Failure of Fertilization Associated with Absence of Zygote Development in the Interspecific Cross of *Fagopyrum tataricum* × *F. esculentum*

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To obtain a desirable hybrid from an interspecific cross between *Fagopyrum tataricum* and *F. esculentum*, the early embryo developmental changes were investigated by light microscopy and transmission electron microscopy (TEM). The nature of pre- and post-fertilization barriers in the hybrid embryo was examined at the ultrastructural level. The ovules were excised at 1 to 5 days after pollination (DAP) and fixed for TEM. In comparison with self-pollinated embryo (*F. tataricum*) or legitimate-pollinated embryo (*F. esculentum*), the interspecific hybrid embryos showed numerous abnormal ultrastructural pre- and post-fertilization phenomena leading to the abortion of embryo. The main abnormal phenomena were failure of fertilization, no zygotic development with a broken cell wall, dispersed endosperm with absence of ribosomes, and defective embryo sac development such as srunkun, small and irregular-shaped embryo sac. The interspecific incompatibility was attributed to the failure of fertilization and the hybrid zygotic embryo showed retardation in cell division, which resulted in cease of development.

Key Words: *Fagopyrum tataricum*, *F. esculentum*, interspecific cross, failure of fertilization, abnormal ultrastructure.

Introduction

Common buckwheat (*Fagopyrum esculentum*), a so-called dictyledonous "cereal", is a self-incompatible species belonging to the Polygonaceae. It possesses a high quality protein (Eggum 1980). However, its low and very unstable seed yield results from dimorphic heterostyly and consequent failure of fertilization (Adachi 1986). Tartary buckwheat (*F. tataricum*) has been used as a parent in interspecific crosses for transferring its self-compatibility, higher seed yield, frost resistance and plant vigour to the common buckwheat (Adachi 1990, Sammimy 1991, Campbell 1995). Attempts to interspecifically hybridize common

buckwheat with tartary buckwheat conventionally have not been successful (Ruszkowski 1980, Adachi *et al.* 1989, Sammimy 1991). Application of protoplast fusion (Lachmann 1991) has not been successful either. Some progress in the understanding of interspecific hybridization barriers has been recently made (Hirose *et al.* 1994, Lee *et al.* 1994, Tsai and Adachi 1994). However, an interspecific hybrid between *F. esculentum* and *F. tataricum* has not yet been achieved (Tsai 1994). In general, it is difficult to obtain interspecific hybrids in buckwheat through conventional techniques due to the presence of strong pre- and post-fertilization barriers. Sammimy *et al.* (1996) developed an infertile plant. Wagatsuma and Un-no (1995) reported the first fertile hybrid from the cross of the diploid *F. esculentum* and *F. tataricum*. The F₁ hybrid of *F. esculentum* and *F. tataricum* proved functionally sterile or had embryo abortion (Wang and Campbell 1998). The nature of the barriers at pre- and post-fertilization stages, among the different species of the genus *fagopyrum* is still largely unknown. The details of interspecific cross compatibility with the pollen tube growth (Hirose *et al.* 1994), and in the early stages of development of the embryo (Adachi *et al.* 1983, Guan and Adachi 1997) are becoming clear through light and electron microscopic studies. Two types of sterility were observed in the embryo sac by using Nomarski's differential interference contrast microscopy, namely (1) enervative sterility of the embryo sac before fertilization (Guan and Adachi 1992a, Guan and Adachi 1994) and (2) abortive sterility of the embryo (Guan and Adachi 1992a, Guan and Adachi 1992b). It is, therefore, apparent that the barriers of interspecific hybridization must be overcome to obtain a hybrid. This, however, requires an understanding of the nature of the barriers. A detailed ultrastructural study was, therefore, undertaken to reveal the pattern of failure of fertilization associated with retarded zygote development, and the nature of post fertilization barriers as well as the degeneration of endosperm and embryo in the interspecific crosses in *Fagopyrum*.

Materials and Methods

Interspecific cross pollination

Fagopyrum esculentum cv. Miyazakizairai (2X) and *F. tataricum* Gaertn. (2X) (Nagatomo 1965) were grown in a phytotron with a 14-hr day/10-hr. night cycle at a temperature of 25°C by day and 20°C by night. The bud pollination

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technique was used to cross *F. esculentum* cv. Miyazakizairai with *F. tataricum*, using the latter as female parent. The self-compatible homostylous species (*F. tataricum*) was carefully emasculated at one day before anthesis. Then tartary buckwheat was pollinated by the pollen grains from newly opened flowers of common buckwheat. The pollinated flowers were bagged to be isolated from foreign pollen grains. Surplus buds and open flowers were removed to prevent accidental self-pollination. As controls, legitimate pollination was performed for the heterostylous species *F. esculentum*, and self-pollination for *F. tataricum*.

Light and transmission electron microscopy

The ovules were excised from the experimental flowers at 1-5 DAP and fixed with 4% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer of pH 7.2 for 5 hr at room temperature. Then the ovules were post-fixed with 1% osmium tetroxide solution in the same buffer at 4°C for 5 hr. After dehydration in a graded ethanol series, ovules were embedded in Spurr resin. Semithin and ultrathin sections were cut on an ultramicrotome (RMC MT-7000, Reichert Ultramicrotome). For observation by light microscopy, sections were made approximately 1.5 µm thick and stained with toluidine blue. Ultrathin sections of 70-80 nm were double stained with uranyl acetate and lead citrate, and then observed under a Hitachi H-800 MV transmission electron microscope at 75 kV.

Results and Discussion

The normal ultrastructural features are presented in Fig. 1. At 1 DAP, the micropylar end of the long and pear-shaped zygote was narrower than its chalazal end and contained a large vacuole while most of the cytoplasm and cell organelles were located at the chalazal pole, as was the nucleus which showed a distinct polarity. The zygote was surrounded by a cell wall, which was thick at the micropylar pole and gradually reduced in thickness towards the chalazal region, where only a plasma membrane was present. Around the perinuclear cytoplasm there were a number of rod-shaped, spherical or oval-shaped mitochondria with well-developed cristae. The cytoplasm also contained polymorphic plastids, with or without starch grains. Many vacuoles of different sizes were found in the chalazal region of the zygote. Abundant single cisternae or clusters of rough endoplasmic reticulum (RER) were distributed near the nucleus in parallel to the cell wall. Ribosomes were freely dispersed in the cytoplasm or attached to the ER. Numerous small lipid bodies and relatively few dictyosomes were also present in the cytoplasm. The rich cytoplasm indicates normal physiological activities and a high rate of metabolism, which is a prerequisite nutrient source for double fertilization and endosperm development. The nucellar cells attached around the embryo sac were filled with dense cytoplasm. The mitochondria of cell cytoplasm were spherical or rod-shaped. A large number of plastids with and without starch grains and

Fig. 1. Longitudinal section through the micropylar part of the embryo sac at 1 day after self pollination of *F. tataricum* showing the zygote (Z), part of endosperm (End) and nucellus (NC). Plasma membrane (arrows). Bar = 100 µm.

few small vacuoles were distributed throughout the cytoplasm. Dictyosomes and RER were abundant.

Failure of fertilization

In the cross between *F. tataricum* and *F. esculentum* at 1 DAP, the cytoplasm of both synergids was degenerated, electron dense and appeared irregular in shape although the cell outline was completed (Fig. 2a & b). The cell wall between the two synergids was still normal on the micropylar side but highly amplified on the chalazal side. In the degenerated synergid cells the cytoplasm showed some ultrastructural abnormalities. Most of the organelles appeared to be aggregated (Fig. 2a). Many irregularly shaped mitochondria were also seen to be aggregated and the mitochondrial cristae had almost disappeared. The degenerated cytoplasm of both synergids became electron translucent and ribosomes were not dispersed uniformly. The membranes of the mitochondria and plastids were darkly stained. The degeneration was accompanied by the electron dense deposition of osmiophilic substances on the membranes of the mitochondria and other organelles observed in the cytoplasm. Most of the organelles in the degenerated cytoplasm could not be identified.

The egg cell attached to this embryo sac wall near the micropyle was still not fertilized (Fig. 2b). It showed characteristics of distinct polarity similar to a normal egg cell. At the chalazal part of the egg cell, the cytoplasm contained a single nucleus, polymorphic mitochondria and plastids, extensive single cisternae of RER and dictyosomes. A large

Fig. 2. Electron micrographs (a & b) of an embryo sac (*F. tataricum* $\times$ *F. esculentum*) at 1 DAP, showing two degenerated synergids (dSy) and a unfertilized egg cell (EC). CW = cell wall. a, Bar = 200 μ m, b, Bar = 100 μ m.

number of vacuoles of different sizes were found in this region. Ribosomes were freely dispersed or attached to the RER. Lipid bodies and plastids with starch grains observed in the cytoplasm were also smaller amount than that of the normal zygote of 1 DAP (Fig. 1). The egg cell was partially surrounded by a cell wall, which was highly amplified in some areas and indicated an abnormal phenomena (Fig. 2b). In this embryo sac, the central cell occupied the largest portion with a large vacuole and most of its cytoplasm was occupied by a fusion nucleus with rich organella (Fig. 3).

Degeneration of both synergids has also been reported in *Triticum aestivum* (You and Jensen 1985) and *Brassica campestris* (Van Went and Cresti 1988) before pollen tube penetration in to stylopodium. Maeda and Maeda (1990) illustrated a rapidly collapsing plasma membrane of a degenerating synergid after the arrival of the pollen tube. In many plants, like *Gosypium hirsutum* (Jensen and Fisher 1968) and *Crepis capillaries* (Kuroiwa 1989), the degeneration of one synergid has been observed prior to pollen tube penetration into ovules. Van Went and Cresti (1988) proposed that synergid degeneration may play a major role in

Fig. 3. Longitudinal section through an embryo sac (*F. tataricum* $\times$ *F. esculentum*) at 1 DAP. The central cell (CC) contains cytoplasm rich in organelles. M = mitochondria, P = plastid, V = vacuole. Bar = 100 μ m.

the process of pollen tube penetration and the discharge of its contents. In this study, the vacuoles of both synergids were normal and the cell walls were completed, but both synergids degenerated simultaneously, and showed identical ultrastructural characteristics. Both the egg cell and central cell contained active cytoplasm, which indicates that the hybrid embryo sac has normal function for fertilization, but the unsuccessful fertilization observed in this study indicates that the pollen tube may not have entered into the synergid. This may be the result of cross incompatibility between these two species. In the previous report on cross incompatibility, Ruszkowski (1980) showed that, the growth of the pollen tube of *F. esculentum* stopped at the base of the *F. tataricum* style. Woo *et al.* (1995) found that 30% of the pollen tubes of *F. esculentum* penetrated into the micropyle of *F. tataricum*. Samimy (1991) also showed that 31% of the pollen tubes of common buckwheat entered the tartary embryo sac and the other pollen tubes penetrated the stigma only. In this study, quite a large portion (60%) of interspecific hybridization between *F. esculentum* and *F. tataricum* consists of such failure of fertilization.

Fig. 4. Light (a) and electron (b) micrographs of an interspecific hybrid embryo sac (*F. tataricum* × *F. esculentum*) at 5 DAP. dSy = degenerated synergid, End = endosperm, ER = endoplasmic reticulum, L = lipid bodies, pSy = persistent synergid, Z = zygote. a, Bar = 50 µm, b, Bar = 100 µm.

No zygotic development

No zygotic development was observed in the cross between *F. tataricum* and *F. esculentum* at 5 DAP (Fig. 4a & b). The receptive synergid degenerated and became electron dense. The persistent synergid was deformed shaped and contained a large vacuole surrounding a thin layer of darkly stained cytoplasm (Fig. 4b). The zygote contained a large vacuole at the micropylar side and a partially fused nucleus at the chalazal side. Nuclear fusion was completed in the zygote and the presence of the male nucleus could be traced by the position of the smaller sperm nucleolus (Fig. 4b, arrow). Although the fusion of the nucleus completed, cell division was stopped and the zygote remained unchanged. Many polymorphic mitochondria, darkly stained plastids with or without starch grains and some lipid bodies were found in the zygote cytoplasm. Many wall ingrowths of the central cell occurred towards the micropylar side. In some places the outer wall of the zygote was broken (Fig. 4b, arrowheads). Dispersed endosperm was identified around the zygote, which was a pattern of collapse. The endosperm was still of a free nuclear type at 5 DAP and also showing abnormal phenomenon. There were

scattered phases of organelle because the ribosome density in the endosperm cytoplasm was decreased. The lack of ribosomes influences protein synthesis and results in low protein content. Most of the ribosomes attached to the RER and the degenerated endosperm cytoplasm appeared electron translucent. Other organelles in the cytoplasm were very sparse. Nucellar cells contain condensed cytoplasm with many normal organelle.

Based on ultrastructure of the fertilized embryo sac, Guan and Adachi (1994) clarified that the zygote absorbed nutrients from its surrounding tissue, through the portion closed to the nucellus, plasmodesma and plasma membrane in the chalazal region of the zygote for generation, development and differentiation. By ultrastructural studies in rice, Maeda and Maeda (1990) also found that, nutrients for early zygote development were efficiently transferred through transmembranal, symplastic and apoplastic ways, from the surrounding cells. In this embryo sac, the zygote could not absorb nutrients properly because of the partially broken outer wall of the zygote and abnormal endosperm. Samimy (1991) found that the embryo development in the ovule of tartary buckwheat was at an early heart-shaped stage at 5 DAP, but ovules of tartary buckwheat after cross pollination with common buckwheat did not contain any embryos. In this study also some interspecific hybrid embryos that were rod-shaped showed slower growth at 5 DAP than the self-pollinated globular shaped embryos. This characteristic of embryo retardation was another major constraint to the interspecific cross.

Fig. 5. Light micrographs (a-d) of defective embryo sacs in *Fagopyrum*. Bar = 50 µm

Defectiveness of embryo sac development

In interspecific crosses, some hybrid ovules contained shrunken or small embryo sac or irregular shaped embryo sac (Fig. 5). Such a defective embryo sac may not contain a normal embryo, which is one of the breeding barriers in interspecific crosses. In some cases some nucellar cells contained condensed cytoplasm with large vacuoles (Fig. 5b). Guan and Adachi (1997) reported that the nucellar cells called "nutritive jacket" is the principal nutritive source of the early developing embryo. The vacuolated nucellar cells found during the present study could not provide sufficient nutrients to the respective embryo in interspecific crosses.

The percentage of fertilization that occur in the interspecific cross between *F. tataricum* and *F. esculentum* (40%) was lower than that of the maternal parents (90%). In the interspecific cross, most of the embryos degenerated after the zygote to rod stage. It seemed that during the early zygote stage the fate of the embryo was already determined. Embryo abortion was associated with retarded zygotic development and early degeneration of endosperm.

Literature Cited

- Adachi, T. (1986) Is it possible to overcome the low yield of buckwheat by means of biotechnology? Some trials in plant genetic manipulation. In "Proceedings of 3rd International Symposium on Buckwheat" Pulawy, Poland. p. 108-116.
- Adachi, T., K. Kawabata, N. Matszaki, T. Yabuya and T. Nagatomo (1983) Observation of pollen tube elongation, fertilization and ovule development in autogamous autotetraploid buckwheat. In "Proceedings of 2nd International Symposium on Buckwheat" Miyazaki, Japan. p. 103-113.
- Adachi, T., A. Yamaguchi, Y. Miike and F. Hoffman (1989) Plant regeneration from protoplasts of common buckwheat (*Fagopyrum esculentum*). Plant Cell Rep. 8: 247-250.
- Adachi, T. (1990) How to combine the reproductive system with biotechnology in order to overcome the breeding barrier in buckwheat. *Fagopyrum* 10: 7-11.
- Eggum, B.O. (1980) The protein quality of buckwheat in comparison with other protein sources of plant or animal origin. In "Proceedings of International Symposium on Buckwheat" Ljubljana, Yugoslavia. p. 115-120.
- Guan, L.M. and T. Adachi (1992a) Reproductive deterioration in buckwheat (*Fagopyrum esculentum*) under summer conditions. Plant Breed. 109: 304-312.
- Guan, L.M. and T. Adachi (1992b) Ultrastructure of the zygote sterility in common buckwheat (*Fagopyrum esculentum*) at high temperature. In "Proceedings of 5th International Symposium on Buckwheat" Taiwan, China. p. 111-121.
- Guan, L.M. and T. Adachi (1994) Ultrastructural changes of the mature embryo sac in buckwheat (*Fagopyrum esculentum*) as a result of high temperature exposure. *Cytologia* 59: 237-248.
- Guan, L.M. and T. Adachi (1997) Ultrastructural studies of embryogenesis in *Fagopyrum esculentum* under temperature conditions. Int. J. Plant Sci. 158: 110-120.
- Hirose, T., A. Ujihara, H. Kitabayashi and M. Minami (1994) Interspecific cross compatibility in *Fagopyrum* according to pollen tube growth. Breed. Sci. 44: 307-314.
- Jensen, W.A. and D.B. Fisher (1968) Cotton embryogenesis: The entrance and discharge of the pollen tube in the embryo sac. *Planta* 78: 158-183.
- Kuroiwa, H. (1989) Ultrastructural examination of embryogenesis in *Crepis capillaris* (L.) Wallr.: I. The synergid before and after pollination. Bot. Mag. (Tokyo) 102: 9-24.
- Lachmann, S. (1991) Plant cell and tissue culture in buckwheat: an approach towards genetic improvements by means of unconventional breeding techniques. In "Proceedings of ICOBB" Miyazaki. p. 145-154.
- Lee, B.S., A. Ujihara, M. Minami and T. Hirose (1994) Breeding of interspecific hybrids in genus *Fagopyrum*. (4) Production of interspecific hybrids through ovule culture among *F. esculentum*, *F. tataricum* and *F. cymosum*. Jpn. J. Breed. 44 (Suppl. 1): 183 (in Japanese).
- Maeda, E. and K. Maeda (1990) Ultrastructure of egg apparatus of rice (*Oryza sativa*) after anthesis. Jpn. J. Crop Sci. 59: 179-197.
- Nagatomo, T. (1965) Buckwheat from Afganistan and Iran. In "Results of the Kyoto University Science Expedition to the Karakoram and Hindukush, 1955". KUSE. p. 192-200.
- Ruszkowski, M. (1980) The possibility of changing the yielding of buckwheat by breeding the homostyle varieties. In "Proceedings of International Symposium on Buckwheat". Ljubljana, Yugoslavia. p. 7-15.
- Samimy, C. (1991) Barrier to interspecific crossing of *Fagopyrum esculentum* with *Fagopyrum tataricum*: I. Site of pollen-tube arrest. II. Organogenesis from immature embryos of *F. tataricum*. *Euphytica* 54: 215-219.
- Samimy, C., T. Bjorkman, D. Siritunga and L. Blanchard (1996) Overcoming the barrier to interspecific hybridization of *Fagopyrum esculentum* with *F. tataricum*. *Euphytica* 91: 323-330.
- Tsai, Q.S. (1994) Studies on interspecific cross incompatibility in Genus *Fagopyrum*. M.S. Thesis, Faculty of Agriculture, Miyazaki University, Japan. 4 p.
- Tsai, Q.S. and T. Adachi (1994) Analysis and overcoming breeding barriers in genus *Fagopyrum*: Heterostylic incompatibility and abnormality of fertilized embryo. Jpn. J. Breed. (Suppl.2): 274 (in Japanese).
- Ujihara, A., Y. Nakamura and M. Minami (1990) Interspecific hybridization in genus *Fagopyrum* properties of hybrids (*Fagopyrum esculentum* Moench $\times$ *Fagopyrum cymosum* Meissner) through ovule culture. Gamma Field Symp. Inst. Radiation Breeding NIAR, MAFF, Japan. 29: 45-51.
- Van Went, J.L. (1970a) The ultrastructure of the synergids of *Petunia*. Acta. Bot. Neerl. 19: 121-132.
- Van Went, J.L. and M. Cresti (1988) Prefertilization degeneration of both synergids in *Brassica campestris* ovules. Sex. Plant Reprod. 1: 208-216.
- Wagatsuma, T. and Y. Un-no (1995) In vitro culture of interspecific ovule between buckwheat (*F. esculentum*) and tartary (*F. tataricum*). Breed. Sci. 45 (Suppl. 2): 312 (in Japanese).
- Wang, Y.J. and C. Campbell (1998) Interspecific hybridization in buckwheat among *Fagopyrum esculentum*, *F. homotropicum* and *F. tataricum*. Adv. Buckwheat Res. (Proc. 6th Intl. Symp. Buckwheat, Ina, Japan): 1-12.
- Woo, S.H., Q.S. Tsai and T. Adachi (1995) Possibility of interspecific hybridization by embryo rescue in genus *Fagopyrum*. In "Current Advances in Buckwheat Research" Matano T. and A. Ujihara (eds.), Shinshu. p. 225-237.
- Woo, S.H., Q.S. Tsai and T. Adachi (1997) Production of interspecific hybrids between *Fagopyrum esculentum* and *F. homotropicum* through embryo rescue. SABRAO J. 29: 89-95.
- You, R. and W.A. Jensen (1985) Ultrastructural observations of the mature megagametophyte and the fertilization in wheat (*Triticum aestivum*). Can. J. Bot. 63: 163-178.