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Towards Intergeneric Hybridization between *Alstroemeria* L. and *Bomarea* Mirb.

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ABSTRACT

There are many interspecific hybrids of *Alstroemeria*. In this study, the possibility of intergeneric hybridization between *Alstroemeria* and *Bomarea* Mirb. was examined through the development of pollination procedures and ovule culture based on the histological observation of embryo and endosperm development after intergeneric pollination. Three methods of pollination (stigmatic, cut-style, and non-style) were combined with four different pollen types (fresh, frozen, non-germinated, and pre-germinated). We observed that the pollen tubes of *Bomarea coccinea* (Ruiz & Pav.) Baker could reach to the ovules of *Alstroemeria aurea* Graham 48 hours after stigmatic pollination with frozen pollen. Histological observations revealed that a primary embryo was formed, but subsequently aborted during development. This study demonstrates the possibility of intergeneric hybridization between *Alstroemeria* and *Bomarea*, but showed that there are post-fertilization barriers between *A. pelegrina* and *B. coccinea*. Further study is needed to investigate the optimum conditions for obtaining hybrid progeny.

Keywords: Alstroemeriaceae, embryo and endosperm development, ovule, pollen tube
Abbreviations: DAP, days after pollination; **FP**, fresh pollen; **FZP**, frozen pollen grains; **HAP**, hours after pollination; **NGP**, non-germinated pollen grains; **PGP**, pre-germinated pollen grains

INTRODUCTION

Alstroemeria L. is a genus of monocotyledonous ornamental plants belonging to the family Alstroemeriaceae that have become popular cut flowers because of their long post-harvest life and wide variety of flower colours. Numerous cultivars have been produced through intra- and interspecific hybridization (Goemans 1962) and mutation breeding (Goemans 1962; Broertjes and Verboom 1974). Some interspecific hybrids of *Alstroemeria* species have also been produced using ovule culture techniques (Buitendijk *et al*. 1995; De Jeu and Jacobsen 1995; Lu and Bridgen 1996; Ishikawa *et al*. 1997, 2001; Shinoda and Murata 2003). Although many cultivars have been produced by these hybridizations, it is still desirable to introduce new cultivars with novel characters. Bridgen *et al*. (2009) mentioned that intergeneric hybridization between *Alstroemeria* and *Leontochir ovallei* Phil. had been achieved, but that the resultant hybrid could not be induced to flower. *Bomarea* Mirb. is another genus in Alstroemeriaceae. The genus consists of about 280 species (Hofreiter and Tillich 2002). Some *Bomarea* species have different flower colours and growth forms compared to *Alstroemeria* (Kashihara *et al*. 2011). Thus, the broad variation within *Bomarea* is considered to be an useful source for further improvement of *Alstroemeria* cultivars. In our previous study, intergeneric crossing between *Alstroemeria* and *Bomarea* was examined (Kashihara *et al*. 2010). However, the plantlets obtained after intergeneric crossing died during *in vitro* culture.

In general, successful interspecific and intergeneric hybridization is difficult due to cross-incompatibility. To overcome interspecific pre-fertilization barriers in *Lilium*; cut-style, grafted-style, and placental pollination techniques have been used (Asano and Myodo 1977; Van Tuyl *et al*. 1991; Janson 1993). With regard to post-fertilization barriers; embryo, ovule, ovary-slice and ovary culture have been shown to be effective in producing progenies (Asano 1982; Van Tuyl *et al*. 1991). Further, ovule culture has been used to achieve intergeneric hybridization in monocotyledonous ornamental crops such as *Sandersonia aurantiaca* Hook. × *Littonia modesta* Hook. (Morgan *et al*. 2001) and *Sandersonia aurantiaca* × *Gloriosa rothschildiana* O'Brien (Nakamura *et al*. 2005).

In the present study, the possibility of intergeneric hybridization between *Alstroemeria* and *Bomarea* was investigated through several pollination methods and ovule culture based on the observation of embryo and endosperm development.

MATERIALS AND METHODS

Plant materials

Alstroemeria aurea Graham, *A. pelegrina* L., and *Bomarea coccinea* (Ruiz & Pav.) Baker were used in this study. These plants were grown in a greenhouse at Hokkaido University. Plants were grown under natural light conditions, and the greenhouse was maintained at a minimum temperature of 15°C by heating during winter.

Methods of pollination

Flowers were emasculated and covered with paraffin paper bags. Following the maturation of pistils within the emasculated flowers, cross-pollination was attempted between *Alstroemeria* and *Bomarea*. Three pollination methods were used: stigmatic, cut-style, and non-style. For cut-style pollination, pistils were excised halfway using a razor blade. For non-style pollination, whole styles were excised.

Four pollen grain types were used for pollination: fresh (FP),

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frozen (FZP), non-germinated (NGP), and pre-germinated (PGP) pollen. For preparation of NGP and PGP, liquid culture medium was used to stimulate pollen germination. Instead of stigma, liquid culture medium helps hydration of pollen grains at the cut place. NGP were mixed in pollen germination medium immediately prior to pollination. PGP were cultured in the medium for 30 minutes prior to pollination. The pollen germination medium consisted of 0.01% (w/v) H_3BO_3, 0.01% (w/v) $CaCl_2$, 0.0007% (w/v) KH_2PO_4, 0.1% (w/v) yeast extract, and 10% (w/v) sucrose, and was adjusted to pH 5.8 before autoclaving at 121°C for 15 min (Hirano and Hoshino 2009). Fresh pollen (FP) was used as a control of the normal pollination technique.

Observation of pollen tube growth

Observation of pollen tube growth followed the procedure of Hoshino *et al*. (2006). Briefly, pistils were harvested 7, 11, 24, 33, 41, 48, 54, 65 and 103 h after pollinations, fixed in a 3:1 solution of ethanol: acetic acid for 24 h, and were maintained in a refrigerator in 70% (v/v) ethanol. Fixed pistils were softened in 1 N NaOH at 60°C for 15 min. In order to stain the pollen tubes, the pistils were treated with a 0.1% aniline blue solution (including 0.1 N K_3PO_4) for 24 h. Prior to observation, the external parts of the ovary were removed. The pollen tubes were observed under a fluorescence microscope (Axiovert 200; Carl Zeiss Micro Imaging Co., Ltd).

Histological observation of embryo and endosperm development

Serial sections of ovules were prepared following the procedure of Hoshino *et al*. (2000). Briefly, ovaries and ovules were fixed in FAA [5:5:90 parts of formaldehyde solution: acetic acid: 50% (v/v) ethanol], dehydrated in a graded series of butyl alcohol [I. 10: 40: 0: 50 parts of butyl alcohol: 95% (v/v) ethanol: absolute ethanol: distilled water, II. 20: 50: 0: 30 parts are same with I, III. 35: 50: 0: 15 parts are same with I, IV. 55: 45: 0: 0 parts are same with I, V. 75: 0: 25: 0 parts are same with I, VI. 100: 0: 0: 0 parts are same with I, VII. 100: 0: 0: 0 parts are same with I], and embedded in paraffin wax. Embedded samples were sectioned at 10 μm using a microtome (HM 315; MICROM GmbH). The sections were mounted on glass slides, dyed with Mayer's hematoxylin solution (Wako Pure Chemical Industries, Japan) for 1 h, and then observed under a light microscope (Primo Star; Carl Zeiss Micro Imaging Co., Ltd).

Ovule culture

Ovaries were harvested 7 days after pollination and surface-sterilized with sodium hypochlorite solution (1% active chlorine) and a few drop of polyoxyethylene sorbitan monooleate (Tween 20) for 15 min, and then rinsed three times in sterilized distilled water. Ovules were excised from the ovaries and then cultured on 2 g l^{-1} gellan gum-solidified MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} or 60 g l^{-1} sucrose. In the crossings of *A. aurea* and *B. coccinea*, 110 ovules from 6 ovaries were cultured on MS medium containing 30 g l^{-1} sucrose. In the crossings of *A. pelegrina* and *B. coccinea*, 128 ovules from 7 ovaries were cultured on MS medium containing 60 g l^{-1} sucrose. Cultures were maintained at 20 ± 1°C under 24-h illumination with fluorescent light.

RESULTS AND DISCUSSION

Pollen tube observation to evaluate pre-fertilization barriers

In our previous study, we observed that pollen tubes reached the ovule 24 h after pollination (HAP) in the self-pollinated pistils of *A. aurea* (Hoshino *et al*. 2006). The frequency of pollen tube entry into ovules 24 HAP was 80%. This result is consistent with the observations of de Jeu *et al*. (1992), who demonstrated that during compatible pollination in *A. aurea* and cultivar 'Jubilee', pollen tubes entered the micropyle of ovules at 24 HAP.

In the present study of intergeneric pollination, four pollen grain types, fresh pollen (FP), frozen pollen (FZP), non-geminated pollen (NGP) or pre-germinated pollen (PGP), were examined in combination with pollination methods (stigmatic, non-style or cut-style pollination) to compare the effects of pollen tube elongation and pollen tube entry into ovules. In the preliminary study, pollen fertility of one year frozen pollen grains was examined. The *in vitro* pollen germination frequencies of *A. aurea*, *A. magenta* and *A. pelegrina* were 8%, 46% and 23%, respectively (unpublished data). The pollen tubes of both FP and FZP grew up to the upper half of the style in stigmatic pollinations within 24 HAP (**Table 1**). Furthermore, stigmatic pollination was examined, the pollen tubes of both FP and FZP were observed in the ovaries during 41 to 103 HAP. Thus, no obvious difference in the competence of pollen tube elongation was observed between FP and FZP. Finally, we observed that the pollen tubes of *B. coccinea* entered the ovules of *A. aurea* 48 hours after stigmatic pollination with FZP (**Fig. 1A, 1B**). To obtain FP for intergeneric pollination was difficult for continuous experiments because the flowering season of *Bomarea* does not always concur with that of *Alstroemeria*. Therefore, we used the FZP for pollination in subsequent experiments.

The effects of non-style or cut-style pollination were shown in **Table 2**. The pollen tubes of *B. coccinea* entered the ovules of *A. aurea* 24 h after non-style pollination with FZP however the pollen tubes of PGP could not enter to the ovary 24 h after non-style pollination. When using non-style pollination, neither FP nor NGP pollen grains had germinated on the surface of ovaries at 33 HAP. The pollen grains of both FP and NGP used in cut-style pollinations had failed to germinate on the cut surface at 48 HAP. Cut-style pollination has been used to overcome pre-fertilization barriers to produce interspecific hybrids in *Lilium* (Asano and Myodo 1977; Van Tuyl *et al*. 1991). However, the styles of *Alstroemeria* are more delicate than those of

Table 1 Pollen germination and pollen tube growth of *B. coccinea* in the pistil and ovarian regions of *A. aurea* with stigmatic style.

Hours after pollination	No. of pistils	Pollen type*	Germinated on stigma or surface of ovary	Pollen tubes growing up to					No. of pollen tube entry per ovule
				Stigmatic region	Upper half of style	Lower half of style	Ovary	Ovule	
7	2	FZP	2/2	2/2	0/2	0/2	0/2	0/2	0/32[1)]
11	2	FZP	2/2	2/2	0/2	0/2	0/2	0/2	0/34[1)]
24	1	FP	1/1	1/1	1/1	0/1	0/1	0/1	-
24	2	FZP	2/2	2/2	2/2	0/2	0/2	0/2	0/21[1)]
33	4	FZP	3/4	3/4	2/4	0/4	0/4	0/4	0/106
41	1	FP	1/1	1/1	1/1	1/1	1/1	0/1	-
48	3	FP	2/3	2/3	2/3	1/3	1/3	0/3	0/53
48	20	FZP	20/20	20/20	20/20	19/20	16/20	2/20	2/451
54	2	FP	2/2	2/2	2/2	1/2	1/2	0/2	0/20
65	1	FZP	1/1	1/1	1/1	1/1	1/1	0/1	0/21
103	1	FP	1/1	1/1	1/1	1/1	1/1	0/1	-

Pollen type: FP, fresh pollen grains; FZP, frozen pollen grains

1) Data obtained from 1 ovary.

Table 2 Pollen germination and pollen tube growth of *B. coccinea* in the pistil and ovarian regions of *A. aurea* with Cut-style and Non-style.

Hours after pollination	No. of pistils	Pollen type*	Style type	Germinated on stigma or surface of ovary	Pollen tubes growing up to					No. of pollen tube entry per ovule
					Stigmatic region	Upper half of style	Lower half of style	Ovary	Ovule	
24	3	FZP	Non-style	2/3	-	-	-	2/3	2/3	2/59[2)]
24	5	PGP	Non-style	3/5	-	-	-	0/5	0/5	0/101
33	1	FP	Non-style	0/1	-	-	-	0/1	0/1	0/16
33	1	NGP	Non-style	0/1	-	-	-	0/1	0/1	0/21
48	1	FP	Cut-style	0/1	-	-	0/1	0/1	0/1	0/19
48	1	NGP	Cut-style	0/1	-	-	0/1	0/1	0/1	0/21
48	2	FZP	Non-style	0/2	-	-	-	0/2	0/2	0/64
54	2	FZP	Cut-style	2/2	-	-	0/2	0/2	0/2	0/36[1)]
103	1	FP	Cut-style	1/1	-	-	1/1	1/1	0/1	-

*Pollen type: FP, fresh pollen grains; FZP, frozen pollen grains; NGP, non-germinated pollen grains; PGP, pre-germinated pollen grains
1) Data obtained from 1 ovary.
2) Data obtained from 2 ovaries.

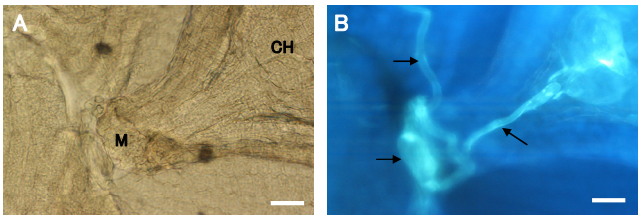

Fig. 1 Pollen tube entry into the micropylar region of *A. aurea* × *B. coccinea*. (**A**) Bright field. (**B**) Fluorescence. Arrows indicates pollen tube. Abbreviations: CH, chalazal side; M, micropylar side. Scale bar = 100 μm.

Lilium and, after cutting, the style withers and immediately dries. Consequently, the cross-sectional diameter of the style becomes narrow and it is difficult to place a sufficient number of pollen grains within the style. Janson *et al*. (1993) and Gurusamy *et al*. (2007) reported that the frequencies of fertilization were lower in cut-style pollination compared with stigmatic pollination. Further, compared with stigmatic pollination, non-style pollination showed low pollen germination on the stigma or the surface of the ovary. Therefore, we used stigmatic pollination in subsequent experiments.

A pre-fertilization barrier is known to exist in certain plants such as *Lilium* (Asano and Myodo 1977; Van Tuyl *et al*. 1991), *Oryza* (Suputtitada *et al*. 2000), and *Gossypium* (Ganesh Ram *et al*. 2008). In the present study, we observed pollen tubes entering into ovules following both stigmatic pollination and non-style pollination in *A. aurea* × *B. coccinea*. This result shows that there are no strong pre-fertilization barriers (such as upper inhibition or lower inhibition in lilies: Van Tuyl and de Jeu 2005) between *A. aurea* and *B. coccinea*.

Histological observation of embryo and endosperm development to evaluate post-fertilization barriers

De Jeu and Garriga Calderé (1997) described embryo and endosperm development in self-fertilized ovules of *A. pelegrina*. According to their research, at 2 and 3 days after pollination (DAP), zygotes undergo the first mitotic division. The proembryo is differentiated into a round-shaped embryo at 6 DAP. Globular stage embryos surrounded by nuclear endosperm were observed at 8 DAP, and these embryos were still observed at 14 DAP, but with cellularization of the endosperm.

In our study, in the intergeneric crossing of *A. pelegrina* × *B. coccinea*, a three-celled embryo was found at the ovule at 3 DAP (**Fig. 2A, 2B**) at which time also the endosperm nucleus was observed (**Fig. 2C**). At 5 DAP, the ovule had grown in the transverse direction (**Fig. 2D**). The embryo differentiated into a round-shaped embryo (**Fig. 2E**), and coenocytic endosperm nucleus division was observed (**Fig. 2F**). At 7 DAP, a loss of contact between the endospermic

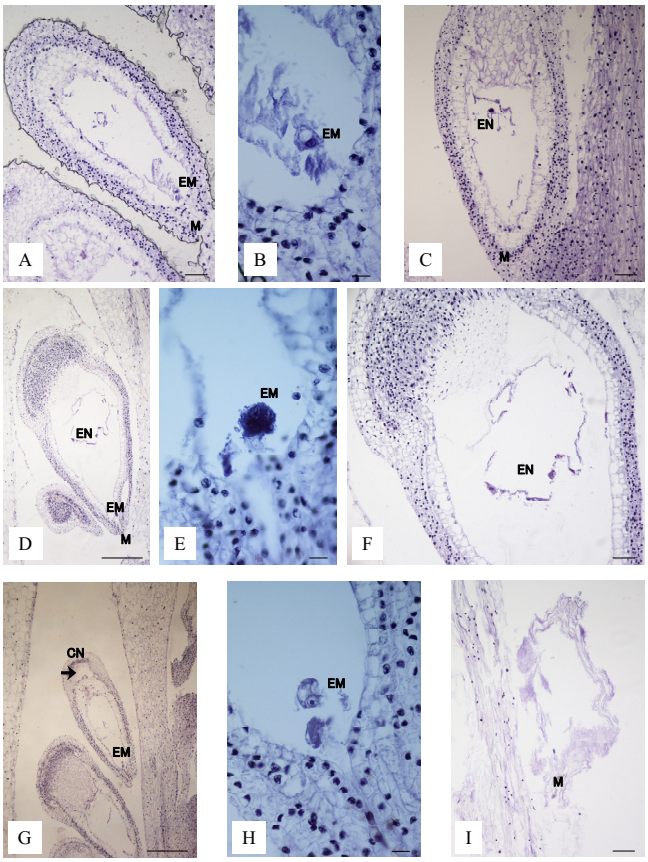

Fig. 2 Histological observation of the ovules of *A. pelegrina* × *B. coccinea*. (**A**) Ovule at 3 DAP, growing ovule. (**B**) Ovule at 3 DAP, three-celled embryo. (**C**) Ovule at 3 DAP, endosperm nucleus. (**D**) Ovule at 5 DAP growing in a transverse direction. (**E**) Ovule at 5 DAP, a round-shaped embryo. (**F**) Ovule at 5 DAP, the coenocytic endosperm nucleus. (**G**) Ovule at 7 DAP, arrow indicates a gap between the endospermic transfer layer and the chalazal nucellus. (**H**) Ovule at 7 DAP, degenerated embryo. (**I**) Ovule at 21 DAP, aborted ovule. Abbreviations: CN, chalazal nucellus; EM, embryo; EN, endosperm nucleus; M, micropylar side. Scale bars equal 500 μm (**D**, **G**), 100 μm (**A**, **C**, **F**, **I**) and 20 μm (**B**, **E**, **H**).

transfer wall and the chalazal nucellus occurred within the ovule (**Fig. 2G**), and the embryo had degenerated (**Fig. 2H**). At 21 DAP, the ovule had aborted (**Fig. 2I**). In a cross between *A. pelegrina* and *A. aurea*, De Jeu and Garriga Calderé (1997) observed that there was no cellularization of the endosperm, and that the ovules shrunk and lost their swollen shape. Aw *et al*. (2010) reported that sperm entry in *Arabidopsis* triggers division of the central cell, but that the paternal genome is required for endosperm development. In order to explain the absence of cellularization, we speculate that the failure of karyogamy in the central cell prevents incorporation of the paternal genome. These histological

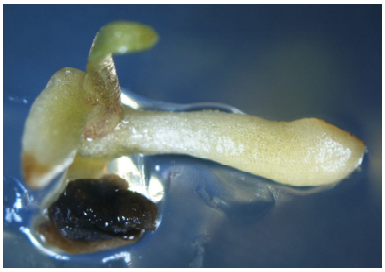

Fig. 3 Germination from cultured ovule of *A. pelegrina* × *B. coccinea*. Scale bar = 1 mm.

observations revealed that there are post-fertilization barriers between *A. pelegrina* and *B. coccinea*.

Ovule culture to overcome post-fertilization barriers

To overcome post-fertilization barriers in *Lilium*, embryo, ovule, ovary-slice and ovary culture are used in interspecific crossings (Asano 1982; Van Tuyl *et al*. 1991). Moreover, some intergeneric hybrids have been produced using ovule culture in monocotyledonous ornamental crops (Morgan *et al*. 2001; Nakamura *et al*. 2005; Amano *et al*. 2009).

In the present study, 1 or 2 germinations were observed using ovule culture after intergeneric pollinations in the crossings of *A. aurea* and *B. coccinea* or in the crossing of *A. pelegrina* and *B. coccinea* (**Fig. 3**), respectively. This indicates that immature embryos may be rescued by ovule culture. We investigated optimum ovule culture conditions (Kashihara *et al*. 2010). At present, however, no surviving seedlings have been obtained. Li *et al*. (1997) demonstrated that hybrid breakdown in rice results from uncoupling of coadapted subspecific gene complexes by recombination. We presume that this indicates that the survivability of seedlings is likely to be low. However, more research is needed to unravel these relationships.

CONCLUSIONS

Cross-pollination for intergeneric hybridization between *Alstroemeria* and *Bomarea* was examined. By observing pollen tubes with aniline blue staining, we confirmed that *Bomarea* pollen tubes could reach and enter ovules. On the basis of histological examinations of embryo and endosperm development after intergeneric pollination, we were able also to confirm the formation of primary embryos. These embryos, however, eventually aborted. Preliminary attempts to rescue embryos after intergeneric pollination revealed the germination of cultured embryos, but subsequent plantlet lethality. Further study should be focused on the development of precise and appropriate embryo or ovule culture systems.

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