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Early *in vitro* selection of winter oilseed rape (*Brassica napus* L.) plants with the fertility restorer gene for CMS *Shaan* 2A via non-destructive molecular analysis of microspore-derived embryos

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Abstract: Cotyledons of 201 microspore-derived oilseed rape (*Brassica napus*) embryos were analysed for the presence of the cytoplasmic male sterility *Shaan* 2A restorer gene using the primer pair 3F/4R. In total, 172 plants regenerated and were grown to the generative stage. Among phenotypically non-haploid flowering plants, there was a broad spectrum of different flower types from normally developed flowers to flowers with short anthers, stamens, or deformed flowers with atypically developed floral organs, often with traces of pollen. For this reason, only 115 typically fertile or sterile accessions were selected for further phenotype-genotype comparisons. In 14 plants (12.2%) the phenotype did not match the genotype: two plants were marked as fertile, but without the *Rf* gene. *Vice versa,* in 12 sterile plants the *Rf* gene was determined. Nevertheless, the two fertile plants did not produce any pods after self-pollination. In total, 47 doubled haploid (DH) lines with good seed yield, carrying the *Rf* gene were harvested.

Keywords: cytoplasmic male sterility; doubled haploids; marker-assisted selection; microspore culture; *orf224-1*; *Rf*-gene

A microspore culture technique has been developed for doubled haploid (DH) production in most important *Brassica* species. Non-destructive marker-assisted selection may provide early identification of all genotypes carrying the desired gene in the selected material, in just three weeks of microspore culture under optimal conditions (Havlíčková *et al.* 2015). For hybrid breeding of oilseed rape (*B. napus* L.), several cytoplasmic male sterility (CMS) systems have been widely used to develop commercial cultivars. *Shaan* 2A CMS was discovered in China in 1976 (Li 1986 in Fu & Yang 1995) and currently it is one of the most important systems used in that country for hybrid production, along with *Pol* CMS and genic male sterility systems (Sang & Lessard 2015 in Bonjean *et al*. 2016). The aim of our study was to introduce a non-destructive analysis of fertility restorer (*Rf*) gene for *Shaan* 2A CMS in microspore-

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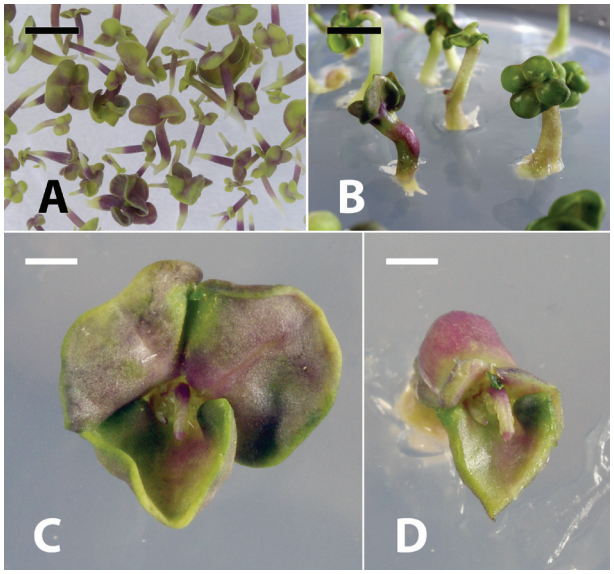

Figure 1. Microspore-derived oilseed rape embryos on culture media; largest embryos (4–5 mm long) on a liquid NLN-13 medium with well-developed cotyledons suitable for cutting of cotyledons (A); smaller embryos after 10 days on a solid DM medium ready for sampling (B); the method of trimming an embryo for the cotyledon removal (C–D); bar = 5 mm (A, B) or 1 mm (C, D)

derived cotyledonary embryos and to regenerate plants with *Rf* gene, applicable in hybrid breeding of winter oilseed rape.

Three F_1 hybrids from crosses of *Rf* lines and three cultivars of oilseed rape were used as microspore donor plants for the *in vitro* androgenesis process in the culture of isolated microspores. Microspore culture technique, cotyledon sampling and related experiments were carried out according to Klíma *et al.* (2004, 2008) and Havlíčková *et al.* (2014) with modified concentration of a doubling agent (trifluralin, 5 µmol/l). Once embryos with well-developed cotyledons reached a length of 4–5 mm (Figure 1A), approx. 3/4 of both cotyledons were cut off with a sharp scalpel (Figure 1C, D). The dissected cotyledons were then placed in a snap cap centrifuge tube, labelled and stored at –19°C prior to DNA isolation. The remaining embryo was transferred to a differentiation medium (DM) solidified with agar (Klíma *et al.* 2004) and further cultivated. Embryos too small for non-destructive sampling were initially cultivated on DM medium for 2–3 weeks (Figure 1B). Plants in the generative stage were initially evaluated for bud and flower morphology, later for self-pollination ability (i.e. the capability to set pods with seeds in technical isolation). Regenerants with properly developed anthers with a sufficient amount of powdery pollen and good seed set were finally marked as fertile. Plants with typical haploid inflorescences, i.e. with small, prematurely falling buds or with small flowers and dwarfed, thin and dry anthers were determined as haploid. Visually non-haploid regenerants with reduced anthers, no powdery pollen and no seed set were finally considered as sterile. Haploids and transient types, i.e. types difficult to determine, were excluded from the phenotype-genotype comparison. All plants except haploids were individually and tightly covered with sacks made of dense fabric, grown to seed maturity in isolation cages and harvested. Genomic DNA was extracted from dissected cotyledons using a modified CTAB method (Havlíčková *et al.* 2014). The concentration and quality of DNA were determined with a BioSpec-nano spectrophotometer (Shimadzu, Japan). PCR amplification was done using a SCAR marker (primer pair 3F/4R, Formanová *et al.* 2010).

Cotyledons of 201 microspore-derived embryos were analysed for the presence of the restorer gene. DNA was isolated preferably from embryos 4.0–5.0 mm in length. Average DNA yield per mg

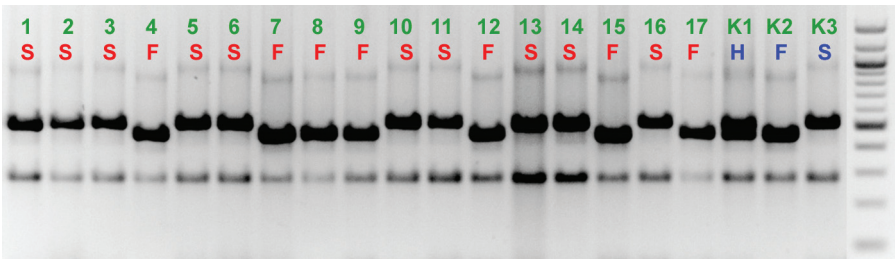

Figure 2. Results of PCR detection of the *Rf* gene in microspore-derived oilseed rape embryos (samples 1–17) and control plants – F_1 hybrid (CMS×Rf – K1), Rf plant (K2), plant without *Rf* gene (K3); primer pair 3F/4R amplified 440 bp fragment in fertility restorer (lower band), 500 bp fragment in non-Rf plants (upper band) and both bands in F_1 hybrid; 280 bp band is an internal standard present in all plants; molecular weight marker – 100 bp ladder (NEB)

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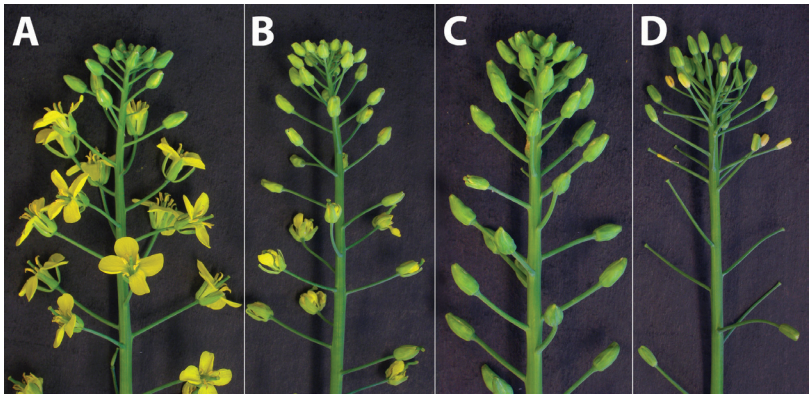

Figure 3. An overview of contrast inflorescence types among microspore oilseed rape regenerants; the inflorescence typical of a fertile plant with *Rf* gene (A); inflorescences typical of sterile plants, without *Rf* gene (B, C); the inflorescence typical of a haploid plant with tiny, prematurely falling and/or yellowing buds (D)

of cotyledon tissue varied between 50 and 150 ng of DNA, and was suitable for PCR detection, giving clear amplification results. Surprisingly, DNA isolation even from smaller embryos (2.0–3.0 mm) provided quite a sufficient amount of material for DNA extraction. However, it may be difficult to obtain such a small sample always in a non-destructive way, without the risk of damage to the embryo tip or loss of the sample during routine manipulation (Havlíčková *et al.* 2014, 2015). The length of the amplified fragment was approx. 440 bp in *Rf* plants and approx. 500 bp in plants without *Rf* gene (Figure 2). *Rf* gene was amplified in 104 (51.7%) accessions, which corresponds to the assumed 1:1 segregation ratio for a single copy gene. It differs from our previous results for *Ogu*-INRA restorers (Havlíčková *et al.* 2015) where the occurrence of genotypes with *Rf* gene was substantially lower (37%). In total, 172 (85.6%) plants were regenerated from the above-mentioned embryos to the generative stage. Inflorescences of 15 plants (8.7%) corresponded to the haploid constitution, mostly with tiny prematurely falling and/or yellowing buds (Figure 3D). Among 157 non-haploid regenerants there was a wide range of different types from normally developed flowers (Figure 3A, 4A) to flowers with short anthers (Figure 4B), stamens (Figure 4C), or deformed flowers with atypically developed floral organs, often with traces of pollen in stunted anthers (Figure 3B–C, 4D–F). Fan *et al.* (2007) also discovered different fertility grades (i.e. fertile, partially sterile, sterile flowers) and malformed floral organs in the double haploid population derived from the cross between two fertility restorer lines for *pol* CMS. Thus, in 42 transient accessions, we were not able to decide clearly whether a plant is fertile or sterile. Among these accessions there were plants, for instance, with a higher amount of sticky pollen in dwarfed and/or watery anthers, or regenerants with traces of pollen occasionally setting pods on individual inflorescences. For this reason, 115 more contrasting (typically fertile or sterile) accessions were selected for phenotype-genotype comparisons. In 14 plants (12.2%) the phenotype did not match the genotype: two plants were initially marked as fertile according to anther morphology, but not carrying the *Rf* gene, and *vice versa* in 12 sterile plants the *Rf* gene was determined. Nevertheless, those two plants resembled maternal CMS components with short anthers but with powdery pollen, and did not set any pods in technical isolation. Twelve, phenotypically sterile plants with the *Rf* gene could be true haploids or plants with genetic defects, caused by *in vitro* diploidization and/or regeneration process. In total, 47 DH lines, with good seed yield, carrying the

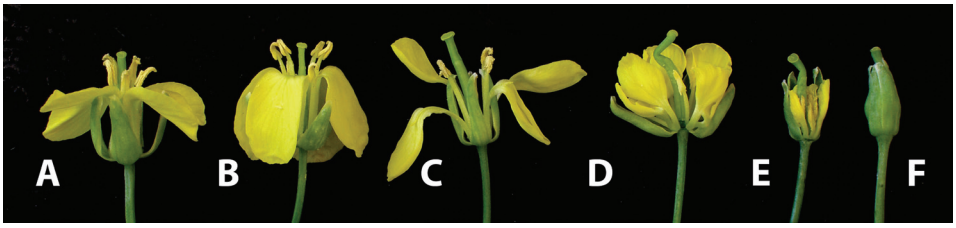

Figure 4. An overview of contrast flower types observed among microspore oilseed rape regenerants; the flower typical of a fertile plant (A); the flower with shorter anthers (B), with different height of the pistil and stamens (C) or deformed, stunted floral organs (D–F)

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Rf gene were harvested. Although 100% genotype-phenotype match was not achieved (mainly due to the occurrence of phenotypically transient types difficult to determine, and most probably unidentified haploids with the *Rf* gene as well), the method proved to be reliable for the identification of fully fertile plants with the *Rf* gene. If a plant phenotypically corresponded to a common fertile cultivar and set the normal amount of pods and seed in technical isolation, it always possessed the *Rf* gene. However, the versatility of the procedure needs to be verified for a broader range of genotypes. It can be concluded that the combination of microspore culture technique and molecular analyses with phenotypic evaluation allowed obtaining a sufficient number of fertile plants with good seed set and the *Rf* gene, applicable in winter oilseed rape breeding programmes.

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