Cyto-histological studies on the male sterility of Satsuma orange (*Citrus unshiu* MARC.)

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Summary

In order to determine the cause of pollen abortion in male-sterile Satsuma orange, certain morphological and cyto-histological studies with anthers were undertaken as compared with the male-fertile Buntan and Natsudaikai. Cyto-histological observations on Satsuma orange treated with temperature and IAA were also carried out.

1. In the early stage of anther development, meiosis and microspore formation were quite similar in fertile and sterile species of *Citrus*.

2. Tapetum in fertile species started to degenerate soon after the liberation of microspores from the wall of tetrads and almost completely degenerated during the disappearance of vacuoles from the cytoplasm of microspore.

3. Tapetum in sterile Satsuma orange occasionally

showed an extraordinary development of large vacuole at the vacuole appearing stage and completely disintegrated at the vacuole enlarging stage of microspore. However, tapetum which remained cellular without large vacuoles persisted unusually long and degenerated at the vacuole enlarging stage of microspore. So it may be suggested that the tapetum in sterile Satsuma orange can not play normal nutritive role and supplies less nutrients to the developing pollens.

4. Abnormal behavior of tapetum in Satsuma orange could be recovered partially and the pollen fertility could be increased by temperature (at 20°C minimum temperature) and IAA treatments (500 ppm) to those plants growing under unfavorable temperature condition.

Introduction

It has been generally considered that tapetum in anther is a nutritive tissue to feed microspores^(4,5,6,9,10,19,20,27,28). In case of the occurrence of abnormality in tapetum, the pollen formation may be disturbed during its development and ultimately abortive pollen will be produced. The abortion of pollen caused by the abnormality of tapetum behavior has been reported in many field crops^(1,2,3,6,8,12,15,16,26,27) as well as in vegetable crops^(7,10,17,18,23,24,29,30,34), but that in fruit trees are relatively few^(4,5,9,14,21,25,33). It has been confirmed that the degenerative period of tapetum cells of Satsuma orange is the vacuole enlarging stage of microspore⁽³³⁾, however, the mechanism of pollen abortion is not yet fully understood. It can be seen that in certain warmer subtropical region of Taiwan, either in glass-house or in the temperature controlled chamber (above 15°C), and when treated with IAA at the young bud stage, the pollen fertility of Satsuma orange could be increased and ultimately produced the seeded fruits^(11,18,22,31,32).

In order to understand the relationship between the degeneration of tapetal cells and of microspores and pollen grains, a comparative study of microsporogenesis and development of male gametophyte in both male-fertile and male-sterile species of *Citrus* plants were carried out. Furthermore, the trees of male-sterile Satsuma orange grown in the temperature controlled glass-house and treated with IAA under natural condition were also investigated to make sure the mechanism of pollen abortion.

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Materials and Methods

Two male-fertile species, Buntan (*Citrus grandis* OSBECK) and Natsudaikai (*C. natsudaikai* HAYATA), and one male-sterile species, Satsuma orange (*C. unshiu* MARC.), were used for this study. All these trees were 25-30 years old, and were growing in the *Citrus* Orchard of Osaka Agriculture Research Center, Osaka Prefecture.

In addition, 5-year-old Satsuma orange trees, each planted in an unglazed pot of 60 cm in diameter, were used for the temperature treatment. Three of them were transferred to the glass-house in which the minimum temperature was controlled at 20°C during the experiment from March 10 to March 30, 1969.

Furthermore, young flower buds about 3 mm in diameter, under field condition, were treated with IAA solution of 500 ppm containing 0.005 % Tween-20.

Six flower buds in each plot were collected at 1-2 days intervals from young to flowering stages and fixed in a solution of one part glacial acetic acid and three parts absolute ethyl alcohol and then preserved in 70% alcohol.

For the preparation of microtome section, usual method of processing in ethanol-xylene dehydration series and embedding in paraffin were employed. Sections of 10-13 μ in thickness were made and stained by Heidenhain's iron-alum hematoxylin procedure. The development of pollen grains and pollen fertility were examined by the smear method with 45% aceto-carmine according to the method of NAKAMURA⁽²²⁾. Pollen grains showing deep staining with aceto-carmine were determined as functional and viable ones, and showing no staining or weak staining were determined as abortive ones.

Observations

The differentiation and development of anthers in male-sterile Satsuma orange were essentially similar to the male-fertile Buntan and Natsudaikai. The anther wall of Buntan and Natsudaikai consists of four parts: an epidermis, two or three endothelial layers, two or three middle layers and a tapetum. The tapetal cells were initially uninucleated. During the course of meiosis in pollen mother-cells (PMCs), the tapetal cells continued to increase in size and contained four or more nuclei which generally varied in size. At the stage of pollen tetrads, not only the tapetal cells increased in size, but also the nuclei of tapetal cells increased in number, and ultimately the tapetal cells became in polynucleated state (Fig. 1). Soon after the liberation of microspores from the wall of tetrads, the nuclei of tapetal cells underwent chromatolysis and decreased in number and remained one or two in cytoplasm. Tapetum at this time became irregular in shape and showed neither any vacuole nor any abnormal behavior, and started to degenerate (Fig. 2 and Table 1). The gradual degeneration of tapetum was always associated with the development of microspores (Figs. 3 and 4). The degeneration of tapetum was almost completed and the tapetum lost all their contents when the vacuole disappeared from the cytoplasm of microspores (Fig. 5 and Table 1). Finally, in anther loculi the tapetum completely disappeared and pollen grains grew completely, showing a rich content of viable pollen grains, after the pollen mitosis stage (Fig. 6). At the time just before anther dehiscence, the male-fertile Buntan and Natsudaikai showed very high fertility, i.e. about 90 and 75%, respectively.

Fig. 1~6. Normal microsporogenesis in male-fertile species of Citrus. $\times 1200$.

Fig. 1. Polynucleated tapetum at the tetrad stage in Buntan.

Fig. 2. Degenerating tapetum soon after the liberation of microspores from the wall of tetrads in Natsudaiddai.

Fig. 3. Degenerating tapetum at the vacuole appearing stage in Buntan.

Fig. 4. Tapetum almost completely degenerated at the vacuole enlarging stage in Natsudaiddai.

Fig. 5. A tracing tapetum in loculus at the vacuole disappearing stage in Natsudaiddai.

Fig. 6. Tapetum completely degenerated after the pollen mitosis stage in Natsudaiddai.

Fig. 7. Degenerating tapetum at the vacuole disappearing stage in Satsuma orange. $\times 300$.

Fig. 8. Tapetum completely disappeared at the young pollen grain stage in Satsuma orange. $\times 300$.

Fig. 9. Tapetum completely disappeared at the young pollen grain stage in Natsudaiddai. $\times 300$.

Figs. 10~18. Abnormal tapetum in male-sterile Satsuma orange. $\times 1200$.

Fig. 10. Cellular tapetum soon after the liberation of microspores from the wall of tetrads (temperature treatment above 20°C).

Fig. 11. Cellular tapetum at the vacuole appearing stage of microspore (temperature treatment-above 20°C).

Fig. 12. Degenerating tapetum at the vacuole enlarging stage (outdoors).

Fig. 13. Degenerating tapetum at the vacuole enlarging stage of microspore (temperature treatment-above 20°C).

Fig. 14. Degenerating tapetum at the beginning of vacuole disappearance from the cytoplasm of microspore (outdoors).

Fig. 15. Tapetum completely degenerated after the pollen mitosis stage (outdoors).

Fig. 16. Vacuous tapetum soon after the liberation of microspores from the wall of tetrads (outdoors).

Fig. 17. Large vacuolous tapetum at the vacuole appearing stage of microspore (outdoors).

Fig. 18. Tapetum completely disintegrated at lost their contents at the vacuole enlarging stage of microspore (outdoors).

MS : microspores, PG : pollen grains, PT : pollen tetrads, V : vacuoles, T : tapetum.

Table 1. Relationship between the degenerating stage of tapetum and the developing stage of pollen grains in *Citrus* species

Species	Degenerating stage of tapetum			
	Beginning	Middle	Late	Consummation
<i>C. grandis</i>	I	II - III	IV	VI
<i>C. natsudaoidai</i>	I	II - III	IV	VI
<i>C. unshiu</i> *	III	III - IV	V	VI

* Only the cellular tapetum is represented here.

I, II.... Showing the respective stages of pollen grains development.

I. Soon after the liberation of microspores from the wall of pollen tetrads.

II. Vacuole appearing stage.

III. Vacuole enlarging stage.

IV. Vacuole disappearing stage.

V. Pollen mitosis stage.

VI. Young pollen grain stage.

found in these anther loculi. In a few anthers, however, the tapetum remained cellular and formed a narrow line and the cytoplasm of the cell took a deep stain with hematoxylin. This tapetum always showed no vacuoles and persisted unusually long even till the vacuole enlarging stage of microspore and then started to degenerate (Fig. 12). The degenerating time of tapetum was too late to develop the microspores. In consequence, most of the vacuous starveling microspores could not absorb the nutrient from the tapetum on time, and became abortive pollens. During the vacuole disappearing stage, most of the tapetum still persisted and showed incomplete degeneration (Figs. 7 and 14). The tapetum, then, almost completely disintegrated after the vacuole disappearing stage and disappeared at the young pollen grain stage (Fig. 15). In this case, a few viable pollen grains appeared in anther locule, and it showed very low fertility as about 15% at anthesis (Fig. 8).

In the cyto-histological studies on the development of tapetum and PMCs and on the course of meiosis, there were no significant differences among IAA- or temperature-treated and untreated Satsuma orange before microspore formation. The major differences, however, between treated and untreated were that the number of cellular type of tapetum without large vacuole was increased, and more repletion of cytoplasm in tapetum showing deep staining was observed

In male-sterile Satsuma orange, the development and degeneration of tapetum and pollen grains have been reported in our previous study⁽³³⁾. The present paper describes herein particular abnormal behavior of tapetum after the microspore formation. Soon after the liberation of microspores from the wall of tetrads, vacuoles began to appear in cytoplasm of tapetal cells in many anther loculi (Fig. 16). These vacuoles of tapetal cells rapidly increased in size (Fig. 17) and then the tapetum with large vacuoles collapsed during the vacuole enlarging stage of microspores. No fertile pollen could be

Table 2. Effect of temperature and IAA treatments on the number of normal and abnormal anther loculi at the vacuole enlarging stage of microspore and the pollen fertility at anthesis

Treatment	No. of loculi observed	No. of normal loculi	No. of loculi		Degree of staining cytoplasm in tapetum	Pollen fertility (%)
			With large vacuoli in tapetum	Without large vacuoli in tapetum		
Control (outdoors)	480	45	372	63	+	14.5
Temperature (above 20°C)	"	49	278	153	++	39.8
IAA (500 ppm)	"	52	266	162	++	42.3

in the treated Satsuma orange (Table 2, and Figs. 10 and 11). The deep-stained, persisted tapetum in the treated Satsuma orange started to degenerate at the vacuole enlarging stage (Fig. 13) and the degeneration of tapetum was mostly completed at the vacuole disappearing stage of microspore. Ultimately, the tapetum completely degenerated and disappeared, and many pollen grains grew fully in anther loculi after the pollen mitosis stage. At anthesis, the pollen fertility was markedly increased by the temperature and IAA treatments as about 39.8%, and 42.3% respectively (Table 2).

Discussion

A comparative study of anther development between the male-fertile Buntan and Natsudaïdai and male-sterile Satsuma orange showed large differences in the behavior of microspores and tapetal cells from the stage of microspore formation to anthesis. The tapetum of most anther loculi, however, developed fully and PMCs normally passed through the course of meiosis and formed pollen tetrads.

OSAWA⁽²⁵⁾ in Washington Navel orange and Satsuma orange, and IWAMASA⁽¹⁴⁾ in the male-sterile hybrids between Satsuma orange and trifoliate orange reported that the tapetal cells showed abnormal behavior and disintegrated during the early resting stage of meiosis in PMCs, and then the PMCs degenerated after this stage. It was shown from the result of the present study, however, that the degeneration of tapetum in male-sterile anther occurred at the vacuole enlarging stage and in male-fertile anther it occurred soon after the liberation of microspores from the wall of tetrads.

During the vacuole appearing stage of microspore, an extraordinary development of large vacuoles occurred in tapetum of most of the anther loculi of Satsuma orange, but it was not found in tapetum of Buntan and Natsudaïdai. ARTSCHWAGER⁽²⁾ described that the large vacuoles may be the places of storage for the metabolic waste products, and the degeneration of microspores may be a sudden release of metabolic waste products from the large vacuole due to the disintegration of periplasmodium. In general, the hypertrophic occurrence and periplasmodium formation in tapetum are characteristic for male-sterile anthers^(2,7,10,12,17,18,21,24,27,29,34). However, these phenomena could not be observed in Satsuma orange except the large vacuoles in tapetum. On the other hand, the unbalanced timing between the development of microspores and the process of degeneration of tapetum is also very common feature in male-sterile anthers. In this case, the microspore abortion is due to the unusually long persistence of tapetum after the development of the microspores into pollen grain^(1,6,10,15,17,23,24,27), or it is due to the very rapid degeneration of tapetum and sudden loss of their cytoplasm during the stage of pollen maturation⁽⁸⁾. The similar result was observed by the present authors in male-sterile Satsuma orange; that is, the tapetum of few anther remained cellular persisted long even till the vacuole enlarging stage of microspore and then degenerated. On the contrary the tapetum of male-fertile Buntan and Natsudaïdai degenerated gradually soon after the liberation of microspores from the wall of tetrads. It was in accordance with the observation in male-fertile anthers of many crops by many workers^(6,10,14,16,24). Therefore, the cause of pollen abortion in Satsuma orange can be considered in the light of two possible explanations based on the behavior of tapetum. First, the extraordinary development of large vacuoles occurs in tapetal cells during

the vacuole appearing stage of microspores, and the tapetum does not play normal nutritive role. Secondly, the tapetum persists long even till the vacuole enlarging stage of microspores. So the young starveling microspores can not absorb the nutrient from the tapetum on time and become abortive pollens.

The degeneration of pollen in anther associated with abnormal behavior of the tapetum in many crops may be caused by the unfavorable environment which has been reported by SAKAI⁽²⁶⁾ and FUJISHITA⁽¹⁰⁾. In case of Satsuma orange, growing in the temperature controlled glass-house (above 20 °C) during winter, the percentage of fertility was increased and the number of deep-stained tapetum without large vacuoles remaining cellular was increased as compared with those under the natural condition. A similar trend was obtained when the young buds were treated with IAA.

As above stated, the pollen fertility at anthesis remarkably recovered in both treatments may be due to the increase in number of cellular type of tapetum, the repletion of cytoplasm in tapetum resulting more deep staining and the complete degeneration of tapetum. These facts suggest that the tapetum can play relatively normal nutritive role and supplies more nutrients to the developing pollens.

From the basis of the observation on the fertile and sterile species of *Citrus*, it can be concluded that the degeneration of microspores in male-sterile Satsuma orange is caused by the insufficient supply of essential nutrients, ascribing the abnormal behavior of tapetum. It may be also said that such abnormal behavior of tapetum can be recovered partially by the temperature or IAA treatments to those plants growing under unfavorable temperature condition.

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温州ミカンの雄性不稔に関する細胞組織学的研究

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摘 要

温州ミカンの花粉退化の原因を追求するため、葯および花粉の発育に伴う、形態学的、細胞組織学的な研究を行なった。なお稔性種文旦および夏ミカンを用いてその差異を比較するとともに、温州ミカンの温度処理および IAA 処理についても同様の調査をした。

1. 葯発育の初期では、花粉母細胞の減数分裂および小孢子形成のいずれにおいても、稔性、不稔性種の間に差異は認められなかった。

2. 稔性種のタペート細胞は、4 分子から小孢子が遊離した直後より退化しはじめ、小孢子の細胞質から液胞が消失する時期までに完全に退化してしまう。

3. 不稔性の温州ミカンのタペート細胞は、小孢子の液胞発現期に、異常に拡大した液胞を生ずることがあり、

その後、小孢子の液胞拡大期には完全に崩壊した。しかし、細胞状のままで大きな液胞をもたないタペート細胞は、通常はそのままの状態で崩壊せず、小孢子の液胞拡大期になつて始めて退化した。従つてこのような場合、タペート細胞は花粉発育のための栄養源としての働きをせず、また花粉に栄養を与えるとしても量的に少ないと思われる。

4. このような温州ミカンのタペート細胞の異常は、温度処理 (1969 年 3 月 10 日より 30 日まで、最低温度 20°C)、または IAA 処理 (露地、花蕾の直径約 3 mm, 500 pp) を行なうことにより、ある程度まで回復し、花粉稔性を高めることができた。