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Pollen-tube Behavior and Embryo Development in Interspecific Crosses Among the Genus *Fagopyrum*

Sun-Hee Woo[1†], Soo-Hwan Kim[2†], Kei Sei Tsai[3], Keun-Yook Chung[4], Seung-Keun Jong[1], Taiji Adachi[3] and Jong-Soon Choi[5]*

[1]Department of Crop Science, Chungbuk National University, Cheongju 361-763, Korea
[2]Department of Biological Science, Yonsei University, Wonju 220-710, Korea
[3]Venture Business Laboratory, Center for Advanced Science and Innovation, Osaka University, Yamadaoka, Suita, Osaka, 565-0871 Japan
[4]Department of Agricultural Chemistry, Chungbuk National University, Cheongju 361-763, Korea
[5]Proteome Research Team, Korea Basic Science Institute, Daejeon 305-333, Korea

Common buckwheat (*Fagopyrum esculentum* Moench) is an agriculturally and pharmaceutically valuable crop due to its well-balanced essential amino acids and rutin content. However, global mass production of buckwheat is limited because its genetic self-incompatibility results in low seed sets and poor grain yield. Therefore, this study was conducted to classify the modes of pistil-pollen interaction between species belonging to the genus *Fagopyrum* and to determine the optimal combination of outcrosses for the most successful pollinations. Based on the interaction between pistils and pollen, we classified the modes of pollen tube growth during interspecific crosses of *Fagopyrum* species into five categories: (i) Highly compatible: normal pollen tube elongation and style penetration within 6~24 hours of pollination, (ii) Slightly compatible: delayed (for 1~6 hours) pollen tube elongation and normal style penetration, (iii) Incompatible type I: pollen tube inhibition at the stigma, (iv) Incompatible type II: pollen tube inhibition at the style, and (v) Incompatible type III: pollen tube inhibition at the stylodium. Based on the observed pollen tube elongation and the following embryo development, highly compatible pollinations were found to be crosses between *F. esculentum* x *F. cymosum* and between *F. esculentum* (*thrum*) x *F. homotropicum*.

Keywords: embryo development, genus *Fagopyrum*, pistil-pollen interaction, pollen tube, self-incompatibility

Common buckwheat is a self-incompatible species in the genus *Fagopyrum* belonging to *Polygonaceae* that is primarily grown in Asia, the United States, Canada, Russia and Eastern Europe. Buckwheat is a nutritious plant that also contains unique proteins with special biological activities such as cholesterol-lowering and anti-hypertension effects. Additionally, buckwheat is known to improve constipation and obesity (Li and Zhang, 2001). Therefore, common buckwheat is widely used as a nutritional and pharmaceutical resource. Rutin, which is an antioxidant that is found within most parts of the buckwheat plant, including the leaf, stem, flower, root and hull (Choi et al., 1996; Shim et al., 1998), has been used for the treatment of patients who need intensive health care. In addition to these agricultural and pharmaceutical purposes, buckwheat is a major raw material used in the production of porridges, soups and alcoholic and nonalcoholic soft drinks (Mazza, 1993).

There are approximately 15 known species in the genus *Fagopyrum*, most of which are diploid plants (2n=2x=16), although a few species have greater than 15 chromosomes (Ohnishi, 1998; Chen, 1999). Additionally, 2 major species that can be cultivated, *F. esculentum* and *F. cymosum*, carry tetraploid chromosomes (2n=4x=32) and are autopolyploids. Cytological studies of the F_1 hybrids produced by crosses between *F. esculentum* x *F. cymosum* revealed that both species share 50~70% homology in chromosomal organization and pollen viability based on meiotic pairing (Adachi et al., 1982).

One of the major constraints of buckwheat production is the low rate of seed formation, which results in poor grain yield. This poor yield often results in local production being limited by persistently low and unstable yields, which prevent local demands from being met. In addition, grains are often shattered by unwanted trashing during harvest (Alekseeva and Malikov, 1992; Oba et al., 1998). Production is also hampered by apomixes and sterility, which result from combinatory interactions between genetic and environmental factors. Many genetic breeding programs have been developed to overcome these problems in an attempt to improve buckwheat production. One such program involved an effort to avoid the phenomenon of self-incompatibility, which is specific to the reproductive biology of the genus *Fagopyrum*. To achieve a successful buckwheat breeding program, interspecific hybridization has been conducted by introducing valuable genes and transferring desirable traits from related species.

Common buckwheat is essentially self-fertile and contains two types of flower, pins and thrums. The pin-type flower has a long style and short stamens, while the thrum type has a short style and long stamens. These typical heteromorphic flower organizations result in dimorphic heterostyly, followed by the failure of fertilization (Adachi, 1990). To date, self/cross-incompatibility of the crop has been considered to be a major cause of low seed productivity in common buckwheat. However, self-incompatibility can be rescued by the

*Corresponding author; fax +82-42-865-3419
e-mail jschoi@kbsi.re.kr

†Authors contributed equally to the article.

introduction of a single dominant gene (Sharma and Boyes, 1961), and it has been shown that the homomorphic sporophytic system is controlled by a single S^h gene (Brennan, 2003). The incompatible reaction presumably happens at the papillal cells of the stigma, which is similar to the sporophytic system of self-incompatibility (Woo et al., 1997, 1999). However, pre-fertilization barriers are also important factors in interspecific cross-incompatibility, and the pistil-pollen interaction and embryo development in response to different crosses are important for successful fertilization to occur. Therefore, this study evaluated different combinations of interspecific crosses in the genus *Fagopyrum* to determine the site and time at which inhibition as a result of the formation of incompatible pollen tubes occurs, as well as to identify highly compatible interspecific combinations and the optimal time for fertilization. Taken together, our observations indicate that crosses between *F. esculentum* x *F. cymosum* and between *F. esculentum* (*thrum*) x *F. homotropicum* result in highly compatible pollinations.

MATERIALS AND METHODS

Plant Materials

Both homomorphic self-compatible and dimorphic self-incompatible individuals of five *Fagopyrum* species were used for this study. These plants included *F. esculentum* (2n=16, 32), *F. tataricum* (2n=16), *F. cymosum* (2n=16, 32), *F. homotropicum* (2n=16) and *F. giganteum*. In particular, *F. giganteum* was an induced amphidiploid derived from *F. esculentum* and *F. cymosum*. Common buckwheat (*F. esculentum* Moench) is a widely cultivated wild type species that produces either short-style (thrum) or long-style (pin) plants in approximately equal frequencies. The other wild species evaluated in this study are similar to common buckwheat in morphology, but are distinguished by the presence of homomorphic flowers and fragile premature seeds, as well as by their ability to self-fertilize. All plants were grown in pots in a pollinator-free greenhouse and growth chamber.

Manual Pollination

Legitimate combinations were performed for the heterostylous species *F. esculentum* and *F. cymosum*. For crosses between heterostylous species and homostylous species such as *F. homotropicum*, *F. tataricum* and *F. giganteum*, all possible pollen-pistil combinations were examined. In addition, both heterozygotic and homozygotic pollinations of *Fagopyrum* species were conducted. Additionally, dimorphic species were used as female parents as well as pollen donors, while homomorphic species were used only as pollen donors. All possible reciprocal crosses between *F. esculentum* and the remaining five species were made using hand-pollination, which was conduced by using fine forceps to collect fresh pollen from the pollinator in the morning and then rubbing the pollen against the fresh stigma of the female parent when the flowers were in full blossom. The emasculation of self-compatible cleistogamic homostylous species was carefully conducted one day prior to anthesis under a magnifying glass. The pollinated flowers were then marked, after which they were immediately isolated and stored in cellophane bags for microscopic observations. Only flowers that had opened at the beginning of the flowering period were used for pollination.

Microscopic Observation of Pollen Tubes

Pollinated flowers were detached at 24 h after pollination and then fixed with Carnoi's fixative for microscopic observations. The legitimately pollinated flowers of *F. esculentum* x *F. cymosum* and the self-pollinated flowers of *F. homotropicum*, *F. tataricum* and *F. giganteum* were also fixed at the same time as controls. To observe and assign the mode of pollen tube growth, at least ten pollinated pistils for each time interval indicated in the text were collected and fixed in an acetic acid:alcohol solution (1:3, v/v) after pollination. The collected pistils were then softened in 1N NaOH at 60°C for 10 min, stained with 0.1% (w/v) decolorized aniline blue solution, and examined using a fluorescence microscope (AH2-PC type, Olympus Co., Japan) as previously described (Adachi, 1990). The lengths of the styles and pollen tubes were measured using a Nikon microscope equipped with a micrometer, with the length of the longest pollen tube in each pistil being recorded.

Miscroscopic Observation of Embryos and Ovules

Approximately twenty pollinated flowers were collected at 1, 2, 3, and 5 d after pollination (DAP) and then kept at 4°C prior until used. To examine the size and morphology, the ovules were carefully dissected from the ovaries using fine forceps under a stereo microscope after measuring the size of the ovaries using a micrometer. The dissected ovule was then directly transferred into benzyl benzoate-four-and-a-half (BB-4 1/2) clearing mixture (Herr, 1982) and incubated for 24 h at room temperature. The cleared ovules were then mounted on a Raj slide with a cover glass and subsequently the morphology and embryonic development of the samples were examined using Nomarski's Differential Interference Contrast (DIC) optics. The size of the embryo and ovule was then measured using a micrometer. Photographs were taken with a photoautomate (MP48, Leica-Wild, Germany).

Germination of Interspecific Crosses

Enlarged ovaries were removed at 3 to 5 DAP and then surface-sterilized with 70% (v/v) ethanol for a few seconds, after which they were submerged in 2% (v/v) sodium hypochlorite solution for 5 min and subsequently rinsed three times with sterile distilled water. The ovaries were then dissected aseptically and cultured for 5-6 d on MS medium (Murashige and Skoog, 1962). Thereafter, all ovules were checked for the presence of embryos. Ovules containing an embryo were gently placed vertically on an agar-solidified MS medium (0.8 %, w/v) containing 3% (w/v) Suc and 0.1% (w/v) casein hydrolysate in the presence or absence of hormones and then supplemented with 0.2 mg/L IAA (Indole-3-acetic acid) and 2 mg/L BA (6-Bentylaminopurine). The ovules were then independently cultured in liquid MS medium supplemented with 3% (w/v) Suc at different initiation times (3 and 5 DAP) and at different temperatures (25

and 30°C). The culture media was kept at 25°C under white fluorescent light adjusted to 2000 Lux under a 16-h light and 8-h dark period for one month. The resulting calli with germinated seedlings were then transferred to hormone-free MS medium containing 3% (w/v) Suc and 0.1% (w/v) casein hydrolysate for further development. After an additional month, each plantlet was transferred to a Magenta box that contained sterile peat-vermiculite growth medium and 11 holes with a diameter of 5 mm in the top. The perforated area was initially covered with clear plastic. The tops were then punctured at 20 d after the transfer and the plants were allowed to grow for an additional 10 d. The plantlets were usually hardened 30 d after the transfer, at which time they were placed in pots and grown at 20°C under a 15-h light and 9-h dark cycle.

RESULTS AND DISCUSSION

Pollen Tube Growth and Modes of Pollen-Pistil Interactions

During male gametophyte development in angiosperms, microspore mother cells produce microspores that mitotically result in the formation of pollen grains that each contain one tube cell and one generative cell. Upon arrival on the surface of the stigma, the generative cell mitotically produces two sperm cells and one tube nucleus. Each sperm cell is then guided to the inside of an embryo sac by a pollen tube that is generated by the tube cell, in which it grows into the micropyle. This results in double-fertilized sperms with two polar nuclei and one egg, which in turn develop into an endosperm (3n) and an embryo (2n), respectively

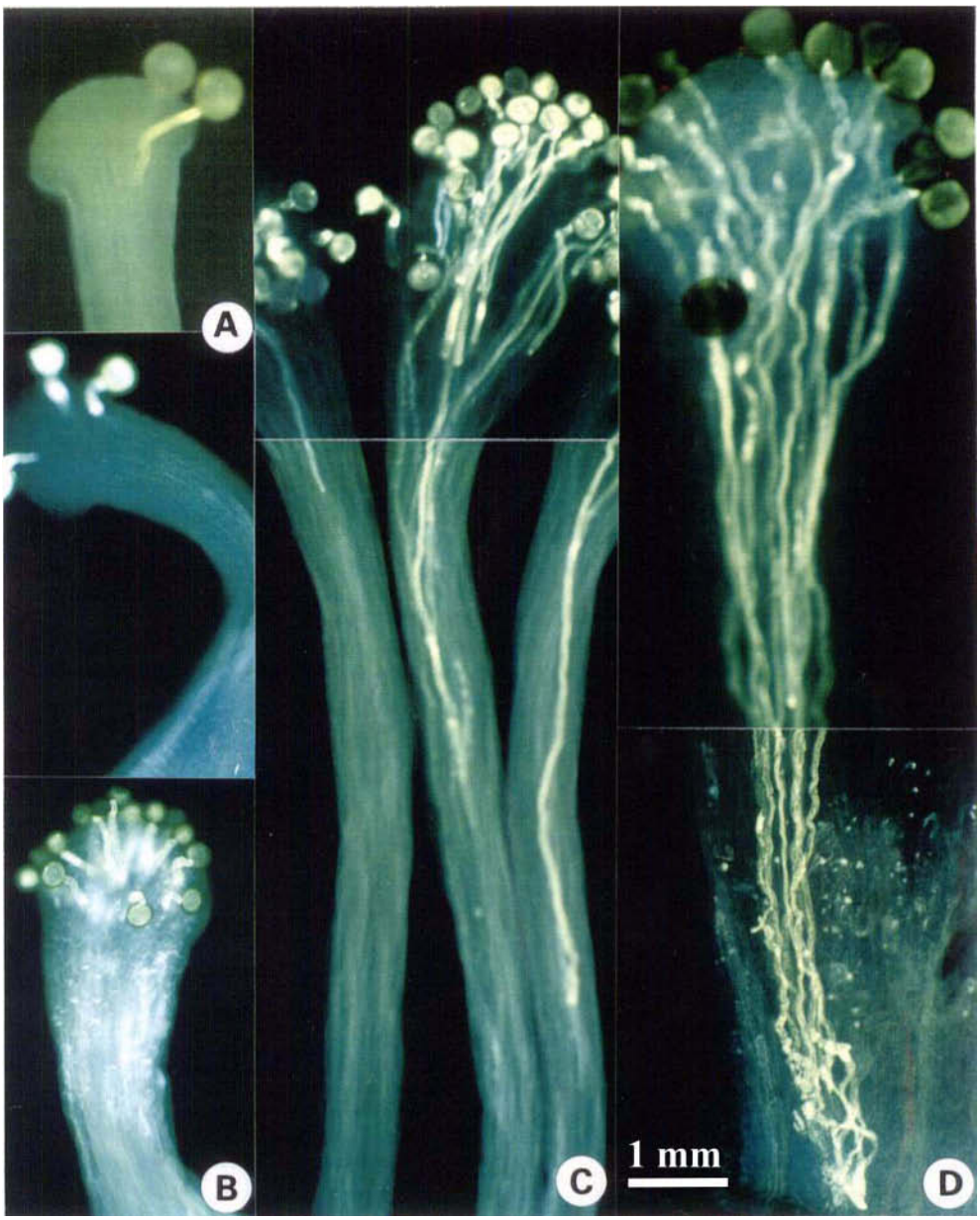

Figure 1. Microscopic observations of pollen tube growth at 6 h after incompatible cross-pollination. (**A**) *F. homotropicum* x *F. esculentum* (2x, thrum), pollen tubes stop in stigma. (**B**) *F. esculentum* (4x, thrum) x *F. tataricum*, pollen tubes stop in stigma. (**C**) *F. esculentum* (2x, pin) x *F. tataricum*, pollen tubes stop in style. (**D**) *F. homotropicum* x *F. esculentum* (4x, pin), pollen tubes stop in stylodium.

(Faure, 2001).

To evaluate compatible pollination crosses and the subsequent embryonic development of compatible crosses, we examined pollen tube growth in the stigmatic area and into the style, calculated the frequency of pollen tube penetration into the micropyle, and morphologically dissected the embryos during different stages of development. Six hours after pollination, all flowers examined had successfully generated pollen tubes that had penetrated into the stigmatic surface area (Fig. 1A-1D). The source of the pollen had no apparent effect on the pollen tube behavior of the stigma. In addition, use of the pin- or thrum-styled *F. esculentum* or *F. tataricum* as a pollen donor had no effect on the penetration of pollen into the stigmatic surface. Taken together, these findings indicate that the source of pollen was not a major incompatible barrier in these combinations of crosses. However, further growth of the pollen tube into the style in the crosses of *F. homotropicum* x *F. esculentum* (2x, thrum) and *F. esculentum* (4x, thrum) x *F. tataricum* were strictly restricted immediately below the stigmatic surface (Fig. 1A, 1B). In addition, in the cross of *F. esculentum* (2x, pin) and *F. tataricum* the pollen tube penetrated deeply into the style before it stopped (Fig. 1C). Additionally, pollen tube growth was inhibited in the upper part of the style when thrum flowers of *F. esculentum* and homostylous species were reciprocally used as pollinator and pistillate, respectively (not shown). However, when the flowers of *F. homotropicum* were pollinated with incompatible pollens of *F. esculentum* (4x, pin), a large number of the pollen tubes were successfully grown, and these tubes penetrated into the base of the style and the ovary at 6 h after pollination (Fig. 1D).

Based on of the point that the pollen tube reached before being inhibited, we classified the interspecific crosses of *Fagopyrum* plants into five types of compatible and incompatible patterns (Table 1). Interestingly, the frequency of hypertrophy that occurred at the pollen tube tips was closely correlated with the pollen tube growth. The pollen tube growth in interspecific crosses of plants belonging to different groups stopped at the upper part of the style with hypertrophy. However, when plants within a group were crossed, the pollen tube grew into the middle of the style or into the micropyle without hypertrophy. As shown in Table 1, highly compatible hybrids were obtained when *F. esculentum* x *F. cymosum* and thrum type of *F. esculentum* x *F. homotropicum* were crossed. Conversely, their reciprocal crosses were found to be only slightly compatible (Table 1, group II) and incompatible (Table 1, group III), respectively. The length of the longest pollen tube in the compatible combinations varied greatly. Though an interspecific cross between *F. esculentum* and *F. homotropicum* and the analysis of a refined linkage map for characterization of the buckwheat genome and phenotypic traits has been previously reported (Yasui, 2004), our systematic interbreeding experiment of common buckwheat is the first case to our knowledge.

Table 1. Classified types of compatibility in various interspecific crosses of *Fagopyrum*.

I.	Highly compatible
	F. esculentum x F. cymosum
	F. esculentum (thrum) x F. homotropicum
II	Slightly compatible
	F. cymosum (thrum) x F. esculentum (pin)
	F. cymosum (4x, pin) x F. esculentum (thrum)
	F. homotropicum x F. esculentum (2x, pin)
	F. tataricum x F. esculentum (pin)
III	Incompatible : pollen tube inhibited at stigma
	F. esculentum (thrum) x F. tataricum
	F. homotropicum x F. esculentum (thrum)
	F. tataricum x F. esculentum (thrum)
IV	Incompatible : pollen tube inhibited in style
	F. esculentum (pin) x F. tataricum
	F. esculentum x F. giganteum
	F. giganteum (pin) x F. esculentum (pin)
V.	Incompatible : pollen tube inhibited at stylodium
	F. esculentum (pin) x F. homotropicum
	F. cymosum (2x, pin) x F. esculenteum (thrum)
	F. homotropicum x F. esculentum (4x, pin)
	F. giganteum x F. esculentum (thrum)

Pollen-Pistil Interaction

We expanded our investigation of pollen tube behaviors and pollen-pistil interactions by examining the 52 possible combinations between the five species of homomorphic self-compatible and dimorphic self-incompatible *Fagopyrum*. In these experiments, we measured the frequency of pollen tube penetration into micropyle. The lengths of *F. homotropicum* and *F. giganteum* pollen tubes in the thrum pistils were shorter than those in the pin pistils. However, over half number of all the pollinated *F. esculentum* thrum pistils showed long pollen tubes that penetrated into micropyle. Moreover, the length of the pollen tubes in the pin pistils was longer than those in the thrum pistils. Furthermore, the pollen tubes of all of the homostylous species did not arrive at the micropyle in the pin pistil of *F. esculentum*. In addition, the pollen tube penetration of *F. tataricum* was different from those of *F. homotropicum* and *F. giganteum*, and was inhibited in the stigma of the thrum pistil in *F. esculentum*. When the three self-compatible homostylous species, *F. homotropicum, F. tararicum,* and *F. giganteum*, were used as the pistillate plant, varying degrees of obstruction from maternal plants to the *F. esculentum* thrum and pin pollen tubes was observed (Table 2). In addition, all plants showed very low penetration of the pollen tube into the micropyle in the pistils of the three homostylous species generated by crosses using either the pin or thrum of *F. esculentum* when compared to self-pollinated species. The length of the pollen tubes produced when the pin type of the *F. esculentum* was crossed with pistils from *F. homotropicum* and *F. tataricum* varied greatly, and several of them reached the micropyle. In contrast, the pollen tube generated by crosses of *F. giganteum* x *F. esculentum* did not reach the micropyle. In addition, the length of the pollen tubes of the thrum flower of *F. esculentum* was shorter than those of the pin flower in *F. homotropicum* and *F. tataricum* (data not shown). This

Table 2. Frequency of pollen tube penetration into micropyles at 24 h after pollination.

Pollen	Pistil	E2X		E4X		C2X		C4X		H	T	G
		P	T	P	T	P	T	P	T			
E2X	P	-	10/10 (100)	-	-	-	9/9 (100)	-	10/10 (100)	0/10 (SD)	0/8 (SL)	0/10 (SL)
	T	10/10¶ (100)	-	-	-	10/10 (100)	-	10/10 (100)	-	6/9 (66.7)	0/10 (SM)	0/10 (SL)
E4X	P	-	-	-	10/10 (100)	-	12/12 (100)	-	10/10 (100)	0/10 (SD)	0/10 (SL)	0/10 (SL)
	T	-	-	10/10 (100)	-	10/10 (100)	-	6/6 (100)	-	6/10 (60.0)	0/10 (SM)	0/10 (SL)
C2X	P	-	0/10 (SD)	-	0/10 (SD)	-	8/10 (80.0)	-	-	-	-	-
	T	2/9 (22.2)	-	4/15 (26.7)	-	8/10 (80.0)	-	-	2/9 (22.2)	-	-	-
C4X	P	-	9/25 (36.0)	-	7/13 (53.8)	-	-	-	10/12 (83.3)	-	-	-
	T	1/10 (10.0)	-	1/10 (10.0)	-	-	-	1/10 (10.0)	-	-	-	-
H		2/10 (20.0)	0/10 (SM)	0/10 (SD)	0/10 (SM)	-	-	-	-	10/10 (100)	-	-
T		3/10 (30.0)	0/8 (SM)	4/13 (30.8)	0/10 (SM)	-	-	-	-	-	10/10 (100)	-
G		1/10 (SL)	0/10 (SD)	0/10 (SL)	0/10 (SD)	-	-	-	-	-	-	10/12 (83.3)

¶The number of pistils from the pollen tube that penetrated into the micropyle divided by the total number of pollinated pistils. The numbers in parenthesis represent the percent value of the pistils penetrated by pollen tubes.
*Abbreviations, E2X, *F. esculentum* (2x); E4X, *F. esculentum* (4x); C2X, *F. cymosum* (2x); C4X, *F. cymosum* (4x); H, *F. homotropicum*; T, *F. tataricum*; G, *F. giganteum*; P. pin; T, thrum.
**SM, SL and SD represent the longest pollen tube stopped at the stigma, style and stylodium, respectively.
Hyphenation (–) indicates no crossing experiment conducted in the present study.

suggests that the obstruction from pistils of homostylous species must be more critical to the pollen tubes of the thrum than to those of the pin in *F. esculentum*.

There have been many studies conducted to evaluate pollen tube growth and self-incompatibility of various types of flowers. For example, Adachi et al., (1983) reported that the growth of pollen tubes stopped at the lower part of the style in pin x pin crosses and at the stigma in thrum x thrum crosses of *F. esculentum*. In addition, fairly highly compatible crosses of buckwheat were reported when homostylous long-styled flowers with large pollen grains were crossed with homostylous short-styled flowers with small pollen grains (Molchan, 1988). Fesenko (1989) proposed that incompatibility in a pin x pin cross could be determined by differences in pollen grain size and style length because the small pollen grains had insufficient growth to reach the base of the long styles. In this study, the large pollen grains of the thrum flowers produced fairly thick pollen tubes that could not be easily penetrated through the less dense tissue of the long styles in the thrum x thrum cross. In addition, the specific soluble proteins and enzymes are known to be present in the stigmas of *Lolium multiforum* treated with self or non-self pollen, which suggests that a possible interaction between the pollen and pistil that occurs at the molecular level is responsible for self-incompatibility (Kalinowski, 2006).

The results of this study indicated that the barriers involved in interspecific hybridization between *F. esculentum* and other major species in the genus *Fagopyrum* were set prior to fertilization and after pollination. A recent study evaluating pollen tube growth revealed that the tube cell developed as it progressed from the stigma to the ovary, where it acquired the ability to respond to the last guidance event at the micropyle (Matsui, 2004). However, the mechanism by which the tube cells are guided through the style into the ovary remains to be elucidated.

Observation of Embryo Development

Normal embryogenesis during the early developmental stage can be characterized as distinguishable embryo shapes (club-shaped, early-globular, globular and early-heart contour) designed by a large number of cells and nuclei in an embryo sac. We found that crosses of pin-styled and thrum-styled *F. esculentum* produced a normal shaped embryo that properly connected with the micropyle via a suspensor, regardless of whether a pin or thrum was used (Fig. 2). Conversely, many abnormalities were found in various hybrid embryos, including irregularly shaped embryos, isolated suspensor, obscured and/or vacuolated cells without any nuclei and stranded endosperms and nuclei. These abnormalities were divided into the five groups described below.

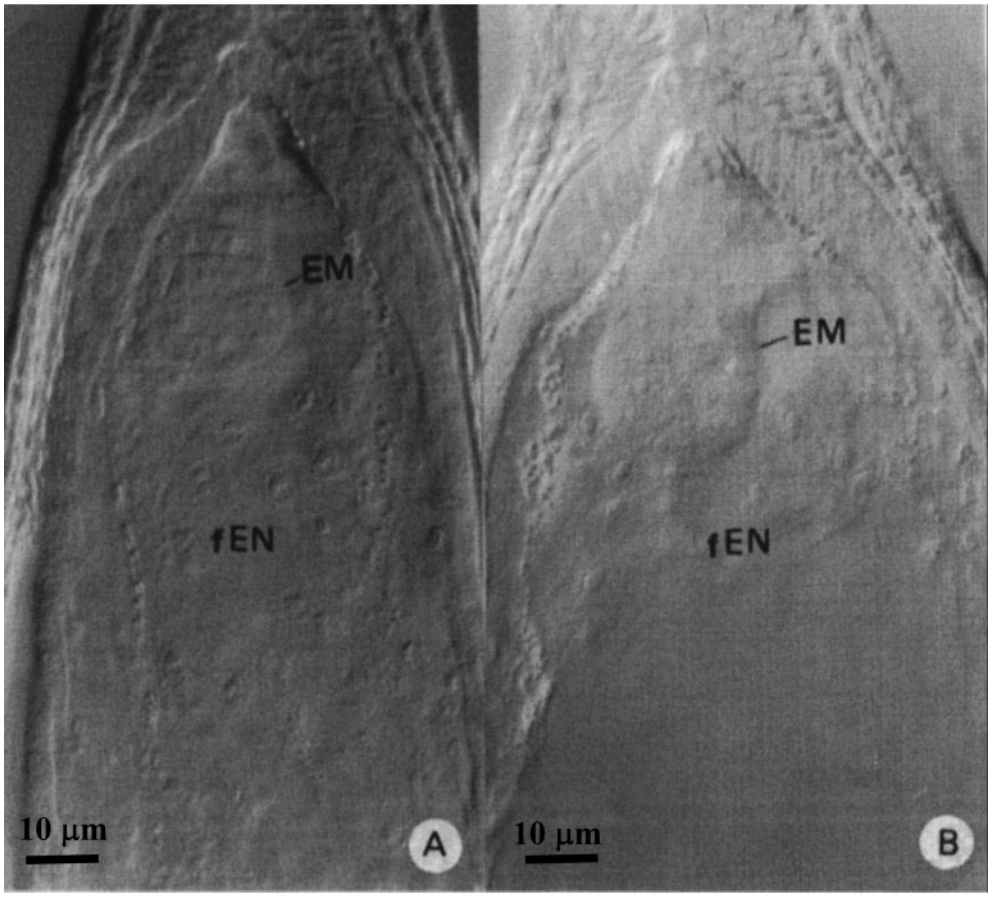

Figure 2. DIC micrographs of embryo development show normal embryo (EM) and free endosperm nuclei (fEN). (**A**) *F. esculentum* (pin x thrum) at 2 DAP. (**B**) *F. esculentum* (thrum x pin) at 3 DAP.

A. Undivided zygote: The fertilized egg did not divide normally although it was surrounded by normal free endosperm nuclei (Fig. 3A). This abnormal phenomenon was observed at both 2 DAP and 5 DAP.

B. Degenerated and collapsed endosperm: Young hybrid embryos developed normally. The cells were clearly distinguished, but no free endosperm nuclei were observed in the embryo sac. In some cases, nurse cells in the nucleus and integuments are also degenerated or collapsed (Fig. 3B).

C. Degenerated embryo and endosperm: Stranded endosperms and shrunken embryos with abnormal cells were present. These characteristics were observed in most crosses between the inter-group members described in Table 1. A representative embryo produced by a cross between *F. esculentum* (2x, thrum-styled) and *F. giganteum* is shown in Fig. 3C.

D. Collapsed embryo and endosperm: This type of abnormal embryo and endosperm was completely collapsed and crumbled so that they could not be identified (Fig. 3D). This distortion started to occur early, at 3 DAP, and peaked at 5 DAP.

E. Floating embryo with no suspensor: Development of a suspensor from the fertilized egg was defective in this group, therefore the embryo was not properly connected to the micropyle. Free nuclei of cells were usually not yet detected in the endosperm when this occurred (Fig. 3E).

In summary, we found that the degeneration and collapse of the endosperm occurred most frequently in hybrid embryos. This indicates that the most important factor causing embryonic failures of the hybrids is abnormal nuclear division, which leads to impaired development of endosperms (Adachi, 1986, 1990)

Disrupted Hybrid Development During the Early Developmental Stage

Normal interactions between the pollen and egg appear to occur in hybrid embryos before further development fails, and the ratio of degenerated embryos was found to be greater during the early stages of embryonic development. All pollen tubes formed as a result of interspecific hybridizations between *F. esculentum* and *F. cymosum* and more than 50% of the pollen tubes formed as a result of crosses between thrum flowers of *F. esculentum* and *F. homotropicum* penetrated into the micropyle (Table 2). This indicates that the observed failure of seed formation may be caused by barriers at the post-fertilization stage. This is also supported by the finding that the *F. esculentum* x *F. cymosum* cross showed the highest percentage of ovules with micropylar penetration, but that only 3 normal embryos in 14 fertilized ovules were observed at 2 DAP and no normal embryos were observed at 5 DAP (Fig. 3E).

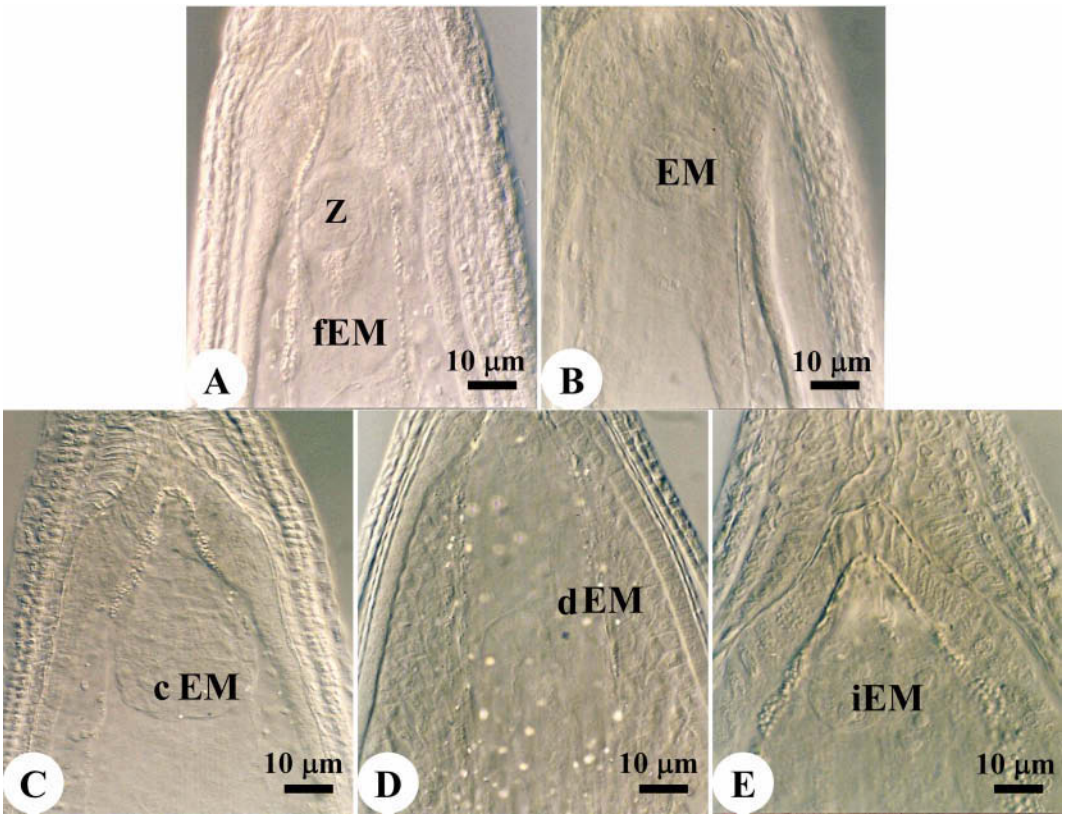

Figure 3. DIC micrographs of hybrid embryo development after interspecific hybridization. (**A**) Undivided zygote, *F. esculentum* (2x, thrum) x *F. giganteum* at 2 DAP. (**B**) Collapsing endosperm, *F cymosum* (2x, thrum) x *F. esculentum* (2x, pin) at 5 DAP. (**C**) Degenerating endosperm and embryo, *F. esculentum* (2x, thrum) x *F. giganteum* at 5 DAP. (**D**) Collapsing endosperm and embryo, *F. esculentum* (2x, thrum) x *F. cymosum* (4x, pin) at 3 DAP. (**E**) Embryo isolated from the micropyle, *F. esculentum* (2x, pin) x *F. cymosum* (2x, thrum) at 5 DAP. (cEM: collapsing embryo and endosperm, dEM: degenerating embryo and enosperm, iEM: embryo isolated from the micropyle, z: undivided zygote).

Table 3. Number of pollinations from stimulated ovules excised in crossing experiments of the genus *Fagopyrum*.

Cross	No. of pollinations	No. of ovules excised	% of ovule formation
F. esculentum (t) x *F. esculentum* (p, 2x)	91	48	53
F. esculentum (p) x *F. esculentum* (t, 2x)	170	85	50
F. esculentum (t) x *F. cymosum* (p, 2x)	211	96	45
F. esculentum (p) x *F. homotropicum* (2x)	151	26	17
F. esculentum (p) x *F. giganteum* (4x)	101	13	13

*Abbreviations, p, pin ; t, thrum ; 2x, diploid ; 4x, tetraploid.

The frequency of ovules with normal embryos at 5 DAP was higher when *F. esculentum* was used as the female parent than when other species were used as the female parent or when *F. esculentum* was used as the male parent. In addition, the number of normal embryos was generally lower in interploid crosses than in intraploid crosses. These findings indicate that the optimum combination of intraploid crosses producing the highest number of normal embryos at 5 DAP was *F. esculentum* (thrum) x *F. homotropicum* (Table 1).

Suitable Combinations and Stages for Embryo Rescue

Genetic barriers that occur prior to fertilization and result in cross-incompatibility appear to be controlled by parental genotypes rather than by the genotype of the hybrid zygote itself (Matsubara, 2003). Genetic barriers can also occur as a result of parental divergent relations or disparity between the style and the pollen. Overcoming the cross-incompatibility is easier in the latter case than in the former case because of the low disparity between the style and the pollen. In this study, interspecific hybridization between *F. esculentum* and homostylous species, such as *F. esculentum* (thrum) x *F. homotropicum* and *F. esculentum* (thrum) x *F. giganteum* or *F. cymosum* (intraploid crosses), were most successful.

It is interesting to note that there was no difference in ovule formation when the pin or thrum type was used as the female parent or pollen donor in *F. esculentum* (Table 3), with the percentage of ovule formation when the pin or thrum type was used as a female parent in the cross between *F. esculentum* being 53% or 50% respectively. However, the percentage of ovule production in interspecific crosses was only 13 to 45%, with the highest percentage of ovule formation among interspecific crosses being

observed in *F. esculentum* (thrum) x *F. cymosum* (pin, 2x) crosses (45%) and the lowest percentage being observed in *F. esculentum* (pin) x *F. giganteum* (4x) crosses (13%). It is well known that the production of interspecific hybrids in buckwheat using conventional crossing techniques is very difficult due to strong pre- and post- fertilization barriers that exist in the genus (Adachi et al., 1982; Adachi, 1990; Hirose et al., 1994, 1995). The results of this study indicate that post-zygotic barriers are the primary cause of the reproductive isolation, whereas post-fertilization failure was commonly associated with endosperm abortion, which consequently resulted in abnormal differentiation and starvation of the hybrid embryo.

In this study, abnormalities during the early stage of hybrid embryo formation and during the course of development were successively revealed by Nomarski's interference micrography. Abnormalities such as endosperm degeneration, endothelial proliferation and abnormal embryo cell division were observed in the hybrid ovules at 5 DAP (Fig. 3C). Because the endosperm completely degenerated at approximately 5 DAP, endosperm cells were not observed in most of the ovules at that time. Our findings are consistent with those of a previous report that found that endothelial proliferation is not a causal factor in endosperm degeneration in interspecific crosses of *Lycopersicon* (Barbano and Topoleski, 1984). However, it is likely that the retarded development of the hybrid embryo is a contributing factor because the endothelium can get nutrients from the endosperm that can not be consumed by the failing embryo. Hybrid embryos that did not contain any endosperm did not simply stop growing, but continued to develop for an additional 5 d (data not shown). This indicates that embryo rescue through ovule culture could be used to overcome breeding barriers such as self- and/or cross-incompatibility between *F. esculentum* and *F. cymosum*. Interspecific hybrids that are generated using ovule culture have been successfully produced in several genera, including *Allium* (Gonzalez and Ford-Lloyd, 1987).

The results of this study indicate that it is possible to overcome the barrier of crossincompatibility in a *F. esculentum* (pin) x *F. homotropicum* cross using wild selection of genotypes and mutation of genetic variations in the architecture of the flowers. In addition, the barrier caused by degeneration of young hybrid embryos can be overcome by embryo or ovule-embryo culture. However, to facilitate embryo rescue, the appropriate time to begin the embryonic culture must be determined. This can be accomplished by determining the time at which development of the hybrid embryo stops or the time at which the embryo degenerates, as well as the number of surviving embryos and the technical ability to excise the young embryos. In a *F. esculentum* (thrum) x *F. homotropicum* cross it may be possible to increase the number of surviving embryos by up to 3 times if embryo rescue occurs during the early-heart stage in *F. esculentum* (2X thrum) x *F. homotropicum* crosses and at the globular stage in *F. esculentum* (4X thrum) x *F. homotropicum* crosses at 5 DAP instead of during the torpedo stage, which occurs much later. In summary, this study revealed that crosses between *F. esculentum* (thrum) x *F. cymosum* and between *F. esculentum* (pin) x *F. homotropicum* are highly compatible.

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