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Identification and characterization of a putative light-harvesting chlorophyll a/b-binding protein gene encoded at a fertility restorer locus for the *Ogura* CMS in *Brassica napus* L.

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Abstract The nuclear-encoded fertility restorer locus for *Ogura* radish cytoplasmic male sterility (CMS) was previously transferred from radish into *Brassica napus* through intergeneric crosses, with the putative restorer gene(s) being expressed in leaves and flower buds. To identify transcripts corresponding to this restorer locus, we performed a PCR-selected cDNA subtraction using leaf mRNA from the restorer line and its recurrent parent. The resulting library was differentially screened using the subtracted cDNAs for the library itself and the cDNAs from the reverse subtraction (recurrent parent subtracted against the restorer line). Forty-eight cDNA clones were isolated based on their specific hybridization with cDNAs for the library construction. Sequence analyses revealed that these 48 clones correspond to only three different genes: two light-harvesting chlorophyll a/b-binding protein (*Cab*) genes and one chloroplast-encoded gene (a chloroplast 50S ribosomal protein gene). Of the two putative *Cab* genes, one showed polymorphism on the Southern blot with the *Eco*RI-digested genomic DNA from the restorer line and its recurrent parent. Segregation analyses confirmed that this putative *Cab* gene comes from radish DNA and co-segregates with the restorer locus. A sequence comparison of this putative *Cab* gene with its homeolog in *Brassica napus* revealed that (1) their coding regions share a 94% similarity, (2) they differ in the size of the intron, and (3) they are identical in the deduced amino acid sequences, except that the one encoded at the restorer locus contained an additional alanine at position 34.

Keywords *Brassica napus* · *Raphanus sativus* · cDNA subtraction · *Cab* gene · Restorer gene

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Introduction

Plant growth and development involve the coordinate function of three genetic compartments: the nucleus, the plastids, and the mitochondria. It has been well-established that the nuclear genes play an important role in the regulation of organellar development and function since a majority of plastid and mitochondrial proteins are nuclear-encoded and imported into these organelles after their synthesis (Mackenzie et al. 1994; Robinson and Klospen 1994). Cytoplasmic male sterility (CMS), a widespread phenomenon in flowering plants, provides an excellent system to study the nuclear-organelle interaction. CMS is defined as the inability of plants to produce or release functional male gametophytes, which is caused by the incompatibility between nucleus and cytoplasm (Hanson and Conde 1985). While mitochondrial (mt)DNA and chloroplast (cp)DNA are both inherited in a maternal fashion, as is CMS, the determinants of CMS have been found to be associated with the mitochondrial genome (Hanson and Folkerts 1992). In CMS plants, alterations in mitochondrial genome organization have been identified and found to lead to the production of chimeric transcripts that encode novel proteins (Schnable and Wise 1998). In maize, for example, the mitochondrial protein, *T-urf13*, which is present in CMS plants but absent in the fertile cytoplasm, is responsible for the male sterility (Levings and Brown 1989). In many CMS systems, there are nuclear genes that are able to restore male fertility by suppressing the mitochondrial defects. In rice, sunflower, and sorghum, for example, nuclear restoration of CMS acts at the post-transcriptional level by reducing or editing the altered mitochondrial transcripts (Iwabuchi et al. 1993; Monéger et al. 1994; Pring et al. 1998). To

date, only one restorer gene has been cloned – a maize aldehyde dehydrogenase gene (Cui et al. 1996) – and even in this case the molecular mechanisms by which this nuclear gene restores male fertility to CMS plants is not clearly understood.

Various male-sterile cytoplasms have been found in *Brassica* species, with one of the most extensively investigated being the *Ogura* cytoplasm of radish (*Raphanus sativus* L.) in which the abortion of male gametogenesis occurs at the early microspore stage owing to the degeneration of the tapetal tissues (for review see Shiga 1980; Delourme and Budar 1999). The mitochondrial genome in *Ogura* CMS plants is highly rearranged, generating a specific DNA fragment containing two open reading frames (*orf138* and *orfB*) transcribed as a bicistronic mRNA (Bonhomme et al. 1992; Krishnasamy and Makaroff 1992). The *orfB* transcripts have been detected in both sterile and fertile plants. The transcripts for *orf138*, however, are present only in *Ogura* CMS plants, suggesting a role for *orf138* in *Ogura* CMS (Bonhomme et al. 1991; Krishnasamy and Makaroff 1992; Bellaoui et al. 1997, 1998). Nuclear fertility restorer genes for *Ogura* CMS have been found frequently in European radish varieties, but not in Japanese varieties. When the restorer gene(s) is present, the abundance of *orf138* transcripts is not affected, but the *orf138* protein is reduced significantly in concentration in the flower and/or leaf, but not in root (Krishnasamy and Makaroff 1994; Bellaoui et al. 1999). It has been suggested that the restorer gene(s) affects *orf138* at a translational or post-translational level and in an organ-specific manner (Krishnasamy and Makaroff 1994; Bellaoui et al. 1999).

Ogura CMS has been introduced from radish into *Brassica* species by interspecific crosses (Bannerot et al. 1974). Male-sterile lines without obvious defects have been generated using protoplast fusion (Pelletier et al. 1983). The nuclear fertility gene(s) has also been transferred from radish to *Brassica* species through intergeneric crosses (Heyn 1976). The transfer of restorer gene(s), however, was accompanied by a number of linked genes that lead to an increase in glucosinolate production and a large decrease in seed set. To improve the restorer lines and to clone the restorer gene(s), a considerable effort has been made to identify molecular markers that are closely linked to the restorer gene(s) (Delourme et al. 1994, 1998; Hansen et al. 1997). These markers have greatly facilitated the selection of restorer plants with less radish DNA, and restorer lines with low glucosinolate and good female fertility have been developed. To date, however, after many backcross generations, it is neither clear what genes are still present in this introgressed radish DNA nor which of these is the restorer gene(s). We have initiated a project to clone this introgressed radish DNA, including the restorer gene(s) (Wu et al. 2000). Here we report the identification of a putative light-harvesting chlorophyll a/b-binding protein (*Cab*) gene present within the introgressed radish chromosomal segment using a polymerase chain reaction (PCR) select cDNA subtraction approach (Diatchenko et al. 1996).

Materials and methods

Plant materials

The restorer locus for the *Ogura* CMS was introduced into a spring-type *Brassica napus* variety (Cyclone) and a winter-type variety (MN1717) by a series of backcrosses. All plants were grown in a growth chamber at 23°/17°C (day/night) under a 16-h daylength. The cDNA subtraction was performed using the spring-type restorer plants from the sixth generation of backcross. A segregating F_2 population of 120 plants was used for linkage analysis. A radish variety (Yamagishi and Terachi 1994) which carries the restorer locus was also included in this linkage analysis.

cDNA subtraction

Total RNA was isolated as previously reported (Wu et al. 1996). Poly(A)$^+$RNA was purified using a mRNA Purification Kit (Pharmacia). cDNA synthesis and subtraction were performed using a PCR Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. The cDNA that contains the unique transcripts is referred to as the ''tester'', and the reference cDNA is referred to as the ''driver''. The tester and driver cDNAs are digested with *Rsa*I, yielding short and blunt-ended molecules. The tester cDNA is then divided into two groups, and each is ligated with a different cDNA adaptor. Two hybridizations are then performed using an excess of driver cDNA. After the ends of the hybridized cDNAs are filled in, the entire cDNA population is subjected to PCR amplification, which greatly enriches the differentially expressed sequences.

TA vector cloning and differential screening

The subtracted cDNA population was cloned directly into the pGEM-T Easy vectors (Promega). About 50 ng secondary PCR-amplified products was ligated into 25 ng vectors and the ligation mixture was transformed into JM109 competent cells (Promega) by heat shock. The transformants were plated on LB plates containing 100 μg ml^{-1} ampicillin, 0.5 m*M* IPTG (isopropyl β-D-thiogalactopyranoside) and 80 μg ml^{-1} X-gal. The resulting library was differentially screened using probes made from two different subtractions (the restorer line subtracted against its recurrent parent and the recurrent parent subtracted against the restorer line). Duplicate blots were made according to Sambrook et al. (1989), and the hybridization conditions were as previously reported (Wu et al. 1996).

Southern blot and Northern blot analyses

Total DNA and RNA were isolated from young leaves as previously described (Wu et al. 1996). For Southern blot analysis, 10 μg of DNA was digested by restriction enzymes, separated on 0.8% agarose gels, and blotted onto nylon membranes (Hybond™-N$^+$, Amersham) according to standard procedures (Sambrook et al. 1989). For Northern blot analysis, 10 μg of total RNA was used. The hybridization conditions for both the Southern blot and Northern blot analyses were as previously reported (Wu et al. 1996).

Construction of a subgenomic library and screening

A subgenomic library containing the 4.8-kb *Eco*RI fragment cosegregating with the restorer locus was constructed using genomic DNA from a winter-type restorer line (N1717). Genomic DNA was completely digested using *Eco*RI and separated by electrophoresis on 0.8% agarose gels. The region containing DNA fragments between 4.5 and 5.0 kb was cut out, and the DNA was purified using a QIAEX II kit (Qiagen). The DNA fragments were ligated with *Eco*RI-digested and dephosphorylated *pBuescript KS*

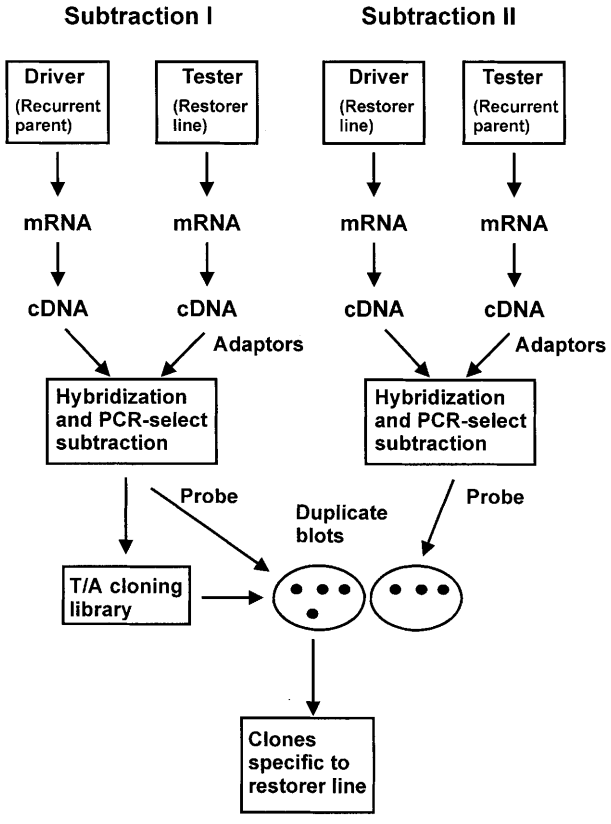

Fig. 1 A schematic representation of the cDNA subtraction and differential screening

vectors overnight at 4°C. The ligation mixture was transformed into the ElectroMAX DH10B cells (BRL, USA) by electroporation using a Gene Pulser system (BioRad, USA) following the manufacturer's instructions. Transformed cells were cultured at 37°C for 1 h in 1 ml SOC medium and then plated on LB plates containing X-gal, IPTG, and ampicillin. The transformation efficiency for this subgenomic library was about 15,000 recombinants per microliter of ligation mixture. Filter lifting and library screening were performed according to Sambrook et al. (1989). Twelve positive clones were obtained by screening about 1.5×10^5 colonies.

PCR analyses of *Cab* genes

PCR analyses of *Cab* genes in *Brassica napus* and radish genome were performed using primers P1 (5'-GTTCTTGTTTGGAG CTAAAG-3') and P2 (5'-GCGCGTGGATCAAGTTAGGG-3'). The PCR reaction (25 µl) contained 50–70 ng of template DNA, 20 ng of primers, 0.1 m*M* dNTPs, 1 × PCR buffer and 0.5 U of *Taq* polymerase. The PCR amplification conditions were 94°C for 4 min for the initial denaturation followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplified products were separated on a 2% agarose gel.

DNA sequence analysis

DNA sequencing was performed using an ABI PRISM DNA Sequencer (model 377), and the sequences were then analyzed using PCGene (IntelliGenetics). Similarity searches on DNA and predicted peptide sequences were carried out using the BLAST searching method (Altschul et al. 1990) against Genbank, EMBL, and SWISSPROT databases.

Results

cDNA subtraction and differential screening

Leaf mRNA was used in the cDNA subtraction experiments since (1) the putative restorer gene(s) is expressed in leaf and (2) developmentally regulated gene expression in leaf tissues is less complex than that in flower buds or anthers. This complexity can lead to differences in the mRNA populations, due to slight differences in the collection of plant materials from these two lines. The flow diagram of the cDNA subtraction and differential screening is shown in Fig. 1. The ''tester'' is defined as the mRNA population that contains the transcripts of interest, whereas the ''driver'' is the mRNA population that will be eliminated during subtraction. The cDNA subtraction was performed using a PCR select subtraction method (see Materials and methods). One important feature of this method is that sequences unique to the tester mRNA are amplified exponentially, whereas the amplification of identical sequences in the two populations is suppressed. The efficiency of subtraction can be determined by monitoring the depletion of sequences common to both populations and the enrichment of sequences specific to one population. Two different subtractions have been performed in order to eliminate the false positives generated by PCR amplification and to avoid missing low-abundance transcripts. In subtraction I, the leaf mRNA from the restorer line (tester) was subtracted against the leaf mRNA from its recurrent parent (driver). Subtraction II is a reverse subtraction in which the original tester (restorer line) mRNA was used as the driver and the driver (recurrent parent) mRNA was used as the tester. A subtracted library was constructed using the subtracted cDNA from the restorer line (subtraction I). This subtracted library was then hybridized with ''itself'' (cDNA probe from subtraction I) and with the ''reverse-subtracted'' cDNA (cDNA probe from subtraction II). By screening about 5,000 recombinant clones, we were able to identify 48 clones that clearly hybridized with the subtracted probe from the restorer line (subtraction I), but not with the subtracted probe from the recurrent parent (subtraction II). Seventy-eight clones were found to hybridize preferentially with the subtracted probe from the restorer line. Approximately 30% of the total clones did not show hybridization signal with both probes.

Sequence analyses of selected cDNA clones

The subtracted cDNA library was primarily analyzed by sequencing 78 cDNA clones including all of the 48 clones that specifically hybridized to the subtracted probes from the restorer line and 30 ''cold'' clones that did not show detectable hybridization signal with either of the subtracted probes. Sequence data was sent to Genbank for a homology search using BLASTX (Alschul et al. 1990). Of the 48 cDNA clones that are specific to

Table 1 Sequence information evaluation of the selected cDNA clones

Clone type	Clone number	Clone size (bp)	Sequence homology (accession no.)	Position pointed to the homologous sequence	Poly-morphism detec ted
I	11	353	*Cab* (P27494)	Central	Yes
II	7	163	*Cab* (P27494)	5’ end	Yes
III	3	550	*Cab* (P27494)	5’ end and central	Yes
IV	17	196	*Cab* (P0478)	5’ end	No
V	10	121	Chloroplast 50S protein (P42345)	Central	Not determined

the restorer line, 38 cDNA clones showed significant homology to different light-harvesting chlorophyll a/b binding proteins (*Cab*), and 10 cDNA clones showed homology to the chloroplast-encoded 50S ribosomal proteins. The 30 ‘‘cold’’ clones showed homology to different sequences, and some of them showed no similarity to any known sequence. Detailed sequence analyses of the 38 cDNA clones that are homologous to the *Cab* genes revealed that they correspond to four different types of sequences. Eleven cDNA clones belong to the first type of sequence, which is 353 bp in size, 7 cDNA clones belong to the second type of sequence, which is 163 bp in size, and 3 clones belong to the third type of sequence which is 550 bp in size. The first type corresponds to the central part of a *Cab* transcript, whereas the second type contains the 5’ untranslated region. These two sequences are separated by 42 bp, with the third type containing sequences which overlap with the first two. Thus, these three sequences are in fact overlapping regions derived from the same transcript, which shows the highest homology with a *Cab* gene from tobacco. Seventeen cDNA clones belong to the fourth type of sequence, which is 196 bp in size and which is derived from a different *Cab* gene that has its highest homology with a *Cab* gene from *Arabidopsis thaliana*. The detailed sequence analysis of some selected cDNA clones is summarized in Table 1.

Northern and Southern analyses using the *Cab* cDNAs

Each of the four types of cDNA sequences that are derived from two different *Cab* genes has been analyzed by Northern and Southern blot hybridizations. Even though these cDNA sequences showed specific hybridization with the subtracted probe from the restorer line during differential screening, in Northern blot analyses they hybridized to a similar extent to the *Cab* mRNA isolated from either the restorer line or the recurrent parent. In order to determine whether the specific hybridization seen in the differential screening is due to efficient subtraction, we performed Southern blot analyses with the subtracted cDNA. In these blots, the hybridization signals in the lanes containing the subtracted cDNA from the restorer line were much higher than those in the lanes containing subtracted cDNA from the recurrent parent, suggesting that these four types of cDNA sequences have been enriched during the PCR-selected cDNA subtraction. An example of this type of analysis is shown in Fig. 2.

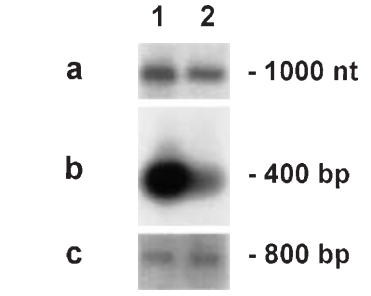

Fig. 2a–c Enrichment of the first type of *Cab* sequence by cDNA subtraction. **a** Northern blot with total RNA (10 µg) from a restorer line (*1*) and its recurrent parent (*2*), probed with the first type of *Cab* sequence and exposed for 2 days. **b** Southern blot with subtracted cDNA (50 ng) from a restorer line subtracted against its recurrent parent (*1*) and from the recurrent parent subtracted against the restorer line (*2*), probed with the first type of sequence and exposed for 5 h. **c** Duplicate blot of **b**, probed with a constitutively expressed protease gene as a loading control, exposed for 3 days

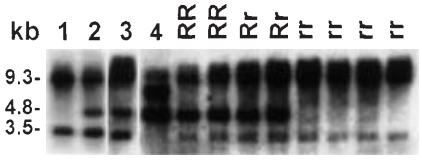

Fig. 3 Segregation analysis of the first type of *Cab* sequences. Southern blot containing *Eco*RI-digested genomic DNA (10 µg) from a recurrent parent (*1*), a spring type restorer line (*2*), a winter type restorer line (*3*), a radish restorer line (*4*), and a segregating population including plants with a homozygous restorer locus (*RR*), plants with heterozygous restorer locus (*Rr*), and plants with a homozygous recessive restorer locus (*rr*)

If these *Cab* cDNAs are really derived from genes present in the introgressed radish DNA and present only in the restorer line, it should be possible to distinguish them from their related members by genomic DNA blot analyses. Genomic DNA from both the restorer line and its recurrent parent were subjected to different restriction enzyme digestions and probed with the four types of cDNA sequences. The first three types of cDNA sequences that were derived from the same *Cab* gene showed a clear polymorphism on the blots containing genomic DNA digested with *Eco*RI, with an extra 4.8-kb fragment present only in the restorer line (Fig. 3, lanes 1 and 2). The only difference amongst these results was

that the smallest cDNA fragment (type-2 cDNA corresponding to a region near the 5' end of the *Cab* transcript) did not hybridize with a 3.5-kb band present in both the restorer line and the recurrent parent (data not shown). However, the fourth type of cDNA sequence derived from a different *Cab* gene did not show any polymorphism on the blot with the genomic DNA digested with eight different restriction enzymes, namely *Bam*HI, *Bgl*II, *Hin*dIII, *Eco*RI, *Eco*RV, *Nco*I, *Pst*I, and *Xba*I (data not shown).

To confirm that the *Cab* gene detected by the first three probes is associated with the restorer locus and is thus present in the introgressed radish DNA, we performed Southern blot analyses using the genomic DNA from individual plants in a segregating F_2 population (a spring-type *Brassica napus* line), a winter-type restorer line, and a radish variety that contains the restorer locus. Twenty-four F_2 plants were tested, including 6 plants homozygous for the restorer locus (RR), 6 plants heterozygous for the restorer locus (Rr), and 12 plants that are homozygous for the recessive allele of the restorer locus (rr). The 4.8-kb band was present in all the plants containing the restorer locus but absent from the plants that are recessive for the restorer locus. This band was also present in the winter-type restorer line and the radish variety containing the restorer locus. An example of this analysis is shown in Fig. 3. Thus, this *Cab* gene segregates with the restorer locus. Furthermore, there is considerably less of the introgressed radish DNA in the winter-type line than in the spring-type line since most of the molecular markers closely linked to the restorer gene(s) present in the spring-type line have been lost in the winter-type line (unpublished data). We conclude that this putative *Cab* gene is closely linked to, or is part of, the restorer locus.

Sequence comparison of the radish *Cab* gene with its homeolog in *Brassica napus*

To further characterize this putative radish *Cab* gene encoded at the restorer locus, we cloned and sequenced both the putative full-length cDNA and the 4.8-kb polymorphic genomic band. The putative full-length cDNA was cloned by PCR amplification of the cDNA population from the restorer line using a cDNA synthesis primer provided by the PCR Select™ cDNA Subtraction Kit (Clontech) and a sequence-specific primer corresponding to the 5' untranslated region of the partial cDNA clone. Sequence analyses of 12 PCR clones revealed two types of highly homologous sequences. One obvious difference between these two types of sequences is the presence of three nucleotides (TGC) in one type, which are absent in the other. The 4.8-kb polymorphic genomic band was cloned from the subgenomic library constructed specifically for this fragment (see Materials and methods). A sequence comparison of this 4.8-kb genomic clone with the cDNA clones revealed that this genomic sequence matched the cDNA sequence that contains the three additional nucleotides (TGC). One intron with 197 bp was also found in this genomic sequence. To analyze the allelic distribution of this C*ab* gene in the *Brassica* genome, we designed specific primers from the genomic sequence that can clearly distinguish between the radish allele and its corresponding *Brassica* allele (Fig. 4). Using this PCR approach, we screened an additional 96 F_2 plants segregating for the restorer locus. No recombination between this *Cab* gene and the restorer locus was found.

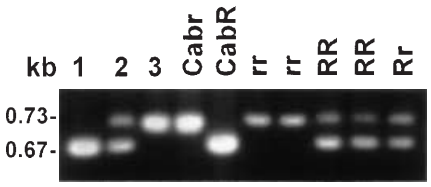

Fig. 4 PCR analyses of *Cab* genes in radish and *Brassica napus*. The putative *Cab* genes encoded at the introgressed radish chromosomal segment and at the *Brassica* genome are distinguishable from each other by PCR reaction. The PCR amplification was performed on DNA samples from a radish restorer line (*1*), a *Brassica napus* restorer line (*2*), and its recurrent parent (*3*), a genomic clone encoded at the restorer locus (*CabR*) and its corresponding *Brassica* homologous clone (*Cabr*), and a segregating population including plants with a homozygous recessive restorer locus (*rr*), plants with a homozygous restorer locus (*RR*), and plants with a heterozygous restorer locus (*Rr*)

To compare the genomic sequences of the radish *Cab* gene encoded at the restorer locus (*CabR*) with its homeolog in *Brassica napus* (*Cabr*), we screened a binary vector-based large-insert DNA insert library constructed for *Brassica napus* (Wu et al. 2000); and 68 positive clones were found. PCR analyses of these 68 clones indicated that 8 overlapping clones which contained a 15-kb *Eco*RI band hybridizing with the cDNA probes showed a PCR product that is about the same size as that present in all the *Brassica napus* plants; this product is not in the radish plants. These 8 clones were inferred to be the *Brassica* homologous clones corresponding to the radish one. A part of this analysis is shown in Fig. 4. Of these 8 clones 1 was selected and the 15-kb *Eco*RI fragment was subcloned into *pBluescript KS*. The region containing the *Cab* gene was sequenced. Sequence analyses revealed that this genomic sequence matched the cDNA sequence that does not contain the three nucleotides (TGC) that are present in the radish *Cab* gene encoded at the restorer locus. One intron was also found in this genomic sequence, which is 263 bp in length. A sequence comparison of this genomic clone with the radish one revealed that their coding regions share a 94% similarity and that the sequence homology extends into the 5' untranslated regions and the promoter regions. The deduced amino acid sequences of these two genomic clones are identical except that an additional alanine is present at position 34 in the radish one, which resulted from the addition of the three nucleotides (TGC). A sequence analysis of these two deduced amino acid sequences using PCGene (Intelli Genetics) revealed the presence of both a chloroplast

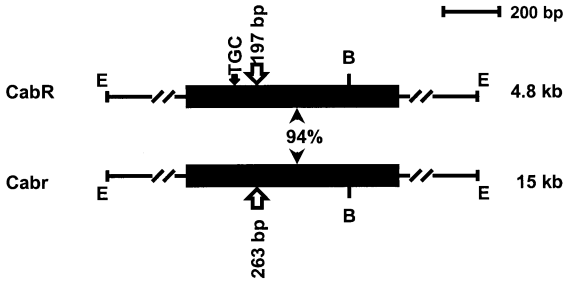

Fig. 5 Physical structure of the putative *Cab* gene encoded at the restorer locus (*CabR*) and its homeolog in *Brassica napus* (*Cabr*). The coding regions which share a 94% similarity are indicated as *black boxes*. The positions of introns are marked with *open arrows*. The addition of the three nucleotides (TGC) in CabR is marked with the *solid arrow*. Restriction enzyme sites: *E Eco*RI, *B Bam*HI

transit peptide and a potential mitochondrial transit peptide (Gavel and von Heijne 1990a, 1990b). After cleavage of the putative transit peptide, the predicted mature proteins for both clones are identical. The physical structures of these two genomic clones are shown in Fig. 5.

Discussion

One method that can be used to identify genes whose products are unknown is the cloning of cDNAs based on their presence and expression profiles in individuals expressing the phenotype. To identify the differences between two mRNA populations, several techniques have been developed including differential display (Liang and Pardee 1992), representational difference analysis (RAD) (Lisitsyn et al. 1993), enzymatic degradation subtraction (Zeng et al. 1994), linker capture subtraction (Yang and Sytkowski 1996), and other subtraction methods that involve the physical removal of common sequences (Akopian and Wood 1995; Deleersnijder et al. 1996). Although all of these techniques have been used successfully in the isolation of differentially expressed genes, each method has its uncertainties and drawbacks. For example, differential display picks up the differences only at the 3' end of the cDNA, so that the differences at the 5' end of a cDNA are not detected. RAD requires multiple rounds of subtractions and is prone to the production of false positive clones. In some cDNA subtraction methods, the removal of common sequences is inefficient. To overcome these difficulties, a technique termed subtraction suppression hybridization (SSH) has been developed which combines a high subtraction efficiency with an equalized representation of differentially expressed sequences (Diatchenko et al. 1996).

In this report, we describe a method that combines the advantages of SSH with differential screening using subtracted probes. The aim of this investigation was to isolate transcripts corresponding to the restorer locus for the *Ogura* CMS. It was expected that a number of genes that are specific to the restorer line would be identified after cDNA subtraction and differential screening. However, in our experiment, all of the cDNA sequences obtained corresponded to only three different genes: two *Cab* genes and one chloroplast gene (a chloroplast-encoded 50S ribosomal protein gene). Other genes present in the introgressed radish DNA may not have been identified due to their low expression levels in leaf relative to the *Cab* genes. The isolation of the chloroplast-encoded gene by subtraction may be due to the fact that the restorer line and its recurrent parent have different cytoplasms. Of the two putative *Cab* genes, one did not show any polymorphism on the Southern blot with the genomic DNA digested by eight different restriction enzymes. This may be due to the evolutionary conservation of these restriction enzyme sites around this gene or it may not be linked to the restorer gene. The other putative *Cab* gene has been found to be closely linked to or part of the restorer locus.

Chlorophyll deficiency and male sterility are the two most common abnormalities in plant development caused by functional incompatibility between nucleus and cytoplasm (Grun 1976; Stubbe and Herrmann 1982). Sometimes both of these abnormalities occur simultaneously, and both are maternally inherited. For example, when the *Ogura* cytoplasm was introduced into *Brassica* species, the male-sterile progenies exhibited severe chlorophyll deficiency (Bannerot et al. 1977). *Cab* proteins constitute the major protein component of the chloroplast thylakoid membrane and are important for the accumulation and stability of chlorophyll molecules (Buetow et al. 1988; Chitnis and Thornber 1988). After being synthesized as precursor polypeptides in the cytoplasm, *Cab* proteins are transported into the chloroplast where they bind non-covalently to chlorophyll a, chlorophyll b, and carotenoid molecules and function to harvest light energy for photosynthesis. In our study we found some DNA sequence differences in the *Cab* genes between radish and *Brassica napus*. It would be very interesting to test whether these differences in *Cab* genes cause the chlorophyll deficiency. Since male-sterile lines with normal chlorophyll content were developed by replacing the radish chloroplasts with the *Brassica* ones through protoplast fusion (Pelletier et al. 1983), and since one would expect that the restorer gene product would be targeted into the mitochondrion, the *Cab* gene would not seem to be a likely candidate for the restorer gene. However, one cannot rule out entirely the possibility that the *Cab* gene is involved in the fertility restoration since functional plastids are also necessary for microspore development. For example, the tapetum cells in *Brassica napus* contain abundant globuli-filled plastids whose lipids are discharged and deposited onto the surface of the developing microspores (Wu et al. 1997). Further, the expression pattern of the *Cab* gene exactly matches that of the putative restorer gene: highest in leaf tissue, also present in flower buds, but not found in roots (Krishnasamy and Makaroff 1994). We also found that this putative *Cab* gene is expressed in young anthers (data not shown). We are currently using this putative *Cab* gene as a landing marker for the positional cloning of the restorer locus.

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