Production of Intergeneric Hybrids of *Eutrema wasabi* Maxim. and *Armoracia rusticana* ph. Gaertn., B. Mey. et Scherb. by Ovule Culture

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Summary

Embryo development in ovules of intergeneric hybrids between *E. wasabi* cv. Takai (*2n = 14*) and *A. rusticana* ‘Akame’ (*2n = 32*) proceeded up to 30 days after pollination. In contrast, in the reciprocal cross of *A. rusticana* × *E. wasabi*, the embryo development was retarded and nearly all hybrid embryos degenerated by the 30th day after pollination. The hybrid ovules derived from *E. wasabi* × *A. rusticana* eventually yielded plantlets, whereas none were obtained in the reciprocal cross. We found that the MS medium for our ovule culture was superior to White's medium. The analysis of esterase isozyme and the chromosome numbers in the root tip cells, *2n = 23*, confirmed that the plants obtained by ovule culture were true hybrids of *E. wasabi* × *A. rusticana*. The morphological characteristics of the hybrid plants resembled those of *E. wasabi*.

Introduction

*Eutrema wasabi* Maxim. (wasabi) is a plant unique to Japan. It is an important vegetable condiment for Japanese dishes because of its piquancy. The environment conducive for the production of large succulent, enlarged stems is limited to flooded gravel and sandy fields along streams or near springs. On the other hand, *Armoracia rusticana* ph. Gaertn., B. Mey. et Scherb. (horseradish) contains sinigrin which has a hot, biting quality like wasabi and adapts to diverse soil conditions; however, its flavor properties are inferior to those of wasabi. The purpose of this experiment was to produce an intergeneric hybrid which has the desirable traits of both species. Under natural conditions, viable seeds are not easily obtained between intergeneric and interspecific crosses because the hybrid embryo degenerates in its early developmental stage (Chung et al., 1988). Ovule culture (embryo rescue) has been used to overcome such cross-incompatibility (Hossain et al., 1988; Imanishi, 1988; Iwai et al., 1985). In a preliminary experiment previously carried out, when *E. wasabi* was used as the female parent, the embryos failed to develop.

In the present experiment, ovule culture was used to obtain intergeneric hybrids, and the development of hybrid embryos was observed after pollination to estimate the optimal time of embryo rescue. The characteristics of hybrids obtained by ovule culture are reported herein.

Materials and Methods

Strains in *E. wasabi* cv. Takai (*2n = 14*) and *A. rusticana* cv. Akame (*2n = 32*) were used. *Armoracia rusticana* may be classified into two groups, i.e. ‘Akame’ and ‘Aome’ which do not clearly show the varietal differentiation of *A. rusticana* yet in Japan. Generally, the flowering of *A. rusticana* is about one month later than *E. wasabi*. Therefore, the plants of *A. rusticana* were cultured in a growth chamber to control the flowering. Twenty-four plants of *E. wasabi* and 14 plants of *A. rusticana* were used for self-pollination and reciprocal crosses. Selfed and crossed ovaries were collected between 10 and 30 days after pollination. The ovaries were fixed in FAA, embedded in paraffin, sectioned at 10 μm. After staining the sections with safranine and first base green, the embryo development was observed. The styles, sampled 50 hr after pollination, were fixed in FAA, embedded.
in paraffin, and sectioned at 10 μm thickness; the sections were stained with aniline blue and examined. Thereafter, the elongation of the pollen tube was observed by fluorescence microscope. The hybrid ovules were excised from ovaries on the 20th and 30th day after pollination, and their development was examined macroscopically.

Hybrid ovules of reciprocal crosses of *E. wasabi* and *A. rusticana* excised on the 10th, 20th and 30th day after pollination were cultured on MS medium and White's medium. These media, containing 30 g·liter⁻¹ sucrose (pH 5.8) and 6 g·liter⁻¹ agar were autoclaved at 121 °C under 1.2 kg·cm⁻² for 13 min. The ovaries obtained by reciprocal crosses were collected on each date after pollination. They were surface-sterilized by dipping in 70% ethanol for 30 sec and 1% sodium hypochlorite for 10 min; they were rinsed three times with sterile water. The hybrid ovules were excised from the ovaries and transferred to both MS medium and White's medium. The ovules were cultured in scattering light at 18 °C. Plantlets obtained by the culture were transplanted to MS medium and cultured at 18 °C with 16 hr photoperiod of ca. 3,000 lx. Plantlets which developed normally in the culture were transplanted to pots containing sand and vermiculite (1:1, v/v) and grown in a greenhouse maintained at 12 ° to 20 °C.

All of the hybrid plants which survived after potting were used for counting their chromosomes and isozyme analysis. To count chromosomes, at least 3 root tips per plant were collected. Those materials were kept at 0 °C for 24 hr in distilled water. Thereafter, they were fixed in an ethanol-acetic acid mixture (3:1, v/v) and kept overnight at 4 °C, then stained by the Feulgen method. The chromosome number was counted in at least 5 cells per root. Esterase isozymes extracted from a mature leaf were analyzed by the electrofocusing method (Nakai, 1970).

### Results

**Development of hybrid ovules and selfed and hybrid embryos**

In the observation of the hybrid ovules at 20 and 30 days after pollination, the globular ovules without wrinkles were judged to be the normally developed ovules. The number of normally developed ovules per ovaries obtained from crosses of *E. wasabi* × *A. rusticana* slightly outnumbered those from the reciprocal crosses (Table 1).

Examination of selfed and hybrid embryos in *E. wasabi* and *A. rusticana* (Fig. 1) revealed that among the selfed embryos of *E. wasabi* obtained at 10 days after pollination, 43.0% of them was developing into normal, globular embryos. The survival rate of embryos decreased gradually thereafter, until it reached 26.8% on the 30th day after pollination. In the selfed embryos of *A. rusticana*, the percentage of normally developed embryos was 40.0% on the 10th day after pollination, but the survival rate of embryos decreased rapidly until 30 days after pollination. The embryos, which

<table>
<thead>
<tr>
<th>Cross-combinations</th>
<th>Days after polli.</th>
<th>No. of flowers pollinated (a)</th>
<th>No. of ovules developed (b)</th>
<th>b/a</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. wasabi</em> ×</td>
<td>20</td>
<td>1691</td>
<td>159</td>
<td>0.09</td>
</tr>
<tr>
<td><em>A. rusticana</em></td>
<td>30</td>
<td>1608</td>
<td>107</td>
<td>0.07</td>
</tr>
<tr>
<td><em>A. rusticana</em> ×</td>
<td>20</td>
<td>1533</td>
<td>70</td>
<td>0.05</td>
</tr>
<tr>
<td><em>E. wasabi</em></td>
<td>30</td>
<td>1516</td>
<td>46</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Fig. 1. Survival rate of selfed and hybrid embryos after pollination.  
□ : selfed embryos of *E. wasabi*  
■ : selfed embryos of *A. rusticana*  
△ : hybrid embryos of *E. wasabi* × *A. rusticana*  
▲ : Hybrid embryos of *A. rusticana* × *E. wasabi*
barely survived in the ovules, developed into a globular stage on the 20th day after pollination.

The examination of hybrid embryos of *E. wasabi* and *A. rusticana* (Figs. 1 and 2) show that the pollen tubes reached the ovules 50 hr after pollination. The cells of embryos of *E. wasabi* × *A. rusticana* divided more than once on the 3rd day after pollination. On the 10th day, oval-shaped proembryos were observed and the percentage of normally developed embryos became 32.8%. The sur-

![Fig. 1. Elongation of pollen tubes and development of hybrid embryos.](image1)

**A.** A pollen tube in an ovule at 50 hr after pollination. **B.** Globular stage (arrow) 20 days after pollination. **C.** Heart-shaped stage (arrow) 30 days after pollination. **D.** A pollen tube in an ovule at 50 hr after pollination. **E.** Degenerated ovules 20 days after pollination. **F.** Wrinkled ovules (arrows) 30 days after pollination.

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![Fig. 2. Elongation of pollen tubes and development of hybrid embryos.](image2)

**A.** B, C are ovules and embryos derived from *E. wasabi* × *A. rusticana*. **D.** E, F are ovules and embryos derived from *A. rusticana* × *E. wasabi*. **A.** Pollen tubes in ovule at 50 hr after pollination. Arrow indicates pollen tubes. **B.** Globular stage (arrow) 20 days after pollination. **C.** Heart-shaped stage (arrow) 30 days after pollination. **D.** Pollen tubes in ovule at 50 hr after pollination. Arrow indicates pollen tubes. **E.** Degenerated ovules 20 days after pollination. **F.** Wrinkled ovules (arrows) 30 days after pollination.
vival rate of embryos, however, decreased rapidly to 12.0% on the 20th day after pollination. The hybrid embryos which survived up to 20 days after pollination entered a globular stage, after which the endosperm degenerated. Almost all of the surviving embryos attained a heart-shape on the 30th day after pollination; however, the survival rate of embryos fell to 6.0%.

On the other hand, when the pollen tubes reached the ovules on the 10th day after pollination in crosses between A. rusticana × E. wasabi, the percentage of normally developing embryos was 27.0%, the lowest among the four combinations. On the 30th day after pollination, almost all of the hybrid embryos degenerated, and the ovules became empty.

We found that the development of hybrid embryos between E. wasabi × A. rusticana was retarded compared with that of selfed embryos of E. wasabi. Furthermore, many hybrid embryos aborted between the early globular and the heart-shaped stages. In the reciprocal cross, few embryos advanced to the heart-shaped, which can be attributed to the abortion of the endosperm at the early developmental stage.

Ovule culture of hybrids

On the basis of histological observation, ovules on the 10th day after pollination having the high survival rates were cultured. In both reciprocal crossed of E. wasabi and A. rusticana, some hybrid ovules enlarged after being transferred to the MS or White's medium. However, no plantlets were obtained from the ovules. Therefore, ovules extracted on the 20th and 30th day after pollination were cultured, and these results are shown in Table 2.

Ovules excised from ovaries from crosses between E. wasabi and A. rusticana sampled at 20 days after pollination germinated and grew continuously in MS medium from 110 to 120 days after excision and developed into plantlets. The numbers of germinated ovules and plantlets obtained through ovule culture were 5 (3.2%) and 4 (2.6%), respectively. Furthermore, plantlets were also obtained from the ovules which were transferred to MS medium on the 30th day after pollination. The ovules germinated and grew continuously from 50 to 70 days after the onset of their incubation. The numbers of germinated ovules and plantlets obtained through ovule culture were both 9 (7.3%). In contrast, on White's medium, no plantlet was obtained from the ovules which were transferred at 20 days after pollination; whereas, a few plantlets were rescued from ovules which were excised at 30 days after pollination.

In the cross of A. rusticana × E. wasabi, no plantlet was obtained from ovules which were ex-

<table>
<thead>
<tr>
<th>Cross-combinations</th>
<th>Days after poll.</th>
<th>Media</th>
<th>No. of ovules inoculated</th>
<th>No. of ovules germinated (%)</th>
<th>No. of plantlets obtained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. wasabi</td>
<td>10</td>
<td>MS</td>
<td>216</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>White</td>
<td>165</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>A. rusticana</td>
<td>20</td>
<td>MS</td>
<td>156</td>
<td>5 (3.2)</td>
<td>4 (2.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>White</td>
<td>82</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>MS</td>
<td>124</td>
<td>9 (7.3)</td>
<td>9 (7.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>White</td>
<td>90</td>
<td>2 (2.2)</td>
<td>1 (1.1)</td>
</tr>
</tbody>
</table>

Total number of ovules germinated up to 150 days after inoculation.

Total number of plantlets obtained up to 150 days after inoculation.
cised and cultured at 20 and 30 days after pollination. Although a few hybrid embryos survived and developed normally when excised at 20 days after pollination based on their histology, these embryos germinated but appeared abnormal because they lacked cotyledons and shoot apices.

Fourteen plantlets derived from a hybrid ovule from the cross of *E. wasabi* × *A. rusticana*, when transferred to a MS medium, became acclimated and grew.

**Characteristics of hybrids**

A hybrid plant delivered from *E. wasabi* × *A. rusticana* is shown in Fig. 3. Eleven hybrid plants which were transferred to a greenhouse appeared phenotypically uniform. Almost all of the morphological characteristics of the hybrid plants resembled to *E. wasabi*, that is, the petioles were long and the leaves were heart-shaped. The leaves of the hybrid plant were thicker and the color were deeper than those of the parents. Of 11 hybrid plants, 3 degenerated at acclimatization stages without developing a shoot apex and 8, which survived, grew very slowly as compared with their parents. The investigation on ecological characteristics of the hybrid plants is being conducted currently in the experimental field.

The number of chromosomes in the root tip cells in 9 of 11 hybrid plants was 23 (Fig. 3); whereas, the parental *E. wasabi* and *A. rusticana* stocks used in this experiment had 14, and 32, respectively. Two hybrid plants exhibited aneuploid chromosomes i.e. \(2n = 25\) and \(2n = 26\), but they appeared phenotypically like the 9 with \(2n = 23\). The number of chromosomes in the cells of each hybrid plant was the same. The zymogram of esterases (Fig. 4) confirm that the isozyme patterns of 8 hybrid plants can be classified into 3 types showing hybridity of *E. wasabi* and *A. rusticana*.

**Discussion**

The hybrids could not be obtained when the ovules were isolated and cultured at 10 days after pollination. Considerable works have gone into the production of interspecific and intergeneric hybrids in various genus (Bajij et al., 1986; Neal and Topoleski, 1985; Pattee and Mohapatra, 1987). In most of these reports, the hybrids which were obtained by ovule culture and embryo rescue technique when the embryo advanced to the heart-shape stage. We obtained the same results in the present experiment, hybrids could not be obtained from very young ovules.

Shizukuda and Nakajima (1982), Chung et al. (1988), and Chen and Adachi (1992) reported that the interspecific hybrid embryos excised in the proembryo to globular stage could be rescued by a nutritionally adequate culture medium which would replace the endosperm. In this experiment, the hybrid ovules at proembryo stage could not be rescued, even though the culture media were nutritionally adequate. It may be considered that the discrepancy is due to the difference of materials. In the cross between relatively allied species, hybrid ovules in an early stage may be made to grow by substituting essential nutrients for the endosperm. Between more distant crosses, e.g. intergeneric one, the rescue of young ovules is not achieved by nutrients in the culture media; other factors are involved. However, these factors are not elucidated in the present experiment. Ishizaka and Uematsu (1992) have reported in the ovule culture of *Cyclamen*, MS medium was superior to White's medium for germinating hybrid ovules. They pointed out that the hybrid embryos of cyclamen might have a strong ammonium ion requirement in their development process in vitro, because the major difference between these media was the presence of ammonium ion in the MS medium. Similarly, we found the MS medium to be superior to White's medium for obtaining the hybrid plantlets. Therefore, the hybrid embryos of *E. wasabi* × *A. rusticana* may also have a strong ammonium ion requirement.

No hybrid plantlets in a cross between *A. rusticana* × *E. wasabi* was obtained. Maruhashi et al. (1988) reported that selfed plants of *A. rusticana* could be obtained by ovule culture but the success rate was low. We conclude that very few seeds of *A. rusticana* are generally produced under natural conditions. Thus, we attribute the inability to obtain hybrid plants from a cross between *A. rusticana* × *E. wasabi* to the self-sterility of *A. rusticana*.

In the present experiment, we obtained hybrid plants from crosses of *E. wasabi* × *A. rusticana*, albeit, the percentage of rescued embryos was very small. The cross-incompatibility between the local varieties of *E. wasabi* and *A. rusticana* was overcome by ovule culture, even though the num-
ber of chromosomes of *E. wasabi* used in this experiment (*2n = 14*) differed from that of common cultivars of *E. wasabi* (*2n = 28*).

From the results of present experiment, it is difficult to predict if more hybrids can be obtained, provided common cultivars of *E. wasabi* (*2n = 28*) are used as one parent. Ishizaka and Uematsu (1992) reported that the interspecific hybrids in the genus *Cyclamen* were produced even when parents having different number of chromosomes, *2n = 48* and *2n = 96* were crossed. We are, presently, attempting intergeneric crosses between the
common cultivars of *E. wasabi* and *A. rusticana*.

**Literature Cited**


胚珠培養による$Eutrema$ wasabi と$Armoracia$ rusticana の属間雑種の作出

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摘 要

$E. wasabi$（高井, $2n=14$）と$A. rusticana$（赤芽, $2n=32$）の正逆交配による雑種胚の発達と崩壊を組織学的に観察した。交配後 50 時間での花粉管の伸長を蛍光顕微鏡で観察したところ、いずれの組み合わせとも、花粉管は胚珠に達していた。$E. wasabi \times A. rusticana$の場合、交配後 10 日目から崩壊する雑種胚が徐々に増加し、交配後 30 日での生存率は 6.0% となった。これに対して、$A. rusticana \times E. wasabi$では、雑種胚の発達は$E. wasabi \times A. rusticana$に比べて遅く、また、崩壊も著しく急速に進み、交配後 30 日には生き残った雑種胚を含む胚珠はほとんど観察されなかった。

胚珠培養にあたっては、交配後、10, 20 および30日目の胚珠を培地に置床した。培地は MS 培地および White 培地を用いた。いずれの培地とも 3% ホク糖と 0.6 g 磺天を加え、pH 5.8 に調整した。交配後 20 日目および 30 日目に置床した$E. wasabi \times A. rusticana$を用いた。得られた植株を、合計 14 個体の植物体が得られた。しかし、$A. rusticana \times E. wasabi$では植物体は得られなかった。また、本実験で使用した材料の場合は、MS 培地が White 培地より適しているように思われた。