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Micrografting of Secondary Somatic Embryos and Seedling Tissues of Winter Oilseed Rape, *Brassica napus* ssp. *oleifera*

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The first successful micrografting was carried out on *Citrus*[1]. Later on the technique was applied to other plant species such as *Eucalyptus*[2], *Prunus*[3] and apple[4]. Micrografting was used to obtain virus-free plants[1], to rejuvenate materials[5,6] and to regenerate plants from somatic embryos[7]. There is no report on using micrografting techniques to investigate the transmissibility of somatic embryogenic potential in tissue cultures.

The establishment of secondary embryogenic cultures of *B. napus* was reported earlier[8,9]. The cultures were maintained on phytohormone-free Murashige and Skoog's medium[10]. Secondary somatic embryos (hereafter referred to as secondary embryos) developed mainly from the hypocotyl surface. The secondary embryogenic potential could be maintained for years in culture[9]. However, seeds germinated under similar conditions did not give rise to any somatic embryos. The objective of this paper is to investigate whether such highly embryogenic potential as exhibited by secondary embryos is transmissible to non-embryogenic seedling tissues through micrografting.

Diploidised secondary embryogenic cultures[11] of *B. napus* ssp. *oleifera* cv. Primor were maintained as described previously[9]. For Experiments 1 and 2, secondary embryos of 3-4 mm length were used. For Experiment 3, secondary embryos of 4-7 mm were used.

Seeds of the same cultivar were germinated aseptically on Murashige and Skoog's medium[10] containing 3% sucrose and 0.8% Difco Bacto agar. The medium was adjusted to pH 5.8 and then autoclaved at 1.1 kg/cm^2 for 20 min. This medium was also used for all the experiments described below. For Experiment 1, seedlings with hypocotyls of 1-2 cm length were used. For Experiments 2 and 3, seedlings with hypocotyls of 1.5-3 cm length were used. Seedling shoot apical explants (2-3 mm height) less cotyledons and radicular explants cut at about 4 mm above hypocotyl/root junction were used for micrografting. **Fig. 1** shows the scheme of micrografting.

Experiment 1. (a) The seedling radicular explant was cultured vertically on the medium. A shoot apical explant (1-2 mm height) of secondary embryo was excised and placed on the cut surface of the rootstock. (b) The excised radicular explant of the secondary embryo was cultured vertically on the medium. A seedling shoot apical explant was excised and carefully placed on the cut end of

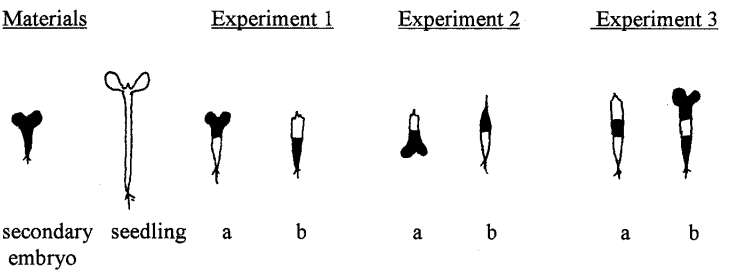

Fig. 1 Schematic drawings of micrografting of secondary embryos with seedling tissues.

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the secondary embryo explant.

Experiment 2. (a) The apical explant of a secondary embryo was planted with cotyledons inserted into the medium. A seedling shoot apical explant was placed on the secondary embryo explant. (b) The excised seedling rootstock was cultured vertically on agar medium. A secondary embryo radicular explant was placed on it.

Experiment 3. (a) A seedling radicular explant was planted upright on the medium. A hypocotyl segment (2 mm length) of secondary embryo was placed on the cut end of the rootstock. A seedling shoot apical explant was placed on top of the secondary embryo hypocotyl segment. (b) A hypocotyl segment (2 mm length) of a seedling was placed in between apical and radicular explants of the secondary embryo.

All experiments were carried out initially in 9 cm petri dishes containing 20 m*l* culture medium. Two weeks later, the cultures were transferred to GA 7 vessels (Magenta Corp. Chicago, IL) of 7 cm×7 cm×10 cm contatning 100 m*l* culture medium. There were three cultures per petri dish or per GA 7 container. All cultures were incubated at 26±2°C under a 16 h light photoperiod from white fluorescent lamps with a light intensity of 30.6±5.0 μE m^{-2} S^{-1}. All cultures that were successfully grafted survived. Tissues that dropped off after grafting were considered unsuccessful and were not scored. For certain cultures, newly formed somatic embryos were observed two weeks after treatment but assessments were made only four weeks after micrografting.

Results in Experiment 1 showed that intact seedlings and seedling explants did not give rise to somatic embryos, whereas 83-100% of the secondary embryogenic cultures showed further secondary embryogenesis (**Table 1**). The frequencies of further secondary embryogenesis in apical and radicular explants of secondary embryos were comparable to that of the intact secondary embryos. When the apical explant of the secondary embryo was grafted to the seedling radicular portion (**Fig. 2-A**), secondary embryogenesis was observed only from secondary embryo portions. Seedling rootstocks, although grafted with apices of secondary embryos, did not produce any somatic embryos (**Table 1**). When the reciprocal micrografting was made between the seedling shoot apical explant and the radicular explant of the secondary embryo, the seedling portion continued to grow

Table 1. Morphological responses after micrografting of apical explant of secondary embryo with radicular explant of seedling and apical explant of seedling with radicular explant of secondary embryo.

Cultures	Total number of cultures	Number of cultures with somatic embryos*
Intact seedling	30	0(0.0)
Intact secondary seedling	30	29(96.7)
Apical explant secondary embryo	24	20(83.3)
Radicular explant of seedling	33	0(0.0)
Apical explant of secondary embryo grafted to radicular explant of seedling	36	
Apical embryo portion		25(69.4)
Radicular seedling portion		0(0.0)
Apical explant of seedling	19	0(0.0)
Radicular explant of secondary embryo	33	33(100.0)
Apical explant of seedling grafted to radicular explant of secondary embryo	36	
Apical seedling portion		0(0.0)
Radicular embryo portion		32(88.9)

* Percentage of total in parenthesis

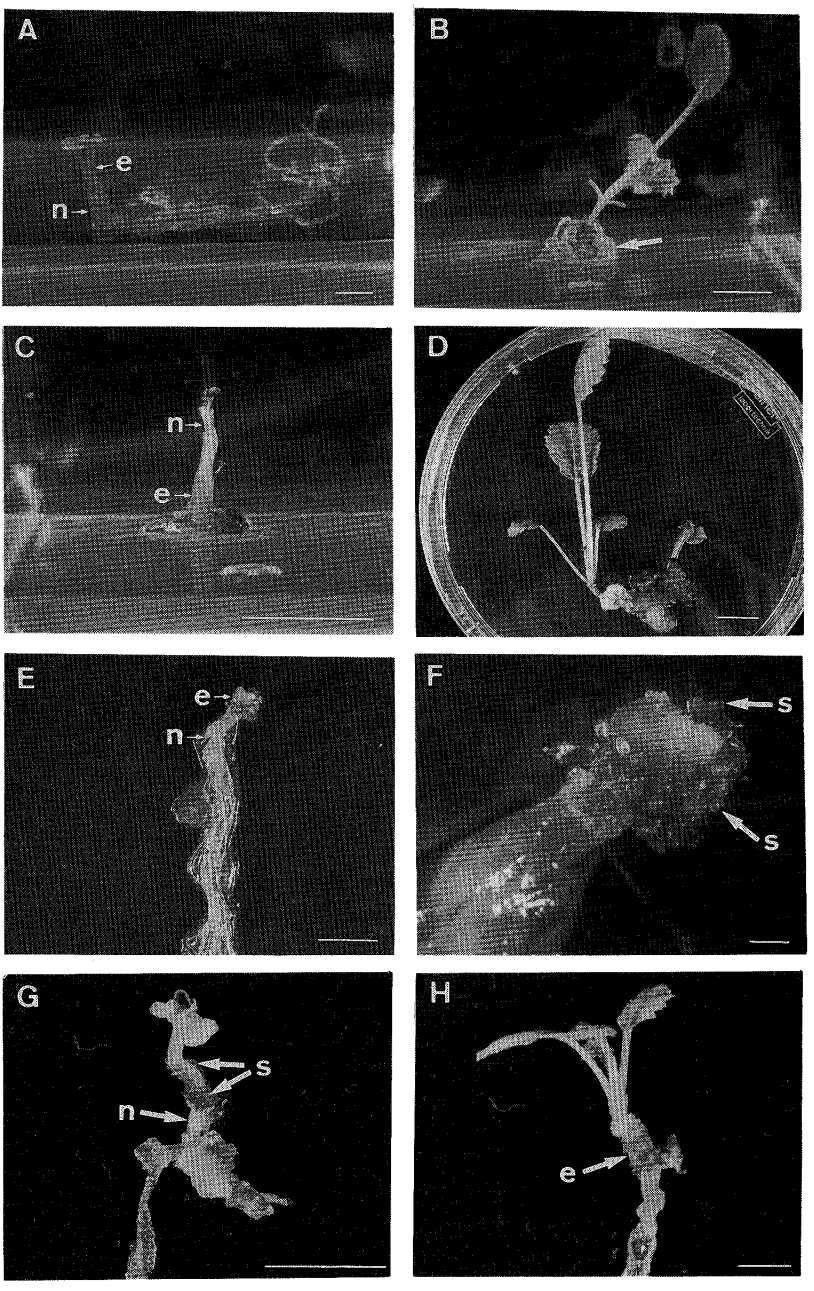

Fig. 2 Morphogenetic responses after grafting between secondary somatic embryos and seedlings.

A: One week after micrografting of an apical explant of a secondary embryo (e) to seedling rootstock (n). Scale bar = 5 mm.

B: Three weeks after micrografting of apical explant of a seedling to radicular explant of a secondary embryo. Note the normal development of the apical seedling portion with the production of normal leaves. The radicular explant of a secondary embryo (arrow) became swollen. Scale bar = 10 mm.

C and D: One week (C) and four weeks (D) after micrografting of an apical explant of a seedling (n) onto an apical explant of a secondary embryo (e). Note the cotyledons of secondary embryo were inserted into the culture medium. Scale bar = 10 mm.

E: Four weeks after micrografting of radicular explant of secondary embryo (e) to seedling rootstock (n). Scale bar = 10 mm.

F: A close-up of **Fig. 2-E**. Note the numerous somatic embryos(s). Scale bar=2 mm.
G: Four weeks after micrografting of hypocotyl segment(n) of a seedling sandwiched between apical and radicular explants of a secondary embryo. Note the formation of somatic embryos(s). Scale bar=10 mm.
H: Four weeks after micrografting of hypocotyl segment(e) of a secondary embryo sandwiched between apical and radicular explants of seedling. Scale bar=10 mm.

and produce leaves(**Fig. 2-B**) but no somatic embryo was observed(**Table 1**).

In Experiment 2, over 90% of the embryo apical explants showed further secondary embryogenesis. Grafting of the apical portion of the seedling to the apical explant of the secondary embryo(**Figs. 2-C** and **2-D**) did not induce the seedling tissues to produce somatic embryos(**Table 2**). Grafting of radicular explants of seedlings to radicular explants of secondary embryos resulted in 94% of the embryo portions producing secondary embryos but none of the seedling portions producing any somatic embryos(**Table 2, Figs. 2-E** and **2-F**).

In Experiment 3, no somatic embyro was observed from hypocotyl segments of seedlings grafted between apical and radicular explants of secondary embryos(**Fig. 2-G, Table 3**). However, hypocotyl segments of secondary embryos remained embryogenic even when they were grafted between apical and radicular explants of non-embryogenic seedlings(**Fig. 2-H, Table 3**).

There are more than a hundred species(belonging to a few dozen families) in which somatic embryogenesis has been described[12]. However, some species or cultivars are recalcitrant to somatic embryogenesis[12]. The plant materials used in the present study were from the same cultivar. The non-embryogenic material was grown from seeds and is zygotic in origin. The highly secondary-embryogenic tissue was originally derived from anther culture[8] but was diploidised by colchicine[11]. The present results demonstrated that such embryogenic potential in *Brassica napus* is not transmissible to the seedling cells by simple micrografting.

Experience from the studies of carrot showed that cultures impaired in somatic embryogenesis could be rescued by the addition to the medium of certain conditioning factors secreted by the embryogenic cells[12]. For example, De Vries *et al.*[13] reported that addition of excreted, high molecular wieght, heat labile cell factors from an established embryogenic culture considerably

Table 2. Morphological responses after micrografting of apical explant of seedling with apical explant of secondary embryo and radicular explant of secondary embryo with radicular explant of seedling.

Cultures	Total number of cultures	Number of cultures with somatic embryos*
Apical explant of seedling	30	0(0.0)
Apical explant of secondary embryo	36	33(91.7)
Apical explant of seedling grafted to apical explant of secondary embryo	37	
Seedling portion		0(0.0)
Embryo portion		29(78.4)
Radicular explant of secondary embryo	37	37(100.0)
Radicular explant of seedling	20	0(0.0)
Radicular explant of secondary embryo grafted to radicular explant of seedling	17	
Embryo portion		16(94.1)
Seedling portion		0(0.0)

* Percentage of total in parenthesis

Table 3. Morphological responses after micrografting of seedling hypocotyl segment sandwiched between apical and radicular explant of secondary embryo and micrografting of secondary embryo hypocotyl segment sandwiched between apical and radicular explants of seedling.

Cultures	Total number of cultures	Number of cultures with somatic embryos*
Apical explant of seedling	24	0(0.0)
Hypocotyl segment explant of secondary embryo	28	22(78.6)
Radicular explant of seedling	21	0(0.0)
Hypocotyl segment of secondary embryo grafted between apical and radicular explants of seedlings	28	
Apical seedling portion		0(0.0)
Middle embryo hypocotyl portion		17(60.7)
Radicular seedling portion		0(0.0)
Apical explant of secondary embryo	30	30(100.0)
Hypocotyl segment explant of seedling	62	0(0.0)
Radicular explant of secondary embryo	19	19(100.0)
Hypocotyl segment of seedling grafted between apical and radicular explants of secondary embryo	13	
Apical embryo portion		13(100.0)
Middle seedling hypocotly portion		0(0.0)
Radicular embryo portion		13(100.0)

* Percentage of total in parenthesis

accelerated the acquisition of embryogenic potential in starting cultures. Somatic embryogenesis of carrot cells which were inhibited by tunicamycin could be restored by the addition of glycoproteins which had been secreted into the culture medium[14]. A similar effect was reported for a temperature sensitive mutant which regained its efficiency to produce somatic embryos at non-permissive temperature by adding a glycoprotein secreted from a wild type culture[15]. Kreuger and Van Holst[16] also reported that carrot cells secrete arabinogalactan proteins into the medium. Addition of these proteins into a young cell line increased the embryogenic potential of the cell line. They therefore postulated that proteins secreted by the somatic embryogenic cells, which are soluble and diffusable, play a role as messengers in cell-cell interactions during differentiation[16]. All these imply that signals (conditioning factors) are produced by somatic embryogenic cells and these signals are able to enhance the embryogenic potential of other cells impaired in somatic embryogenesis.

It is not clear whether the *Brassica napus* secondary embryogenic culture secretes any extracellular proteins of other soluble, diffusable conditioning factors. However, if there are any conditioning factors, they may be transmitted from embryogenic cells to non-embryogenic cells because micrografting would allow close contact between them. Thus cell (somatic embryogenic) -cell (non-embryogenic) interaction might be possible through such graftings with possible translocation of signals.

Results from the present study showed that secondary embryogenic tissues remained embryogenic and seedling tissues remained non-embryogenic after grafting. There are a few possible explanations for this observation: 1) No conditioning factors were produced by the secondary

embryos; 2) Conditioning factors produced were unable to be translocated to the non-embryogenic cells through micrografting; 3) Insufficient conditioning factors were translocated and were therefore unable to induce a response in the non-embryogenic seedling tissues. Further research is required to clarify these points.

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