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Identification and expression analysis of a novel phytocystatin in developing and germinating seeds of triticale (×*Triticosecale* Wittm.)

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Abstract

In this paper the complete cDNA sequence of a newly identified triticale phytocystatin, TrcC-7, was analyzed. Because *TrcC-7* transcripts were present in seeds, we hypothesized that it may regulate storage protein accumulation and degradation. Therefore, changes in mRNA and protein levels during the entire period of seed development and germination were examined. Expression of *TrcC-7* increased during development and decreased at the end of maturation and subsequently increased during seed germination. Based on these results, TrcC-7 likely regulates cysteine proteinase activity during the accumulation and mobilization of storage proteins.

Keywords: phytocystatin; cysteine proteinase inhibitor; seed development; germination

Introduction

In cereal seeds, germinating embryos use accumulated storage materials, which are primarily starch, proteins and lipids. Most protein accumulation occurs during the middle and late maturation stages. The largest group of proteinases responsible for degradation and mobilization of storage proteins during germination and seedling growth are cysteine proteinases [1]. One mechanism of controlling the activity of these enzymes involves specific inhibitors, phytocystatins (PhyCys).

To date, 5 PhyCys have been identified in triticale (×*Triticosecale* Wittm.), and one (TrcC-4) has been shown to have inhibitory activity against endogenous cysteine proteinase EP8, what may be related to pre-harvest sprouting tolerance [2–4]. Therefore, we examined another triticale phytocystatin. Because the transcripts of *TrcC-7* were present in developing and germinating seeds, we postulated that it may be involved in seed development and germination. To verify this hypothesis, gene expression analysis was performed.

Material and methods

Plant material

Two cultivars of triticale that differ in their resistance to pre-harvest sprouting, Hortenso (more resistant) and Leontino (less resistant), were analyzed. The seeds were provided by Danko Plant Breeders Ltd. (Laski, Poland).

RNA extraction

The total RNA from seeds was extracted according to the Chomczynski and Sacchi method [5], which was preceded by an extraction in 50 mM Tris-HCl pH 9.0, 200 mM NaCl, 1% sarcosyl, 20 mM EDTA, 5 mM DTT and a further extraction in phenol:chloroform:isoamyl alcohol (24.5:24.5:1). Total RNA was treated with RNase-free DNase (Applied Biosystems, USA) according to the manufacturer's protocol.

Sequence identification

First-strand cDNA was synthesized with a Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. PCR primers were designed with the gene sequence of barley HvCPI-8 phytocystatin (Gen Bank: CAG38129), which do not have known homologues in wheat or rye. This sequence was aligned with known triticale phytocystatin sequences: TrcC-1 (GU395200); TrcC-4 (GU395201); TrcC-5 (GU395202); TrcC-8 (JX003861); TrcC-9 (HO068312) and regions characteristic exclusively for HvCPI-8 were selected. PCR with primers complementary to those regions resulted in one-band product. After obtaining full *TrcC-7* gene sequence single nucleotide change (A to G) in the region recognized by forward primer was revealed. However, this variation did not prevent primer hybridization. The 5′ and 3′ ends of *TrcC-7* sequence were amplified using a GeneRacer Kit (Invitrogen, USA) with specific and GeneRacer primers (Tab. 1).

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Tab. 1 PCR primers used for identification and expression analysis of *TrcC-7*.

Gene	Product size (bp)	Type of reaction	Forward primer (5′–3′)	Reverse primer (5′–3′)
TrcC-7	215	PCR; rqRT-PCR	ATCCCGGACGTGAAGGAC	GTCCAGGACTGCTCGTAG
EF1α	109	PCR; rqRT-PCR	GATCAGCAACGGCTATGCC	CTCAATCTCCTTGCCAGACC
TrcC-7	470	5′RACE	GeneRacer 5′ Primer	GGTCCAGGACTGCTCGTAGACCTC
TrcC-7	534	3′RACE	CGAGCAGCAGGTCGTCTCCG	GeneRacer Nested 3′ Primer

Relative Quantitative RT-PCR

The mRNA level of *TrcC-7* was quantified with a Titanium One-Step RT-PCR kit (Clontech Laboratories Inc., USA). In all reactions, 20 ng of RNA was used as a template. As an internal control for RNA quantity, the *EF1α* (*elongation factor 1α*) gene was amplified. (Tab. 1).

Bioinformatic analysis

Primer sequences were designed with Primer3 v.0.4.0 software [6]. The nucleotide and amino acid sequences of TrcC-7 were analyzed with the following tools: MAFFT v7.130b [7], EMBOSS Transeq [8] and ProtParam [9]. To identify signal peptide, SignalP 4.1 was used [10].

Western Blotting

12 µg of protein extracts from seeds were used for SDS-PAGE. Proteins were transferred onto PVDF membrane (0.2 µm; Merck, Germany). For detection of TrcC-7, rabbit anti-TrcC-7 polyclonal antibodies against 16 aa peptide H-VALGGRGARVGGWGPI-NH_2 (Eurogentec, Belgium) which distinguishes TrcC-7 from other known triticale PhyCys were used. Anti-rabbit IgG alkaline phosphatase conjugated secondary antibodies (Sigma-Aldrich, USA) were used for visualization with BCIP/NBT.

Results

Identification and sequence analysis of new phytocystatin

In embryos of the Hortenso and Leontino varieties of winter triticale, unique phytocystatin transcripts are present. The cDNA fragment (215 bp) of *TrcC-7* was obtained by reverse transcription of mRNA extracted from embryos at 8 hours after imbibition. This sequence was extended in the 5′ and 3′ directions using RACE. The complete gene sequence of phytocystatin, along with non-coding regions was 826 bp long. The open reading frame (ORF) was 369 bp. The resulting phytocystatin gene sequence was named *TrcC-7* (GenBank: KJ209713). Gene sequence alignment with other Poaceae phytocystatins is shown in supplementary material (Fig. S1). This sequence was identical in the Hortenso and Leontino varieties. Neither gene had introns, as the PCR products from cDNA and genomic DNA templates were the same lengths. The predicted amino acid sequence of the new triticale phytocystatin was used for further bioinformatic analysis. *TrcC-7* cDNA encodes a protein of 123 amino acids with a molecular mass of 13.0 kDa and a theoretical pI 9.50, which shows the highest identity to HvCPI-8 (89.34%) from barley. Sequence analysis (Fig. 1) demonstrated that this inhibitor have 3 characteristic cystatin motifs, which are responsible for enzyme-inhibitor interactions and a motif with unknown function, LARFAVxEHN-like, which is characteristic of plant cystatins. Also TrcC-7 most likely has signal peptide. Phylogenetic analysis showed that TrcC-7 belongs to phylogenetic group C, identified by Martinez et al. [11], along with another triticale PhyCys TrcC-5. However, TrcC-7 was assigned to subgroup C1 and TrcC-5 to subgroup C2 (Fig. S2).

Analysis of expression

TrcC-7 mRNA levels were examined during seed development, which lasts approximately 50 days. The gene was expressed during the entire period, but transcript levels changed depending on the stage of seed development (Fig. 2a). *TrcC-7* expression pattern showed a rapid increase in expression between 4 and 10 DAP, maximum at 14 DAP after which the transcripts progressively decreased until the seeds reached maturity. There were no significant differences in gene expression in the plant varieties examined. These results were confirmed by the presence of TrcC-7 protein in developing seeds during the same stages (Fig. 3a). Changes in gene expression were also observed during seed germination in both embryos (Fig. 2b, Fig. 3b) and endosperm (Fig. 2c, Fig. 3c). In the Hortenso cultivar, the initially low *TrcC-7* mRNA level increased starting at 12 HAI, decreased at approximately 24 HAI and reached its maximum at the last examined time point. A similar expression pattern was observed in the Leontino cultivar, but the increase in transcripts started earlier, at approximately 8 HAI. Protein levels also increased during germination, but starting at 36 HAI for Hortenso and 24 for Leontino. *TrcC-7* expression in the endosperm was low and constant for both mRNA and protein.

Discussion

Numerous phytocystatins are present in several Poaceae family species. There are 13 known phytocystatins in barley [11], 12 in rice [12,13], 10 in maize [14] and 6 in wheat [15,16], 5 in triticale [2]. The new PhyCys described in this paper, TrcC-7, is characterized by conserved regions that are present in most cystatin superfamily inhibitors and most likely has signal peptide (Fig. 1). Bioinformatic analysis also suggested that the signal peptide directs the PhyCys to ER and, eventually, to the extracellular space. This is consistent with the results presented for barley [17], wheat [18] and rice [19] PhyCys.

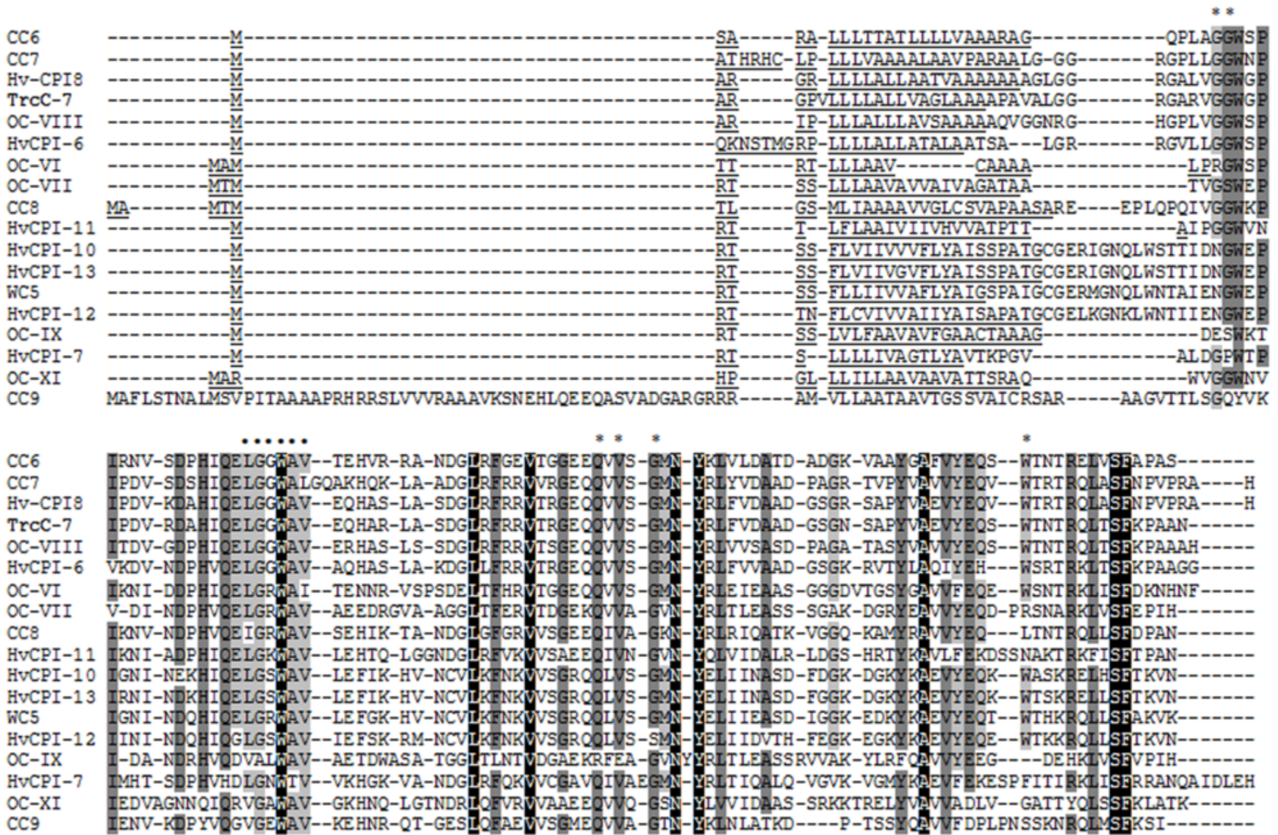

Fig. 1 Alignment of deduced amino acid sequence of TrcC-7 with phytocystatins from phylogenetic group C (subgroup C1 and C2) created by MAFFT. The conserved sequences of the cystatin superfamily are marked by asterisks. Region characteristic for phytocystatin family is marked by dots. Highly conserved amino acids (100% identity) are white letters on black, less conserved (more than 80% identity) are black letters shaded in dark gray. Amino acids less conserved are shaded in light gray. Putative signal peptides are underlined.

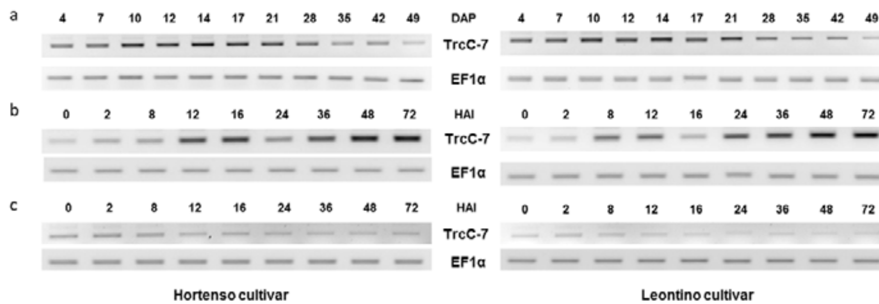

Fig. 2 *TrcC-7* expression patterns. The constitutively expressed *EF1α* was used as a control. **a** Whole seeds during development. **b** Embryos during germination. **c** Endosperms during germination. DAP – day after pollination; HAI – hour after imbibition.

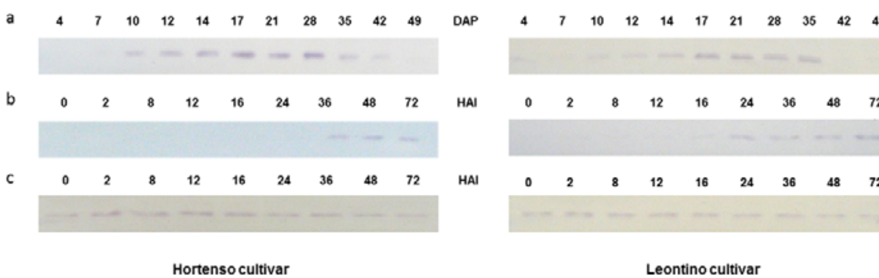

Fig. 3 TrcC-7 protein expression patterns. **a** Whole seeds during development. **b** Embryos during germination. **c** Endosperms during germination. DAP – day after pollination; HAI – hour after imbibition.

TrcC-7 is expressed throughout seed development (Fig. 2a, Fig. 3a). Its transcripts increased during seed development between 10 and 28 DAP. Thus, TrcC-7 may possibly control proteolysis during embryo development and the accumulation of storage proteins. In the final stages of seed maturation, *TrcC-7* mRNA and protein decreased. They were low also in mature seeds, in both embryo and endosperm, and through the first 8 HAI (mRNA) and first 24–36 HAI (protein). This result is similar to *CC6* and *CC7* (subgroup C1) and *CC8* and *CC9* (subgroup C2) [14]. These genes are expressed during seed development, but their expression decreases during filling and maturation stages. This indicates they are not crucial for pre-harvest sprouting tolerance, but for protein accumulation. Other expression patterns are observed for barley PhyCys: *HvCPI-6* and *HvCPI-8* from subgroup C1 [11]. In conclusion, PhyCys in group C (subgroups C1 and C2) exhibit considerable variation in expression during seed development and most likely play various functions during this process. During germination, *TrcC-7* mRNA and protein levels increased only in embryos and remained unchanged in endosperms. Such expression patterns are similar to barley *HvCPI-6* and *HvCPI-8* from subgroup C1 [11]. Although their expression in embryos peaked at 8 HAI and then significantly decreased, it gradually increased from 16 HAI.

These results suggest that the newly identified triticale phytocystatin, TrcC-7, may be involved in the control of cysteine proteinase activity during embryo development and the accumulation and processing of storage proteins in developing seeds. It is also most likely essential during germination, when storage proteins degradation occurs.

Acknowledgments

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Authors' contributions

The following declarations about authors' contributions to the research have been made: identification of a new phytocystatin, bioinformatic analysis, mRNA expression analysis, western blotting: JS; writing the manuscript: JS; revising and final approval of the manuscript: WB.

Competing interests

No competing interests have been declared.

Supplementary material

The following supplementary material for this article is available online at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.2015.011/0:

1. Fig. S1: comparison of gene sequences encoding phytocystatins from phylogenetic subgroup C1 with *TrcC-7*.
2. Fig. S2: unrooted phylogenetic tree of Poaceae phytocystatins, including 6 from triticale.

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