Expression profile of two *CENH3* genes in different tissues of the *Secale cereale*

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Abstract: The assembly site for the kinetochore complex of active centromeres is defined by the chromosomal location of the centromeric modification of histone H3 (CENH3). The loss of CENH3 from centromeres leads to improper chromosomal segregation during cell division. Most of the diploid plant species, in which the structure and copy number of CENH3 genes have been determined, have this gene as a singleton. However, it has been found that some diploid species in the tribe Triticeae have two forms of CENH3. In this work we study the expression dynamics of the αCENH3 and βCENH3 forms in different tissues of the cultivated rye (*Secale cereale* L.) by performing of a comparative RT-PCR analysis.
Key words: centromeric histone CENH3; rye; gene expression.

1. Introduction

In most species, centromere identity is defined by the presence of the centromere-specific variant of histone H3 denoted in plants as CENH3. In most diploid plant species, including cereals, maize and rice, CENH3 is encoded by a single gene (Talbert et al., 2002; Zhong et al., 2002; Nagaki et al., 2004). But some diploid species in the tribe Triticeae (*Triticum*, *Hordeum, Aegilops*) possess two CENH3 forms, αCENH3 and βCENH3 (Yuan et al., 2015; Sanei et al., 2011). Previously we showed the occurrence of two main forms of protein for most rye (the genus *Secale*) species and subspecies. In the rye species, two *CENH3* genes have different intron-exon structures and the nucleotide identity between *αCENH3* and *βCENH3* is 81–83 %, with the main amino acid sequence difference in NTT domain and in α1-helix and loop 1 of the HFD domain (CATD) (Evtushenko et al., 2017). Because of the acquisition of the second form of the histone CENH3 (βCENH3) by some Triticeae species, it is of great interest to explore the functions of the two distinct forms of this central component of centromere identity. We suppose that a comparative study of the expression levels of *αCENH3* and *βCENH3* in different tissues will shed light on this problem.

2. Materials and methods

Plant material. Leaves from tiller, stem, anther, carpel and grain tissue samples were collected from vernalized *Secale cereale* cv. 'Imperial' plants. Radicle, coleoptile and 3rd leaf tissue samples were collected from germinated grains. Tissues were characterized using the Zadoks two-digit code system (Zadoks et al., 1974).

RNA isolation and cDNA synthesis. Total RNA was isolated from individual plants using the TRIzol method (TRI Reagent, MRC) and treated by a DNA-free TM Kit (Invitrogen) according to the protocol. First-strand cDNA was synthesized from 3.6 μg of total RNA by FireScript Reverse Transcriptase (BiolabMix).

RT-PCR analysis and primer design. 25 μl of PCR mixture contained 1 μl of diluted cDNA template, 12.5 μl of BioMaster HS-qPCR SYBR Blue (2×) (BiolabMix) and 0.3 mM of the each forward and reverse primers for each gene, except βCENH3 reverse primer (0.2 mM). Reactions were performed in triplicate by the LightCycler ® 480 Instrument II (Roche) using the following conditions: 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s, at the annealing temperature of 62 °C for 20 s, and at 72 °C for 30 s. Melting curves were performed to control primer dimers. Three reference genes were used for normalization of the level of *αCENH3* and *βCENH3* transcripts. References were selected according to the study of *T. aestivum* reference genes (Paolacci et al., 2009) (Table 1). Primers for amplifying reference transcripts were selected using contigs of the*S. cereale* genomic sequences of orthologous genes (BioSample: SAMEA3928734 BioProject: PRJEB13501).

Primers for amplifying α*CENH3* and β*CENH3* transcripts were designed based on the nucleotide sequences of *Secale cereale* alpha and beta centromeric histone H3 mRNA previously obtained in our laboratory (MG384772.1, MG384763.1, MG384780.1, MG384775.1). Primers were selected preferentially on the boundaries of exons to avoid the influence of genomic DNA. Sequencing reactions of cloned amplicons were carried out to check the PCR products. The efficiency of both αCENH3 and βCENH3 primers was determined by qRT-PCR using 5X-dilution series of cDNA template. Melting curves were performed to control primer dimers. Experimental data were treated by LC480 software. Relative Quantification analysis with the high-confidence 2nd Derivative max method was used.

3. Results and discussion

Previously two main variants of centromere-specific histone H3 proteins, αCENH3-v1 and βCENH3-v1, and their two minor variants, αCENH3-v2 and βCENH3-v2, were characterized in *Secale* species, according to the differences in size and amino acid substitutions (Figure 1) (Evtushenko et al., 2017). The *αCENH3-v1* cDNA sequence in the cultivated rye *Secale cereale* is 501 bp in length and the associated protein consists of 166 amino acids. *βCENH3-v1* is distinct from α*CENH3-v1* in that the former has several deletions in the NTT and the insertion of three nucleotides in the HFD. Thus, β*CENH3-v1* has an overall length of 456 bp and encodes a protein made up by 151 amino acids. Most of the NTT amino acid sequences in αCENH3 and βCENH3 do not align well with each other.

Table 1
List of references used in the work and their functions according to the Identification and validation of reference genes for quantitative RT-PCR normalization in wheat (Paolacci et al., 2009)

Ref. gene	*T. aestivum* UniGene cluster	*T. aestivum* gene annotation	GO Biological Process	GO Molecular Function
1	Ta2776	Similar to RNase L inhibitor-like protein	Transport, protein folding	ATP-binding, ATPase activity, Electron carrier activity (ABC transporter)
2	Ta54227	Cell division control protein 48 homolog (AAA-superfamily of ATPases)	Protein transport, cell division	ATP binding, Nucleoside-triphosphate activity
3	Ta53967	Vacuolar ATP synthase 16 kDa proteo-lipid subunit	Proton transport (ATP synthesis coupled proton transport)	Hydrogen ion transporting ATPase activity, rotational mechanism

Figure 1. Alignment of the amino-acid sequences of two αCENH3 and βCENH3 variants existing in *Secale cereale*.

The α*CENH3-v2* sequences were 492 bp in length, that is, they were shorter α*CENH3-v1*. These two αCENH3 variants have different amino acids at some positions. The sequence of βCENH3-v2 is 6 bp longer than βCENH3-v1, so that it possesses two additional amino acid residues of threonine in the NTT domain, and furthermore both variants have different amino acids at some positions. Taking into account all the above, primers for comparative RT-PCR analysis in this work were designed in that manner to distinguish the αCENH3 and βCENH3 forms but to consider the expression ratio of both respective variants, v1 and v2 of αCENH3 and v1 and v2 of βCENH3.

The RT-PCR assay shows that both forms of the centromeric histone H3, *αCENH3* and *βCENH3*, are expressed in all investigated tissues of the rye *Secale cereale* subsp. *cereale* 'Imperial'. The highest level of expression of both forms, *αCENH3* and *βCENH3,* was observed in carpel tissue, where the value for the *αCENH3* product was nearly two times the value for *βCENH3* – 30 % for αCENH3 and 17 % for βCENH3 (values in percentage represent average transcription levels normalized to the geometric mean of three references). The second place in terms of transcription level of *CENH3* belongs to anther tissue, where expression level of *αCENH3* was equal to that of *βCENH3* on average, unlike the carpel tissue (11 % for αCENH3 and 11 % for βCENH3). Expression is also relatively high in radicle tissue (7 % for αCENH3 and 3 % for βCENH3) and tissue of grain (embryo and endosperm) that is just starting to develop (11 % for αCENH3 and 4 % for βCENH3). In the other tissues investigated, namely coleoptile, tissue of third leaf, leaves from tillering plants and stem tissue, the lowest expression levels of both *CENH3* forms were observed (not higher than 4 %).

It was previously shown that both αCENH3 and βCENH3 forms are included in barley centromeres during cell division (Ishii et al., 2015). Similar results were obtained for rye centromeres in our laboratory. Thus, both these forms are involved in the process of cell division. Moreover, it was demonstrated that the levels of expression of two CENH3 forms vary across different tissues of barley (Ishii et al., 2015). Similarly, we demonstrate that the expression ratio of *αCENH3* and *βCENH3* of rye varies depending on the type of tissue. However, our RT-PCR assay does not show any dramatic differences in the expression of *αCENH3* and *βCENH3* that are characteristic of barley, where the transcription level of *βCENH3* significantly exceeded the transcription level of *αCENH3* in all tissues. The expression levels of both *CENH3* forms show not equal but comparable values in most rye tissues. Probably the variations in the expression ratio reflect tissue-specific requirements for CENH3 forms in rye.

4. Conclusions

We demonstrate that the expression ratio of *αCENH3* and *βCENH3* of rye varies depending on the type of tissue. The expression levels of both *CENH3* forms show not equal but comparable values in most *Secale cereale* tissues.

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Conflict of interest. The authors declare no conflict of interest.