Pak. J. Bot., 48(4): 1585-1591, 2016.

GROWTH AND DNA METHYLATION LEVEL OF *TRITICUM AESTIVUM* SEEDLINGS TREATED WITH 5-AZACYTIDINE

HONG YINGDUAN, WEN XIAO LIU, JING YUN LI, WEI KAI DING, YANQIU ZHU, HUINA WANG, LI NA JIANG AND YAN QING ZHOU*

College of Life Sciences, Henan Normal University, Xinxiang 453007, China
**Corresponding author e-mail: dxdhy@126.com, Luckyqing2004@126.com*

Abstract

In this study, two wheat varieties were used to study effects of DNA methylation inhibitor 5-azacytidine on wheat seedling growth, and found that the high concentration of 5-azacytidine (over 100 μm) significantly affected growth of wheat seedling root, especially for wheat AK58. When the concentration of 5-azacytidine was between 50 to 250 μm, plant height of wheat AK58 significantly reduced, and the minimum dwarf phenotype was obtained when treated by 250 μm 5-azacytidine. Different with wheat AK58, plant height of wheat XM13 increased after being treated by 5-azacytidine. In addition, leaf area and chlorophyll content of two wheat varieties both increased under low concentration of 5-azacytidine (10-50 μm), and the increase magnitude of wheat AK58 was far more compared with wheat XM13, indicating that moderate methylation could promote development of wheat leaf and photosynthesis, and photosynthesis of wheat AK58 might be closely related with methylation status of genomic DNA. This study also found that proline content of wheat AK58 and soluble sugar content of two wheat varieties increased after being treated by 5-azacytidine, and showed a concentration-dependent increase, further more, soluble sugar content of wheat AK58 was far higher than that of wheat XM13 under normal conditions, thus 5-azacytidine might be conducive to accumulation of soluble sugar in wheat and could obviously influence osmotic adjustment ability of wheat AK58. Further analysis showed that DNA methylation level of wheat AK58 was higher compared with wheat XM13, and was both lower in leaf genomic DNA of two wheat varieties than that in root genomic DNA. Although 5-azacytidine significantly reduced DNA methylation level of leaf and root genome, the decrease amplitude of leaf was more obvious. Collectively, these results suggest that there are some differences in seedling growth, physiological characteristics and DNA methylation level of two wheat varieties, furthermore, DNA methylation inhibitor 5-azacytidine could influence growth of wheat seedling, especially for wheat AK58.

Key words: DNA methylation, 5-azacytidine, *Triticuma estivum*, Seedlings, Growth.

Introduction

DNA methylation, an important kind of epigenetic inheritance (Santos *et al*., 2005), would lead to alteration of chromatin structure, DNA conformation, DNA stability and DNA-protein interaction (Buryanov& Shevchuk, 2005; Firestein, 2012). Plant DNA methylation occurred mainly in the nuclear genome with specific tissue and growth stage (Flavell, 1994), and plays an important role in regulating gene expression during development and differentiation of plant (Vanyushin, 2006). Changes of genomic DNA methylation would affect plant phenotype (Chan *et al*., 2005), such as flowering, plant height and disease resistance (Chen & Wang, 2011). Besides, the methylation status of plant genomic DNA could be affected by external environment (Dowen *et al*., 2012; Migicovsky & Kovalchuk, 2013). Under environmental stress, DNA methylation and demethylation of specific gene would result in difference of plant-specific gene expression, suggesting that DNA methylation might possibly be applied in resistance breeding of crop (Hamayun *et al*., 2010). Up to now, many research reports have focused on the relationship between epigenetic phenomenon and plant stress resistance (Boyko & Kovalchuk, 2008), for example, the demethylation in coding region of high salt and low temperature stress-induced anti-adversity gene could increase expression of these genes in response to environmental stress (Choi & Sano, 2007; Khan *et al*., 2011), and the demethylation of *metl* genewould also lead to specific expression of 31 stress response genes (Wada *et al*., 2004), showing that alteration of DNA methylation plays a vital role in the process of plant response to environmental stress. Therefore, the phenotypic variations produced by methylation variation could provide raw materials for breeding selection.

5-azacytidine (5-azaC) is one of DNA methylation inhibitors which could reduce genomic DNA methylation level, and had been widely used to regulate DNA methylation level of plant, such as *Linum usitatissimum* (Fieldes *et al*., 2005), *Oryza sativa* (Akimoto *et al*., 2007), *Arabidopsis thaliana* (Jeddeloh *et al*., 1998; Finnegan *et al*., 2000), and so on. Therefore, it is possible to create mutant materials and cultivate new resistant germplasm through DNA methylation inhibitor, which would be a favorable way to plant resistance breeding (Yang *et al*., 2013). Wheat (*Triticum aestivum* L.) is an important food crop, but environmental stress could seriously affect growth and development of wheat, and then lead to its yield reduction. Horváth *et al*. (2003) found that the low-temperature vernalization in promoting wheat flowering could be partially replaced by DNA methylation inhibitor. However, there are few studies about effect of DNA methylation inhibitor on stress tolerance of wheat. In this study, wheat AK58 and XM13 were taken as test materials, seed germination, seedling growth, physiological characteristics and DNA methylation level of wheat seedlings were studied after treated by DNA methylation inhibitor 5-azacytidine, and the correlation among growth, physiological characters and DNA methylation of wheat seedling was also analyzed in order to provide scientific basis for breeding and cultivation of wheat variety with stress tolerance.

Materials and Methods

Germination of wheat seeds: In this study, two wheat cultivars AK58 and XM13 were chosen, and seeds of wheat cultivars AK58 and XM13 were provided by Henan Institute of Science and Technology and Xinxiang Academy of Agricultural Science, Henan, P. R. China, respectively. After being surface-sterilized for 10 min with 0.1% HgCl2, seeds were washed for 50 min with sterile water, then respectively placed in the Petri dish at whose bottom two layers of filter papers were laid out. Seeds of wheat were cultured at 25°C under 12 h photoperiod of 1000-1200 lux illumination intensity and were irrigated with 3 ml water solution every two days, the germination rate, root length, root number, coleoptile length of wheat were counted after cultured for 7 d. In this study, the concentration of 5-azacytidine in water solution was 0 μm (CK), 10 μm (T1), 30 μm (T2), 50 μm (T3), 100 μm (T4) or 250 μm (T5), further more, 300 seeds were treated in each group, and there were three replicates per group.

Cultivation of wheat seedlings: After treated for 7 d under different concentration of 5-azacytidine, wheat seedlings were transplanted in pots (diameter of 15 cm) with nutrition soil and cultured at 24 ± 1°C under 12 h photoperiod of 1000-1200 lux illumination intensity. When cultured for 21 d (three-leaf stage), physiological characteristics of wheat seedlings were observed and analyzed, such as plant height, fresh weight (FW) and leaf area of first true leaf. In each group, 300 seedlings were treated, and there were three replicates per group. Further more, contents of chlorophyll, proline and soluble carbohydrate in seedling leaves were determinated, and genomic DNA methylation of wheat seedlings was also analyzed.

Determination of chlorophyll, proline and soluble carbohydrate in seedling leaves: The chlorophyll in leaves of wheat seedling was extracted according to the following steps, 0.1 g FW of leaves were placed in 25 ml tube and 80% acetone extraction liquid was added to 15 ml, then leached for 24 h at 40°C and in dark. After cooling at room temperature, the leaching solution was supplied to 10 ml and determinated after blending at 645 nm or 663 nm, respectively, and then content of chlorophyll a, chlorophyll b, and total chlorophyll were calculated. Further more, determination of chlorophyll was repeated three times.

The content of proline in seedling leaf was assayed by ninhydrin colorimetry, 0.5 g FW of leaves was put into 15 ml tube and mixed with 5 ml 3% sulfosalicylic acid solution, then transferred into boiling water for 15 min and then the extract was cooled to ambient temperature. After coloration and extraction of leaching solution, the toluene layer was detected by spectrophotometer at 520 nm, and then proline was calculated according to a standard curve. There were three replications per treatment group.

Soluble carbohydrate in seedling leaf was extracted as follows: 0.1 g FW of leaves was ground together with 5 ml distilled water at 80°C, and transferred to a test tube. Subsequently, the test tube was sealed with plastic film and placed in 80°C water for 30 min, and the extract was filtered into 10 ml volumetric flask and diluted with sterile water to volume. After centrifugation for 15 min at 10 000g, the supernatant in the centrifuge tube was transferred to 10 ml volumetric flask, and diluted with sterile water to volume. The 1:4 volume ratio of extract and anthrone reagent were mixed and placed in boiling water for 10 min, then cooled, and the content of soluble carbohydrate was determined with spectrophotometer at 620 nm using a standard curve of soluble saccharide (sucrose). In addition, determination of soluble saccharide was repeated three times.

Assay of genomic DNA methylation level: Genomic DNA from leaves and roots of wheat seedlings was extracted by CTAB (cetyltriethyl-ammonium bromide) method, yield and purity of genomic DNA were determined with spectrophotometry at 260 nm, and the integrity of genomic DNA was determined by agarose gel electrophoresis. In addition, genomic DNA was hydrolyzed by DNase I, nuclease P1 and alkaline phosphatase, and filtered with 0.45 μm microporous membrane and subsequently determined by HPLC (high performance liquid chromatography). HPLC conditions were as follows: analytical column was Agilent C18Zorbax XDB column (4.6 ×150 mm, 5 μm particle size), column temperature was 30°C, mobile phase was 50 mM KH_2PO_4 and 8% methanol with pH 3.7 and 0.4 ml/min velocity, and detection wavelength of UV detector was 285 nm. In addition, the precision, repeatability and stability of HPLC were tested to guarantee reliability of experimental data, and the determination of genomic DNA methylation level was repeated three times.

Data processing and analysis: In this research, the significance level, ANOVA (analysis of variance) and multiple comparisons of Duncan's multiple range test on root length, leaf area and FW of wheat seedlings were performed by DPS7.5 (data processing system). Content of chlorophyll, proline and soluble carbohydrate in seedling leaves, and the level of 5-methyl cytosine in genomic DNA of wheat seedlings were calculated and analyzed by Excel and DPS7.5.

Results

As shown in Fig. 1, effects of 5-azacytidine with low concentration (below 30 μm) on primary root number of two wheat varieties were not obvious, but root growth of wheat seedlings was significantly inhibited when treated by high concentration of 5-azacytidine (over 100 μm), with few primary roots (3-4), and the number of primary root further decreased with the increase of 5-azacytidine concentration, especially for wheat AK58. Additionally, Fig. 2 showed that primary root length of wheat XM13 was shorter than the control group except for the one treated by 10 μm 5-azacytidine, the primary root length

became even shorter with the increase of 5-azacytidine concentration and was about 2.15 cm when treated by 100-250 μm 5-azacytidine. Different from wheat XM13, the primary root length of wheat AK58 under the treatment of 5-azacytidine was all shorter than the control group, and showed a concentration-dependent decline, furthermore the primary root length of wheat AK58 was only 1.65 cm or so at 100-250 μm 5-azacytidine and had extremely significant difference compared with the control group. In addition, the coleoptile length of wheat XM13 was shorter than the control group, and the inhibition of 5-azacytidine on coleoptile showed an increasing trend with the increase of 5-azacytidine concentration, however the variation of coleoptile length was not obvious (Fig. 2), representing effect of 5-azacytidine on coleoptile growth of wheat XM13 was lesser. Different from wheat XM13, the coleoptile length of wheat AK58 treated by 100-250 μm 5-azacytidine was about 1.95 cm, and was significantly shorter than the control group (Fig. 2).

After being cultured for 21 d, most of wheat seedlings entered into three-leaf stage, yet seedlings of wheat AK58 treated by 250 μm 5-azacytidine had four leaves and the leaf area of its first true leaf was only 2.6 cm^2, smaller than the control group and other treatment groups (Table 1). As listed in Table 1, when treated by the low concentration of 5-azacytidine (10-50 μm), the leaf area of wheat Ak58 was relatively large, but the increasing rate of leaf area became slow with the increase of 5-azacytidine concentration, for example the leaf area of wheat AK58 treated by 10 μm, 30 μm and 50 μm 5-azacytidine was 5.3 cm^2, 4.7 cm^2 and 4.4 cm^2, respectively. In addition, while treated by 10-100 μm 5-azacytidine, the leaf area of wheat XM13 increased at first and then decreased, and the biggest leaf area was about 4.0 cm^2 when treated by 30 μm 5-azacytidine. However, the leaf area of wheat XM13 treated by 250 μm 5-azacytidine showed little difference compared with the control group. Further more, Table 1 also showed plant height of wheat seedlings at three-leaf stage. Under normal conditions, plant height of wheat XM13 was about 23.0 cm and higher than that of wheat AK58. Treated by 5-azacytidine, plant height of wheat XM13 represented an increasing trend, and could reach 26.0 or 27.0 cm when treated by 10-100 μm 5-azacytidine. However, when the concentration of 5-azacytidine was 250 μm, plant height of wheat XM13 decreased to 23.4cm. Different from wheat XM13, the plant height of wheat AK58 treated by 10-30 μm 5-azacytidine had small difference with the control group. However, plant height of wheat AK58 significantly reduced with the increase of 5-azacytidine concentration (50-250 μm). Especially treated by 250 μm 5-azacytidine, wheat seedling of AK58 was very low and the plant height was only 14.0 cm. In addition, the fresh weight of wheat seedling XM13 after being cultured for 21 d was about 0.47 g, and higher than that of wheat AK58 (Table 1). Under the treatment of 5-azacytidine, the seedling fresh weight of two wheat varieties both reduced, especially decrease of wheat AK58 was more obvious, and fresh weight of wheat AK58 was only 0.15 g when treated by 250 μm 5-azacytidine.

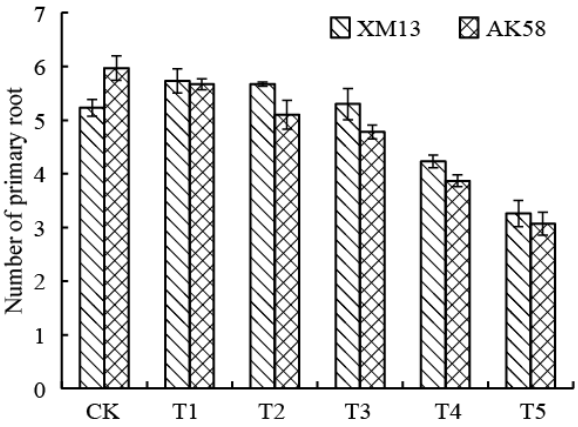

Fig. 1. The number of primary root in wheat seedlings treated by 5-azacytidine. CK, T1, T2, T3, T4 or T5 respectively represents number of primary root in wheat seedlings treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.

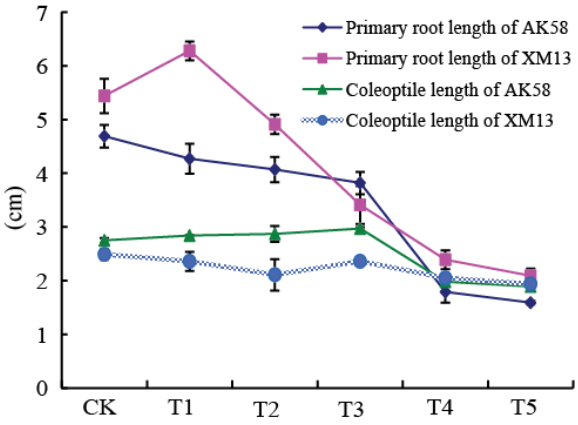

Fig. 2. The primary root length and coleoptile length of wheat seedlings treated by 5-azacytidine. CK, T1, T2, T3, T4 or T5 respectively represents primary root length and coleoptile length of wheat seedlings treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.

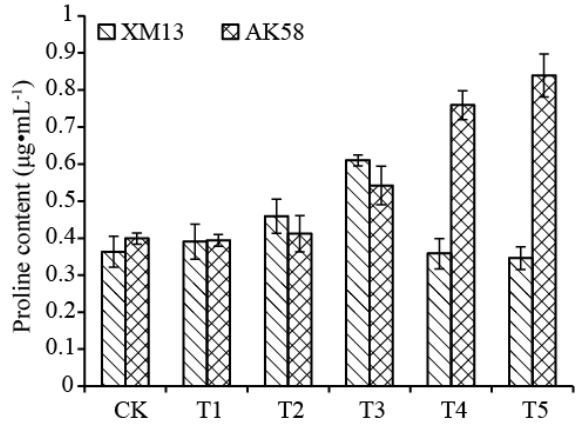

Fig. 3. The accumulation of proline in leaves of wheat seedlings treated by 5-azacytidine. CK, T1, T2, T3, T4 or T5 respectively represents proline content in leaves of wheat seedlings treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.

Proline is one of important osmotic adjustment substances in plants, its accumulation could decrease osmotic potential of cell and improve retention ability of tissue, and then would protect enzyme and membrane in plant (Shen *et al*., 2004). Thus, proline could be used as a physiological index of plant resistance. In this study, when treated by the low concentration of 5-azacytidine (below 50 μm), proline content in seedling leaf of wheat XM13 showed a concentration-dependent increase (Fig. 3), and was 0.61 μg•mL-1 as treated with 50 μm 5-azacytidine, which was significantly higher than the control group. However, proline content of wheat XM13 decreased after being treated by 100-250 μm 5-azacytidine, and had no significant difference compared with the control group. Similarly, proline content of wheat AK58 treated by 5-azacytidine also increased in a concentration-dependent manner, and showed significant difference compared with the control group in the scope of 50-250 μm 5-azacytidine. Further more, the proline content of wheat AK58 was about 0.839 μg•mL-1 at 250 μm 5-azacytidine, and reached extremely significant level. Therefore, the effect of 5-azacytidine on proline content of wheat AK58 was obviously larger than that of wheat XM13.

Soluble sugar is also vital osmotic adjustment substance, could effectively decrease water potential and enhance water retaining capacity of cell (Zhou*et al*., 2013), and then would maintain the water required for plant growth under environmental stress, so the adverse resistance of plant should be improved. As shown in Fig. 4, under normal conditions, the content of soluble sugar in seedling leaf of wheat AK58 was about 4.7%, and obviously higher than that of wheat XM13. After being treated by 5-azacytidine, the content of soluble sugar in two wheat varieties showed different trends (Fig. 4). Compared with the control group, the content of soluble sugar in wheat AK58 showed a concentration-dependent increase, and was obviously higher than the control group when treated by 30-50 μm 5-azacytidine. When the concentration of 5-azacytidine was 100 μm or 250 μm, the content of soluble sugar in wheat AK58 was respectively 7.5% or 8.5%, reached extremely significant difference with the control group. In addition, the content of soluble sugar in wheat XM13 also significantly increased after treatment of 5-azacytidine, and showed a bell curve with the increase of 5-azacytidine concentration. As treated by 30 μm 5-azacytidine, the content of soluble sugar in wheat XM13 was the maximum (5.5% or so), but decreased to 2.0% at 250 μm 5-azacytidine, and the above values both had extremely significant difference with the control group.

Chlorophyll plays an important role in absorption, transmission and transformation of light energy during photosynthesis (Yi *et al*., 2008). Besides, the variation of chlorophyll content also shows the sensitivity of plant under environmental stress (Zhou *et al*., 2013). Fig. 5 showed that the chlorophyll content of wheat XM13 increased after being treated by 10-50 μm 5-azacytidine, especially was higher than the control group at 30 μm or 50 μm 5-azacytidine, and was 13.264 mg•L-1or 12.566 mg•L-1, respectively. However, after being treated with 250 μm 5-azacytidine, the chlorophyll content of wheat XM13 was obviously lower than the control group. Different from wheat XM13, the chlorophyll content of wheat AK58 was significantly higher than the control group when treated by low concentration of 5-azacytidine (below 50 μm), and was the maximum (15.076 mg•L-1) when treated by 10 μm 5-azacytidine. In addition, when the concentration of 5-azacytidine exceeded 100 μm, the chlorophyll content of wheat Ak58 was far below the control group, and had extremely significant difference.

As shown in Figs. 6 and 7, under normal conditions, the ratio of 5-methylcytosine in root genomic DNA of two wheat varieties was far higher than that in leaf genomic DNA, and the ratio of 5-methylcytosine was about 50.0% in root genomic DNA of wheat AK58 and was higher than that of wheat XM13 (Fig. 6), butthe differences were not statistically significant between ratios of 5-methylcytosine in leaf genomic DNA of two wheat varieties (Fig. 7). After being treated by 5-azacytidine, the ratios of 5-methylcytosine in root and leaf genomic DNA of two wheat varieties significantly decreased, and showed a concentration-dependent decline (Figs. 6 and 7), for example, the ratio of 5-methylcytosine in root genomic DNA of wheat XM13 reduced by 9% or so at 10-30 μm 5-azacytidine, and was lower (17 to 26%) at 50-250 μm 5-azacytidine (Fig. 6). Similarly, after being treated at 10-50 μm 5-azacytidine, the ratios of 5-methylcytosine in leaf genomic DNA of wheat AK58 were 18.0-20.0%, and showed extremely significant difference compared with the control group, furthermore, the ratios were even lower (14.0% or so) at 100-250 μm 5-azacytidine and had extremely significant difference compared with the control group. In addition, the decrease amplitude of 5-methylcytosine in root genomic DNA was more than that in leaf genomic DNA. When treated by 100-250 μm 5-azacytidine, the ratio of 5-methylcytosine in leaf genomic DNA of wheat AK58 represented a larger decline, and reduced by 8% or so (Fig. 7), yet the decrease was even obvious in root genomic DNA and was about 25% (Fig. 6).

Discussion

DNA methylation is essential for growth and development of plant, so the insufficient DNA methylation level would result in abnormal growth and phenotype of plant (Kenneth *et al*., 2006; Marfil *et al*., 2009). Besides, plant could quickly adapt to different environments via changing genomic DNA methylation (Chinnusamy & Zhu, 2009). DNA methylation inhibitor 5-azacytidine could decrease the methylation level of genomic DNA, and the reduction of plant genomic DNA methylation plays pleiotropic role in regulating specific tissue or specific stage of plant development (Finnegan *et al*., 1996). Yamamoto *et al.* (2005) found that the low concentration of 5-azacytidine could promote embryos formation of *carrot*, but the high concentration of 5-azacytidine inhibited occurrence of somatic embryos. Currently, 5-azacytidine has been used to induce some useful new traits, such as dwarf and early-maturing characteristic of *Linum usitatissimum* (Fieldes *et al*., 2005), bacterial blight resistance of *Oryza sativa* (Akimoto *et al*., 2007), and these traits could all be applied in plant breeding. In this research, the effects of 5-azacytidine on growth, physiological characteristics and DNA methylation level of wheat seedlings were studied.

Table 1. Effects of 5-azacytidine on growth of wheat seedlings.

	XM13			AK58		
	Plant height/cm	**Leaf area/cm^2**	**FW/g**	**Plant height/cm**	**Leaf area/cm^2**	**FW/g**
CK	22.79±0.951bC	3.159±0.065bC	0.473±0.044aA	21.04±0.444aA	3.468±0.057cdBC	0.330±0.007aA
T1	26.46±0.266aAB	3.618±0.104abAB	0.429±0.022abA	21.9±0.107aA	5.332±0.238aA	0.221±0.040cBC
T2	26.44±0.469 aAB	3.955±0.465aA	0.389±0.031bA	20.21±0.647bA	4.706±0.307bcABC	0.248±0.014bcAB
T3	27.13±0.006 aAB	3.719±0.426abA	0.399±0.03 bA	19.4±0.698 bA	4.413±0.283abAB	0.291±0.025abAB
T4	27.61±0.076aA	3.494±0.177abAB	0.377±0.029 bA	18.10±0.049 bA	3.352±0.404bcABC	0.227±0.049bcB
T5	23.42±0.084bBC	3.14±0.188cB	0.390±0.021 bA	14.0±0.471 bA	2.606±0.588dC	0.132±0.134dC

CK, T1, T2, T3, T4 or T5 respectively represents seedlings treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.
The different lower cases and capital letter separately stand for significant difference ($p<0.05$) or extremely significant difference ($p<0.01$).

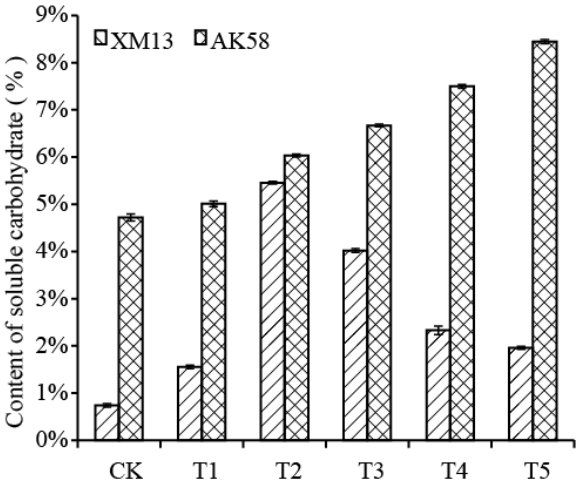

Fig. 4. The content of soluble carbohydrate in leaves of wheat seedlings treated by 5-azacytidine. CK, T1, T2, T3, T4 or T5 respectively represents content of soluble carbohydrate in leaves of wheat seedlings treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.

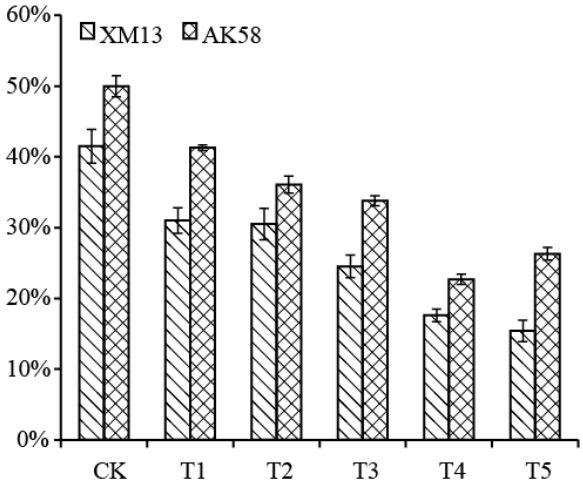

Fig. 6. The level of genomic DNA methylation in roots of wheat seedlings treated by 5-azacytidine. CK, T1, T2, T3, T4 or T5 respectively represents the ratio of 5-methyl cytosine in root genomic DNA of wheat seedlings treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.

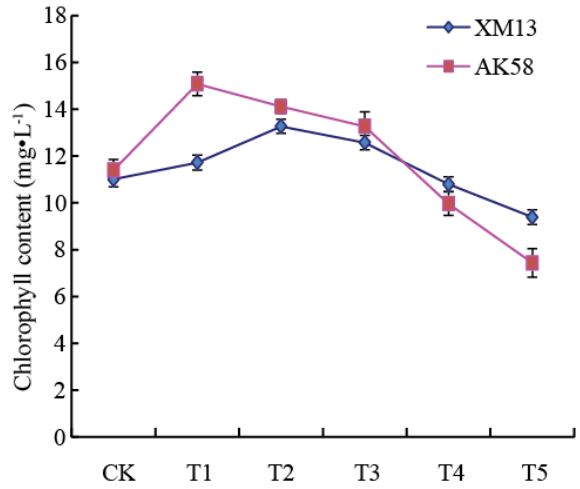

Fig. 5. The chlorophyll content in leaves of wheat seedlings treated by 5-azacytidine. CK, T1, T2, T3, T4 or T5 respectively represents chlorophyll content in leaves of wheat seedlings treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.

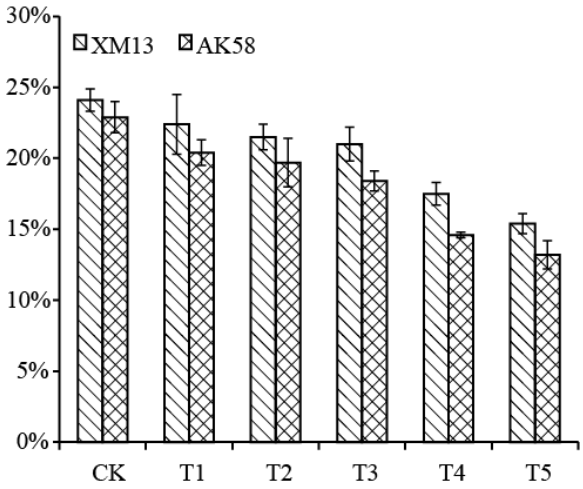

Fig. 7. The level of genomic DNA methylation in leaves of wheat seedlings treated by 5-azacytidine. CK, T1, T2, T3, T4 or T5 respectively represents the ratio of 5-methyl cytosine in leaf genomic DNA of wheat seedling treated with 0 μm, 10 μm, 30 μm, 50 μm, 100 μm and 250 μm 5-azacytidine.

As is well known, root is the main organ to absorb water and mineral nutrients, plays a decisive role in plant growth and development, and is even crucial to respond to environmental stress (Christmann *et al*., 2007). In this study, the high concentration of 5-azacytidine (over 100 μm) significantly inhibited growth of wheat seedling roots, the number of wheat primary roots was few and became even fewer with the increase of 5-azacytidine concentration, further more the decreasing amplitude of wheat AK58 was more than that of wheat XM13. In addition, under the treatment of 5-azacytidine, the length of primary root in wheat seedlings was all shorter than the control group except for wheat XM13 treated by 10 μm 5-azacytidine, and significantly decreased with the increase of 5-azacytidine concentration, especially when wheat seedlings were treated by 100-250 μm 5-azacytidine, the length of primary root showed extremely significant difference compared with the control group. Nie & Wang (2007) also found that a certain concentration of 5-azacytidine could influence production and growth of *chrysanthemum* roots. Therefore, the above researches showed that the high concentration of 5-azacytidine could significantly inhibit growth of wheat primary root, especially for wheat AK58.

Some researches found that DNA methylation level of *cabbage* and *Oryza sativa* treated by 5-azacytidine decreased, and there were some abnormalities in growth and development of plant, such as small leaf, dwarf plant and plexiform plant (Sano *et al*., 1990; King, 1995). This study also showed that plant height of wheat seedling AK58 significantly decreased after being treated by 50-250 μm 5-azacytidine, and presented a concentration-dependent decrease, especially the dwarf phenotype of wheat AK58 appeared at 250 μm 5-azacytidine, however the plant height of wheat seedling of XM13 increased when treated by 5-azacytidine. Further more, after being treated by the low concentration of 5-azacytidine, leaf area of two wheat varieties both increased, especially for wheat AK58. In addition, the chlorophyll content could reflect photosynthetic ability of plant (Zhao *et al*., 2003). This study showed that the chlorophyll content of two wheat varieties increased when treated by10-50 μm 5-azacytidine, while decreased at 100-250 μm 5-azacytidine. Especially, the amplitude variation of chlorophyll content in wheat AK58 was obviously higher than that of wheat XM13. Thus, moderate demethylation could promote development of wheat leaf and photosynthesis, and the photosynthesis of wheat AK58 might be closely related to genomic DNA methylation status.

The osmotic adjustment in plant is an important physiological and biochemical response to environmental stress, and could improve water absorption and retention capacity of plant to guarantee normal physiological metabolism (Guo, 2010). The osmotic adjustment abilities of different plants are diverse, the substances involved in osmotic adjustment are also various, and soluble sugar and proline are important osmotic adjustment substances in plants (Wang *et al*., 2007; Zhou *et al*., 2013). In this study, the proline content of two wheat varieties had few differences, whereas the content of soluble sugar in wheat AK58 was far higher than that of wheat XM13, indicating the osmotic adjustment ability of wheat AK58 via soluble sugar was stronger compared with wheat XM13. When treated by 5-azacytidine, the content of soluble sugar and proline of wheat AK58 and soluble sugar of wheat XM13 significantly increased, and had a concentration-dependent increase, yet the proline content of wheat XM13 only increased when treated by the low concentration of 5-azacytidine (below 50 μm). Therefore, DNA methylation inhibitor 5-azacytidine could promote the accumulation of soluble sugar in wheat seedlings, and significantly affected the osmotic adjustment ability of wheat AK58.

Previous researches showed that in the nuclear genome of higher plant, about 20-30% of cytosine was in methylation status (Flavell, 1994; Richards, 1997), and DNA methylation level had large differences in different development stages, organs and tissues (Finnegan *et al*., 1998; Chen *et al*., 2007; Solís *et al*., 2012). In this study, DNA methylation level in genomic DNA of wheat seedlings was about 33-36%, and was obviously lower in leaf genomic DNA than that in root genomic DNA, which was verified in previous researches (Ruiz *et al*., 2005; Akimoto *et al*., 2007; Wang *et al*., 2011; Yang *et al*., 2013). Some researches also found that DNA methylation level decreased after being treated by DNA methylation inhibitor (Fieldes *et al*., 2005; Akimoto *et al*., 2007). This study showed that DNA methylation inhibitor 5-azacytidine significantly decreased DNA methylation level of wheat genome in a concentration-dependent manner, and the decreasing magnitude of DNA methylation level in root genomic DNA was more than that in leaf genomic DNA.

Conclusion

The seed germination, seedling growth, physiological characteristics and DNA methylation levels of two wheat varieties were somewhat different. Besides, DNA methylation inhibitor 5-azacytidine had certain effect on growth and development of wheat seedlings, yet these effects on two wheat varieties were different, in which the effect of 5-azacytidine on wheat AK58 was larger compared with wheat XM13. However, it is required to further study whether 5-azacytidine could make wheat with good traits. With the development of epigenetics research, it is possible that DNA methylation inhibitor would be widely used in the future.

Acknowledgment

This research was supported by National Science-technology Support Plan Projects (2013BAD07B14) in P. R. China, and wheat seeds were kindly provided by Xinxiang Academy of Agricultural Science andHenan Institute of Science and Technology, Henan, P.R.China.

References

Akimoto, K., H. Katakami, H.J. Kim, E. Ogawa, C.M. Sano, Y. Wada and H. Sano. 2007. Epigenetic inheritance in rice plants. *Ann. Bot*., 100(2): 205-217.

Boyko, A. and I. Kovalchuk. 2008. Epigenetic control of plant stress. *Environ. Mol. Mutagen.*, 49: 61-72.

Buryanov, Y.I. and T.V. Shevchuk. 2005. DNA methyltransferases and structural-functional specificity of eukaryotic DNA modification. *Biochem.*, 70(7): 730-742.

Chan, S.W.L., I.R. Henderson and S.E. Jacobsen. 2005. Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat. Rev. Genet.*, 6: 351-360.

Chen, F. and Z.C. Wang. 2011. Effects of 5-azaC on development and DNA methylation in wheat. *Journal of Henan University (Natural Science)*, 41(1): 61-66.

Chen, X.Q., C.G. Wang, X.L. Li, W.Q. Songand R.Y. Chen. 2007. DNA methylation in plants and its epigentic function. *Chinese Journal of Cell Biology*, 29: 519-524.

Chinnusamy, V. and J. Zhu. 2009. Epigenetic regulation of stress responses in plants. *Current Opinion in Plant Biology*, 12(2): 133-139.

Choi, C. and H. Sano. 2007. Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. *Molecular Genetics and Genomics*, 277(5): 589-600.

Christmann, A., W.W. Elmar and G. Erwin. 2007. A hydraulic signal in not-to-shoot sign aling of water sho~age. *The Plant Journal*, 52: 167-174.

Dowen, R.H., M. Pelizzola, R.J. Schmitz, R. Lister, J.M. Dowen, J.R. Nery, J.E. Dixon and J.R. Ecker. 2012. Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl. Acad. Sci. USA*, 109(32): E2183-2191.

Fieldes, M.A., S.M. Schaeffer, M.J. Krech and J.C. Brown. 2005. DNA hypomethylation in 5-azacytidine-induced early-flowering lines of flax. *Theor. Appl. Genet.*, 111(1): 136-149.

Finnegan, E.J., R.K. Genger, W.J. Peacock and E.S. Dennis. 1998. DNA methylation in plants. *Ann. Rev. Plant Mol. Biol.*, 49: 223-247.

Finnegan, E.J., W.J. Peacock and E. Dennis. 2000. DNA methylation, a key regulation of plant development and other processes.*Curr. Opin. Genet. Dev.*, 10: 217-223.

Finnegan, E.J., W.J. Peacock and E.S. Dennis. 1996. Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc. Natl. Acad. Sci. USA*, 93(16): 8449-8454.

Firestein, G.S. 2012. P2-082 DNA methylome signature in rheumatoid arthritis. *Nihon Rinsho Meneki Gakkai Kaishi*, 35(4): 367b.

Flavell, R.B. 1994. Inactivation of gene expression in plants as a onsequence of specific sequence duplication. *Proc. Natl. Acid. Sci. USA*, 91: 3490-3496.

Guo, H.J. 2010. Research progress on osmotic adjustment material under water stress. *J. Anhui Agri. Sci.*, 38(15): 7750-7753.

Hamayun, M., S.A. Khan, Z.K. Shinwari, A.L. Khan, N. Ahmed and I.J. Lee. 2010. Effect of Polyethylene glycol induced drought stress on physio-hormonalattributes of soybean. *Pak. J. Bot.*, 42(2): 977-986.

Horváth, E., G. Szalai and T. Janda. 2003. Effect of vernalisation and 5-azacytidine on the methylation level of DNA in wheat (*Triticum aestivum* L.,cv.Martonvásár 15). *Plant Sci.*, 165: 689-692.

Jeddeloh, J.A., J. Bender and E.J. Richards. 1998. The DNA methylation locus ddml is required for maintenance of gene silencing in *Ababidopsis*. *Genes Dev.*, 12: 1714-1725.

Kenneth, M., T. Mahmut, M. Poole, Y. Hong, A.J. Thompson, G.J. King, J.J. Giovannoni and G.B. Seymour. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat. Genet.*, 38(8): 948-952.

Khan A.L., M. Hamayun, S.A. Khan, Z.K. Shinwari, M. Kamaran, S.M. Kang, J.G. Kim and I.J. Lee. 2011. Pure culture of *Metarhizium anisopliae* LHL07 reporgrams soybean to higher growth and mitigates salt stress. *World J. Microb. Biotech.*, 28(4): 1483-94.

King, G.J. 1995. Morphological development in *brassica oleraceais* modulated by *In vivo* treatment with 5-azacytidine. *J. Horticul. Sci.*, 70(2): 333-342.

Marfil, C.F., E.L. Camadro and R.W. Masuelli. 2009. Phenotypic instability and epigenetic variability in a diploid potato of hybrid origin, Solanum ruiz-lealii. *BMC Plant Biol.*, 9: 21.

Migicovsky, Z. and I. Kovalchuk. 2013. Changes to DNA methylation and homologous recombination frequency in the progeny of stressed plants. *Biochem. Cell Biol.*, 91(1): 1-5.

Nie, L.J. and Z.C. Wang. 2007. The molecular mechanism and application of DNA methylation inhibitor in the developmental biology of plants. *Journal of Nuclear Agricultural Sciences*, 21(4): 362-365.

Richards, E.J. 1997. DNA methylation and plant development. *Trends in Genetics*, 13: 319-323.

Ruiz, G.L., M.T. Cervera and Z.J.M. Martine. 2005. DNA methylation increases throughout *Arabidopsis* development. *Planta*, 222: 301-306.

Sano, H.V. and I.V. Kamada. 1990. A single treatment of rice seedlings with 5-azacytidine induces heritable dwarfism and undermethylation of genomic DNA. *Molecular Genetics and Genomics*, 220: 441-447.

Santos, K.F., T.N. Mazzola and H.F. Carvalho. 2005. The primadonna of epigenetics: the regulationof gene expression by DNA methylation. *Brazilian J. Medical. Biol. Res.*, 38: 1531-1541.

Shen, Y. and Y.Z. Xie. 2004. Infect of chloropnyll content and WSD of Alfalfa and Corrblated relations under drought stress. *Journal of Ningxia Agricultural College*, 25: 25-28.

Solís, M.T., M. Rodríguez-Serrano, M. Meijón, M.J. Cañal, A. Cifuentes, M.C. Risueño and P.S. Testillano. 2012. DNA methylation dynamics and MET1a-like gene expression changes during stress-induced pollen reprogramming to embryogenesis. *J. Exp. Bot.*, 63(18): 6431-6444.

Vanyushin, B. 2006. DNA Methylation in Plants DNA Methylation: Basic Mechanisms. *Current Topics in Microbiology and Immunology*, 301: 67-122.

Wada, Y., K. Miyamoto, T. Kusano and H. Sano. 2004. Association between up-regulation of stress-responsive genes and hypomethylation of genomic DNA in tobacco plants. *Mol. Gen. Genomics*., 271: 658-666.

Wang, J., D.G. Yang, F.M. Ma and J.L. Chang. 2007. Effects of water stress on soluble sugar and proline contents in *Maize* leaves. *J. Maize Sci.*, 15(6): 57-59.

Wang, W., Y. Pan, X. Zhao, D. Dwivedi, L.H. Zhu, J. Ali, B.Y. Fu and Z.K. Li. 2011. Drought-induced site-specific DNA methylation and its association with drought tolerance in rice (*Oryza sativa* L.). *J. Exp. Bot.*, 62(6): 1951-1960.

Yamamoto, N., H. Kobayashi, T. Togashi, Y. Mori, K. Kikuchi, K. Kuriyama and Y. Tokuji. 2005.Formationof embryogenic cell clumpsfrom carrot epidermal cells is suppressed by 5-azacytidine, a DNA methylation inhibitor. *J. Plant Physiol.*, 162: 47-54.

Yang, M.N., G.L. Yang, R.Z. Liu, J.G. Zhang, Z.Q. Chen and H. Wang. 2013. DNA methylation under stresses and its prospects in plant drought-resistant breeding. *Chinese Agricultural Science Bulletin*, 29(6): 6-11.

Zhao, T.H., X.Y. Shen, D.G. Yang and X.F. Ma. 2003. Effects on chlorophyll content and photosynthetic rate of *Maize* leaves under water stress and rewatering. *Rain Fed Crops*, 23(1): 33-35.

Zhou, T.J., T. Deng, W.X. Tang, L.L. Zhou, Z.C. Xiong and S.X. Huang. 2013. Physiological responses of seedlings of *Hopea chinensis* to PEG simulated drought stress. *Hubei Agricultural Science*, 52(24): 6079-6083.

(Received for publication 12 October 2015)