004) and barley (Morell et al. 2003), the lack of Sgp-1 (SSIIa) proteins produced similar effects on amylose content and amylopectin structure.

In this work we have used chemical mutagenesis with EMS in order to induce novel allelic variation at the *waxy* and *Sgp-1* loci in the bread wheat cultivar Cadenza.

Materials and methods

Development of a mutagenised population of wheat

A mutagenised population of the bread wheat cultivar Cadenza was created by treating dry seeds with aqueous solution of EMS at 0.6% (v/v) or 0.9% (v/v) for 16 h. The seeds were then rinsed six times in distilled water, surface dried on filter paper and sown either in the field at Rothamsted Research or in the glasshouse. Single ears of approximately 6,000 M_1 plants were harvested and one seed from each ear was sown to generate the M_2 population. Leaf material (5 cm of young leaf blade) for DNA extraction was sampled from individual plants of the M_2 population into deep-well microtitre plates, freeze dried and stored at −70°C until processed. A single mature ear from each self-fertilized M_2 plant that set seed (4,244 lines) was sent to Martonvásár in early 2006 and the remaining ears from each plant were threshed and archived at 6°C under controlled humidity. M_3 head rows were grown in Martonvásár during 2006 and an aliquot of M_4 grain sent to Viterbo for biochemical analysis.

Genomic DNA was extracted from freeze-dried samples of M_2 leaf material using a sodium bisulphate-based extraction protocol in 96-well plates (Van Deynze and Stoffel 2006). The DNA was dissolved in 200 µl TE buffer; DNA concentrations were estimated using Quant-iT PicoGreen (Invitrogen) assays carried out on 50-fold dilutions using a Beckman DTX 880 fluorimeter integrated into a Beckman Biomek NX Span-8 liquid handling robot. The Biomek NX was also used to normalize the genomic DNA concentrations to 10 ng/µl and to create twofold pooled plates for TILLING.

Primer design

Gene- and homoeologue-specific primers for TILLING were designed using the PRIMER PREMIER 5 program. PCR primers for TILLING analysis were first validated using ditelosomic lines of the bread wheat cultivar Chinese Spring lacking the short arm of chromosomes 7A, 7B and 7D (Sears and Sears 1978). Genomic DNA was extracted from 0.2 g of green tissue as reported in Tai and Tanksley (1991). PCR reactions were carried out in 50 µl final volume using 50-100 ng of genomic DNA, 1× Red Taq ReadyMix PCR reaction mix (1.5 U Taq DNA Polymerase, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, 0.2 mM dNTPs) and 0.5 µM of each of the two primers.

Amplification conditions for testing *Waxy* and *Sgp-1* primers included an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60–65°C for 1 min and 72°C for 1 min, followed by a final incubation at 72°C for 5 min. Genome specific primers for *Waxy* genes were those reported by Slade et al. (2005); for *Sgp-1* genes those identified by Chibbar et al. (2005) and Shimbata et al. (2005) were supplemented with additional pairs (Table 1) designed using the PRIMER PREMIER 5 program (Premier Biosoft International). Primers shown to be homoeologue-specific were then re-synthesised labeled with IRDye 700 dye (forward primers) or IRDye 800 dye (reverse).

Mutation detection

High throughput TILLING for mutation discovery was carried out essentially as described by Till et al. (2006). PCR amplification was carried out in a 10 µl volume using 5 ng of individual or pooled genomic wheat DNA, 0.5× ExTaq buffer (Takara), 0.2 mM dNTPs, 0.17 µM primers, 1.5 mM $MgCl_2$ and 0.05 U ExTaq HotStart (Takara). PCR conditions included an initial denaturation step at 95°C for 2 min, followed by 8 cycles of touchdown PCR (94°C for 20 s, an annealing step starting to 73°C for 30 s and decreasing 1°C/cycle, a temperature ramp increasing 0.5°C/s to 72°C, and an extension step at 72°C for 1.30 min), then 20 cycles of PCR (94°C for 20 s, 65°C for second, a temperature ramp increasing 0.5°C/s to 72°C and 72°C for 1.30 min) and a final extension at 72°C for 5 min. PCR amplicons were denatured at 99°C for 10 min and reannealed by decreasing the temperature at 0.3°C per cycle for 99 cycles.

Heteroduplexes were cleaved using nuclease CelI (Surveyor, Transgenomic, Inc.) and after desalting on

Table 1 Gene specific primers, used for molecular characterization of *Sgp-1* mutants and TILLING analysis

Primer	Nucleotide sequence	Amplicon (bp)	Reference
Sgp-A1 F1	CCT TCG GAC AAG AAG TTG	711	This paper
Sgp-A1 R1	GGA GTC CAG CGT GCT CAG		This paper
Sgp-A1 F2	ATG TTC TCT TCA CCG GCG C	825	This paper
Sgp-A1 R2	CCA CAC ACA GAC ACA CACATA C		This paper
Sgp-A1 F3	GCG TTT ACC CCA CAG AGC	451	Shimbata et al. (2005)
Sgp-A1 R3	ACG CGC CAT ACA GCA AGT CAT A		Shimbata et al. (2005)
Sgp-A1 F4	ACC AAC TTC TCC CTG AGC AC	800	Chibbar et al. (2005)
Sgp-A1 R4	GGA CCA GAT CGA GAT CGG A		Chibbar et al. (2005)
Sgp-B1 F1	GGC TCA AAT TTC GTG CCC	715	This paper
Sgp-B1 R1	GCG TGG TTA TCA GCG TTC		This paper
Sgp-B1 F2	ATT TCT TCG GTA CAC CAT TGG CTA	671	Shimbata et al. (2005)
Sgp-B1 R2	TGC CGC AGC ATG CC		Shimbata et al. (2005)
Sgp-B1 F3	CAT CGT ATC ACG ATC ACC CAC	805	This paper
Sgp-B1 R3	GGA AGC AGA AGC CGA GGG CAC		This paper
Sgp-B1 F4	CTG GGG ACG CTG GAC TC	780	Chibbar et al. (2005)
Sgp-B1 R4	GCT ACG GAC CAG ATC GGA A		Chibbar et al. (2005)
Sgp-D1 F1	GGG AGC TGA AAT TTT ATT GCT TAT TG	558	Shimbata et al. (2005)
Sgp-D1 R1	TCG CGG TGA AGA GAA CAT GG		Shimbata et al. (2005)
Sgp-D1 F2	CCG CGA ACC GTA CCA TCT C	798	This paper
Sgp-D1 R2	GAG CAG AGG CCG AGG ACT C		This paper
Sgp-D1 F3	TTT CGA GTC CTC GGC CTC TG	776	This paper
Sgp-D1 R3	TCC TTC TTT GTG AAA TCT GGC		This paper
Sgp-D1 F4	CAC CAA CTT CTC CCT GAG GAC	700	Chibbar et al. (2005)
Sgp-D1 R4	GCG CAA TGC AGT TCC AT		Chibbar et al. 2005)

Sephadex G-50 the products were separated on denaturing polyacrylamide gels using a Li-Cor 4300 DNA analyzer. Cleaved heteroduplexes in reactions containing a mutation within the amplicon produced two smaller molecular weight products, one labeled with IRDye 700 and the other with IRDye 800. Positive reactions in which cleavage products were observed in both dye channels were identified and re-amplified from each individual in the pool for sequence analysis.

DNA sequence analyses were conducted by a commercial sequencing service (MWG BioTech, Ebersberg, Germany). The comparative analyses of nucleotide and aminoacid sequences were performed by DNAMAN program. The comparison with known sequences in databanks was carried out using a online blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

SDS–PAGE analysis

Preparation of starch granule proteins was carried out on half seeds and their electrophoretic separation by SDS–PAGE was according to the method described by Zhao and Sharp (1996) with some modifications, as in Mohammadkhani et al. (1999). Protein bands were visualized by silver staining.

Results

Screening and characterization of EMS-mutagenized bread wheat lines

In order to identify new mutations for starch granule proteins, seeds of the M_4 population (500 lines) of the bread wheat cultivar Cadenza, obtained after EMS

treatment in Rothamsted, UK, and grown in Martonvásár, Hungary, were analyzed. Results of SDS–PAGE analysis of starch granule proteins from half-grains led to the identification of different null lines lacking 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. 1A, B). SDS–PAGE permitted only the identification of null lines and no other major changes were observed in the protein profiles of the analyzed material. In order to determine the cause of the absence of the proteins DNA analysis of corresponding gene regions was performed on PCR amplified fragments using gene-genome specific primer pairs. In particular, one mutant line for each of the three *Wx* and *Sgp-1* loci was analysed. Waxy *null* lines were analyzed using the primer pairs described by Slade et al. (2005), whereas the primers reported in Table 1 were used for analysis of *Sgp-1* genes.

In order to check genome specificity, the Sgp-1 primers designed to amplify each homoeoallele were first tested by PCR analysis using genomic DNAs extracted from ditelosomic lines of the wheat cultivar Chinese Spring, lacking the short arm of the homoeologous group 7 chromosomes (Fig. 2).

By sequencing different gene regions, mutations responsible for loss of the gene product were identified. In the two lines lacking the Wx-A1 and Wx-D1 protein, nucleotide substitutions (C-T and G-A) resulting in the introduction of stop codons, respectively, in positions 1,978 and 1,497 of the coding sequence, was observed (TAG and TGA). The absence of the Wx-B1 protein, is caused by a substitution (G-A) involving the 5′ splice junction site (GT) between the IV exon and IV intron. Finally, a premature stop codon (TAG) in position 6,302 has been identified in a line lacking the Sgp-A1 protein. A nucleotide insertion in II exon, differently, is responsible for reading frame shifting and causes the silencing of the *Sgp-B1* gene; this has occasionally been observed (Greene et al. 2003). For the Sgp-D1 null no mutation was detected in the gene region analyzed.

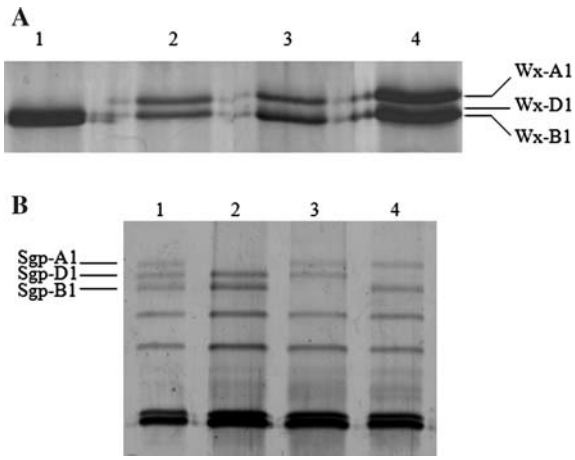

Fig. 1 SDS–PAGE separation of starch granule proteins extract from *Waxy* and *Sgp-1* mutants. **A** *1* Wx-A1^{-}; *2* Wx-B1^{-}; *3* Wx-D1^{-}; *4* Cadenza (wt). **B** *1* Cadenza (wt); *2* Sgp-A1^{-}; *3* Sgp-B1^{-}; *4* Sgp-D1^{-}

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

Three primer pairs, one for each homoeoallele, reported in Table 1, were used for TILLING analysis of the mutagenised lines of the bread wheat cultivar Cadenza using DNA from M_2 leaf material). 1,344 lines were used for Sgp-A1 and Sgp-D1 and 256 for Sgp-B1. Gene regions targeted are indicated on Fig. 3. PCR was performed on pools of genomic DNA (each pool formed by two individual DNAs) and identified 33 possible mutants. Mutations, visible as dark bands on acrylamide gel, are shorter than full-length PCR product (Fig. 4). Eighteen putative positive pools were identified using primers Sgp-A1 F1 and Sgp-A1 R1, but only eight samples yielded confirmed mutant sequences. In the line CAD-R1-4H (27B) the mutation (G for A) fell on the splice junction site between the intron VII and exon VIII (Fig. 5). The line CAD-R8-3C (22A) had a premature stop codon, due to substitution of a guanine with an adenine on the exon VIII (Fig. 5). The substitution of a guanine with an adenine resulted in the substitution of the amino acid Glycine with Aspartic acid in position 481 of the Sgp-A1 protein in the sample CAD-R1-9D (12A). The other nucleotide changes did not produced any variations as they were detected on the third base of the codons or on the intron sequences.

From analyses carried out using the primers Sgp-D1 F1 and Sgp-D1 R1, twelve pools were identified, but only seven of them contained mutant lines. In total, using this primer pair, four missense changes at the Sgp-D1 locus were identified (Fig. 6).

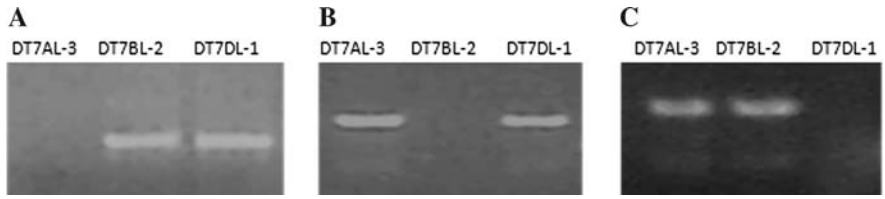

Fig. 2 PCR assay on genomic DNA of ditelosomic lines lacking, respectively, the short arm of chromosomes 7A, 7B or 7D to test specificity of primers designed for each *Sgp-1* homoeoallele gene

Silent mutations were also identified at the Sgp-A1 (5) and Sgp-D1 locus (3). Best conditions for TILLING analysis were found for two primer pairs specific for the *Sgp-B1* gene (Sgp-B1 F1 and Sgp-B1 R1; Sgp-B1 F2 and Sgp-B1 R2). Two pools were identified using the second pair, but only the line CAD-R6-10F (32B) resulted with a substitution (C for T), responsible for a amino acid change in position 494 (Serine for Proline).

All mutations observed were transitions (G/C to A/T) as expected from alkylation by EMS treatment.

Seeds of mutant lines were analysed by SDS–PAGE. The absence of the Sgp-A1 protein was observed in the two lines in which mutation involving the splice junction site and introduction of a premature stop codon was detected. Seeds of the other lines identified showed a normal protein pattern for Sgp-1 proteins.

Discussion and conclusion

Mutagenesis has been widely used in crop plant both for functional genomic studies and practical breeding purposes. The use of mutagenesis in plant breeding has resulted in the production of more than 2,250 crop varieties, officially released, with improved yield and quality traits and about 70% of these were released as direct mutants (Ahloowalia et al. 2004). Introduction of agronomic traits, such as semi-dwarfness and lodging resistance, through induced mutations, has also resulted in major economic impact in cereals such as durum, bread wheat, barley and rice.

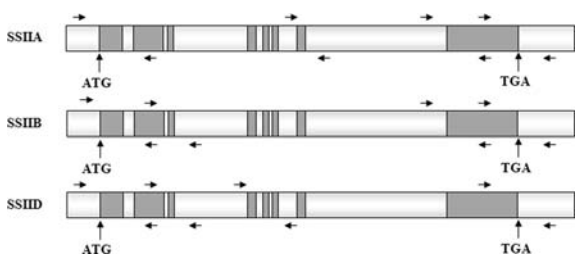

Fig. 3 Schematic representation of the localization of gene-genome specific primer pairs for three *SSII* genes, reported in table 1. *Gray boxes* correspond to exon regions. The *arrows* indicate the annealing sites and the direction of the primers

More recently, the application of mutagenesis in association with reverse genetics approaches has provided a new powerful approach for both functional genomics and crop breeding applications; a combination of chemical mutagens with PCR detection of point mutations in a gene of interest has resulted in the development of the TILLING strategy (McCallum et al. 2000). Chemical mutagens such as EMS have been shown be very effective in inducing single nucleotides changes high in density and randomly distributed in genomes of many different organisms (Greene et al. 2003); this leads to the generation of allelic series of mutations which includes mis-sense changes, with amino acid substitutions that have a range of effects on protein function and non-sense or splice site changes that cause truncation of the gene product and, depending on the location, probable loss of function.

The potential of the TILLING technology for wheat genetic improvement has been shown by Slade et al. (2005) using bread and durum wheat cultivars mutagenized with EMS. These authors were able to identify 196 new alleles in the *waxy* genes in 1,152 individuals of a bread wheat TILLING population and 50 new alleles analysing 768 lines of a durum wheat TILLING population. The new allelic series detected included all types of mutations including non-sense (truncation), splice junction as well as mis-sense (aminio acid substitution mutations, demonstrating that the use of chemical mutagens can generate a wider spread of genetic diversity than

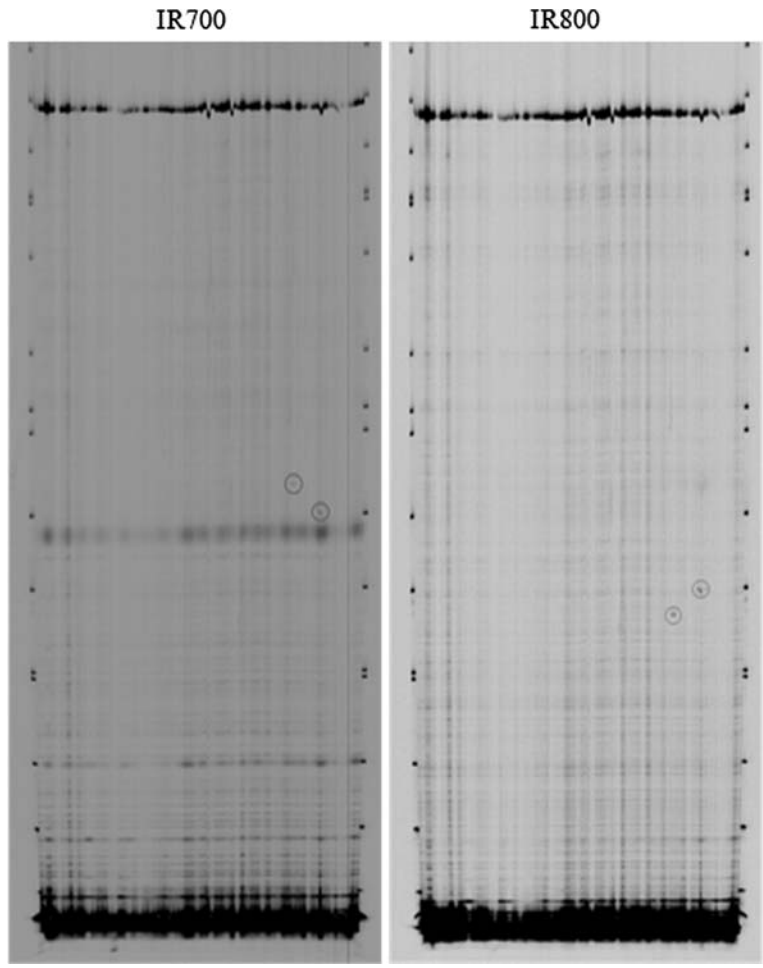

Fig. 4 EMS-induced mutation identified by TILLING using primer pair Sgp-A1 F1 and Sgp-A1 R1. In the *left* and in the *right* there are, respectively, the images of IR700 and IR800 channels. CelI-cleaved products (*circled*) appear as *dark bands* shorter than expected full amplicon. The addition of the bands in same well corresponds to expected size of full-length product in the *top* of panel

have been identified in collections of bread and durum wheat cultivars, landraces and wild relatives. Similarly, also in wheat, Feiz et al. (2009) screened an M_2 population after EMS treatment of the soft white wheat cultivar Alpowa to identify new alleles of the puroindoline genes *Pina* and *Pinb*, present at the *Hardness* (*Ha*) locus, located on the distal end of chromosome 5DS that controls grain texture and many end-use properties of wheat (*Triticum aestivum* L.). Eighteen new *Pin* alleles, including eight missense alleles, were identified and the impact of four of the new *Pin* alleles was assessed on the seeds of a segregating population obtained crossing the mutant lines with the parent cultivar Alpowa. The new mutations were responsible for between 28 and 94% of the grain hardness variation and seed weight and vigour of all mutant lines was restored among the F_2 populations.

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. Different approaches have recently been described, in order to increase the amylose content of cereal grains, as a means to increase resistant starch in foods; these comprise both transgenic and conventional methods (Rahman et al. 2007). The use of transgenic technology still has major problems associated with the lack of acceptance by consumers of GM (genetically modified) crops and foods derived from these. On the other hand, classical breeding approaches have to face, in some cases, the problem of limited genetic variation in adapted material and the genetic drag associated with the use of exotic germplasm or wild relatives. In this context, the use of mutagenesis represents a powerful tool to increase genetic

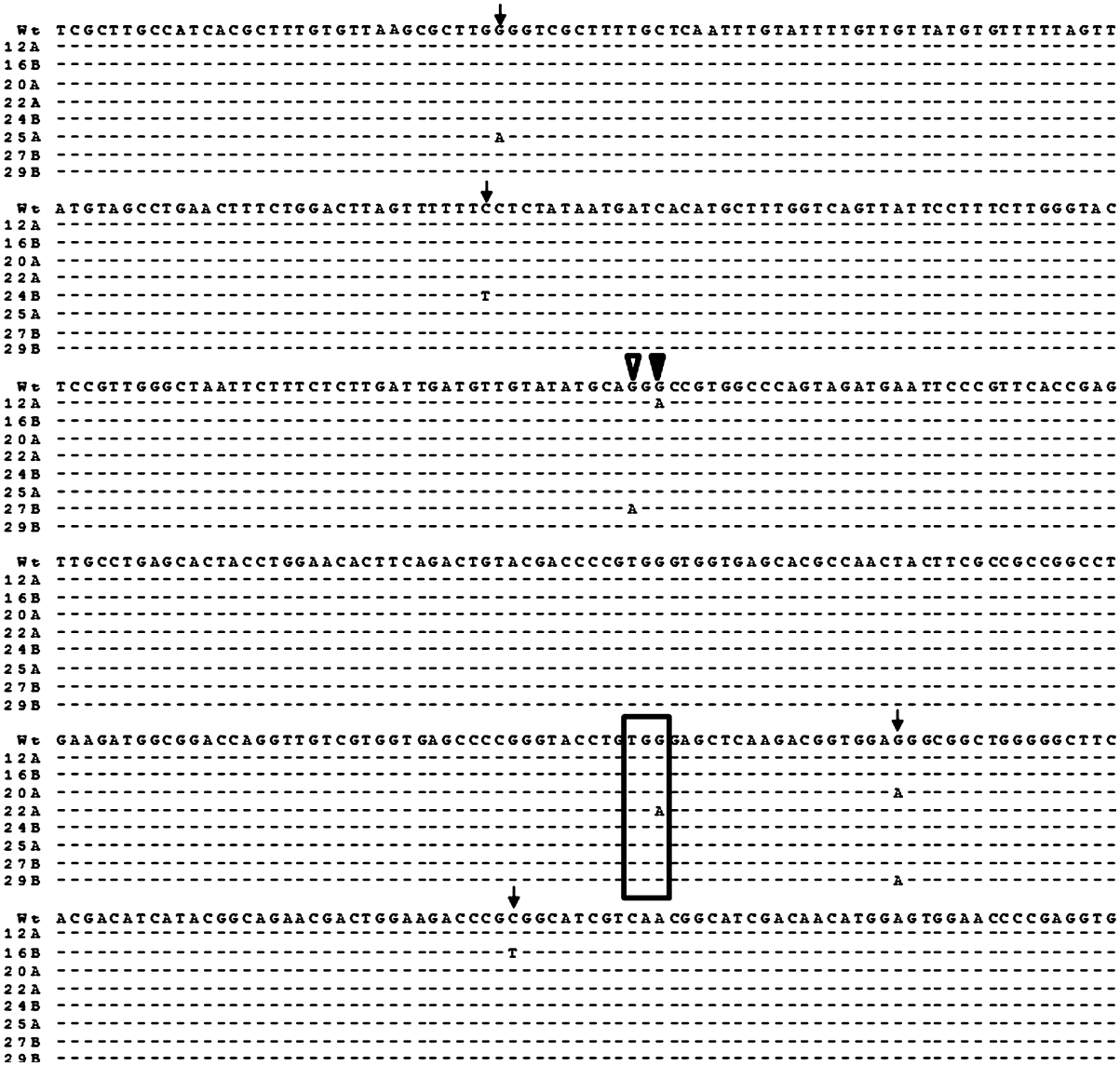

Fig. 5 Nucleotide alignment of *Sgp-A1* region amplified with primers Sgp-A1 F1 and Sgp-A1 R1 and comparison between mutants and wild type (*wt*). *Black arrow-head* indicates the mutation on the splice junction site between the intron 7 and exon 8. *Blank arrow-head underlines* the mutation responsible for amino acid substitution (Glycine with Aspartic acid). The *box* put in evidence the premature stop codon in the mutant 22A. *Arrows* indicate synonymous substitutions or mutations falling on introns

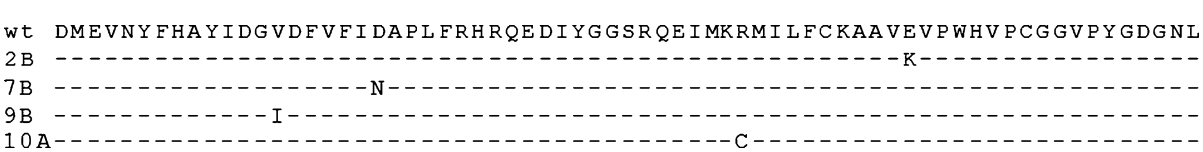

Fig. 6 Alignment of deduced amino acid region amplified with primers Sgp-D1 F1 and Sgp-D1 R1 and comparison between mutants and wild type

diversity and is particularly suitable for starch synthase genes for which limited variation exists in the wheat gene pool. Additionally, as already stressed by Slade et al. (2005) and Feiz et al. (2009), mutagenesis can be directly applied to superior elite cultivars and a few backcrosses with the parent cultivar to remove unlinked mutations will result in the novel allele introduction into the parental background.

Our work has led to the identification of induced mutants for *Sgp-1* and *Waxy* genes by combining traditional and reverse genetic approaches in the elite bread wheat cultivar Cadenza. Analysis of mutagenized lines by SDS–PAGE analysis of grain proteins allows the identification of nulls with complete loss of the encoded protein due to the introduction of stop codons or splicing errors; such null mutants are of scientific and commercial interest for both gene targets. TILLING technology provides greater information as besides identifying null alleles it has the ability to uncover mis-sense mutations resulting in amino acid substitutions that confer a wide range of effects on protein function. In addition, the TILLING approach is particularly advantageous in case of crops such as wheat where polyploidy can hinder the detection of mutations, as the homoeologous copies are likely to complement mutations in a single gene. TILLING allows targeting of the three homoeologues of any given gene, through the use of homoeologue-specific primer pairs, and the incorporation of the identified homoeoalleles into the same genetic background by crossing the mutations generated to be assessed for their effect on the trait. Additionally gene dosage effect can be properly modulated with the possibility to generate partial mutants with an array of different functionalities. Thus, the null alleles that we have identified in the *Waxy* and *Sgp-1* genes will be crossed to obtain double and triple null lines thus expanding the available genetic variation in amylose content in an elite bread wheat cultivar.

The availability of gene sequence information along with informatics tools that maximise the probability of recovering useful mutations will make this approach a strong contributor to genomic studies and crop breeding in the near future (Parry et al. 2009). In the few years since TILLING technology has emerged its application to crop plants has steadily increased and it is predictable that this trend will continue thus impacting on plant breeding significantly as already witnessed in the past century.

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