Development of molecular markers specific to the Ogura fertility restorer gene *Rfo* in canola (*Brassica napus* L.)

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Abstract

Ogura cytoplasmic male sterility (CMS) and its corresponding nuclear fertility restorer gene, *Rfo*, have been introduced from radish to *Brassica* species by interspecific crosses. *Rfo* restores male fertility by altering the translational expression of *Orf138*, a mitochondrial gene, whose expression results in the male sterile phenotype. This system has been extensively investigated and breeding restorer lines for the Ogura CMS has become a major objective for hybrid seed production in many canola breeding programs. In this study, we have sequenced genomic clones of *Rfo* and its orthologs amplified from a rapeseed restorer line R2000, obtained from INRA, France, and a non-restorer canola line Nex 705 using primers designed from the radish *Rfo* sequence (GenBank accession AJ550021). Sequence alignment revealed three orthologs of *Rfo*. Two of the orthologs were present in both R2000 and Nex 705 but the third one was present only in R2000. These results suggested that the first two sequences could be the orthologs of *Rfo* already existing in the canola genome and the third one could be the radish *Rfo* introduced into canola. Based on the sequence differences between the restorer and non-restorer lines, *Rfo* allele-specific PCR markers were developed. Linkage analysis revealed a co-segregation between the markers and the phenotypes for fertility restoration. These markers have been proven very useful for direct selection of *Rfo* alleles for fertility restoration during marker-assisted introgression of the Ogura restorer gene for hybrid development in canola.

Key words: *Brassica napus*, Ogura CMS, *Rfo*, Allele-specific marker

Introduction

Breeding of restorer lines for Ogura CMS system as a means of developing rapeseed hybrids has been a major objective in many rapeseed or canola breeding programs since the Ogura CMS and restorer gene was introduced from radish into rapeseed through interspecific crosses and subsequent protoplast fusion (Heyn 1976; Pelletier et al. 1983). However, its possible linkage with negative traits, such as high glucosinolate content, introgressed from the radish genome (Delourme et al. 1998; Giancola et al. 2003) has been a major problem in breeding restorer lines in rapeseed. INRA, France, has developed a low glucosinolate restorer line, R2000, with a shorter radish segment through gamma ray irradiation (Primard-Brisset et al. 2005). Dow AgroSciences (DAS) has obtained R2000 from INRA and used it as a donor of restorer gene, *Rfo*, in DAS canola breeding program for the development of Nexera hybrids. Although several markers flanking the *Rfo* locus have been developed by INRA, these markers were developed from the introgressed radish sequence rather than the *Rfo* gene itself and less useful for linkage drag analysis for breaking the linkage drag associated with the *Rfo* locus. The objective of this study was to develop *Rfo* allele-specific markers for the direct selection of the *Rfo* alleles for fertility restoration in canola hybrid development.

Materials and Methods

Plant Material: The fertility restorer line R2000 used in this study was a rapeseed (*B. napus*) line obtained from INRA, France. It was developed by INRA and contained the Ogura fertility restorer (*Rfo*) gene introgressed from radish (Primard-Brisset et al. 2005). The non-restorer lines used in this study were Dow AgroSciences Nexera canola lines Nex 705, Nex 715, Nex 830 CL and a winter rapeseed line, Boston.

Cloning and Sequencing: DNA was extracted from the leaves of 2-week-old greenhouse grown plants using DNeasy 96 Plant Test Kit (QIAGEN, Valencia, California) per manufacturer's instructions. Three pairs of primers were designed from the radish *Rfo* (PPR-B) gene sequence (GenBank Accession AJ550021). PCR was performed in 96-well PCR plates, with each reaction containing 10-20 ng of genomic DNA, 0.125 µM of forward and reverse primers each, 2.5 mM $MgCl_2$, 0.1mM of each dNTP, 1X PCR buffer, 0.4% PVP and 1 unit of *Taq* DNA polymerase. Amplification program was 30 cycles of 30 sec at 94 ^{0}C, 45 sec at 50 or 55 ^{0}C, 30 sec at 72 ^{0}C and ending with 10 min at 72 ^{0}C. The PCR amplification products were separated in 1.5% agarose E-gels produced by Invitrogen (Carisbad, California) and visualized under UV light. The bands of the *Rfo* fragments amplified by the primer pairs BnRFO-F1/BnRFO-R1 and BnRFO-F3/BnRFO-R3 from R2000 and Nex 705 were excised from the gel and were cloned using a TA-cloning kit (Invitrogen Corp, San Diego, Calif) per manufacturer's instructions. The positive clones containing the insert were sequenced by Lark Technologies (Houston, Texas). The sequences were analyzed and aligned by using Sequencher v.4.1.4 (Gene Coded Corporation, Ann Arbor, Michigan) and a web-based SeqWeb (version 2) sequence analysis software present in GCG software package (University of Wisconsin).

Allele-Specific PCR: Allele-specific PCR was performed in 96-well PCR plates, with each reaction containing 10-20 ng of genomic DNA, 0.125 µM of forward and reverse primers each, 2.5 mM $MgCl_2$, 0.1mM of each dNTP, 1X PCR buffer, 0.4% PVP and 1 unit of *Taq* DNA polymerase. Amplification program was similar to that described in the section of Cloning and Sequencing except that annealing temperature was set at 50 ^{0}C and 55 ^{0}C, respectively. The PCR amplification products were separated in 4% agarose E-gels produced by Invitrogen (Carisbad, California) and visualized under UV light.

Results and Discussion

Sequence analysis of *Rfo* cloned from *B. napus* lines: In order to develop *Rfo* allele-specific markers, we implemented cloning and sequencing of the *Rfo* gene and its orthologs from the restorer line R2000 and non-restorer canola lines to identify the sequences that are unique to the restorer line. As a result, the *Rfo* sequence can be used to develop allele-specific marker to detect *Rfo* in canola restorer trait introgression. Three pairs of primers were designed from the radish *Rfo* gene sequence retrieved from NCBI GenBank (Accession AJ550021) and were used to amplify the genomic DNA of the restorer line R2000 and two non-restorer, Nexera canola lines (Nex 705 and Nex 830 CL). Two primer pairs (BnRFO-F1 + BnRFO-R1 and BnRFO-F3 + BnRFO-R3) amplified a single band of the expected size in all three lines, while the primer pair BnRFO-F2 and BnRFO-R2 amplified a single fragment present only in the restorer line R2000 but absent in the two non-restorer lines (data not shown). The primer pairs BnRFO-1F/BnRFO-1R and BnRFO-3F/BnRFO-3R were used to amplify the genomic DNA of the restorer line, R2000 and the non-restorer line, Nex 705, for cloning and sequencing. The fragments amplified by these two primer pairs were isolated from the gel and cloned. Approximately 40 clones (10 clones from each line and each primer set) were submitted to Lark Technologies (Huston, Texas) for sequencing. The alignment of 20 sequences of the fragment amplified by each of the primer pairs BnRFO-F1/BnRFO-R1 and BnRFO-F3/BnRFO-R3 revealed at least three orthologs of *Rfo* sequences for each fragment based on the sequence similarities. Two of the orthologs were found in both restorer (R2000) and non-restorer (Nex 705) lines and the third one was found only in the restorer line unique to R2000. This ortholog could be the *Rfo* sequence integrated into R2000 from radish.

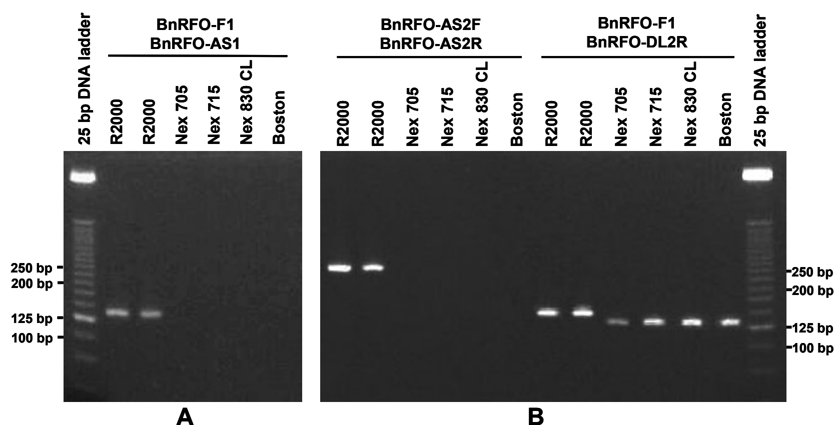

Figure 2. PCR products amplified by the allele-specific primers (BnRFO-AS1 and BnRFO-AS2) and the primers flanking the 9-bp deletion (BnRFO-DL) for the *Rfo* gene in canola. Primers BnRfo-AS1 and BnRfo-DL2R were each paired with BnRFO-F1 and BnRFO-AS2F was paired with BnRFO-AS2R for the amplification. The PCR products were separated by electrophoresis in 4% E-gels produced by Invitrogen (Carisbad, California).

Development of the *Rfo* allele-specific markers: The R2000 sequence-specific primer BnRFO-AS1 was first designed to confirm the uniqueness of the R2000 sequence. The R2000 sequence-specific primer BnRFO-AS1, when paired with the primer BnRFO-F1, amplified a fragment of the expected size only in R2000 but not in the non-restorer lines (Figure 1A), confirming that this copy was unique to the restorer line. To further confirm that this sequence is unique to the restorer line, three more R2000 sequence-specific primers, BnRFO-DL2R, BnRFO-AS2F and BnRFO-AS2R, were designed and used to amplify DNA from R2000 and non-restorers lines. The primer BnRFO-DL2R is positioned at the downstream of a 9 bp insertion at the nucleotide location of 106 to 114 in the sequence amplified by the primer pair BnRFO-1F/BnRFO-1R. When it was paired with the primer BnRFO-F1, the primer pair amplified a larger fragment in R2000 than in non-restorer lines (Figure 1B), indicating that the presence of the 9 bp insertion in the *Rfo* gene of the restorer line R2000. The primer pair BnRFO-AS2F and BnRFO-AS2R, which was specific to the radish *Rfo* sequence present in R2000, amplified a single fragment present in R2000, but was absent in all the non-restorer lines (Figure 1B). The results indicated that the R2000 sequence-specific primers amplified unique fragments in the restorer line R2000 and confirmed that the unique sequence in the restorer line R2000 was the *Rfo* sequence introgressed into canola from radish. A population of 172 individual plants from a cross between CMS sterile line and a R2000 plant suspected to be heterozygous for the *Rfo* locus was screened using the *Rfo* allele-specific PCR marker. At flowering, the plants were scored for presence or absence of pollen as a predictor for the presence or absence of the restorer gene. Except for 4 instances, PCR results and the observed phenotypic pollen shed scores were in agreement. When there was a discrepancy, 3 out of the 4 times, it involved a negative PCR result with an observed pollen shedding. Since there was no control endogenous band, it is possible that these were false negatives due to the failure of

PCR reactions. These results indicated that allele-specific markers are very useful for the selection of the *Rfo* allele introgressed from the restorer line R2000 for fertility restoration in canola hybrid development.

In conclusion, we have sequenced genomic clones of *Rfo* and its orthologs amplified from the restorer line R2000 and non-restorer line Nex 705 using primers designed from the radish *Rfo* sequence (GenBank accession AJ550021) and developed *Rfo* allele-specific PCR markers based on the sequence differences between the restorer and non-restorer lines. The *Rfo* allele-specific markers have proven very useful for direct selection of *Rfo* alleles for fertility restoration during marker-assisted introgression of the Ogura restorer gene for hybrid development in canola.

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