Effects of the cytidine analogue zebularine on wheat mitotic chromosomes

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Received: August 31, 2011 / Accepted: October 5, 2011
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Abstract

Zebularine, cytidine analog is known as DNA methylation inhibitor such as 5-azacytidine and 5-aza-2’-deoxycytidine, and it is more stable in aqueous solution than 5-azacytidine and 5-aza-2’-deoxycytidine. We investigated effects of zebularine on plant mitotic chromosomes. A wheat disomic addition line carrying a pair of alien chromosomes (Leymus racemosus chromosome ℓ) was treated with zebularine at various concentrations. The alien chromosomes were discriminated from the wheat chromosomes by genomic in situ hybridization, facilitating observation of any rearrangements between the wheat and alien chromosomes. Root growth was obviously inhibited by zebularine because of reduction of the mitotic division cells. Rearrangements such as ring chromosomes, insertions, deletions, and translocations were observed in the treated mitotic chromosomes. The aberrations were increased in a concentration-dependent manner of zebularine.

Keywords: zebularine, wheat, chromosomal rearrangements, chromosome breakage, alien chromosome addition line

Introduction

Zebularine is a cytidine analog like 5-azacytidine and 5-aza-2’-deoxycytidine (Jones and Taylor 1980; Harris 1982; Gowher and Jeltsch 2004). Zebularine lacks the amino group at position 4 of the pyrimidine ring (Champion et al. 2010) and was originally known as the cytidine deaminase inhibitor 1-β-D-ribofuranosyl-2(1H)-pyrimidinone (McCormack et al. 1980; Kim et al. 1986). In addition to inhibiting cytidine deaminase, zebularine is known as DNA methylation inhibitor (Zhou et al. 2002; Ben-Kasus et al. 2005). Zebularine is stable in aqueous solution, with a longer half-life (Marquez et al. 2005) and lower toxicity than 5-azacytidine (Rao et al. 2007). Zebularine can sustain demethylation for a long time and prevent remethylation (Cheng et al. 2004).

The first discover of the methylated base 5-methylcytosine is in calf thymus (Hotchkiss 1948). DNA methylation occurs by the addition of a methyl group to the 5 position of cytosine in higher eukaryotic cells, vertebrates, plants, and many lower eukaryotes (Attwood et al. 2002; Castiglione et al. 2002; Clark and Melki 2002). Between 60 and 90% of CpGs are methylated at the 5 position on the cytosine ring in vertebrate genome (Bird 1986). In plants, the methylated cytosine can be at a variety of cytosine-containing nucleotides such as C-G, C-A, C-T, C-C, C-A-G, C-T-G, and C-A-T sites (Gruenbaum et al. 1981).

In this study, we investigated effect of zebularine on wheat mitotic chromosomes. The genome size of wheat is large, approximately 16,000 Mb and it is about 40 times larger than that of rice (Arumuganathan and Earle 1991). Wheat can survive even under the chromosome deletion in homozygous state (Endo and Gill 1996) because it is an allohexaploid. Homoeologous genes on the other genomes with similar function complement the genes on the lost chromosomes (Kikuchi et al. 2009). Hence, the wheat is suitable material for studying on chromosomes. We used a wheat disomic addition line carrying one pair of chromosomes from Leymus racemosus. The alien chromosomes were used as marker chromosomes (Kikuchi et al. 2009) because they can be distinguished from the wheat chromosomes by Genomic in situ hybridization (GISH). In addition, we investigated various concentrations of zebularine and treatment times for wheat, and documented the types of chromosome rearrangements induced by this substance.

Materials and methods

Plant materials

For all of the experiments in this study, we used a wheat (Triticum aestivum L. cv. Chinese Spring) disomic addition line carrying one pair of chromosomes ℓ from Leymus racemosus (2n = 42+2, AABBDD+ℓ ℓ, Kishii et al. 2004). This line combined with GISH as described later...
is adequate to observe chromosome aberration in detail (Kikuchi et al. 2009).

**Treatment of seeds with zebularine**

Seeds of the wheat-Leymus racemosus disomic addition line were incubated for 3 days at 4°C on wet filter paper and then transferred to dark condition at 20°C to germinate. The 15 seeds selected by the same length of coleoptile (about 2 mm) were treated on the wet Petri dishes with various concentrations of zebularine (lower concentrations: 2.5, 5.0, 7.5, and 10.0 μM and higher concentrations: 25, 50, 75, and 100 μM) in dark condition at 20°C. The Petri dishes were wrapped with parafilm to prevent evaporation. The length of roots was measured after treatment of zebularine for 24, 36, 48, and 60 h. Zebularine (Wako) was first dissolved in dimethyl sulfoxide (DMSO) and then further diluted in water. The final concentration of DMSO was 0.5% for all concentrations of zebularine.

**Preparation of slides with mitotic chromosomes**

To prepare slides for in situ hybridization, at least 10 roots were selected from each treatment concentration after treatment for 36 h. Primary roots were incubated for 24 h in ice water to accumulate mitotic metaphase cells and then fixed in ethanol: acetic acid (3:1 v/v) fixative solution at room temperature for 5 days. Root tips were stained using 2% acetocarmine solution then squashed in 45% acetic acid on microscope slides and then covered with cover slips. The slides were frozen at -80°C to remove the cover slips and air-dried for GISH treatments. In case of mitotic index, primary roots were prepared without ice-water pretreatment and fixed in fixative solution. Slides were prepared by the same process described above.

**Cytological analysis**

In mitotic index, cells undergoing mitotic division were counted to assess the effects of zebularine. A total number of 1,000 cells were scored per slide, and measurement of mitotic index was performed 3 times. Mitotic index was measured for the 5 and 10 μM zebularine treatments at 24 and 36 h of treatment.

For measurement of chromosome aberrations in metaphase, a total number of 500 cells per slide were scored, and 10 slides were scored for each concentration after in situ hybridization. The ratio of cells in metaphase was calculated as the number of cells in metaphase divided by the total number of cells. The ratio of metaphase cells with chromosome aberrations was calculated as the number of cells with abnormal structure chromosome by chromosome aberrations between wheat and alien chromosomes in metaphase divided by the total number of metaphase cells.

**Labeling of DNA probes**

Genomic DNA of L. racemosus and L. mollis was labeled with tetramethyl-rhodamine-5-dUTP (Roche) and fluorescein-12-dUTP (Roche) by random-primer labeling to distinguish between euchromatic and heterochromatic regions of chromosomes of L. racemosus. This is possible because the chromosomes of L. racemosus have heterochromatic regions at the chromosome ends, but L. mollis chromosomes do not (Kishii et al. 1999).

In addition to the probes of genomic DNAs, we used the wheat centromere probe to detect the centromeric regions. The sequences of wheat centromeric region were amplified by oligonucleotide primers (Fukui et al. 2001), forward (5’-ATGATACTGATTTCAAAGAT-3’) and reverse (5’-ACCATAACAATTTCAGG-3’). The PCR product was cloned using pGEM®-T and pGEM®-T Easy Vector Systems (Promega) in ligation reaction and then transformed using E. coli DH5 α Competent cell (TaKaRa). The clone DNA was labeled with tetramethyl-rhodamine-5-dUTP by the random-primer method and used as probe of fluorescence in situ hybridization (FISH).

**Sequential in situ hybridization**

Sequential GISH and FISH were performed to distinguish the pair of alien chromosomes in the wheat-Leymus racemosus disomic addition line and to identify centromeric region on wheat chromosomes following Dou et al. (2009). Briefly chromosome slides prepared by the squash method were denatured by using 0.2 M...
NaOH in 70% ethanol at room temperature for 10 min. After denaturation, the slides were dehydrated in cold ethanol series (70, 90, and 99.5% ethanol, for 5 min at each concentration). In the first hybridization for FISH, hybridization solution (50% formamide, 10% dextran sulfate, 50–500 ng labeled probe, 2 x SSC) was applied to the slides, which were then incubated for at least 20 h at 37°C in a humid box. After hybridization, the slides were washed for 5 min in 2 x saline sodium citrate (SSC) with 0.1% Triton X-100, followed by 5 min in 2 x SSC. The slides were briefly dried and then covered with a drop of Vectashield (Vector) containing 1 ng/μl 4',6-diamidino-2-phenylidole (DAPI). Images were captured using a fluorescence microscope (Olympus BX61) with a cooled CCD camera (Photometrics CoolSNAP fx; Roper Scientific) and processed using Meta imaging series 5.0 software (Universal Imaging Corporation). After the first in situ hybridization, the cover glasses were removed and the slides were washed in 2 x SSC for 30 min at room temperature and air-dried. The second hybridization was performed by the same process of FISH described above for GISH.

Results

Root growth under various concentrations and treatment times

We first treated 25-100 μM of zebularine and observed the root growth at 24-60 h (Fig. 1a). After treatment for 24 h, root growth was similar to untreated control. However, at 36 h and beyond, root growth was seriously inhibited for all concentrations. Although the roots of the untreated control elongated over the time, even in the concentration of zebularine at 25 μM, the roots were significantly inhibited in t-test (P<0.01).

To know the specific effect of zebularine, we lowered the concentration to 2.5 to 10.0 μM and observed root growth (Fig. 1b): The root in concentration of 2.5 μM was slightly inhibited over the time of treatment. According to increase of zebularine, the roots were significantly inhibited (P<0.01). At 7.5 μM, the length of roots were about half of control.

Mitotic index of treated root tips cells

The mitotic index was affected by both treatment time and concentration. The mitotic index of control cells was 27.4 ± 0.35% (± standard error) after 24 h. At 5.0 and 10.0 μM, the indices were reduced to 24.2 ± 2.29% and 23.8 ± 2.35%, respectively, but these were not significantly different from the control in t-test (P>0.01). After 36 h, the indices at 5.0 and 10.0 μM were 20.9 ± 0.66% and 10.9 ± 1.25%, respectively, while the control index was 24.3 ± 1.63%. The index at 10.0 μM was significantly less than that in the control (P<0.01).

Chromosome rearrangements caused by zebularine

After 36 h of zebularine treatment, chromosomes exhibited abnormal structures such as gaps (Fig. 2a, arrowhead) and abnormally short chromosomes (Fig. 2a, hollow arrowhead). GISH/FISH analyses revealed more precise rearrangements. For example, as for the cell in Fig. 2a and b, the structure of one alien chromosome was normal but another chromosome was highly rearranged: the euchromatic region was inserted into a wheat chromosome losing subtelomeric heterochromatin regions. The subtelomeric regions are remained as a small chromosome (Fig. 2b). In the cell shown in the images of Figs. 2c and d, two alien chromosomes were separated into four parts, a deleted chromosome lacking one of the telomeric heterochromatin and three wheat-alien translocated chromosomes of

Figure 2. Chromosome rearrangements between wheat and Leymus racemosus chromosomes in metaphase after zebularine treatment for 36 h.

a, c, and e: Different interference images of the chromosomes. b, d, and f: The same cells as in a, c, and e, respectively, after GISH/FISH. Centromeric regions (green) of wheat chromosomes (blue), heterochromatic regions of the alien chromosomes (red) and the euchromatic regions (white) were observed. Arrows in each pair images indicate the same positions on the same chromosomes. Arrowheads and hollow arrowheads are remarkable chromosomes with rearrangement(s) (See text). Scale bar, 10 μm.
Wheat chromosome aberrations induced by zebularine

which two carries only the portion of heterochromatin. In images of Figs. 2e and f, one very long chromosome was shown (Fig. 2e, hollow arrowhead). Sequential GISH/FISH revealed that this chromosome was a resultant of fusion of an alien chromosome and wheat chromosomes, including several centromeric regions (Fig. 2f). Here another alien chromosome seemed to be normal. In addition to these alien chromosomes, a chromatid-type aberration was observed (Fig. 2e, arrowhead). Figures 3 shows examples of chromosome aberrations observed in the treated plants. Chromosomes in Figs. 3c-e are truncated of which those in c are interstitial deletion of the alien chromosome, those in d may be of one arm, and that in e is produced wheat-alien translocation carrying a heterochromatic region of the alien chromosome and euchromatic wheat chromatin. Chromosomes in Fig. 3f are ring form: Upper chromosome loses the heterochromatic region, whereas lower one carried a heterochromatic region. Figures 3g-n are translocations between wheat and alien chromosomes. The lengths of the translocated regions were various in chromosome. Some chromosome might be produced by a single translocation event (g-i), whereas others produced by complex events of chromosome aberrations, and as a result, those caused insertions or long and multicentric chromosomes.

Frequency of cells with abnormal chromosomes in metaphase

We observed cells with abnormal structure chromosomes by chromosome aberrations between wheat and alien chromosomes in metaphase. The frequency of cells with abnormal structure chromosomes as a percentage of total metaphase cells was approximately 0.5% in control (Fig. 4). At concentrations of zebularine ranging from 2.5 to 10.0 μM, the frequency of cells with abnormal structure chromosomes increased from 3.9 to 16.9% of total cells. The frequency of cells with abnormal structure chromosomes was roughly proportional to the dose of zebularine used. Reduction of metaphase cells frequency was also observed with increasing concentration (Fig. 4).

Discussion

We found in wheat that elongation of roots was inhibited after 36 h of treatment at more than 5.0 μM of zebularine. Recently, it is reported that growth of Arabidopsis thaliana is inhibited on medium with 20, 40, and 80 μM of zebularine (Baubec et al. 2009). Most of the roots did not elongate at all concentrations above 10.0 μM of treatment even after 36 h in wheat. In addition, the proportion of dividing cells (mitotic index) decreased with increasing of concentration and treatment time. In human breast cancer cells, the cell viability is inhibited by zebularine treatment in dose and time dependently (Billam et al. 2010). In addition, zebularine induces DNA damage in human leukemic T cells (Ruiz-Magaña et al. 2011) and causes cell death in Escherichia coli (Betham et al. 2010). In wheat disomic addition line, the concentration over 10.0 μM seemed to be too strong to observe real effect of zebularine on mitotic cell division.

Here, in this study, we found chromosome breakage on wheat mitotic chromosomes, treated zebularine using well-established system to observe chromosome breakage. Previously Kikuchi et al. (2009) used this system to observe effect of heavy ion beam on chromosome aberration. The chromosome breakage is reported in DNA-protein kinase-
deficient human glioblastoma cells under zebularine treatment (Meador et al. 2010). We investigated cells in metaphase after zebularine treatment and identified various types of chromosomal aberrations (Fig. 3). The chromosome breakage occurred in both euchromatic and heterochromatic regions. No specific fragile chromosome regions susceptible to zebularine were observed. Any expected types of chromosome aberration appeared in the treated cells. In this study, we mostly paid attention to the fate of alien chromosomes to elucidate result clearly, but we could observe occurrence of chromosome aberration also in wheat chromosomes. Chromosomes carrying more than one centromere were the remarkable examples. The frequency of chromosome rearrangements increased with increase of the concentrations (Fig. 4). At concentrations higher than 25 μM, metaphase cells seldom appeared, thus it was difficult to observe chromosomes. With increasing treatment time, the number of cells observed in metaphase clearly diminished. Therefore, treatment of 36 h or less, with less than 10 μM zebularine, may be most suitable for root growth and cell survival to observe chromosome rearrangement in this system using wheat-ailen chromosome addition line.

The results reported here demonstrate that zebularine causes chromosome breakage. Chromosome breakage is known to be caused by factors such as radiation (Kikuchi et al. 2009), chemicals (Kato and Shimada 1975), and transposable elements (McClintock 1950). Transposable elements are transcriptionally silenced by DNA methylation (Gehring and Henikoff 2008), so one possible mechanism for the chromosome breakage might be that zebularine induces demethylation of transposable elements, and this function of zebularine causes the release of transposable elements, which then cause chromosome breakage.

Another possible mechanism for the chromosome breakage by zebularine may be related to Gametocidal gene (Gc), genes causing chromosome breakage. The mechanism of the chromosome breakage by Gc is supposed to be that like methylation-mediated restriction-modification system in bacteria (Tsujimoto 2005). Zebularine may inhibit the modification enzyme, allowing the restriction enzyme to be expressed. Expression of the restriction enzyme might cause chromosome breakage.

The phenomenon of zebularine-induced chromosome breakage might be useful for cultivated wheat, or introducing useful genes from wild relatives, to improve wheat breeding. If mechanism of the chromosome breakage at the molecular level can stably control genetic recombination, it will be possible to improve the genetic diversity of wheat for breeding.

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