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Starch metabolism mutants in barley: A TILLING approach

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Abstract

In this study, the targetting-induced local lesions in genomes approach was used to identify mutants for genes related to starch metabolism in barley. Starch is the major reserve of plants and serves as primary carbohydrate component in human and livestock diets and has also numerous industrial applications. Mutants for biosynthetic or regulatory genes of starch metabolism often produce starch granules with abnormal morphological and molecular features that could be of interest for technological applications. We report the identification of 29 mutations in five starch-related barley genes (*Bmy1*, *GBSSI*, *LDA1*, *SSI* and *SSII*) through the molecular screening of TILLMore, a sodium azide-mutagenized population. Almost all the mutations detected were CG–TA transitions and several (*c.* 60%) implied a change in amino-acid sequence and therefore possible phenotypic effects. Four mutants showed non-sense or splice-junction alterations, which could drastically affect the protein function.

Keywords: *Hordeum vulgare*; reverse-genetics; starch; TILLING

Introduction

Starch is the major reserve of plants and the major source of food calories for humans, as well as an important raw material for the food and processing industries (James *et al.*, 2003). The major component of starch granules is amylopectin, which forms partially crystalline structures, while amylose constitutes the amorphous portion of the granule (Hizukuri, 1996; Lemke *et al.*, 2004). The different molecular features of starch polymers (i.e. chain length, frequency of branching, abundance of amylose, etc.) influence both the morphology of the granule and the technological properties of starch as a raw material or foodstuff (Jobling, 2004). The European starch market is substantial and the interest of both the scientific community and industry in starch biosynthesis and technology is strong. Although the genetic and physiological bases of starch biosynthesis in plants are well known, the regulatory machinery controlling the formation of the complex and ordered structure of the starch granule is still not fully understood. Mechanisms of post-translational regulation are likely to play a major role in starch metabolism (Michalska *et al.*, 2009; Valerio *et al.*, 2011; Zeeman *et al.*, 2010). Mutants for biosynthetic or regulatory genes of starch metabolism often produce starch granules with abnormal morphological and molecular features (Sehnke *et al.*, 2001; Asano *et al.*, 2002).

We describe the utilization of TILLMore (http://www.distagenomics.unibo.it/TILLMore/), a barley targeting-induced local lesions in genomes (TILLING) resource (Talamè *et al.*, 2008) to identify new alleles involved in starch biosynthesis and degradation in seeds. TILLING has already been successfully applied to identify starch mutants in wheat (Slade *et al.*, 2005). The long-term goal of this research is the identification of barley mutants with starch granules of peculiar morphological, structural

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and molecular features eventually leading to novel technological properties.

Materials and methods

For the TILLING screening, five genes involved in starch metabolism were selected based also on the genomic sequence availability in barley cv. 'Morex', provided by Dr. Edward Schiefelbein (University of Minnesota, St. Paul, MN, USA). The genes chosen for the analysis are: *β-amylase 1* (*HvBMY1* accession no. EF175470), *granule-bound starch synthase I* (*HvGBSSI* accession no. AB089162), *limit dextrinase 1* (*HvLDA1* accession no. AF122050), *starch synthase I* (*HvSSI* accession no. AF234163) and *starch synthase II* (*HvSSII* accession no. AY133250). Primers were designed with Codons Optimized to Discover Deleterious LEsions (CODDLE; http://www.proweb.org/coddle), a tool facilitating the selection of gene regions for TILLING purposes, and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The CODDLE program is able to identify regions where point mutations are most likely to result in deleterious effects on the gene's function (Till *et al.*, 2003). The following primer sequences were used for PCR and sequencing of the population and the putative mutants:

*HvBMY1*_For, TTTGCCTTCCGGGAGACCATGT;
*HvBMY1*_Rev, CGCGTTTTCGGATGCCACATTT;
*HvGBSSI*_For, GAGCACCCAGCCACCCACACA;
*HvGBSSI*_Rev, CTGCAGCATACGCCCAGACCA;
*HvLDA1*_For, CTCGTGTGCAGCTGACGGGAAA;
*HvLDA1*_Rev, GTGCCATCGTGGGCGCTGTAAT;
*HvSSI*_For, TGTCGCGTTCCCCATTCTGATA;
*HvSSI*_Rev, TGGCATGGCTACAGTTCACCAAGC;
*HvSSII*_For, CCGATTCGATGTATGCCGGCAAT;
*HvSSII*_Rev, CCAGATCGGAATCAGCGTCTCA.

The TILLING analyses were implemented using the procedure described by McCallum *et al.* (2000); DNA

Table 1. Details of the mutants identified in the five genes analyzed

Gene	Plant code	Nucleotidic substitution	Position	Effect of mutation	Aminoacid substitution	PSSM[a]	SIFT[b]
HvBMY1	1513	C to T	Exon	Silent	Y to Y	–	–
	2253	G to A	Exon	Missense	D to N	10.2	0.30
	2682	G to A	Exon	Missense	E to K	–	–
HvGBSSI	94	A to T	Intron	–	–	–	–
	570	T to A	Intron	–	–	–	–
	1090	G to A	Exon	Missense	G to E	20.5	0.00
	2209	C to A	Intron	–	–	–	–
	2733	A to T	Intron	–	–	–	–
	5214	G to C	Intron	–	–	–	–
HvLDA1	250	G to A	Exon	Missense	R to K	–	–
	905	G to A	Exon	Missense	V to I	–	–
	1020	C to T	Exon	Missense	S to F	8.5	0.02
	1139	C to T	Exon	Missense	T to I	–	–
	1317	G to A	Intron	–	–	–	–
	1550	C to T	Exon	Missense	P to S	–	–
	1696	C to T	Intron	–	–	–	–
HvSSI	662	G to A	Exon	Non-sense	W to *SC*[c]	–	–
	877	C to T	Intron	–	–	–	–
	1089	G to A	Splice jun.	–	–	–	–
	1132	C to T	Exon	Missense	T to I	–	–
	1284	G to A	Exon	Missense	G to E	–	–
	1808	G to A	Intron	–	–	–	–
	1963	C to T	Intron	–	–	–	–
	2822	G to A	Splice jun.	–	–	–	–
	5758	G to A	Intron	–	–	–	–
	5850	G to A	Exon	Missense	G to D	–	–
HvSSII	1039	G to A	Exon	Missense	G to R	–	–
	1517	G to A	Exon	Non-sense	W to *SC*	–	–
	2273	C to T	Exon	Silent	L to L	–	–

jun, junction.
[a,b] PSSM and SIFT are values used to rank the missense mutations on the basis of their probability of affecting protein function. PSSM and SIFT can be calculated only if the missense mutation occurs in a conserved domain of the protein. Mutations are considered to be deleterious for PSSM values above 10 and SIFT values below 0.10.
[c] Stop codon.

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samples of eight M_3 lines were pooled and subjected to gene-specific PCR amplification using properly designed labelled-primers (MWG-Biotech). The PCR reaction and cycling were performed as described in Colbert *et al.* (2001). The PCR products were then digested with a commercial endonuclease, the Surveyor® Mutation Detection Kit (Transgenomics, Omaha, NE, USA), according to the manufacturer's directions. The digested PCR products were analyzed using a detection method based on denaturing electrophoretic gels (LI-COR-4200; LI-COR Biosciences; Lincoln, NE, USA). The final validation of the results was performed by sequencing using an Applied Biosystems' 377 DNA Analyzer (Applied Biosystems, Forster City, USA). Finally, the sequences were analyzed with the PARSESNP (Taylor and Greene, 2003) and sorting intolerant from tolerant (SIFT) (Ng and Henikoff, 2003) programs.

Results and discussion

The molecular screening was achieved on five genes involved in starch metabolism, using a cell-based heteroduplex assay, coupled with gel electrophoresis on DNA sequencers. A total number of 4906 DNA samples from individual M_3 plants were screened. The analyses identified an allelic series for each of the genes examined with a total number of 29 mutations and an average of *c.* five mutations/gene (Table 1). The estimated mutation density was of one mutation/520 kb screened, which compares well with what was previously reported by Talamè *et al.* (2008) on the same collection. The value of the mutation density was computed by dividing the total number of identified mutations by the number of base pairs screened and corrected, considering the effective screened window. In fact, a limitation of the TILLING procedure is that mutations can escape identification when present in the terminal 80 bp of both ends of the amplicon as a result of PCR priming and electrophoresis artifacts. In our case, a correction on the effective screening window was applied by subtracting 160 bp from the length of each amplicon (Greene *et al.*, 2003).

Almost all the mutations detected were G/C to A/T transitions. Since a previous study proposed that NaN_3 causes mutations of transition type (Olsen *et al.*, 1993) and because almost all of our mutations were G/C to A/T transitions, the possibility that the polymorphisms identified in TILLMore are naturally occurring as a result of seed contamination of our starting 'Morex' seed stock can be ruled out.

Among the 29 alleles, 13 silent mutations occurred in non-coding regions or affected the third base of a codon which does not change the aminoacid encoded by that codon; 12 mutations were classified as missense alleles, causing changes in one of the aminoacids of the protein. In four cases, non-sense alleles (two truncation mutations and two splice junction mutations) were identified. All the non-sense mutations occurred in *starch synthase I* and *II*, two genes with a crucial role in the elongation of the amylopectin chains. Severe mutations in these genes are expected to drastically reduce the content of amylopectin, hence conferring a clear phenotype (Umemoto *et al.*, 2002; Fujita *et al.*, 2006; Sestili *et al.*, 2009). As to the missense mutations, identified for all the genes analyzed, bioinformatic tools were applied to estimate the impact of mutations on protein function. In particular, PARSESNP and SIFT programs were utilized to identify the mutations that more likely will have a deleterious effect on protein function. In our case, the mutations *GBSSI* 1090 and *BMY1* 2253 showed position specific scoring matrix (PSSM) values of 20.5 and 10.2, respectively (mutations are considered to be deleterious for PSSM values above 10). The application of the SIFT algorithm predicted a possible deleterious effect for the mutations *GBSSI* 1090 (SIFT 0.0) and *LDA1* 1020 (SIFT 0.02). Mutations are predicted to be deleterious for SIFT values below 0.05 or even below 0.10. Since all other missense mutations were predicted to be located outside conserved domains, PSSM and SIFT values could not be calculated.

In conclusion, we detected at least one interesting allele for all the five genes analyzed in our study. These findings provide valuable genetic materials for studies on the regulation of starch biosynthesis in barley and for applications in mutation breeding.

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