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Enhanced Seed Development in the Progeny from the Interspecific Backcross (*Fagopyrum esculentum* × *F. homotropicum*) × *F. esculentum*

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Abstract - To facilitate the introgression of *F. esculentum* into the traits of *F. homotropicum*, several accessions of the hybrids between these two species were pollinated with *F. esculentum* as the recurrent parent. The embryo *in vitro* rescue was performed to increase the recovery of backcross progenies. The F_2 generation was more amenable than F_1 hybrids to produce backcross progenies. The F_1 hybrids were backcrossed twice with common buckwheat (pin-type *F. esculentum*) (recurrent backcrossing). Also, alternate backcrosses with common buckwheat and *F. homotropicum* (congruity backcrossing) were carried out. Pollen tube growth of BCF_1 × *F. esculentum* (thrum) and *F. homotropicum* × BCF_1 was the disturbed penetration exceeded for all initial interspecific hybrids, and its requirement was proportionally lower when the common buckwheat was used as the recurrent parent and as the last parent of congruity hybrids. Effects of both common buckwheat and *F. homotropicum* on seed success rate for hybridization were observed. Growth of hybrid embryos before rescue, regeneration of mature hybrids all increased recurrent and congruity backcrosses and inter-crosses between F_1 plants and selected fertile plants of the second congruity backcrosses.

Key words - *Fagopyrum esculentum* Moench. *F. homotropicum* Ohnishi, interspecific backcrosses, incompatibility, embryo rescue

Introduction

Wide hybridization is important for the improvement of many crop species. The family Polygonaceae, which includes the genus *Fagopyrum*, comprises many wild species that form good resource of genes imparting resistance/tolerance to biotic and abiotic stresses. Buckwheat is a crop species of considerable importance and its use may become more widely in future due to its high nutritive value (Marshall and Pomeranz, 1982). The consumption of buckwheat has increased tremendously over the past several years. However, local production in Korea was often troubled by persistently low and unstable yield and, therefore, did not meet the demand, Thus, imports of buckwheat from other countries have been increasing in recent years.

The major constraint to buckwheat production was low seed set resulting, low grain yield. To improve buckwheat yield same programs are further fraught with numerous problems. Prominent among them was the self/cross-incompatibility phenomenon peculiar to the reproductive biology of this seed propagated genus (Kreft, 1983; Adachi, 1990; Guan and Adachi, 1992; Woo *et al*., 2008). In buckwheat, these breeding barriers have in no small measures contributed to the intractable difficulty and recalcitrance to conventional improvement techniques. For instance, the transfer of valuable traits like self-fertility and another agronomic characters has so far proved abortive due to cross-incompatibility. These pose a challenge to researchers working on buckwheat. In order to broaden the genetic basis, several interspecific hybrids have been attempted (Morris, 1951; Nagatomo, 1961; Krotov and Dranenko, 1975; Samimy 1991; Campbell, 1995; Wagatsuma and Un-no, 1995; Samimy *et al*., 1996; Wang and Campbell, 1998). Through such interspecific crosses, tolerant and resistant genes to abiotic and biotic stresses could be transferred from wild species to

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cultivated forms. However, reports on successful interspecific hybrids between *F. esculentum* (pin) and *F. homotropicum* are rare (Campbell, 1995).

The objective of this study is to transfer desirable agronomic traits from *F. homotropicum*, a wild annual species, into elite lines of the cultivated common buckwheat, *F. esculentum*. The efficiencies of recurrent and congruity backcrossing with respect to increased hybrid embryo development *in vitro* as well as hybrid fertility and pollen tube growth were investigated.

Materials and Methods

Plant material: The common buckwheat and *F. homotropicum* were used for inter- specific hybridization (Woo *et al*., 1999). For the initial cross and subsequent recurrent and congruity backcrosses, common buckwheat and its hybrids were used as females. All interspecific crosses were made in Phytotron by hand-pollination to prevent selfing or contamination from foreign pollen.

Pollen tube growth: Flower samples were fixed six hours after pollination to allow the growth of pollen tubes (Adachi, 1990; Guan and Adachi, 1992). Pollen tube growth in styles and ovaries was observed after staining with aniline blue fluorescence (Woo *et al*., 1995).

Embryo development: The pollinated flowers were collected about 2 days after pollination, and placed into the fixative solution at 4℃ in a refrigerator. To observe embryo development, ovules were removed carefully with forceps and needle, and placed in BB solution according to the method of Herr (1982), after soaking on filter paper. After 24 hours of treatment with BB solution, transparent samples were observed by Nomarski`s differential interference contrast optics.

***In vitro* embryo rescue:** For ovule culture, pistils were excised from the interspecific hybrids 3, 5 and 7 days after pollination (DAP). All ovaries of these flowers were still green. After removal of perianth, the ovaries were sterilized by submerging for 30 seconds in 70% ethanol, for 2 min in a 2% solution of sodium hypochlorite with one drop of detergent (Tween 20). And washed three times with distilled sterile water. The ovule culture technique (Woo *et al*., 1997) was used to rescue the developing embryos. Plants were grown on basal MS medium (Murashige and Skoog, 1962) and Gamborg's (1968) B5 medium supplemented 30 g/L sucrose, 0.02 mg/L IAA + 2.0 mg/L BA and 500 mg/L of casein hydrolysate. The pH of the medium was adjusted to the range of pH 5.5～5.8 and 0.8% agarose was added to make the semi-solid medium before autoclaving. For shoot proliferation, developed embryos were transferred after 14～16 days to solid MS medium supplemented with 2% sucrose, 1.0 mg/L 6-benzyladenine or no growth regulators, 2.2 g/L gelrite and pH 5.7. The culture was maintained at 25℃, 2000 Lux light and 16 hour light and 8 hour dark period. Rooted plants were transferred to sterilized soil and grown for about 2 weeks in Phytotron.

Results

Stylar morphology and seed fertility in the progeny analysis

Reciprocal crosses were made between *F. esculentum* and *F. homotropicum* and their hybrid was selfed. Both reciprocal

Table 1. Stylar morphology and seed fertility in the progenies of interspecific hybrids (*F. esculentum* × *F. homotropicum*)

Generation(stylism)	No.of plants	No.of Seed / pollinated flower (%)
Homostyle		
F_1 selfing	5	23/223 (10.3)
F_2 selfing	6	77/880 (8.8)
Thrum style F_1		
$BCF_1(Ep \times F_1(th))$	2	4/45 (8.9)
$(F_1(th) \times E_P)$	6	24/196 (12.2)
$BCF_2(Ep \times BCF_1)$	2	13/85 (15.3)
$(BCF_2 \times Ep)$	4	22/142 (15.5)

FE : *F. esculentum*

P : Pin, Th : Thrum.

crosses between heterostyle (thrum-type) F_1 and *F. homotropicum* produced fertile plants. When *F. esculentum* (pin) was used as female, fertility was decreased. Self-pollination of homostyl plants resulted in favorable seed set. Seeds of the progenies produced in the reciprocal crosses, i.e., *F. esculentum* (pin) × F_1 hybrids (thrum) and F_1 hybrids (thrum) × *F. esculentum* (pin), were similar to those of common buckwheat, *F. esculentum* in their morphological characters (Table 1).

Pollen tube growth

The frequency of pollen tube penetration into micropyle and their inhibition part in pistil at six hours after pollination were used as a criterion to distinguish compatibility and incompatibility (Table 2 and Fig. 1). Both thrum-type BCF_1 × pin-type *F. esculentum* and its reciprocal cross, i.e., pin-type *F. esculentum* × thrum-type BCF_1, were compatible, but the degree of compatibility in reciprocal combinations was slightly different. The length of pollen tubes and their micropylar penetration in

Table 2. Seed fertility and variation of incompatibility in the progenies of interspecific hybrids of *F. esculentum* × *F. homotropicum*

Cross combination	No. of seeds in selfing and backcrossing			Pollen tube penetrated at micropylar region(%)
	No. of flowers pollinated	No. of seeds formed	No. of seeds/ pollinated flower(%)	
Homostyle				
F_1 self	223	23	10.3	30.0
F_2 self	880	77	8.8	20.0
Thrum heterostyle				
BCF_1 × FE(P)	142	22	15.5	30.0
BCF_1 × FE(Th)	56	0	0	0
BCF_1 × FH	142	1	1.4	10.0
FE(P) × BCF_1	45	5	11.4	20.0
FE(Th) × BCF_1	85	6	7.0	10.0
FH × BCF_1	32	0	0	0

FE : *F. esculentum*. FH : *F. homotropicum*
P : Pin, Th : Thrum.

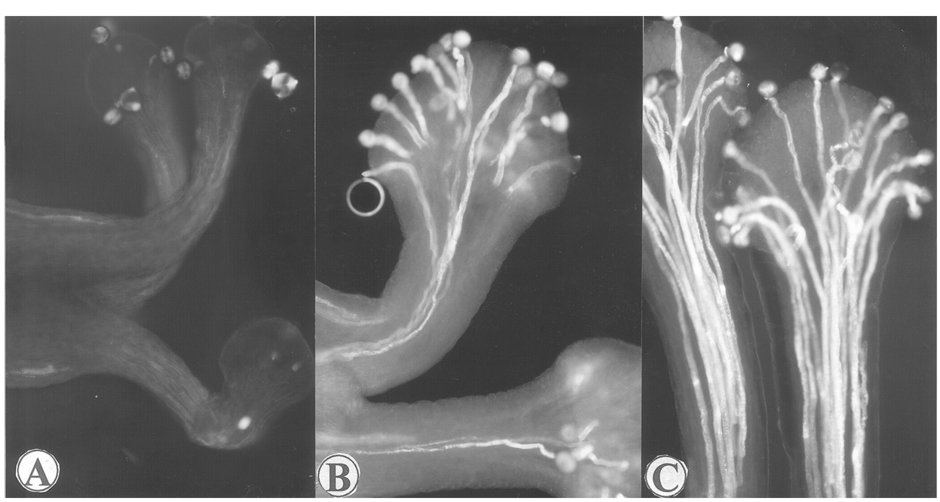

Fig. 1. Typical pollen tube growth in interspecific crosses in *Fagopyrum.*
A : *F. esculentum* (pin) × BCF_1 (thrum)
B : BCF_1 (*thrum*) × *F. homotorpicum*
C : BCF_1 (*thrum*) × *F. esculentum* (pin) (×100)

slightly compatible combinations were greatly variable in different pollinated pistils and penetration in these combinations must be limited.

In incompatible combinations, the growth of pollen tubes was inhibited in three different parts of pistils (Fig. 1 - A, B and C). In most cases, pollen tubes with thickened wall and burst tip stopped to grow in stigma. The growth of pollen tubes in reciprocal crosses between thrum-type BCF_1 and homostylous *F. homotropicum* was inhibited in styles due to swollen tips and heavy callus deposition. It was noted that the combinations in different style types, even in the same combinations, showed different degrees of campatibility.

Embryo development

Under the Nomarski`s interference microscopy, normal embryos at early development stage was distinguishable as regular shape, club-shape, early-globular, globular and early-heart shape. Normal embryos showed a detectable amount of divided cells and nuclei, an increased number of free endosperm nuclei in embryo sac, and connection with micropyle by suspensor (Fig. 2). The stages of embryo development were similar to past these reports (Adachi, 1990; Guan and Adachi, 1992).

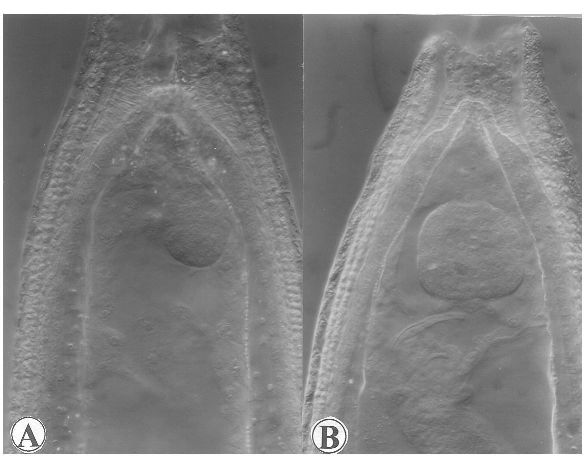

Fig. 2. DIC micrographs of embryo development showing normal embryo and free enderpserm nuclei.
A: BCF_1(thrum) × *F. homotropicum* at 2 DAP.
B: BCF_1 (thrum) × *F. esculentum* (pin) at 3 DAP.

In vitro embryo rescue

The results of ovule culture and embryo rescue in backcross of F_1 (*F. esculentum* × *F. homotropicum*) to *F. esculentum* were summarized in Table 3. Although ovaries were swollen in ovule culture, most of them were shriveled when ovaries were opened. From 3 to 7 days after pollination, the collection of ovaries appeared to give the most promising result. The ovaries were excised five days after pollination and cultured for 14 days. The regenerated plants were excised from the embryo and rooted on hormone free MS medium with 3% sucrose.

Discussion

Pre-zygotic interspecific barriers

This is the first study on pre-fertilization barrier by investigating pollen tube growth and embryo development at early stages in interspecific hybridizations in genus *Fagopyrum*. The growth of *F. esculentum* pollen tubes in styles of interspecific hybrids of *F. esculentum* × *F. tataricum* and *F. esculentum* (pin) × *F. homotropicum* was greatly disturbed. Disturbed and disoriented growth and callus plug formation most likely accounted for the failure of pollen tubes to reach to the lower half of styles and penetrating to micropyles. Therefore, fertilization and subsequent development of embryo and seed were very limited. Similar descriptions of disturbed pollen tube behavior affecting seed production in various interspecific crosses in *Fagopyrum* have been reported by Morris (1951), Nagatomo (1961), Krotov and Dranenko (1975), Adachi *et al*. (1983), Ujihara *et al*. (1990) and Samimy (1991), Samimy

Table 3. Embryo development and its regeneration from backcrossing to *F. esculentum*

DAP	No. of cultured ovules	No. of formed embryos (%)	No. of regenerated plants (%)
3	20	2 (10)	0 (0)
5	20	5 (25)	2 (10)
7	20	3 (15)	1 (5)

DAP : Days after pollination.

et al. (1996) and Hirose *et al*. (1995).

The micropylar penetration was greatly reduced in comparison to the incidence of greening and swelling of ovaries. Also, these traits turned out to be a marker inadequate to identify a successful fertilization or embryo and seed development. Evidently, stylar incongruity appeared to be strong pre-zygotic barriers restricting ovules to develop into viable progenies. The high recovery ratio of offsprings through embryo rescue and ovule culture in interspecific cross between *F. esculentum* and *F. homotropicum* was remarkable.

Post-zygotic embryo abortion and embryo rescue

Application of *in vitro* techniques to overcome breeding barriers in *Fagopyrum* was restricted for a long time before the use of embryo rescue. Ujihara *et al*. (1990), Samimy (1991) and Samimy *et al*. (1996) have exploited the possible utilization of ovule culture. We reported the first successful production of interspecific hybrid between common buckwheat, *F. esculentum* (thrum) and *F. homotropicum* through embryo rescue (Woo and Adachi, 1995; Woo *et al*., 1999). According to our results we want to report the stylar differences and variations in compatability, seed set and seed development. Before the any further breeding work, the nature of interspecific hybrids must be clarified (Campbell, 1995). This is especially important when wild species are used as female parent. For technical reasons, manual castration of wild species is almost impossible and, therefore, self-fertilization cannot be excluded.

On the other hand, the reciprocal crosses between *F. esculentum* and *F. homotropicum* were not obtained in this study. Samimy (1991) failed to produce the hybrids through embryo rescue when *F. tataricum* was used as a female. This study indicates that it is very difficult to obtain interspecific hybrids between *F. esculentum* and *F. homotropicum*, which is used as a female parent. It was also speculated that embryo rescue was very difficult because of the small flower of this species. However, it is reasonable that embryo rescue is necessary to obtain the interspecific hybrids.

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