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Isolation and characterization of an isoamylase gene from rye

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ABSTRACT

Genomic DNA and cDNA sequences of an isoamylase gene were isolated and characterized from the rye genome. The full-lengths of the rye isoamylase gene are 7351 bp for genomic DNA and 2364 bp for cDNA. There are 18 exons and 17 introns in the genomic sequence, which shares a similar organization with homologous genes from *Aegilops tauschii*, maize, rice and *Arabidopsis*. Exon regions of rye and other plant isoamylase genes are more conserved than the introns. High sequence similarity (>95%) was observed in mature proteins of isoamylase genes originating from rye, *Ae. tauschii*, wheat and barley. The transcript profile revealed that rye isoamylase is mainly expressed in the seed endosperm with a maximum level at the middle developmental stage (15 DPA). A phylogenetic tree based on the deduced aa sequences of mature proteins from rye and other plant isoamylases indicated that rye isoamylase is more closely related to *Ae. tauschii* wDBE1 and wheat iso1. This is the first report on identification and characterization of the isoamylase gene from rye, making it possible to explore the roles of this enzyme for amylopectin development in rye and triticale.

1. Introduction

Rye (*Secale cereale* L.) is an important cereal crop worldwide. Rye grain is mainly used for animal feed, to make beer, whisky or vodka, and is also milled into flour for bread, pumpernickel or crisp bread [1]. Compared to other cereal crops such as wheat (*Triticum aestivum* L.), barley (*Horderum vulgare* L.) and oat (*Avena sativa* L.), rye has a number of positive and special attributes, such as outstanding cold hardiness, excellent drought tolerance and strong disease resistance. Apart from its use as a minor cereal crop and a donor of the R genome to triticale (×*Triticosecale*), it has also been extensively used as an important germplasm source to introgress resistance genes into wheat [2]. Some rye attributes are conserved in triticale, an artificial hybrid species made by crossing wheat and rye [3]. Triticale is being explored for use as a novel bioindustrial crop in Canada.

Starch synthesis is a complicated process in plants. The first step takes place inside and/or outside amylopasts via ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27) for synthesis of ADP glucose, an activated glucosyl donor for starch synthesis [4–6]. Subsequent steps lead to two separate pathways

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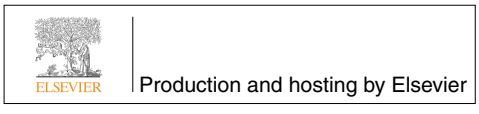

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for amylose or amylopectin synthesis. Granule-bound starch synthase (GBSS, EC 2.4.1.21), also known as waxy protein, is responsible for the synthesis of amylose polymers [6–8]. Amylopectin synthesis results from the elongation of glucan chains with both α-(1,4)-linkage and α-(1,6)-linkage synthesized by the multiple subunits or isoforms of starch synthase (SS, EC 2.4.1.21), starch-branching enzyme (SBE, EC 2.4.1.18) [9,10] and starch debranching enzymes (DBE). According to their different substrate specificities, DBEs are divided into two types: isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) [9,11]. Genotypic mutants with low starch but high water-soluble polysaccharides were identified in maize (*Zea mays* L.) [5,12], rice (*Oryza sativa* L.) [13], barley [14] and *Arabidopsis thaliana* [15,16], demonstrating that DBEs, in conjunction with SS and SBE, play an essential role in development and accumulation of amylopectin [8,17]. Characterization of barley mutants, transgenic potato and rice also indicate that isoamylase plays a crucial role in initiating the development of starch granules [14,18,19].

Starch is the most important carbohydrate in crop grains, but gene interaction in starch synthesis and accumulation in polyploid crops has not been well explored. Since rye has contributed one third of the hexaploid triticale genome, rye isoamylase must be one of the essential enzymes for amylopectin synthesis in triticale grains. However, there is no scientific report about the molecular features of rye isoamylase genes available in public databases. In this study, we isolated genomic and cDNA sequences of a rye isoamylase gene, characterized its structure, domains and expression profiles, and its predicted protein, and also analyzed the evolutionary relationship of isoamylase proteins from rye and other plant species. This is the first report on identification and characterization of an isoamylase gene from the rye genome.

2. Materials and methods

2.1. Plant materials

Hexaploid spring wheat (*Triticum aestivum* L.) cv. Chinese Spring and diploid spring rye (*Secale cereale* L.) cv. Rogo were grown under controlled environmental conditions (24 °C day, 20 °C night with a 16 h photoperiod of 240 μmol m^{-2} s^{-1}) in the same growth cabinet. Various plant materials (stem, leaf, root, seed) were sampled, flash frozen in liquid nitrogen, and stored at −80 °C until used.

2.2. Preparation of genomic DNA and RNA

Genomic DNA was extracted from young leaf tissue at Zadoks growth Stage 22 [20] using a DNeasy Plant Mini Kit (Cat. No. 69104, Qiagen Inc., Mississauga, ON, Canada). Total RNA was isolated from immature seeds (12 days post anthesis, DPA) according to a phenol/SDS protocol [21]. RNA was further purified using the RNeasy Plant Min Kit (Cat. No. 74904, Qiagen Inc., Mississauga, ON, Canada).

2.3. Primers and PCR amplification

Primers for cloning the rye isoamylase gene were designed according to the conserved regions of *Aegilops tauschii* isoamylase gene sequence (GenBank accession no. AF548379) [22], wheat *iso1* mRNA sequence (GenBank accession no. AJ301647) [23] and barley isoamylase mRNA sequence (GenBank accession no. AF490375) [14]. Ten pairs of primers were designed to amplify the overlapping genomic DNA sequences that correspond to the rye isoamylase gene. Furthermore, three pairs of primers were developed to amplify the overlapping cDNA sequences. Typically, 25 μL of PCR mixture contained 20 pmol primers, 30 ng of genomic DNA or 5 μg of cDNA, 1 × buffer, 1 × Q-solution and 1.25 U of Qiagen HotStar HiFidelity Polymerase (Cat. No. 202605, Qiagen Inc., Mississauga, ON, Canada). Reverse transcription (RT)-PCR was performed using total RNA as the template with Superscript III Reverse Transcriptase (Cat. No. 18080-093, Invitrogen, Burlington, ON, Canada). Primer sequences and PCR conditions are listed in Table 1.

2.4. Cloning and sequence analysis

Amplified isoamylase DNA fragments were cloned into the PCR4-TOPO vector (Cat. No. K4575-02, Invitrogen, Burlington, ON, Canada) and at least three independent clones for each fragment were sequenced in both directions by the DNA Sequencing Service Centre, University of Calgary (Calgary, Canada). Rye isoamylase sequences and the corresponding protein were blasted with the NCBI BLASTN tool (http://blast.ncbi.nlm.nih.gov) and aligned with previously reported isoamylase sequences using DNAMAN software v5.0 (Lynnon Biosoft, U.S.A.). The putative encoding regions of transit peptides and mature proteins of isoamylase genes from different plant genomes were predicted using the ChloroP 1.1 server (http://www.cbs.dtu.dk/services/ChloroP/).

2.5. qRT-PCR and gene expression

Total RNAs were isolated from rye leaves, stems, roots and rye seeds at different developmental stages (9, 15, 24 and 33 DPA) with an RNA Extraction Kit (Cat No. 74904, Qiagen Inc., Mississauga, ON, Canada). For RT-PCR, 1 μg of total RNA was transcribed to cDNA in a 20 μL PCR by using the oligo (dT) 18 primer and the SuperScript III Reverse Transcriptase (Cat No. 18080-044, Invitrogen, Burlington, ON, Canada). The cDNA mixture was diluted 20 times in RNA-free water and 2.5 μL of cDNA was used as template in a 10 μL real-time PCR consisting of 1 μL primer pair (final concentrate 500 nmol L^{-1} each), 1.5 μL RNA-free water and 5 μL SYBR Green (Cat. No. 204145, Qiagen, Mississauga, ON, Canada). The primer pair (5′-AAGGAGTGCG AGGGTCTTGG-3′ and 5′-GGTAAGTGGCTGGTGTTGAAGG-3′) was designed by using the Beacon Designer software (v7.0) to detect the transcription level of the rye isoamylase gene identified in this study (GenBank accession no. FJ491379). The real-time PCR was performed on a 7900H qRT-PCR system (Applied Biosystem Canada, Ontario, Canada) and temperature cycling parameters were as follows: 95 °C for 15 min, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Relative expression of rye isoamylase was analyzed by REST software [30]. The ADP-ribosylation factor (Ta 2291, GenBank accession no. AB050957), shown to exhibit a constant transcript level at different plant developmental stages under various environmental conductions [31], was used as a reference gene