PRODUCTION OF NOVEL NUCLEOCYTOPLASMIC COMBINATIONS BY MEANS OF PROTOPLAST FUSION IN BRASSICA NAPUS

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INTRODUCTION

A hybrid system for the production of oilseed rape (Brassica napus) is expected to lead to yield increases (Grant and Beversdorf 1985). As oilseed rape is a facultative open-pollinating species, a workable system for the commercial production of true hybrid seed requires effective pollination control.

Hybridizing systems that have potential applicability include those based on cytoplasmic male sterility (CMS), self-incompatibility (SI) and nuclear male sterility (NMS).

CMS is the most commonly used system for the large-scale production of hybrid seed in crops such as maize, sugar beet, sunflower and rye. Two main CMS sources available for the production of hybrid oilseed rape are the Ogura system (Bannerot et al. 1974) and the polima system (Fu 1981). Physiological problems associated with the Raphanus-derived Ogura system (Bannerot et al. 1977) have been overcome by organelle exchange via protoplast fusion (Pelletier et al. 1983; Jarl and Bornman 1988). However, the limitation of this system lies in the unavailability of nuclear genes for the restoration of male fertility.

In polima, the other main CMS source, although nuclear restorer genes are available (Fang and McVetty 1989), other negative characteristics such as temperature sensitivity (Fan and Stefansson 1986) and lower seed yield and oil content (McVetty et al. 1990) restrict its application. Since B.napus cytoplasm is not regarded as the probable ancestor of CMS polima (Erickson et al. 1986), negative nucleocytoplasmic interactions are likely to cause yield penalties. In an attempt to overcome these barriers in the use of the polima system in hybrid seed production, protoplast fusion between CMS polima x male fertile oilseed rape was carried out. The objectives were firstly, to eliminate potential negative nucleochloroplastic interactions by chloroplast exchange and secondly, to create a variable base population for screening for environmentally stable male sterility.

Abbreviations: CMS - cytoplasmic male sterility, cp - chloroplast, EFF - electric field fusion, MF - male fertile, MS - male sterile, NMS - nuclear male sterility, PEG - polyethylene glycol, SI - self incompatibility.

MATERIALS AND METHODS

Plant Materials

Seeds of CMS polima, backcrossed into double-low winter oilseed rape (Brassica napus L.), as well as B.napus seeds of male fertile (MF) breeding lines of double-low winter forms

were germinated and grown aseptically in 250 ml beakers on Murashige and Skoog (1962) medium without plant growth regulators. Every four weeks, shoot tips were subcultured to fresh medium and maintained as clones. The plants were grown at 22±1 °C under a 16-h photoperiod at a photon fluence rate of 50 μmol m^{-1} s^{-1} supplied by 40 W Osram Fluora fluorescent lamps.

Seeds of CMS polima were either germinated and grown in the dark (etiolated) or in the light under conditions as described above on culture medium containing 100 μm SAN 9789 (photobleached, Bornman et al. 1986). SAN 9789 [4-chloro-5-(methylamino)-2-(α,α,α,-trifluoro-m-tolyl)-3(2H)-pyridazinone], also known as norflurazon, is a pre-emergence, chloroplast-selective herbicide.

Protoplast Isolation, Fusion and Culture

Protoplasts of CMS polima and MF oilseed rape were isolated and fused following methods described by Hahn-Hägerdal et al. (1986) and Jarl and Bornman (1988) using either polyethylene glycol- (PEG) or electric field- (EF) induced fusion. Following fusion, protoplasts were cultured according to Glimelius (1984) and Jarl and Bornman (1988). In total, 43 separate paired fusion experiments each representing ca. 5 x 10^6 protoplasts were performed.

Regenerated Plants

Regenerated plantlets were transferred to a soil-vermiculite mixture, hardened-off and vernalized for 7 weeks at 4±1 °C under 8/16 h light/dark cycle using an irradiance of 100 W m^{-2} (incandescent and Tungsram lamps). After vernalization, the plants were repotted into soil and transferred to a greenhouse (Landskrona, Sweden, 55.52° N lat.). Sunlight was supplemented to a minimum photoperiod of 16 h with an equal mixture of sodium high pressure and metal halogen lamps (Tungsram RS400); temperature was 20±5/14±2 °C day/night.

Flower Morphology

During flowering, plants were scored for male fertility, petal and pistil shape, nectar production and vegetative vigour in the greenhouse. Ploidy level was estimated from the relative bud size before flowering. All scorings were compared to normal, non-fused, seed-grown CMS polima and MF oilseed rape.

Detection of Cybrids

All protoplast-regenerated plants showing the MS phenotype were subjected to RFLP (restriction fragment length polymorphism) analysis of cpDNA. Extraction and purification of cpDNA was carried out using a modification (personal communication, P. Tenning) of the method described by Milligan (1989). Purified cpDNA was digested with restriction enzymes and separated on an agarose (0.8%) gel. Comparison between the restriction enzyme digests of genotypes of CMS polima and genotypes of winter oilseed rape used in our experiments, showed two distinctly different bands for Eco RV and one each for Nco I and Hind III (Fig. 1). Protoplast-regenerated MS plants that displayed a cpDNA restriction pattern similar to that of B.napus were regarded as chloroplast-corrected cybrids.

RESULTS AND DISCUSSION

The rationale underlying the use of both PEG- and EF-induced fusion was the following. The PEG method of fusion had earlier (Jarl and Bornman 1988) been applied successfully albeit at low frequency to the correction of the Raphanus-derived chloroplast deficiency in oilseed rape. On the other

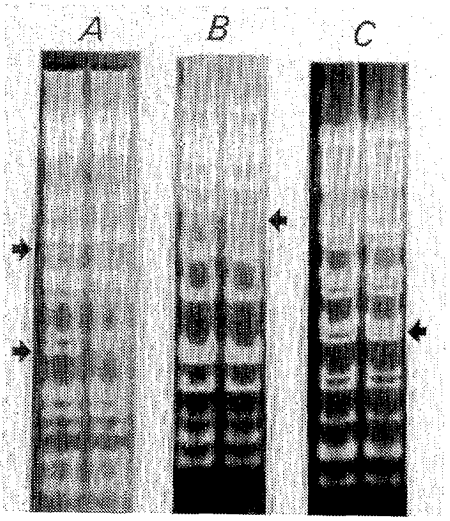

Fig. 1. Restriction enzyme digests of cpDNA of CMS polima and double-low, MF oilseed rape (winter form, breeding line R19655). Left lanes (A-C), CMS polima; right lanes (A-C), MF oilseed rape. A, Eco RV digest; B, Nco I digest; C, Hind III digest. Arrows indicate differences in banding patterns.

hand, EFF allows careful predetermination of the fusion parameters such as duration of pulse and amplitude that govern reversible membrane breakdown. This technique has been shown to result in higher fusion frequencies than can be obtained with PEG (Hahn-Hägerdal et al. 1986) and is better suited to experiments involving bulk fusion.

In the case of CMS polima, differentiation and development of chloroplasts from proplastids was either interfered with by the dark treatment or the entire chloroplast genome was eliminated by using SAN 9789 (Bornman et al. 1986). SAN 9789 inhibits carotenoid biosynthesis and chlorophyll accumulation in visible light (Hilton et al. 1969), resulting in photobleached, albinoid plants.

PEG-Induced Protoplast Fusion

Nine paired isolations of dark-treated CMS polima and MF oilseed rape, representing ca 45 x 10^6 protoplasts, were carried out (Table 1). Altogether 16 plants were regenerated of which only one was male sterile but uncorrected, that is, it did not contain chloroplasts exchanged from MF B.napus. The PEG-induced fusion of SAN-treated CMS polima x MF B.napus resulted in three plants, all of which were male fertile.

EFF-Induced Protoplast Fusion

Fourteen paired fusions (ca 70 x 10^6 protoplasts) of etiolated CMS polima x MF oilseed rape were made, 9 of which gave rise to plants. In total, 101 plants were regenerated of which 9 were male sterile (Table 1). No sterile plants with MF B.napus chloroplasts substituted for those of CMS polima were found.

Thirteen paired fusions (ca 65 x 10^6 protoplasts) between photobleached (SAN-treated) CMS polima x MF oilseed rape resulted in 91 regenerated plants, of which 8 were male sterile. Of the latter, 2 plants displayed the corrected phenotype, i.e. B.napus chloroplasts and CMS polima male sterility.

Flower Morphology

The degree of male sterility was assessed on the basis of visual scoring of flower morphology of male sterile plants.

Table 1. Plants regenerated from polyethylene glycol (PEG)- and electric field (EF)-induced fusion of protoplasts of CMS polima (either etiolated or photobleached) x MF oilseed rape

Fusion	Number					
	Protoplasts x 10^6	Regenerated plants from $7x10^6$ protoplasts	Male fertile plants	Semi-male sterile plants	Male sterile plants	Male sterile cybrid plants
PEG Etiolated polima	45	16	15	0	1	0
PEG Photobleached polima	35	3	3	0	0	0
EF Etiolated polima	70	101	92	0	9	0
EF Photobleached polima	65	91	83	0	8	2

Table 2. Flower morphology of male sterile plants derived from electric field-induced fusion of protoplasts of CMS polima (either etiolated or photobleached) x MF oilseed rape

Character	Number of male sterile plants							
	Etiolated				Photobleached			
	Total	Score			Total	Score		
		1	2	3		1	2	3
Male sterility	9	0	1	8	8	0	0	8
Ploidal level	9	6	0	3	8	4	1	3
Petals	8	1	3	4	7	0	7	0
Pistil	8	1	3	4	7	1	3	3
Nectaries	8	3	1	4	7	3	2	2
Vigour	8	1	3	4	7	0	7	0

Plant and floral morphology were scored as follows: Male sterility - fertile (1), semi-fertile (2), sterile (3); ploidy level - 2n (1), >2n (2), 4n (3); petal development - closed (1), open but disturbed (2), open normal polima (3); pistil shape - deformed (1), partially deformed (2), normal (3); nectaries - absent or very weakly developed (1), small (2), normal (3); vegetative vigour - weak (1), normal (2), strong (3).

Under greenhouse conditions, phenotypic expression of male sterility in CMS polima usually varies between semi-fertile and sterile (Fan and Stefansson 1986), whether at single plant or at line level. With the exception of one semi-fertile plant all protoplast-regenerated plants were phenotypically male sterile (Table 2). However, whether or not the degree of male sterility is sufficiently high for hybrid seed production has to be confirmed by further experimentation.

CONCLUSIONS

In this study the chloroplasts of B.napus were successfully substituted for those of CMS polima via paired fusion of protoplasts. Photobleaching of plants with norflurazon (SAN 9789) is an effective method for the elimination of a particular chloroplast genome and somatic protoplast fusion makes possible the introduction of novel nucleocytoplasmic combinations by organelle reconstitution. The degree of male sterility, nectar production and variation in other flower characteristics differed widely between the protoplast-regenerated plants. Whether or not this variation is heritable awaits testing under different environmental conditions.

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