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Kosena cms-restorer system in *Brassica napus*

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In the breeding of oilseed rape, programs for the commercial production of hybrid seeds by cytoplasmic male sterility/restored fertility (CMS/Rf) systems have been pursued over the past decades. Several types of CMS/Rf systems have been introduced into *B.napus* both by conventional backcrossing and somatic hybridization, for example, Ogura system, polima system, Shiga-Thompson system, and MS-Lembke system, and some of them have been used for commercial production of the hybrid seeds. We have introduced a CMS/Rf system found in *Raphanus sativus* cv. Kosena into *B.napus* by intergeneric protoplast fusions.

Protoplast fusion

The CMS line was originally identified in the Japanese radish cultivar Kosena. A mixed population of CMS and fertility-restored plants was maintained in a greenhouse by open pollination. This population was used as the source of the CMS and restorer genes in fusion experiments. For the cms transfer we used *B.napus* cv. Westar as a cytoplasmic recipient.

"Asymmetrical protoplast fusion" was used both for the cms and restorer gene transfer. The "donor" protoplasts were X-irradiated with over lethal dose and the "recipient" protoplasts were treated with iodoacetamide, and the treated protoplasts were fused with polyethylene glycol. For the detailed methods of the protoplast fusion and regeneration of the fusion products were described elsewhere (Sakai T. and J.Imamura, 1992 Theor Appl Genet 84:923-929)

The CMS *B.napus* cybrid plants produced by the cell fusion have been backcrossed and maintained with spring and winter types of oilseed varieties. The stability of the male sterility has not been broken on the different genotype of maintaining lines in high or low temperature conditions. All the *B.napus* cultivars tested are worked as maintainers and no restoration of the fertility has been observed.

Kosena CMS specific *orf125* gene and its expression

We found that Kosena CMS mitochondria contained a sequence homologous to Ogura CMS specific sequence, *orf138*. We cloned and sequenced a 1.5kb *Spe*I-*Sac*I fragment and found a sequence homologous to *orf138/orfB* with deletions and substitutions of nucleotides. The corresponding sequence of *orf138* encodes 125 amino acids (*orf125*) being equivalent to the entire sequence encoded by *orf138* with deletion of 39bp. The deletion encodes 13 amino acids and it is tandem repeated in ORF138 (Fig. 1).

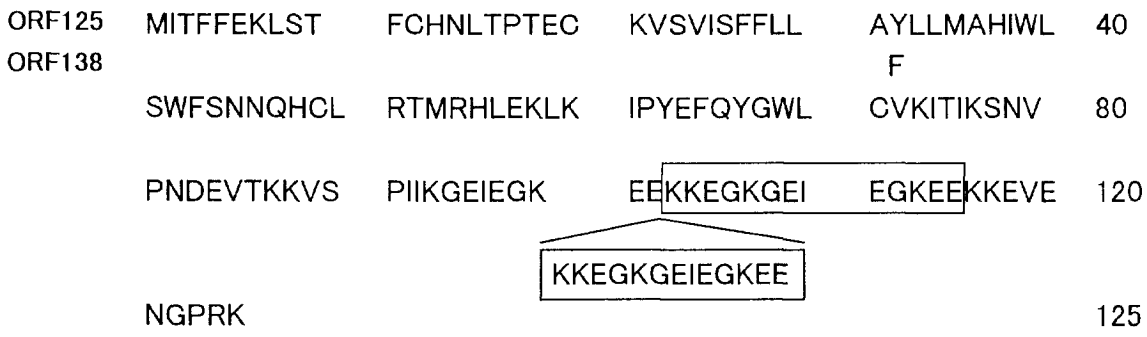

Fig.1. The amino acid sequence of ORF125. Phenylalanine at position 32 of ORF138 (represented a letter under the ORF125) was substituted by tyrosine in ORF125. A sequence consisted of 13 amino acids (boxed) with duplicates tandem in ORF138 is deleted in ORF125.

We examined regulation of expression of *orf125* at both RNA and protein levels in the CMS and fertility-restored *B.napus* cybrids. Total RNAs were extracted floral buds of cms (SW18 and SW12) and restored cybrids (RW18) and characterized by RNA gel blot analysis using the coding region of *orf125* as a probe. *orf125* is transcribed into 1.2kb, and 1.1kb in SW12 and SW18 cybrids, respectively, and the size and amount of the transcript was not changed by the presence or absence of the Rf gene in SW18 (Fig.2).

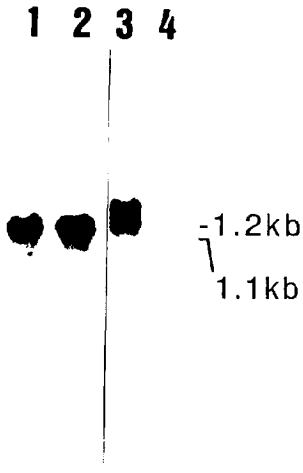

Fig.2. RNA gel bolt analysis of *orf125* in restored cybrid RW18 (lane 1), CMS cybrid SW18 (lane 2), CMS cybrid SW12 (lane 3) and *B.napus* cv. Westar (lane 4). Total RNAs extracted from floral buds were subjected to electrophoresis on 1.2 % agarose gel and the blot was hybridized with the coding region of *orf125*.

Mitochondrial proteins were extracted from floral buds of *B.napus* cv. Westar, a CMS cybrid (SW18) and fertility-restored cybrids. Expression of ORF125 protein in floral bud, leaf, and root were examined by Western blotting (Fig.3a). The 17-kDa protein was detected in all the tissues tested and the amount was reduced in the presence of the restorer gene. The results indicated that accumulation of the 17-kDa protein is down regulated specifically by the fertility-restorer gene in all the organs tested. We further analyzed the level of accumulation of ORF125 protein in floral tissues, namely, petal, style, sepal, and anther (Fig.3b). Accumulation of the protein was observed in all the tissues analyzed and the levels were also reduced in all the tissues of the fertility-restored flower. The reduction of the protein was most abundant in anther tissue.

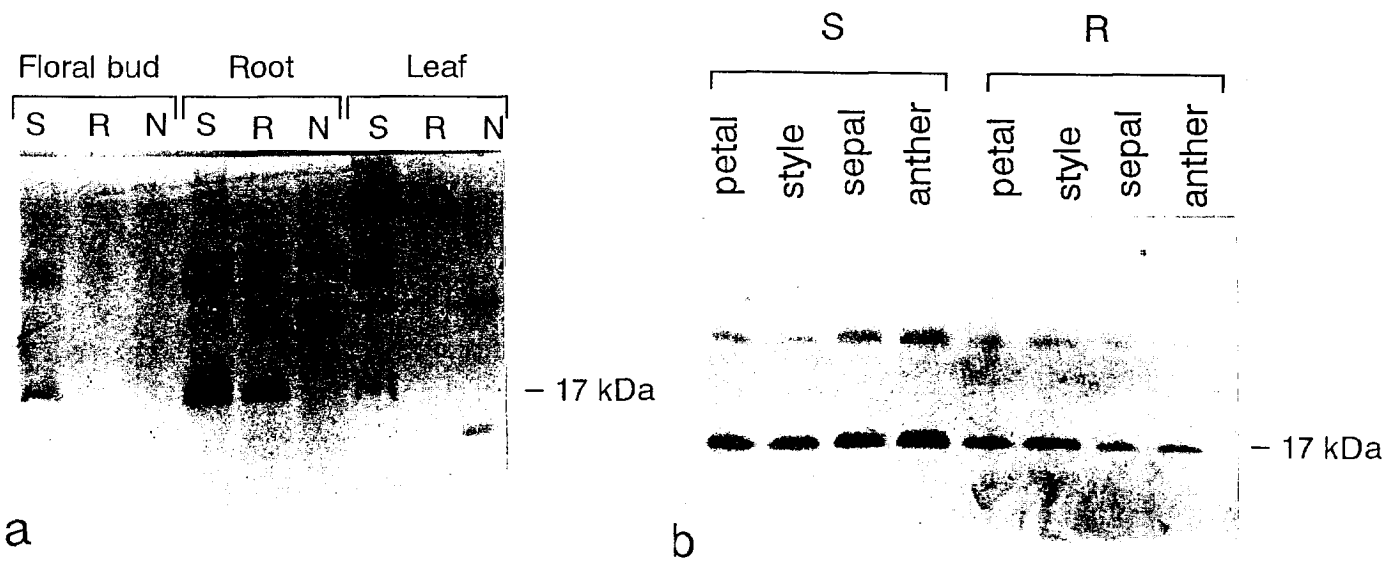

Fig.3. Immunoblot analysis of ORF125 protein in organs (a) and floral tissues (b)
Each lane contains 60 μ g and 5 μ g of total cellular proteins extracted from organs and floral tissues, respectively, of *B.napus* cv. Westar(N), SW18(S) and fertility-restored SW18(R), and proteins were subjected to electrophoresis on 16% SDS polyacrylamide gels. The blots were probed with the anti-ORF125 antibodies.

Production of *B.napus* restorer line by protoplast fusion

The restorer line that counteracts the CMS Kosena cytoplasm was originally identified in the Japanese radish cultivar Kosena. A mixed population was used as the source of the restorer gene in the protoplast fusions. The cms cybrid produced by the protoplast fusion was used as a fusion partner for the restorer-gene transfer. The method used for the protoplast fusion and regeneration of the fusion products was the

same with that of the cms transfer (Sakai T, *et al.*, 1996 Theor Appl Genet 93:373-379)

By the two independent fusion experiments, 301 plants out of 1067 shoots regenerated were grown in soil and 13 of these were male-fertile. Six of the thirteen fertile R_0 plants set seeds after crossing with *B.napus*. A plant (#71) showed male-fertile and aneuploid (2n=47) with white petals was selected for further backcrosses.

Backcrossed progeny of the Rf line

After two times of backcrosses with *B.napus* cms cybrid, BC_2 plant showed normal chromosome number and male-fertile with yellow petals. In BC_2S_1 generation, no female sterility was observed. The expected male-sterile and male-fertile segregation ratio (1:1) was observed with the progeny of BC_4S_1.

DNA markers linked to the restorer gene

We have identified 12 STS (sequence-tagged-sites) markers tightly linked to the restorer gene in *B.napus*. PCR analysis was conducted using F_2 generation. The F_2 family was derived from a selfing of F_1 plants between a fertile restorer plant and a sterile cms plant. All 12 STS markers co-segregated with the fertility-restoration. No recombinant except one was found between any of the 12 STS markers and fertility-restoration among 1300 F_2 plans. The recombinant lacks 7 of the 12 STS markers.

We compared loci of the restorer genes between Kosena and Ogura fertility-restorer lines with the STS markers. Four markers out of the 12 markers were not present in the Ogura restorer line. Different sizes of PCR products were generated by the three STS markers, STSN8, STSN9 and STSN12 between Kosena and Ogura fertility-restorer lines (Fig.4). Taken together, these results indicated that the Kosena restorer locus in *B.napus* derived from Kosena radish was not identical to that of Ogura.

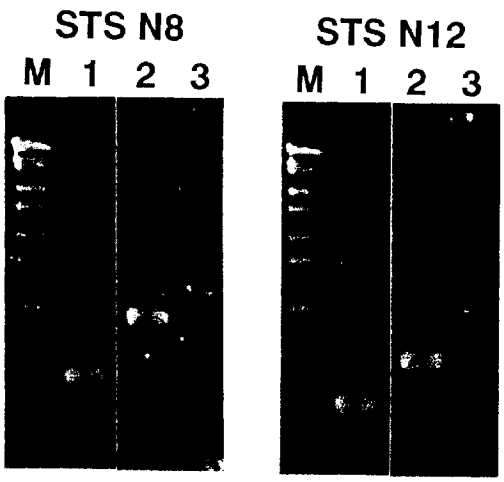

Fig.4. STS markers linked to the restorer locus. Molecular marker λ /EcoT141 (M), *B.napus* Kosena restorer line (lane 1), *B.napus* Ogura restorer line (lane 2), *B.napus* Kosena CMS line (lane 3).