Scientific Reports of the Faculty of Agriculture
Okayama University Vol. 88, 57-63 (1999)

Partial Inhibition of Pollen Degradation by Gibberellic Acid in Male Sterile Tomato Mutants Derived from cv. First (*Lycopersicon esculentum* Mill.)

Youhui Ma, Kei Sakata and Masaharu Masuda
(Department of Eco-physiology for Crop Production)

The young flower buds in three male sterile mutants derived from cv. First were treated with gibberellins to determine whether pollen degradation could be inhibited. Microscopic observations of acetocarmine preparations revealed that the application of gibberellins inhibited pollen degradation for all tested mutants of Fms-1, Fms-2 and Fms-3 in which their breakdown in microsporogenesis occurs at microspore stage, during meiosis and at tetrad formation, respectively. The optimum concentration and treatment time were 10mg/l and 6-12days before flowering. GA_7 proved to be preferable to GA_3 for pollen fertility restoration. With the elongation of stamens by gibberellin application, viable pollens appeared frequently in Fms-2 mutant, being over 200 grains on a 0.5mm equatorial section of anthers in the best case, as compared to the other mutants. Bioassay using dwarf rice seedlings showed that the Fms-2 plants had lower levels of gibberellin in their anthers compared with normal ones.

Key words : tomato, male sterile mutant, gibberellin, fertility restoration

Introduction

While male sterile plants are used in hybrid seed production, one of the problems demanding consideration is how to propagate them. In several crops like rice or onion, the gene-cytoplasmic male sterility has been utilized for heterosis breeding through the system of *mst-mft* restorations[1]. In tomato, using the *ms*-10^{35} mutant in which genic male sterile gene is linked with a gene causing the absence of anthocyanin, F_1 hybrid seeds were produced in Belgium. Although most sterile plants with anthocyanin-less marker can be picked up from the population after crossing *msms* × *Msms* in the nursery, about 5% of fertile plants was unidentified and still remained in the production fields[2]. If *ms* homozygous mutants as female parent could be maintained by artificial restoration of pollen fertility, the marker for identifying heterozygotes or the maintainers would be not required. Pollen fertility in *ms* homozygous tomato mutant was restored partially under low temperature conditions[9], but only limited numbers of seeds were obtained by self-pollination.

On the other hand, regarding anther culture in tomato, Zamir *et al.* (1980)[15] demonstrated that *in vitro*-cultured anthers of a recessive male sterile mutant (*ms*-10^{35}) which blocks microsporogenesis at meiosis readily produced callus, while anthers of the isogenic fertile line rapidly degenerated in culture. A similar phenomenon was also observed in our previous experiments using the mutants originated from cv. First, and

Received October 1, 1998

the Fms-2 mutant in which the breakdown stage of microsporogenesis was earliest, specifically from pre-meiosis to metaphase I, proved to be the most favorable for callus induction in anther culture (Ma *et al.*, in press). However, such mutants are not advisable for the purpose of producing haploid plants, if their fertility can not be improved before excision of anthers from donor plants.

By the application of gibberellin, staminal development and pollen fertility restoration were obtained in a stamenless tomato mutant[6,7,11,13]. Also, fertility restoration by gibberellin treatments proved to be viable in other crops, such as barley or cosmos[3,10], in which each of them was a genic male sterile mutant. In previous studies, male sterile mutants originated from tomato cv. First by gamma irradiation have been classified into three types, depending on their breakdown stages in microsporogenesis[4,5]. To determine the effects of gibberellin types and concentrations on fertility restoration in these mutants, we examined the frequency of pollen-restored fertility by giberrellin treatment. The results as to the levels of endogenous gibberellic acid in Fms-2 are presented in comparison with original cv. First.

Materials and methods

Experiment 1: Effect of gibberellin on inhibition of pollen degradation in three male sterile tomato mutants

Three ms homozygous mutants, Fms-1, Fms-2 and Fms-3 were used in this experiment. They were characterized by the stages of microsporogenesis breakdown which occurred at the microspore stage, during meiosis and at tetrad formation for Fms-1, Fms-2 and Fms-3, respectively[5]. The plants were transplanted in pots and raised in a greenhouse from 29 April, 1995. Flower buds were treated with GA_3 or GA_{4+7} at concentrations of 1, 10 and 100 mg/l on 9 May, 1995. The longest anthers were excised at anthesis with in 0 -20 days after treatment. A cross section of 0.5 mm in length was made from the equatorial part of each anther examined and stained with a drop of acetocarmine. The stained pollens in each section were counted with the help of a microscope.

Experiment 2: Effect of gibberellin on stamen development and pollen fertility restoration in Fms-2 mutant

The plants of Fms-2 mutant were propagated by means of stem cuttings. Young flower buds of 2-3 mm in length were sprayed with 10 mg/l of GA_3 or GA_7 solution on 12 October, 1995. To make observation easy, the equatorial sections were made into 0.25 mm in length from the longest anthers after 6-12 days of treatment. The number of acetocarmine-stained pollen was counted.

Experiment 3: The relationship between stamen and pistil elongation, and number of stained pollen

The inflorescences in Fms-2 mutant were treated with GA_3 or GA_7 at 10 mg/l in autumn, 1995. The length of the longest and the shortest stamen in each flower was measured daily starting from the day of treatment in the order in which the flower came out. Then the equatorial sections were made into 0.25 mm lengths for counting the pollen stained by acetocarmine.

Experiment 4: Activity of gibberellin in flower buds of normal and male sterile plants

Flower buds about 4 mm in length were separately collected from the plants of Fms-2 mutant and cv. First for gibberellin extraction, being 4 g in fresh weight each. The stage of microsporogenesis was in tetrad to microspore for cv. First, while almost all pollen mather cells in Fms-2 mutant collapsed.

1. Extraction

The freezed samples were ground in an earthenware mortar with 40 ml of methanol. The homogenate was filtered under vacuum, then filtrates were concentrated at 40°C in a flash

evaporator. Ninety-ml of solution was made by pouring distilled water into the concentrate and was adjusted to pH 2.5. The solution was extracted three times with 30 ml of ethyl acetate each. Combined ethyl acetate fractions were further extracted three times with 0.5 M phosphoric acid buffer (pH 8.0) on equal volume with ethyl acetate fraction. The combined phosphoric acid buffer fractions (adjusted to pH 2.5) were re-extracted three times with ethyl acetate. Combined ethyl acetate fractions were dehydrated by using 2 g of anhydrous sodium sulfate for 2 h, filtered under vacuum, evaporated to dryness and stored at −18 °C.

2. Refinement

1) Refinement through polyvinyl polypropydone (PVPP)

Dried ethyl acetate extract in an eggplant-shaped flask was dissolved with 30 ml of 0.2 M phosphoric acid buffer (pH 8.0) and with 5 g of PVPP added, stirred and filtered under vacuum. The filtration was further repeated two times under vacuum, by adding 30 ml of 0.2 M phosphoric acid buffer each to the residue. The combined filtrates were acidified to pH 2.5, then extracted three times with ethyl acetate on equal volume with the filtrate. Combined ethyl acetate fractions were dehydrated for 2 h after adding 2 g of anhydrous sodium sulfate, filtered under vacuum and evaporated to dryness.

2) Refinement through Sep-Pak C18 cartridge

After washing a set of Sep-Pak C18 cartridges with 6 ml syringe twice with 4 ml of 100% methanol and once with 4 ml of distilled water, dried ethyl acetate extract dissolved in 5 ml of 80% methanol was placed in the set, further eluted three times with 5 ml of 80% methanol, followed three times with 2 ml of 100% methanol. The elutes that were equivalent to 2 g of samples were collected and evaporated to dryness at 40 °C.

3. Chromatography and bioassay

The dried extract was dissolved with a few drops of ethyl acetate, then chromatographed on Toyo No. 51 filter paper using a solvent of 2-propanol : 28% NH_4OH : water (10 : 1 : 1 v/v). Dwarf rice seeds (cv. Tanginbozu) were surface-sterilized in 10% antiformin for 15 minutes and washed in water, then germinated at 30 °C in the dark. Five seedlings were placed in each test tube on a sheet of filter paper that was divided equally into ten fractions, and moistened with 1 ml of test solution, GA_3 or GA_7 at concentrations of 0.01, 0.1 and 1.0 mg/l, or distilled water (controls). The test tubes with their seedlings were transferred to continuous light at 28 °C. After 5 days of incubation, the length of the secondary leaf sheath was measured.

Results

Experiment 1

Microscopic observations by acetocarmine-staining revealed that the restored anthers in all mutants tested contained normal pollen, after applications of both GA_3 and GA_{4+7} at any concentrations (Fig. 1). In an equatorial section (0.5 mm in thickness), the number of stained pollen was over 100 grains in the best case for Fms-2 mutant, higher as compared to Fms-1 or Fms-3 mutants which were both less than 50 grains. The optimum concentration was 10 mg/l both for GA_3 and for GA_{4+7}, and the GA_{4+7} treatments showed somewhat stronger influence than GA_3. Restoration of pollen fertility appeared on the stamens flowering 6-12 days after application of gibberellin.

Experiment 2

The inflorescences of vegetatively-propagated Fms-2 mutant plants were treated separately with GA_3 and GA_7 at 10 mg/l. The number of acetocarmine-stained pollen per 0.25 mm equatorial section of restored anthers was examined during the flowering, 6-12 days after treatment. Following the number of stained pollen, the observed anthers were placed into four groups, having 0, 1-100, 101-200 and >200 grains. The frequency of anthers having stained pollen was

higher both by GA_3 and GA_7 treatment than in the control. Anthers carrying over 200 stained pollen grains were also observed in the gibberellin treatments, and in this case GA_7 gave a higher frequency than GA_3 (Fig. 2).

Experiment 3

After application of GA_3 or GA_7, anthers with viable pollen developed in treated Fms-2 mutant plants (Fig. 3). In the case of the controls, the majority of the differences between stamen and pistil length were $-2 \sim -1$mm and no stained pollen was observed in these anthers. Although stained pollen appeared in the anthers whose length difference was 1~5mm, it was never

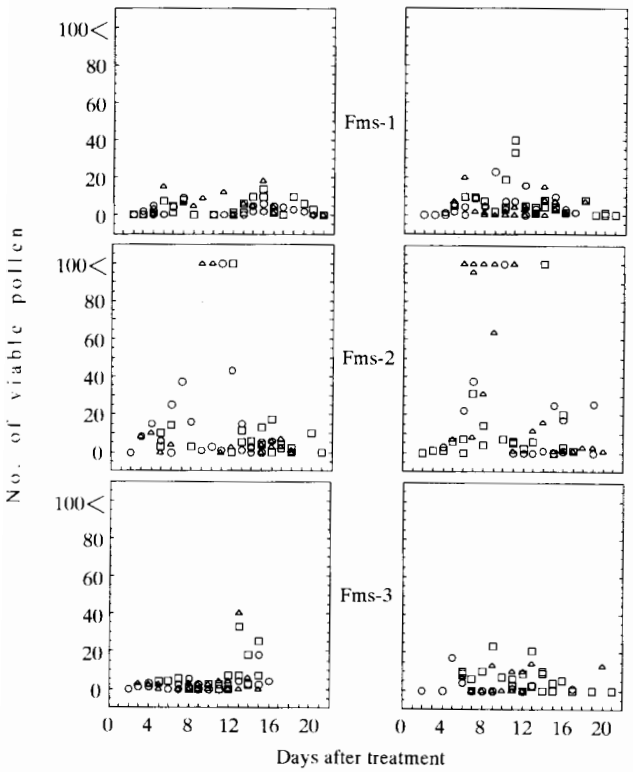

Fig. 1 Restoration of pollen fertility as affected by GA_3 (left) and GA_{4+7} (right) treatments at concentrations of 1 mg/l (○), 10mg/l (△) and 100mg/l (□) in three male sterile lines. Number of viable pollen per 0.5mm equatorial section of anthers was measured at anthesis. Hormones were applied on 9 May, 1995.

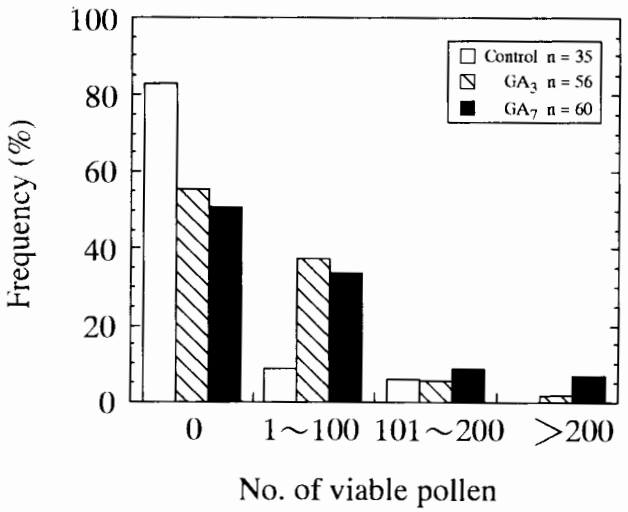

Fig. 2 Effects of GA_3 and GA_7 at a concentration of 10mg/l on restoration of pollen fertility in Fms-2 male sterile line. Number of viable pollen per 0.25mm equational section of anthers was measured on slide. Hormones were applied on 12 Oct., 1995. Investigation was carried out between 18 to 24 Oct., 1995.

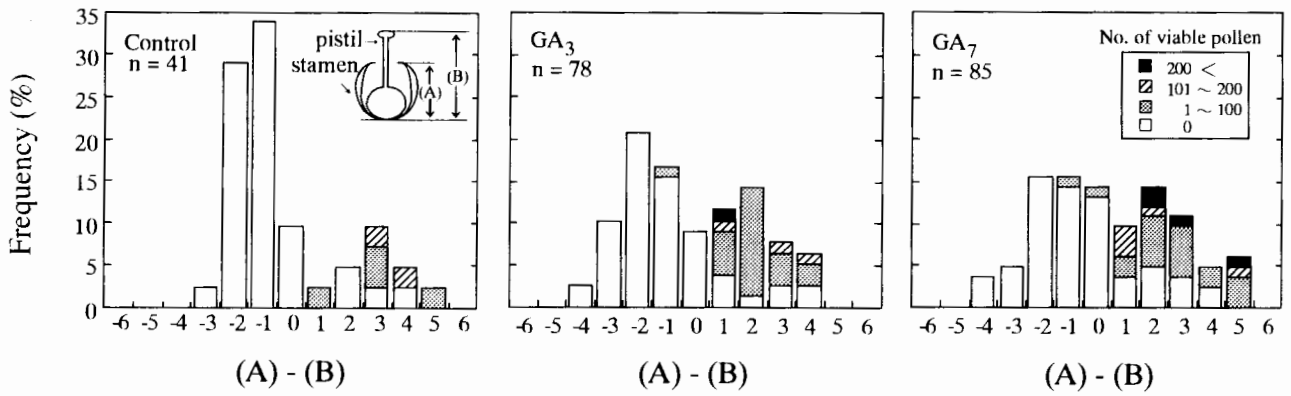

Fig. 3 Relationship between restoration of pollen fertility and difference in stamen (A) and pistil (B) length in Fms-2 male sterile line. Number of viable pollen per 0.25mm equatorial section of anthers was measured on slide. Hormones were applied at a concentration of 10mg/l.

greater than 128 grains even in the best instance. Whereas by applying gibberellins, the anthers whose difference between stamen and pistil length was over 1mm increased and the frequency of anthers containing stained pollen was higher than in the controls. Anthers carrying over 200 stained pollen grains were also observed in the gibberellin treatments, and GA_7 gave a higher frequency than GA_3, which was similar to the results obtained in Experiment 2.

Experiment 4

The ratio of length of the secondary leaf sheath in gibberellin treatment relative to control treatment is shown in Fig. 4, as activity of various fractions of extracts from the flower buds of cv. First and Fms-2 mutant. The fractions between Rf 0.2-0.4 and 0.3-0.4 showed stimulation of leaf sheath elongation both in normal and in sterile plants, and the stimulatory response was weaker in sterile plants than in the normal ones.

Discussion

This study demonstrated that the Fms-2 mutant was most prominent for restoration of pollen fertility by application of gibberellin, as compared to the other mutants. In stamenless-2 mutant, GA_3 application restored fertility and produced seeds after selfing[11]. In the present experiments, application of gibberellin enhanced stamen development and the number of stained pollen, but failed to set seeds by self pollination in all the mutants tested. In general, pollen stained by acetocarmine is regarded as viable pollen in normal plants. In the case of *ms* homozygous plants, it is necessary to distinguish either between acetocarmine-stained pollen and fertility-restored pollen or between pollen fertility and seed fertility. Several reports have suggested that the application of gibberellin may play a determinative part in restoration of pollen fertility in male sterile tomato mutants but the level of the restoration has never been evaluated[6,7,10,11,13]. To evaluate the effects of gibberellin treatment on pollen fertility restoration in this study, therefore, the pollen stained by acetocarmine was regarded as viable pollen. The floral morphological features in Fms-2 mutant are similar to those of sl-2/sl-2 mutant which has

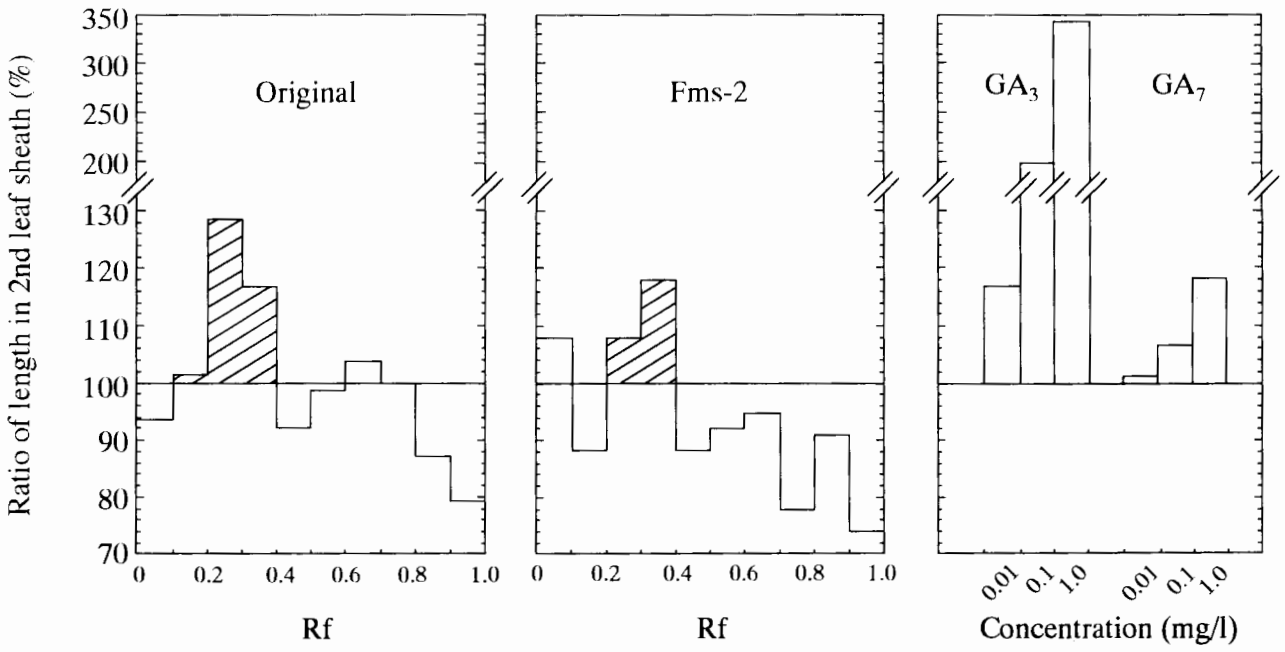

Fig. 4 Activity of gibberellin in the extracts of flower buds of original (cv. First) and Fms-2 mutant in dwarf rice bioassay.

been used as a main source of materials for the purpose of fertility restoration in *ms* homozygous tomato plants. However, their *ms* genes are not similar due to differences in the breakdown stages of microsporogenesis and the response to gibberellin types. In sl-2/sl-2, the breakdown occurs during tetrad formation[12] and GA_3 is favorable for fertility restoration[11]. Whereas in Fms-2, almost all the pollen mother cells degenerated before the completion of meiosis, and fertility restoration was higher by GA_7 or GA_{4+7} than by GA_3. In this study, GA_3 or GA_7 treatments led to the development of nearly normal anthers in Fms-2 mutant and pollen fertility restoration in these elongated anthers. These observations agreed with those obtained in sl-2/sl-2, *ms*-15 or *ms*-33 tomato mutants[7,11,13]. These results suggested that the restoration of pollen fertility was tightly correlated with the development of anthers and the elongation of anthers in *ms* homozygous plants can be considered as an indicator for pollen fertility.

The present study has shown that the activity of GA_3 in flower buds of Fms-2 mutant was much lower than in that of cv. First. This agreed with the results of other authors, who found that either in flowers or leaves of sl-2/sl-2 mutant, GA_3 activity was low[8] and IAA activity was high[14]. Furthermore, under relatively lower temperature conditions the activity of endogenous gibberellin was elevated in many crops. When the plants of sl-2/sl-2 mutant grew at low temperature, they also became partially fertile and showed higher activity of endogenous gibberellin. These observations suggested that the effect of exogenous gibberellin on pollen fertility restoration in ms homozygous mutants may be through changes in endogenous gibberellin levels. Besides, we have also observed that fertility in ms homozygous plants may be promoted by nitrate fertilizer under low temperature conditions. That suggested that the nutritional status of plants may be one of the factors influencing pollen fertility. Therefore, the interaction between gibberellin treatment and other factors with regard to the possibility of seed fertility restoration will need to be further investigated.

Acknowledgment

This work was supported in part by a grant for the specific research "The study of the development of organisms effective to environmental conservation for human life" at Okayama University in 1998-1999.

References

1) Ikehashi, H. : Hybrid rice breeding between Japonica and Indica type by the use of photo-period-sensitive genic male sterility. The Heredity, **42**(5), 1-11 (1998), (in Japanese)

2) Georgiev, H. : Heterosis in tomato breeding. in Genetic Improvement of Tomato (Kalloo, G. ed.), pp. 83-98, Springer-Verlag, Berlin Heidelberg (1991)

3) Kasembe, J. N. R. : Phenotypic restoration of fertility in a male sterile mutant by treatment with gibberellic acid. Nature, **215**, 668 (1967)

4) Masuda, M., T. Furuichi and Y. Takeda : Mutation spectrum of tomato, cv. First, induced by seed radiation with gamma-rays, and the subsequent partial chlorophyll deficiency. J. Japan Soc. Hort. Sci., **67**, 583-588 (1998a), (in Japanese with English summary)

5) Masuda, M., T. Furuichi, Y. Ma and K. Kato : Pollen degradation and inheritance of male sterility in three mutants in tomato, *Lycopersicon esculentum* Mill., cv. First. J. Japan Soc. Hort. Sci., **67**, 93-98 (1998b), (in Japanese with English summary)

6) Phatak, S. C., S. H. Wittwer, S. Honma and M. J. Bukovac : Gibberellin-induced anther and pollen development in a stamenless tomato mutant. Nature, **209**, 635-636 (1966)

7) Rastogi, R. and V. K. Sawhney : Flower culture of a male sterile stamenless-2 mutant of tomato (*Lycopersicon esculentum* Mill.). Amer. J. Bot., **75**, 513-518 (1988)

8) Sawhney, V. K. : Morphogenesis of the stamenless-2 mutant in tomato. 4. Relative levels of gibberellins in the normal and mutant plants. J. Exp. Bot., **25**, 1004-1009 (1974)

9) Sawhney, V. K. : Temperature control of male sterility in a tomato mutant. J. Hered., **74**, 51-54 (1983)

10) Sawhney, V. K. and A. Shukla : Male sterility in flowering plants: Are plant growth substances involved? Amer. J. Bot., **81**, 1640-1647 (1994)
11) Sawhney, V. K. and R. I. Greyson : Morphogenesis of the stamenless-2 mutant in tomato. 2. Comparative description of the flowers and ontogeny of stamens in the normal and mutant plants. Amer. J. Bot., **60**, 514-523 (1973)
12) Sawhney, V. K. and S. K. Bhadula : Microsporogenesis in the normal and male-sterile stamenless-2 mutant of tomato (*Lycopersicon esculentum*). Can. J. Bot., **66**, 2013-2021 (1988)
13) Schmidt, H. and V. Schmidt : Untersuchungen an pollensterilen, stamenless-ähnlichen mutanten von tomato *Lycopersicon esculentum* Mill. II. Normalisierung von *ms*-15 und *ms*-33 mit gibberellin-säure (GA_3). Biol. Zbl., **100**, 691-696 (1981)
14) Singh, S., V. K. Sawhney and D. W. Pearce : Temperature effects on endogenous indole-3-acetic acid levels in leaves and stamens of the normal and male sterile 'stamenless-2' mutant of tomato (*Lycopersicon esculentum* Mill.). Plant Cell and Environment, **15**, 373-377 (1992)
15) Zamir, D., R. A. Jones and N. Kedar : Anther culture of male sterile tomato (*Lycopersicon esculentum* Mill.) mutants. Plant Sci. Lett., **17**, 353-361 (1980)

トマト'ファースト'の雄性不稔突然変異系統における花粉崩壊のジベレリンによる部分的阻害

馬　有会・坂田　圭・桝田　正治
（作物機能調節学講座）

トマト'ファースト'より作出した雄性不稔系統において，その花粉崩壊がジベレリンによって阻害されるかどうかを検討した．ジベレリンの阻害効果は花粉崩壊が小胞子期に始まるFms-1タイプ，減数分裂期に始まるFms-2タイプ，四分子期に始まるFms-3タイプのいずれにおいても認められた．最適濃度は10mg/ℓで散布後6日から12日目に開花してくる葯で認められた．この場合，GA_3よりもGA_7の方が効果は高かった．開花時の葯0.25mm横断切片上のアセトカーミン染色花粉（生存花粉）はFms-2で最も多く200粒以上を示すものもあった．Fms-2花粉の生存には葯の萎縮回復が伴っていた．Fms-2葯のジベレリン活性はオリジナル正常葯のそれより低かった．