RESEARCH ARTICLE

ISOLATION OF A NOVEL CENTROMERE - ASSOCIATED CENH3 GENE AND ITS CHARACTERIZATION FROM SUGARCANE

V.P. Sobhakumari* and P.T. Prathima

Abstract

Centromere specific histone H3 (CENH3) has been used to detect active centromere which is essential for the faithful segregation of chromosomes during cell division. Sugarcane intergeneric hybrids obtained from a cross Co 7201 x (28 NG 210 x IK 76-78) involving multiple genomes were cytologically analyzed and karyotypically stable and unstable types were identified. The hybrids showed a somatic chromosome number range of 2n=88-110 with different levels of chromosome elimination. In order to understand the structure and function of centromere related genes of different genomes of this hybrid, primers were developed from the conserved regions of rice, maize, sorghum, wheat and rye CENH3 sequences available in the NCBI-GenBank database and amplification by RT-PCR has been done in the parents and progenies of the cross. A 450 bp fragment of CENH3 gene was identified, cloned and sequenced which showed 96% homology with sorghum CENH3 sequences and 91% with *Zea mays* CENH3 sequences. Phylogenetic analysis also revealed its close relation with sorghum CENH3. Subsequently, the expression of this gene in clones with different levels of chromosome elimination has been studied. By revealing the role of this novel CENH3 gene in chromosome elimination, it is expected to employ centromere mediated genome elimination in sugarcane.

Key words: Sugarcane, CENH3 gene, centromere, chromosome elimination

Introduction

The centromere is the assembly site for the kinetochore, the protineaceous structure that connects the chromosome to the spindle at mitosis and meiosis, thereby ensuring faithful segregation and transmission of chromosomes during cell division. Centromeres are often associated with mega base sized satellite DNAs (Jin et al. 2004; Lim et al. 2007). The centromere identity and inheritance depends on unique features of centromere chromatin (Black and Basset 2008). Centromeric chromatin is unique due to the presence of a centromere-specific histone variant CENH3, which replaces ordinary histone H3 in the nucleosome of functional centromere and is thought to play a key role in kinetochore assembly. In plants, CENH3 was first identified in *Arabidopsis thaliana* (Talbert et al. 2002) and later in maize (Zhong et al. 2002) and rice (Nagaki et al. 2004). In sugarcane, 140 bp tandem repeats were identified as localizing at the centromeres of all the chromosomes (Nagaki et al. 1998) in addition to sugarcane centromere retrotransposons (Miller et al. 1998). CENH3 has been used to detect active centromeres and to analyse the DNA sequences closely associated with the centromere because they localize only in active centromeres and directly bind to the DNA. In sugarcane, the already reported tandem repeats and centromeric retrotransposons were precipitated with CENH3 antibody which

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showed that these repeats are directly interacting with CENH3 in sugarcane centromeres (Nagaki and Murata 2005).

In higher plants, centromeres vary from 3000 to 9000 kb in size and appear to be composed primarily of tandem arrayed repeats of 150 - 180 bp. These centromeres can retain much of their capacity to segregate chromosomes even at 5% of their original size. Some homology can be detected among the tandem repeats from closely related species such as sorghum, sugarcane (Miller et al. 1998; Nagaki et al. 1998; Zwick et al. 2000), maize and rice (Ananiev et al. 1998; Cheng et al. 2002). It is interesting to know that each of the known CENH3s shares a common histone H3 core sequence but they diverge in the N-terminal and an internal region known as loop 1. Both diverged regions interact with DNA in the nucleosome and show evidence of adaptive evolution, suggesting that CENH3 serves as a linker molecule between the rapidly evolving centromeric DNA and the conserved kinetochore machinery (Malik et al. 2002).

In recent years, it has become apparent that future crop improvement efforts will require new approaches to address the local challenges of farmers while empowering research discoveries across industry and academia. When the practical utility of CENH3 research is considered, it will reveal how basic research can be translated into a potential future tool for plant breeding (Luca Comai 2014). In plants, genome elimination resulting from certain interspecific crosses was described decades ago (Kasha and Kao 1970; Clausen and Mann 1924) but it is limited to occasional natural occurrences. It has been proved that experimental alteration of CENH3 induces genome elimination in *Arabidopsis*. The plant becomes a haploid inducer by knocking out the native CENH3 and complimenting with one encoding altered CENH3 (Ravi and Chan 2010).

Haploidization occurred only when such a haploid inducer was crossed with a wild type plant. The haploid inducer line proved to be stable upon selfing suggesting that competition between modified and wild type centromeres in the developing hybrid embryo would result in the inactivation of the centromeres as the inducer parental genome is lost, and progeny that retain only the haploid chromosome set of the wild type parent can be recovered. Any error in transcription, translation, modification or incorporation can affect the ability to assemble intact CENH3 and cause centromere inactivation (Allshire and Karpen 2008). In barley, a study on the mechanism of selective elimination of parental chromosomes during the development of *Hordeum vulgare* x *Hordeum bulbosum* embryos revealed that centromere inactivity of *H. bulbosum* due to the loss of CENH3 protein leads to chromosome elimination of this species (Sanei et al. 2011). CENH3 could be harnessed for the induction of haploids. Plants with mutation in the centromere targeting domain showed impaired centromere loading in barley, *Arabidopsis* and sugar beet. *Arabidopsis* plants carrying the single point in wild type CENH3 were used as haploid inducer (Karimi-Ashtiyani et al. 2015).

In the present study cytological and molecular analysis has been done in parents and progenies of an intergeneric hybrid Co 7201 x (28 NG 210 x IK 76-78). A gene related to sugarcane CENH3 has been identified and its molecular characterization carried out. The differential expression of this gene in different species and related genera (*Erianthus*) and intergeneric hybrids of sugarcane are also described.

Materials and methods

Plant material

A Sugarcane variety (Co 7201), a *Saccharum officinarum* accession (28 NG 210), related genus

Erianthus arundinaceus accession (IK 76-78), an intergeneric hybrid of 28 NG 210 x IK 76-78 and 13 progenies (IGH clones) from the cross Co 7201 x (28 NG 210 x IK 76-78) were grown in the field at ICAR-Sugarcane Breeding Institute, Coimbatore, Tamil Nadu state, India, and utilized for the present study.

Cytological analysis

The somatic chromosome number of the parents and hybrids was confirmed through root tip mitosis. The chromosome segregation and abnormalities in cell division were studied in pollen mother cells through meiosis.

Mitosis

Root tips (1 cm) from the four parents and 13 progenies of the cross Co 7201 x (28 NG 210 x IK 76-78) were collected from potted plants and pre-fixed in saturated solution of á-bromonaphthalene for 2h. The root tips were washed several times and transferred to freshly prepared ethanol: acetic acid (1:3 v/v) fixative and stored at 4ÚC overnight. The root tips were washed thoroughly and hydrolyzed at 60ÚC in 1N HCl for 13 min. After washing thoroughly, the root tips were treated with basic fuchsine for 30 min. The root caps were removed, squash preparations made from the meristematic tip of the root in 1% acetocarmine and observed under light microscope (Carton Microsystem CM 402 T). Somatic chromosome number of each clone has been confirmed from 10 to 12 well spread mitotic cells.

Meiosis

Meiotic studies were conducted in pollen mother cells of the parents and progenies of the cross Co 7201 x (28 NG 210 x IK 76-78). Young panicles were collected and fixed in Carnoy's fluid overnight and later stored in 70% alcohol. The anthers were teased out from the florets and pollen smear was prepared in 1% acetocarmine solution. Different stages of meiosis, namely diakinesis, metaphase, anaphase and telophase were observed to study the chromosome behavior during microsporogenesis.

Conserved domain identification and primer design

Five cDNA sequences encoding CENH3 from *Sorghum bicolor* (accession number XM-002441245), maize (*Zea mays*) (accession number AF519807.1), rice (*Oryza sativa* (japonica cultivar-group) (accession number AY438639.1), wheat (*Triticum aestivum*) (accession number JF969287.1) and barley (*Hordeum vulgare*) (accession number JF419329.1) were aligned using ClustalX software to determine conserved regions among plant species. A set of primers (C450-F, 5'-ATGGCTCGAACCAAGCAC-3' and C450-R, 5'-TGTATGTCCTTTTGCATGA-3', and C280-F 5' CGCTGCGGGAGATCAGGA3 -' and C280-R 5' GATACGCCTTGCAAGTTGT-3') were designed based on the conserved regions among the monocot CENH3s using Primer3 software and used for amplification.

RNA isolation and RT-PCR

Young leaf tissues were ground to fine powder using liquid nitrogen. Total RNA was isolated using Trizol reagent (Sigma) and treated with DNAse to remove residual DNA. The purity of RNA was checked with Nanodrop DNA, RNA quantifier. Integrity and quantity of the RNA samples were checked by 1.5% (w/v) agarose gel electrophoresis. Two intact bands of 28S RNA and 18S RNA were obtained from the isolated RNA samples. First strand cDNA synthesis was performed as per the instructions given by manufacturers of cDNA synthesis kit (Thermo Fisher Scientific).

The PCR mixture comprised 2 µl of cDNA, 3.2 µl (10mM) of dNTPs, 2 µl (10x) of buffer, 0.4 µl (3U/µl) Taq, 100 ng of each primer and ddH_2O to a final volume of 20 µl. PCR was performed as follows: 94 ÚC for 3 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 7 min. *25SrRNA* gene was used as control to normalize the cDNA synthesized. Agarose gel electrophoresis results on PCR products showed amplicons size of ~280 bp and 450 bp for each of the primer set.

Cloning and sequencing

The PCR product was eluted, cloned using pTZ57R/T vector (TA cloning kit, Thermo scientific) and transformed into *E. coli* DH5 á host. Plasmids were isolated from recombinant colonies and restriction digestion was done with *Eco* R I and *Bam* H I enzymes for confirmation of the presence of insert before sequencing. Sequencing was performed using Sanger's method (Xcelris Genomics, Ahmedabad).

Results and discussion

The parents and hybrids of the cross Co 7201 x (28 NG 210 x IK 76-78) were cytologically analyzed by mitosis and meiosis. The mitotic preparations revealed the somatic chromosome number of the clones (Table 1): the commercial variety Co 7201 had the somatic chromosome number of 2n=110; the *S. officinarum* clone 28 NG 210 was a typical clone with 2n=80 and IK 76-78 was an *Erianthus arundinaceus* clone with 2n=60. The male parent of the cross, i.e. F1 from 21 NG 210 x IK 76-78 showed 2n+n chromosome segregation with 2n=110 (80+30). When the 13 progenies (IGH progenies) obtained from the cross Co 7201 x (28 NG 210 x IK 76-78) were cytologically analyzed, both karyotypically stable and unstable types could be identified. The somatic chromosome number ranged 2n=88-110 with different levels of chromosome elimination (Fig. 1). The combination of two distinct genomes in a hybrid frequently results in aberrant mitotic and meiotic divisions, and most of the times abnormalities lead to chromosome elimination. Despite the fact that many mechanisms have been described and many hypothesis proposed to explain the phenomenon of chromosome elimination (Davies 1974), the exact mechanism is still obscure. Although the mechanism by which chromosomes or chromatids might be eliminated from each of the daughter cells has not yet been defined, it is likely to involve lagging at anaphase. This is a common phenomenon during microsporogenesis in sugarcane hybrids. Reports say that the combination of parental

Table 1. Somatic chromosome number of the parents and hybrids used in the study

Clone	Somatic chromosome number (2n)
Parents	
Co 7201	110
28 NG 210	80
IK 76-78	60
28 NG 210 x IK 76-78	110
Progeny	
IGH-84	96
IGH-87	102
IGH-75	90
IGH-4	96
IGH-5	96
IGH-39	92
IGH-77	98
IGH-8	88
IGH- 86	96
IGH-43	110
IGH-56	96
IGH-99	118
IGH-80	96

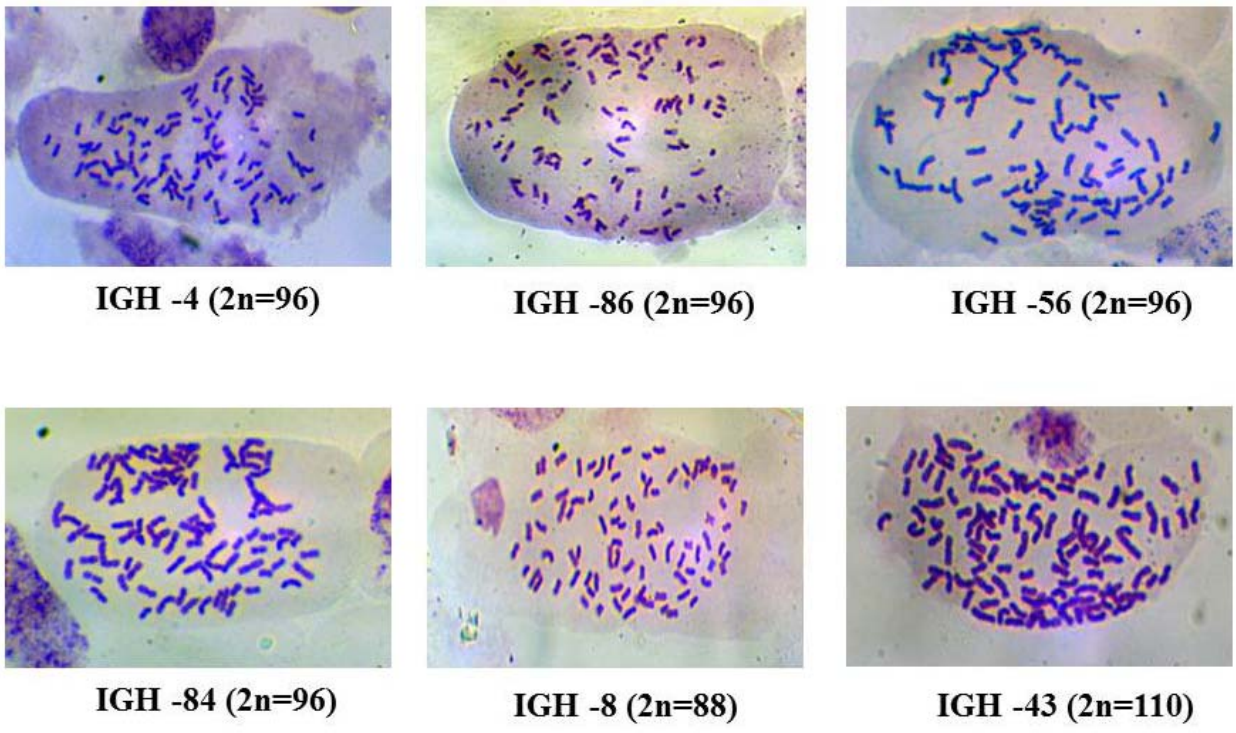

Fig. 1. Somatic chromosome number of different hybrids from the cross Co 7201 x (28 NG 210 x IK 76-78)

species mainly determines the degree of chromosome elimination (Komeda et al. 2007).

An interesting observation in these hybrids was a positive trend between the chromosome number and morphological characters like cane thickness and leaf width. IGH 43 with 2n=110 (expected n+n number of the hybrid) showed thick canes and broad leaves whereas IGH 8 with 2n=88 (elimination of 22 chromosomes) showed very thin canes and narrow leaves. IGH 8 showed profuse flowering also. The same trend was also reported in a comparative study of cytomorphological characters in 30 intergeneric hybrids of sugarcane where the chromosome number showed significant positive association with leaf width, stalk diameter and brix whereas it showed a significant negative correlation with number of millable canes (Sobhakumari 2003).

Meiotic study of Co 7201 showed abnormalities in meiotic divisions. Meiosis in the intergeneric hybrid between 28 NG 210 x IK 76-78 could not be conducted because of its non-flowering nature during the last two years. Meiotic studies of hybrids from Co 7201 x (28 NG 210 x IK 76-78) showed different types of abnormalities like laggards, fragments, higher association of chromosomes, asynchrony of division, etc. This indicates the possibility of development of heterozygosity in the forthcoming generations while using these clones as parents.

The Centromere specific histone H3 (CENH3) gene was amplified in sugarcane population including parents and progenies of Co 7201 x (28 NG 210 x IK 76-78) using degenerate primers. Phylogenetic analysis done with CENH3 gene of different crops of Graminae family revealed that sugarcane CENH3 gene identified in the present study showed 96% homology with sorghum CENH3 gene and 91% homology with maize CENH3 gene (Fig. 2).

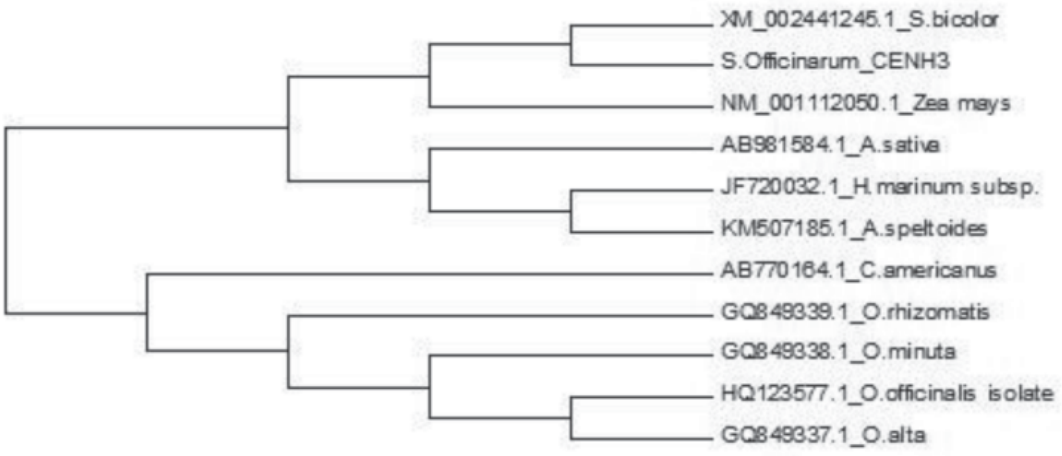

Fig. 2. Phylogenetic tree for various CENH3 gene sequences with isolated sugarcane CENH3 sequences

The earlier reported centromere tandem repeats and centromere retrotransposons of sugarcane also had more homology with maize CENH3 than rice CENH3 (Nagaki and Murata 2005).

For expression studies, primers amplifying a 280 bp from the 450 bp sequence were identified. A 280 bp amplicon was obtained in all the four parents, i. e. Co 7201, 28 NG 210, IK 76-78 and (28 NG 210 x IK 76-78) and also in four progenies, i.e. IGH-87 (2n=102), IGH-39 (2n=92), IGH-86 (2n=96) and IGH-75 (2n=90) out of the total 13 (Fig. 3). Thus, there is a possibility of different CENH3 variants in the centromeres of polyploid hybrids. The degree of cross capability of CENH3 protein between species genomes depends on the ability of centromeres to incorporate different parental CENH3 varieties (Sanei et al. 2011). The expression profiling of CENH3 in the hybrids was characterized by presence or absence of the amplicon. Nagaki et al. (1998) suggested that centromere sequence may vary among species of sugarcane but many of the kinetochore proteins that bind to the centromeres which control the chromosome movement are well conserved. Sequencing analysis of 280 bp and 450 bp fragments of CENH3 gene revealed that the 280 bp gene is a portion of 450 bp gene. The 450 bp CENH3 gene is specific to sugarcane crop and the nucleotide sequence was found to code for 147 amino acids, 10 amino acids short of a full length protein sequence at the C-terminal end.

Studies on functional analysis of sugarcane CENH3 gene, its localization in the chromosomes, identification of different versions of the gene and its role in chromosome elimination are in progress. High degree of evolutionary conservation of the method of induction of haploid plants in *Arabidopsis* and sugar beet by point mutation at centromeric CENH3 site and also by producing tail swap CENH3 offers promising opportunities for application in a wide range of crop species in which haploid technology is of interest. The application of this technology beyond the reported crops needs detailed characterization of endogenous CENH3 gene/genes and its manipulation in the target crop. Since we do not have any previous information on the CENH3 gene and its variants in a polyploid crop like sugarcane, the CENH3 gene identified in this study can be considered a novel sequence and it can be utilized to translate the basic research into a future plant breeding tool for the production of homozygous lines.

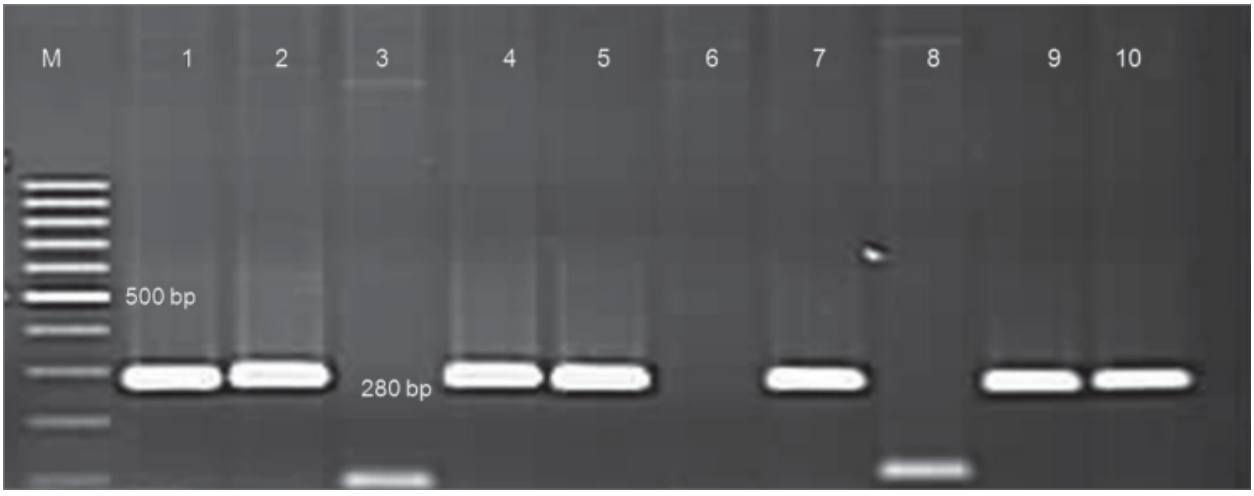

Fig. 3. Expression profiling of CENH3 gene in parents and hybrids of the cross Co 7201 x (28 NG 210 x IK 76-78). M - Ladder 100bp; 1 - IGH-39; 2 - IGH-86; 3 - IGH-80; 4 - 28 NG 210; 5 - IGH- 87; 6 - IGH-56; 7 - IGH-75; 8 - IGH-8; 9 - Co 7201; 10 - 28 NG 210 x IK 76-78

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