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Short communication

Non-destructive *in vitro* selection of microspore-derived embryos with the fertility restorer gene for CMS *Ogu*-INRA in winter oilseed rape (*Brassica napus* L.)

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ABSTRACT

Background: Microspore embryogenesis and cytoplasmic male sterility system (CMS) are two approaches widely exploited in *Brassica napus* breeding for production of homozygous doubled haploid (DH) lines and F1 hybrids respectively. Cytoplasmic male sterility system (CMS) is one of the most important pollination systems for hybrid seed production and utilisation of doubled haploid system to quickly prepare fully homozygous fertility restorer lines for CMS *Ogu*-INRA is very beneficial. Generally, only a small part of microspore-derived embryos is used for plant regeneration, without any knowledge about their properties. Therefore, the possibility of early detection of desirable genotypes bearing a single dominant nuclear fertility restorer (*Rfo*) gene, can double the success of selection and reduce the production costs.
Results: To maximize the efficiency and yield of regenerated microspore-derived embryos (MDEs) with the *Rfo* gene, a protocol for reliable and early, non-destructive selection of desired MDE genotypes was developed. The total amount of 636 cotyledonary embryos was tested by PCR, out of which 37% (237/636) were shown to bear the *Rfo* gene (instead of 50% according to the expected 1:1 segregation ratio for a single copy gene) and 218 of these fertility restorer plants were fully grown to flowering stage. New molecular marker has been demonstrated to have 100% of co-segregation with the phenotypic evaluation.
Conclusion: Technique developed in this study provides early and non-destructive sampling of embryonic tissue and the use of new markers for simple and efficient control of the presence of *Rfo* gene in all accessions.

1. Introduction

Regeneration of doubled haploid (DH) cotyledonary embryos through androgenesis in isolated microspore culture is currently a routine process used in oilseed rape breeding programmes [1,2]. It allows rapid production of fully homozygous genotypes (*i.e.* potentially line cultivars) within one year [3]. Because hybrid cultivars are increasingly being used in rapeseed growing, the utilisation of doubled haploid-based system to quickly prepare their completely homozygous parental components could be very beneficial [4].

One of the most important hybrid systems in oilseed rape is the *Ogu*-INRA based cytoplasmic male sterility (CMS), in which the fertility restorer (Rf) line is homozygously dominant for the restorer gene (*Rfo*) and contains a male-sterile (S) cytoplasm, *i.e.* it is genetically S-*Rfo*/*Rfo* [5]. Quality parameters of Rf lines can be improved by crossing these to the donor of quality (a common male line with fertile cytoplasm, F-*rfo*/*rfo*). If successful, the above F_1 crosses (S-*Rfo*/*rfo*) would generate enhanced Rf lines through the DH method, out of which only half of DH regenerants (*i.e.* with the S-*Rfo*/*Rfo* constitution) should possess the *Rfo* gene according to Mendelian principles. The traditional procedure for selection of Rf lines is based on growth of all F_2 plants to generative stage, crossing individual fertile plants to sterile CMS mothers (S-*rfo*/*rfo*) and checking the ratio of sterile and fertile offspring in each cross [6]. Such process in winter oilseed rape is further prolonged due to vernalization periods and thus requires a lot of space and money for regular maintenance.

Although suitable molecular markers for the *Rfo* gene in *Brassica napus* have been developed [5,7,8], their utilisation in DH Rf line production requires cultivation of DH regenerants up to a stage when there is a sufficiently large leaf area for DNA isolation and subsequent analyses. A method that would enable the non-destructive identification of genotypes carrying the *Rfo* gene at early stages of microspore derived embryos would be of great benefit in terms of significantly reducing the amount of cultivated DH regenerants, and thus decreasing workload, culture space, time and cost.

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The aim of the study was to implement efficient procedures for sample preparation, DNA extraction and PCR detection of the *Rfo* gene from very small quantities of embryo tissue, sampled aseptically and non-destructively from microspore embryos of winter oilseed rape at early stages of *in vitro* cultivation. In addition, direct correlation between genotype (the presence of the *Rfo* gene) and its phenotypic expression (male sterility) was also examined.

2. Materials and methods

Seeds of the F_1 hybrid RD-9/11 (derived from the cross between the Rf line 3358 and the highly embryogenic breeding material MLCH 175) were obtained from SELGEN, Ltd., Czech Republic. The establishment of microspore cultures and early *in vitro* chromosome doubling with trifluralin in the microspore suspension was performed according to the protocol previously published [9].

For the genotyping purposes, ~3/4 of both cotyledons were cut aseptically from NLN medium cultivated embryos with a razor blade on sterile filter paper to avoid damage to the apical meristem (Fig. 1a, b). Cut cotyledons were transferred into a microfuge tube and stored at -20°C until DNA extraction was performed, the remaining embryo was transferred to regeneration medium (RM) [10]. Embryos not large enough for non-destructive cotyledon removal were initially cultivated on DM medium for 2–3 weeks. Further cultivation and handling of trimmed embryos was carried out according to Klíma et al. [9] to obtain rooting regenerants, able to transfer to soil and grown to a generative stadium.

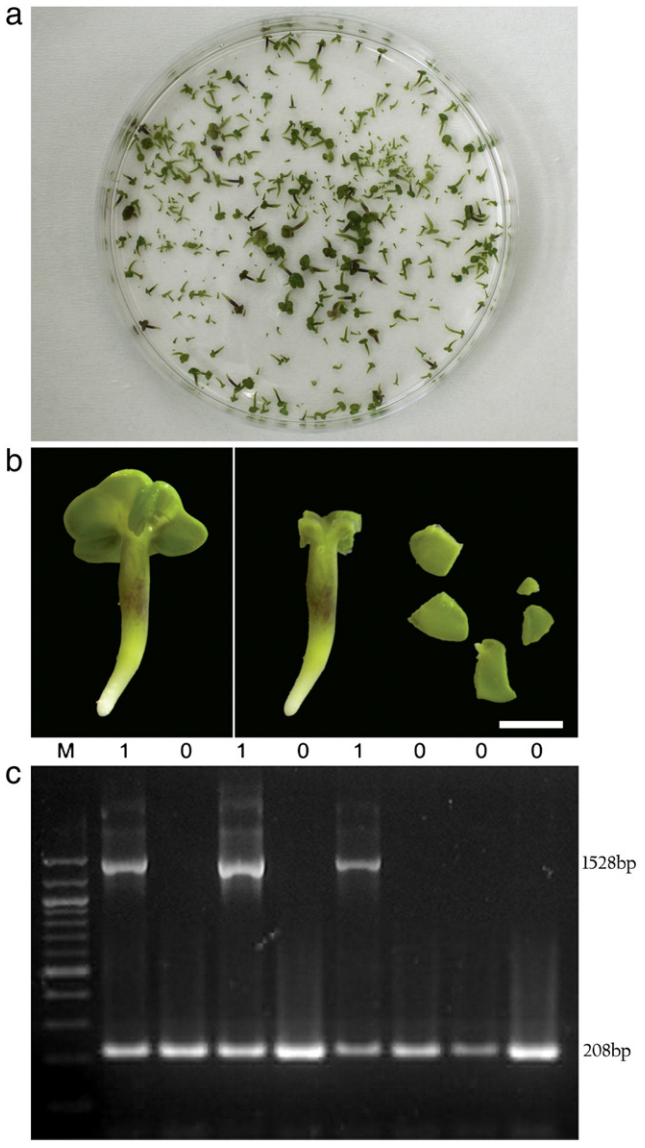

Fig. 1. Microspore embryos and PCR detection of *Rfo* gene. Microspore embryos at different developmental stages on liquid NLN medium (a); microspore embryo of 6 mm (left) and the method of cotyledon cutting (right) (b) and duplex PCR of a 1528 bp fragment of the *Rfo* gene together with internal control amplification of 208 bp fragment in all samples (1 = *Rfo*/*Rfo*; 0 = *rfo*/*rfo*), (c). Bar = 1 mm.

DNA was isolated following the SDS technique [11] from 636 embryonic cotyledons (1–10 mg) and DNA concentration was measured by Qubit Fluorometer (Invitrogen). Duplex PCR assay was performed using primers for the PPR-B region of PPR gene (with a forward primer RsPPRB-F: 5′-^{88504}GAAGCTCTTGCTACCCATCG^{88523}-3′ and a reverse primer RsPPRB-R: 5′-^{90031}TGACATGCTTCGATCTCG TC^{90012}-3′) designed from the publicly available sequence of *Raphanus sativus* (GenBank accession number AJ550021.2). A second primer pair for the noncoding DNA region of *B. napus* derived from AFLP marker (208-F: 5′-TCGGGATGGAAACCATACTC-3′ and 208-R: 5′-GGTCCCAGAT AAGGGGAAAA-3′) was used as a positive control for the presence of DNA in the PCR mix. Duplex PCR reactions were carried out in a volume of 20 μL containing 5–10 ng of DNA template, 10 pmoL of each primer, 1× BSA, 1× PPP Master Mix (with 2.5 mM $MgCl_2$, Top-Bio) and 10 units of Taq DNA polymerase. Amplification was performed using the following programme: 95°C for 5 min; 35 cycles of 95°C for 30 s, 59°C for 45 s, 72°C for 2 min; and 72°C for 10 min and then cooled down to 4°C. PCR products were subjected to electrophoresis on 1.5% agarose gel in 1× TBE buffer and visualized by EtBr staining.

3. Results and discussion

Duplex PCR reaction resulted in amplification of the 1528 bp *Rfo* gene together with a control amplification product of a 208 bp in all 636 samples indicating the reliability of rapid SDS DNA extraction method (Fig. 1c).

Although the average DNA yield per mg of tissue may vary considerably between different length and weight categories of embryos (32.2–268.7 ng DNA per mg of the frozen tissue in embryos from 2 to 10 mm in length; unpublished data, available on request from corresponding author), cotyledons of the smallest tested embryo fraction (2.0–3.0 mm) can provide quite sufficient amount of material for DNA extraction. However, it may be difficult to obtain such small sample always in non-destructive way, without the risk of damage to the embryo tip or loss of the sample during routine manipulation.

In general, the method of sampling and DNA isolation described in this paper proved to be effective for routine and safe (non-destructive) analysis of embryos about the size of 6 mm or more, providing a sufficient reserve of sample for analysis. Moreover, the cutting of cotyledons could be beneficial to promote subsequent growth of the apical meristem, thereby increasing the efficiency of direct regeneration of whole plants [10].

To confirm the specificity of the new primers for the *Rfo* gene, 218 plants identified as DH by flow cytometry with detected Rf genotype were grown to flowering stage. In these plants, 100% efficiency of the marker has been demonstrated by comparing with the phenotypic evaluation of anthers and pollen formation/disruption as described by Li et al. [12]. On average, the *Rfo* gene was amplified in only 37% (237/636) of cotyledonary embryos (instead of 50% according to the expected 1:1 segregation ratio for single copy gene), indicating a possible early mechanism of regulation during meiosis, microspore development or even microspore embryogenesis. This is in agreement with our earlier experiments with six different F_1 combinations of *Ogu*-INRA Rf lines and quality donors, where the frequencies of fertile DH Rf lines were substantially lower (9.2, 12.3, 16.7, 22.2, 29.0 and 35.9%) during phenotypic assessment of fertility restoration

(unpublished data). Similar results were also obtained in a study of Wang et al. [13], where restoring capability test showed that only 33% of doubled-haploid plants possessed restorer genes.

Here we present a reliable, efficient and non-destructive *in vitro* selection procedure allowing early identification of all genotypes carrying the desired *Rfo* gene in the selected material, in just 3 weeks of microspore culture under optimal circumstances. Based on a comparison of genotypic and phenotypic assessment of the *Rfo* gene in 218 flowering regenerants, we clearly demonstrate that the procedure could be useful for routine sampling and genotyping in programmes aimed at hybrid breeding of winter oilseed rape. Application of duplex PCR with novel primers can be used for efficient handling of large numbers of samples and can solve problems of inconsistent results, which may be due to lack of template DNA in the PCR or due to impurities affecting the polymerase activity. Therefore the detection of desired genotypes carrying the *Rfo* gene in combination with internal amplification control, enables early selection for seedling regeneration. This method offers even more effective economic savings in terms of the amount of cultivated regenerants, because the ratio of genotypes with the *Rfo* gene was 37%, which was significantly different from the expected 50% of carriers of a single copy gene. Therefore the protocol hereby describe would enable the early disposal of more unwanted plants. Although our work did not address the causes of the above phenomenon, it opens up space for its further research.

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Conflict of interest

All authors have no conflict of interest to declare.

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