Effect of Phosphoric Amide Herbicide APM on the Structure and Protein Composition of Chromosome in *Triticum durum*

Yongkang Peng, Zhenying Wang, Luogen Cheng* and Hong Chen

(Department of Biology, Tianjin Normal University, Tianjin 300074, P. R. China; *Life Sciences College, Nanjin Normal University, Nanjin 210097, P. R. China)

Abstract: The cytological and biochemical effects of APM on the mitotic cells of root meristems of *Triticum durum* were studied by exposure to concentrations of 2 J1M to 10 J1M for 16 h. The results showed that multipolar spindle cells, chromosome-condensation, bridge-fragments and micronuclei were induced in the cells of root meristems by treatment with 4 J1M APM. Five protein species which have a molecular weight and pI of 40 KD/pI 5.5, 40 KD/pI 5.6, 48 KD/pI 7.0, 88 KD/pI 6.4, 120 KD/pI 6.6 were lost and 12 new protein species which have a molecular weight and pI of 16 KD/pI 6.2, 18 KD/pI 6.7, 19 KD/pI 7.0, 20 KD/pI 6.5, 22 KD/pI 7.0, 23 KD/pI 6.7, 24 KD/pI 5.8, 26 KD/pI 5.8, 88 KD/pI 6.6, 86 KD/pI 6.7, 95 KD/pI 6.5 were induced by 10 J1M APM in the root meristems.

Key words: Amiprophos methyl (APM), Chromosome-condensation, Multipolar division, 2D SDS-PAGE, *Triticum durum*.

Phosphoric amide herbicides are widely used in agriculture around the world, because they are rapidly degraded and offer little residual problem to the environment (Brahma et al., 1985). Amiprophos methyl (APM) is a kind of phosphoric amide herbicide and directly interrupts microtubule dynamics in plant cell (Morejohn et al., 1984; Kierrmayer and Fedlke, 1977). Many experiments demonstrated that cell division could be blocked at the metaphase in root meristems treated with APM. Therefore, APM has been widely used to induce mitotic metaphase synchronization (Dolezel, et al., 1992; Pan et al., 1993; Peng et al., 1999 a,b) in order to isolate mass metaphase chromosomes for further analysis of biochemical composition and morphological structure (Peng et al., 1997 a, b). Although the use of phosphoric amide herbicide has been beneficial to agriculture in China, little information is available regarding the cell and genetic toxicology of root meristems treated with APM. In this study, we investigated the effect of APM on the chromosome structure and protein composition of root meristems of *Triticum durum* in order to obtain valuable information for using the phosphoric amide herbicide safely in the agriculture and to set up a suitable test system at the cytological and biochemical levels.

Materials and Methods

1. Plant materials and culture

Seeds of wheat, *Triticum durum*, were surface sterilized for 5 min with a solution of 0.1% *HgCl*₂ and were then washed two times with tap water, and germinated in petri dishes on filter paper moistened with distilled water for 24 h at room temperature. After that 24 h, the seedlings were treated with a solution of amiprophosmethyl (APM) at 2-10 J1M for 4-16 h. All treatments were performed at 23°C in darkness; at the end of the treatment, root meristems, 0.1-0.2 cm long, were excised from the seedlings. After rinsing three times with distilled water, the excised roots were kept in ice water for 24 h and then fixed with 70% ethanol for cytological analysis. In biochemical analysis, the root segments were directly used without fixation.

2. Cytological observation

The fixative was washed away with distilled water before collecting primary root tips. The meristems was digested with an enzyme mixture (2.5% cellulase RS and 2.5% pectolyase Y-23 diluted in 75 mM KCl and 7.5 mM EDTA; pH 4.0) at 23°C. Thirty minutes after maceration, a single root tip was used as an experimental material for cytological observation. The samples of control and APM-treated root meristems were examined for mitotic division, chromosome behavior and micronucleus formation in Caebol fuchsin–stained preparations. Variation in the structure of metaphase chromosome was expressed as the percentage of nuclei undergoing mitosis to the total number of nuclei scored in a sample. Data are based on a scoring of at least 1,000 cells per treatment.

3. SDS-polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis was performed according to the method of O’Farrell et
Table 1. The number of cells with multipolar spindles induced in the root meristems of T. durum treated with 2-10 μM APM.

| Concentration of APM (μM) | 3-polar | 4-polar | 5-polar | 6-polar |
|---------------------------|---------|---------|---------|---------|
| 0                         | 0       | 0       | 0       | 0       |
| 2                         | 13      | 6       | 0       | 0       |
| 4                         | 97      | 77      | 75      | 15      |
| 6                         | 105     | 91      | 33      | 21      |
| 8                         | 155     | 137     | 37      | 20      |
| 10                        | 150     | 149     | 36      | 19      |

*Data are based on a scoring of at least 1000 cells from root meristems per treatment.

al. (1975), with some modification: the first dimensional 3% polyacrylamide gel, containing 0.98 g of 9 M urea; 40 μl of 2% nonionic detergent pH10, 340 μl of 30% polyacrylamide, 30 μl of ampholytes (pH 5-10) and 60 μl of ampholytes (pH 5-7), 1.09 ml of ddH2O, 2 μl of TEMED, 10 μl of 10% APS, were cast in glass tubes (120 mm x 3 mm). They were electrophoresed for 15 min at 200 V, followed by 30 min at 300 V and 60 min at 400 V. The protein samples were dissolved in the sample buffer which contained 4.0 ml deH2O, 1 ml of 500 mM Tris-HCl (pH 6.8), 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4 ml of β-mercaptoethanol, and 0.2 ml of 10% (w/v) bromophenol blue. After 60 μl protein sample (4-6 mg/ml) was put into each glass tube; the first dimension isoelectrofocusing was run for 14-16 h at 400 V. After electrofocusing, the gels were removed from the tubes by shattering the glass and placed in equilibration buffer containing 6 mM Tris-HCl (pH 6.8), 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4 ml of β-mercaptoethanol, and 0.2 ml of 10% (w/v) SDS for 20 min. The second dimension electrofocusing was performed with a 12.5% SDS-PAGE according to the method of Laemmli (1970). The tube gels were placed on top of the second dimension gels, and then 1% agarose was overlaid and allowed to polymerize. Cylindrical gels were run at a constant voltage of 80 V for 5.5 h in a Bio-Rad unit. Gels were stained with 0.4% AgNO₃ solution (Wary, 1982).

Results

1. Variations in the metaphase chromosome structure

Many variations in the metaphase chromosome structure were observed in the root meristems of Triticum durum treated with APM. Increase in the cells with deformed chromosomes was thought to relate directly to the concentration of APM. Table 2 gives date on multipolar spindle formation, metaphase chromosome condensation, and the formation of bridge-fragments and micronuclei after the treatment with APM (2-10 μM) for 16 h. Data are based on a scoring of at least 1000 cells from root meristems per treatment.

Table 2. Percentage of chromosome condensation, and bridge-fragments in metaphase of root cells treated with 2-10 μM APM.

| Concentration of APM (μM) | Chromosome condensation (%) | Bridge-fragments (%) |
|---------------------------|-----------------------------|---------------------|
| 0                         | 0                           | 0                   |
| 2                         | 0                           | 0                   |
| 4                         | 3.7                         | 3.1                 |
| 6                         | 4.0                         | 3.0                 |
| 8                         | 11                          | 5.1                 |
| 10                        | 55                          | 7.0                 |

Fig. 1. Cells with five polar spindles (5 chromosome groups arranged in a wide ring) in root meristems treated with 4 μM APM.

a, control. b, APM-treated root meristem.

2. Multipolar spindle division

Cells with multipolar spindles were observed very easily. In the normal cells, metaphase chromosomes are arranged along the equatorial plate and the daughter chromosomes are moving toward the poles pulled by spindle fibers, then metaphase cells are entered in anaphase. However, the function of spindle was disturbed when root meristems were treated with APM. In such cells, although the cells were still able to divide, spindle fibers could not pulled the daughter chromosomes toward the two poles, resulting in the formation of multipolar spindles. In the present study, the cells with 3 polar and 4 polar spindles cells are observed in a very high frequency, and those with 5 polar and 6 polar spindles (Table 1). As the concentration of APM increased, the frequency of cells with spindle disturbance increased, however, multipolar spindles have not been found in the controls.

Fig. 1 shows a typical type of multipolar spindle observed in the root meristems treated with 4 μM APM, 1) the chromosomes were not arrangement on the spindle, 2) four or more chromosomes groups were formed in some cells and 3) chromosome groups were arranged in a wide ring or in a star-like shape.

3. Chromosome condensation, and bridge-fragment formation at metaphase

Metaphase chromosome condensation and bridge
fragments at metaphase were observed in the other abnormal cells. Fig. 2 and Table 2 show the chromosome condensation and bridge-fragments at metaphase in some cells of root meristems treated with 4 \( \mu \text{M} \) APM for 16 h. The frequency of chromosome condensation and bridge-fragmentation increased with an increase in the concentration of APM from 2 \( \mu \text{M} \) to 10 \( \mu \text{M} \). The maximum frequency of cells with chromosome condensation was about 55\% in the root meristem treated with 10 \( \mu \text{M} \) APM for 16 h.

4. Micronucleation

In the root meristems treated with 2 \( \mu \text{M} \) APM micronuclei were observed only in 0.2\% of the cells. The percentage of cells containing micronuclei increased with an increase in the concentration of APM; about 3.2, 11, 17 and 27\% in the root meristems were treated with 4, 6, 8 and 10 \( \mu \text{M} \) APM, respectively (Table 3). The number of micronuclei per cell was generally 1~5, however, six micronuclei were also observed in a few cells.

5. Changes in protein species

To identify the changes in protein species in root meristem after the treatment with APM, we analyzed the protein compositions by using 2D SDS-PAGE. More than 100 protein species were detected in the root meristem in the control; their molecular weight ranged from 14 to 120 KD and pI from 4.5 to 7.5. Thirty five protein species abounded in content, but protein compositions were obviously changed by treating with 10 \( \mu \text{M} \) APM for 16 h. Five protein spots, 40 KD/pI 5.5, 40 KD/pI 5.6, 48 KD/pI 7.0, 88 KD/pI 6.4, 120 KD/pI 6. (Fig 3a, indicated by arrows) were lost in treated root meristems, but 12 new protein spots, 16 KD/pI 6.2, 18 KD/pI 6.7, 19 KD/pI 7.0, 20 KD/pI 6.5, 22 KD/pI 7.0, 23 KD/pI 6.7, 24 KD/pI 4.7, 24 KD/pI 5.8, 26 KD/pI

---

Table 3. Percentage of cells with various numbers of micronuclei in the root meristems treated with 2~10 \( \mu \text{M} \) APM*

| Concentration of APM (\( \mu \text{M} \)) | The number of Micronuclei per cell (per 1000 cells) | Percentage of cells with micronuclei |
|-----------------------------------------|-----------------------------------------------|-------------------------------------|
| 0                                       | 0 0 0 0 0 0                                | 0                                   |
| 2                                       | 1 0 1 0 0 0                                | 0.2                                 |
| 4                                       | 15 8 4 0 0                                 | 3.2                                 |
| 6                                       | 67 23 9 11 0                              | 11                                  |
| 8                                       | 57 49 37 27 0                             | 17                                  |
| 10                                      | 110 97 13 29 8 13                        | 27                                  |

*Data are based on a scoring of at least 1000 cells from root meristems per treatment.
Effect of APM on the Structure and Protein Composition of Chromosome in *Triticum durum*

Discussion

APM has been shown to be a specific chemical that directly interrupts with microtubule dynamics in plant cells (Morejohn et al., 1984), and cell division has been found to be blocked at the metaphase in root meristems treated with APM. Some authors indicated that a complete inhibition of the formation or fundation of the spindles was probably the cause of arrested metaphase (Ramulu et al., 1988a, b, 1990, 1991, 1993; Verhoeven, 1990, 1991). In the present study, many cells with multipolar spindles, metaphase chromosome condensation and micronuclei were observed in the root meristems of *T. durum* treated with APM. These effects of APM were thought to be directly related to the disturbance of spindle function by the treatment with APM. In the cells of higher plants, normal spindle function in metaphase through anaphase and cytokinesis at the end of telophase are two key steps in mitosis (Brahma et al., 1985); A metaphase of mitosis, chromosomes attach to the microtubules of the spindle and are oriented at the equatorial plate and the chromosomes are pulled toward the two poles by the spindles; then, two anaphase cells are formed. However, metaphase chromosomes in root meristem cells treated with APM are not arranged regular by along on the equatorial plate, due to the disturbed spindle function. Multipolar spindles were formed in the root meristems by APM treatment, but not the control.

The cells with multipolar spindles slightly increased with an increase in the concentration of APM. The percentage of cells with 3 and 4 polar spindles reached 10.5%, and 9.1%, respectively when root meristems were treated with 6 \( \mu \)M APM. Table 1 shows the number of cells with multipolar spindles in the root meristems treated with APM at various concentration. The number of cells with 3 or 4 polar spindle were reached 15% when treated with 10 \( \mu \)M APM. Chromosome condensation and bridge-fragment formation at metaphase were also observed after 4 \( \mu \)M APM treatment. Thus chromosome-condensation and bridge-fragment formation are related to the APM concentrations. In also the root meristems treated with APM, micronuclei was found only in a few when the concentration of APM, was less than 4 \( \mu \)M, but increased as the concentration increased, the number of micronuclei per cell varied from 1 to 5, but 6 micronuclei were observed in a few cells. Multipolar spindle formation, chromosome condensation and bridge-fragment formation were induced by at 4 \( \mu \)M APM. From this standpoint, 4 \( \mu \)M APM could induce the variation in the structure of metaphase chromosome in the root meristem cells of *Triticum durum*.

Phosphoric amide herbicides have been widely used in agriculture in China, although the induction or variation in the chromosome structure by the treatment with APM and other herbicides has been reported, by the experiments conducted using callus and culture cell lines (Verhoeven, 1990, 1991; Wan et al., 1991; Ramula et al., 1988a; Hoffman et al., 1994). In the present study, we used for the first time, root tip cells of *T. durum*, and found that treatment with 4 \( \mu \)M APM changed the chromosome structure in the root tip cells. This concentration may be a threshold for the action of APM under these experimental conditions.

To identify the changes in protein species induced by APM treatment in the root meristem, we analyzed the protein composition by 2 D SDS-PAGE after treatment with APM at various concentrations (2-10 \( \mu \)M) for
various periods (2–16 h). At least five protein species were lost and 12 new protein species induced by treatment with 10 μM APM for 16 h. In addition, the content of many protein species was increased by the treatment.

Up to now, the cytological and biochemical effects of APM on the cell division and protein composition of T. durum have not been reported in detail. In this study, we found that APM at 4 μM changed chromosome structure, and that, 10 μM APM changed the protein composition in root meristems. These concentrations are thought to be a threshold for each effect. Earlier studies have shown that tubulin assembly can be inhibited and mitotic spindles affected by the treatment with APM in plant cells (Morejohn et al., 1984), and that, abnormal cell mitotic and chromosome structure variation are induced by APM (Hoffman and Vaughn, 1994). In present study, however, the relationship between the chromosome behavior and protein composition, was not examined, and further cytological and biochemical studies concerning the inhibitory effect of APM on plant cells division are necessary.

Acknowledgment

We thank Dr. James Titchener (Hillary College in New Zealand) for critical reading of English manuscript. This work was supported by foundation for university key teacher by the ministry of education of P. R. China.

References

Brahma, B.P. and Umesh, K.S. 1983. Induction of abnormal spindle function and cytokinesis inhibition in mitotic cells of Allium cepa by the organophosphorus insecticide Fensulfothion. Cytobios 42 : 147–15.

Dolezel, J., Gihalkova, J. and Lucretti, S. 1992. A high-yield procedure for isolation of metaphase chromosomes from root tip of Vicia faba L. Planta 188 : 93–98.

Hoffman, J.C. and Vaughn, K.C. 1994. Mitotic disrupter herbicides act by a single mechanism but vary in efficacy. Protoplasma 179 : 16–25.

Kiermayer, O. and Fedtke, C. 1977. Strong anti-microtubule action of amiprophos-methyl (APM) in microtubules. Protoplasma 92 : 163–166.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227 : 680–683.

Morejohn, L.C. and Fosket, D.E. 1984. Inhibition of microtubule polymerization in vitro by the phosphonicamide herbicide amiprophos-methyl. Science 224 : 874–876.

O’Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250 : 4007–4021.

Pan, W.H., Houber, A. and Schlegel, R. 1993. Highly effective cell synchronization in plant roots by hydroxyurea and amiprophos-methyl or colchicine. Genome 36 : 387–390.

Peng, Y.K., Yu, J.C., Zhao, J., Song, W.Q. and Chen, R.Y. 1997. Induction of abnormal cell mitosis of root meristems of Triticum aestivum by amiprophos methyl. Chin. J. Appl. Environ. Bio. 13 : 204–207.

Peng, Y.K., Zhao, J. and Chen, R.Y. 1997. Studies on the metaphase chromosome proteins in Allium sativum. Acta Scientiarum Naturalium Universitatis Nankaiensis 30 : 56–60.

Peng, Y.K., Zhao, J. and Chen, R.Y. 1999. Induction of synchrony and isolation of metaphase chromosomes from mitotic root tip cells in Allium sativum. Bulletin of Botanical Research 19 : 302–3–7.

Peng, Y.K., Zhao, J. and Chen, R.Y. 1999. Biochemical analysis of nonhistone protein scaffold in metaphase chromosome of common wheat Triticum aestivum. Cytologia 64 : 229–239.

Ramulu, K.S., Verhoeven, H.A., Dijkhuis, P. and Gilissen, L.J.W. 1988a. Chromosome behaviour and formation of micronuclei after treatment of cell suspension cultures with amiprophos–methyl in various plant species. Plant Science 56 : 227–237.

Ramulu, K.S. and Dijkhuis, P. 1988b. Mitotic dynamics of micronuclei induced by amiprophos-methyl and prospects for chromosome-mediated gene transfer in plants. Theor. Appl. Genet. 75 : 375–384.

Ramulu, K.S., Verhoeven, H.A., Dijkhuis, P. and Gilissen, L.J.W. 1990. A comparison of APM-induced micronucleation and influence of some factors in various genotypes of potato and nicotiana. Plant Science 69 : 123–133.

Ramulu, K.S., Verhoeven, H.A. and Dijkhuis, P. 1991. Mitotic blocking micronucleation and chromosome doubling by oryzalin amiprophos-methyl and colchicine in potato. Protoplasma 160 : 65–75.

Ramulu, K.S., Dijkhuis, P., Fameraer, I., Cardi, T. and Verhoeven, H.A. 1993. Isolation of sub-diploid microprotoplasts for partial genome transfer in plants : Enhancement of micronucleation and enrichment of microprotoplasts with one or a few chromosomes. Planta 189 : 190–198.

Verhoeven, H.A., Ramulu, K.S. and Dijkhuis, P. 1990. Comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of Nicotiana plumbaginifolia. Planta 162 : 408–414.

Verhoeven, H.A. and Ramulu, K.S. 1991. Isolation and characterization of microprotoplasts from APM-treated suspension cells of Nicotiana plumbaginifolia. Ther. Appl. Genet. 82 : 346–352.

Wary, W., Boulikas, T., Wary, V.P. and Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. 118 : 197–203.

Wan, Y., Duncan, D.R., Rayburn, A.L., Petolino, J.F. and Withholm, J.M. 1991. The use of antimicrotubule herbicides for the production of doubled haploid plants from anther-derived maize callus. Theor. Appl. Genet. 81 : 205–211.