COMPARISON OF CYTOPLASMIC MALE STERILITY BASED ON PET1 AND PET2 CYTOPLASM IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

***Renate HORN*[1], *Antje REDDEMANN*[1], *Miglena DRUMEVA*[2]**

[1]*Institute of Biological Sciences, Department of Plant Genetics, University of Rostock, Albert-Einstein-Str. 3, 18059 Rostock, Germany*
[2]*Technical University of Varna, Department of Plant Production, Studentska Str. 1, 9010 Varna, Bulgaria*

ABSTRACT

Commercial sunflower hybrid breeding is exclusively based on the PET1 cytoplasm. However, diversity on the level of the cytoplasm is desired to reduce the susceptibility to potential pathogens. The PET2 cytoplasm represents a new CMS source with high potential for hybrid breeding. As the PET1 cytoplasm, CMS PET2 originates from an interspecific cross of *H. petiolaris* with *H. annuus*. However, rearrangements observed in the PET2 cytoplasm are totally different from the PET1 cytoplasm. The PET1 cytoplasm is characterized by the co-expression of *atpA* and *orfH522* and the presence of the CMS-specific 16-kDa-protein, whereas in the PET2 cytoplasm due to a duplication of the *atp9* gene, followed by an insertion of 271 bp of unknown sequences, two new open reading frames *orf288* and *orf231* are created. Both *orf*s, which share homology to the *atp9* gene, are co-transcribed and a clear reduction of this co-transcript can be observed in the anthers of fertility-restored hybrids. The *orf*s encode proteins of 11.1 kDa and 7.9 kDa, respectively. New markers linked to the *Rf1* gene and to the *Rf_PET2* restorer gene have been identified by AFLP analyses and have been developed from BAC-end sequences. Comparative mapping using SSR-markers demonstrated that both restorer genes are located on linkage group 13 close to each other, which facilitates cloning of both restorer genes.
Keywords: Cytoplasmic male sterility, fertility restoration, CMS PET1, CMS PET2, marker

INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants in which these fail to produce or shed viable pollen (Horn 2006). In most cases mutations in the mitochondrial DNA lead to new open reading frames (ORFs) that encode CMS-specific proteins that interfere with the pollen development, a process that has a high demand on energy supply (Horn et al. 2014). For hybrid breeding, CMS is of special interest because it allows directed crosses when using CMS lines as mother lines. In addition, CMS can be restored by dominant nuclear genes, so called restorer of fertility (*Rf*) genes. This allows the restoration of fertility in F_1 hybrids. Improvements in yield and yield stability of sunflower hybrids require the development of new lines, which are resistant to diseases, e.g. fungal pathogens, or which have improved oil quality. Marker-assisted breeding can accelerate the back-cross programs and is especially useful if the trait like fertility restoration can only be assessed as late as the flowering stage, a rather advanced period in the plant development (Neuhaus & Horn 2004). Therefore the development of co-dominant markers closely linked to the fertility restorer gene is of great importance for sunflower breeding programs. In addition, the isolation of the fertility restorer gene is of general interest to the research community to understand the molecular mechanism behind fertility restoration (Horn 2006).

The development of co-dominant markers closely linked to the locus of the fertility restorer gene *Rf1* would present a great improvement for marker-assisted breeding in sunflower hybrid production. The selection for the presence of the restorer gene *Rf1* (homozygous or heterozygous) could be performed at a very early stage of plant development.
Worldwide, only one CMS source, the so called PET1 cytoplasm, has been used for commercial sunflower hybrid production, so far (Nichterlein & Horn 2005). This CMS source is the result of an interspecific cross between *Helianthus petiolaris* and *H. annuus* (Leclerq 1969). The PET1 cytoplasm is characterized by the *orfH522* that encodes for a 16-kDa-protein (Horn et al. 1991, Köhler et al. 1991, Laver et al. 1991). However, more than 70 CMS sources have been described for sunflower (Serieys 2005), but only about half of them have been analyzed for the molecular mechanisms leading to CMS (de la Canal 2001, Horn 2002, Horn & Friedt 1999, Horn et al. 2002). However, diversity on the cytoplasm side is desired to avoid the pathogen specialization as the one observed in the maize T-cytoplasm (Miller & Koeppe 1971), exclusively used up to then in maize hybrid production. In sunflower, the PET2 cytoplasm, which was also derived from an interspecific cross of *Helianthus petiolaris* and *H. annuus* (Whelan & Dedio 1980), might be an interesting alternative (Horn & Friedt 1997). However, molecular characterization of the PET2 cytoplasm as well as markers for the restorer gene *Rf_PET2* would be required.
Markers linked to the restorer gene *Rf1*, responsible for fertility restoration of hybrids based on the PET1 cytoplasm, have been identified (Horn et al. 2003, Kusterer et al. 2005). The restorer gene has been placed on linkage group 13 of the sunflower reference map using SSR-markers (Kusterer et al. 2005). In order to isolate the restorer gene *Rf1* by a map-based cloning approach (Kusterer et al. 2004 a, b) a bacterial artificial chromosome (BAC) library has been constructed for the restorer line RHA325 (Özdemir et al. 2002, 2004). Markers were hybridised against high density BAC filters of two BAC libraries (RHA325 and HA383) to identify positive BAC clones. Using BAC fingerprinting, cloning and sequencing of BAC ends, the BAC clones were organized into contigs around the restorer gene *Rf1* (Hamrit et al. 2008, Hamrit 2009).
All markers linked to the restorer gene *Rf1* described up to now are dominant markers, which do not allow distinguishing the homozygous from the heterozygous fertile plants. Here we present the development of a co-dominant CAPS-marker linked to the *Rf1* gene. In addition, the molecular mechanism behind the PET2 cytoplasm will be elucidated as well as the close location of the restorer genes *Rf1* and *Rf_PET2* on linkage 13 by comparative mapping.

MATERIALS AND METHODS

Plant material

HA89 (maintainer line of CMS PET1 and CMS PET2) was used to study the male fertile cytoplasm, CMS line PET2 (Whelan and Dedio, 1980) maintained by RHA265, which is a restorer line of CMS PET1, and CMS line PET1 (Leclercq, 1969), maintained with HA89 and the fertility-restored hybrid PET2 (RHA265) x IH-51 were used for comparing CMS PET1 and CMS PET2 source. Mapping of the *Rf_PET2* was performed in the F2 population RHA265(PET2) x IH-51.
The investigations on the restorer gene *Rf1* were performed using the fertility restorer line RHA325, homozygous for the dominant *Rf1* allele, the fertility maintainer line HA342, homozygous for the recessive allele *rf1*, and bulks of the F2 population of the cross HA342 x RHA325. Each of the F2 bulks consisted of 10 individuals from the F2 population, which were either homozygous for the recessive allele of the fertility restorer gene (S1 and S2) or for the dominant allele (R1 and R2). Total genomic DNA was extracted from leaves according to the protocol of Doyle & Doyle (1987).

Cloning and sequencing mitochondrial DNA

Mitochondrial DNA was isolated using the procedure of Köhler et al. (1991). The *HindIII* digested mtDNA was blotted on Hybond N+ membrane (GE Healthcare) after separation on a 0.8 % agarose gel. Hybridizations with *atp9* as probe were performed according to the manufacturer's instructions using ECL Direct™ Nucleic Acid Labeling and Detection System (GE Healthcare) for the detection of restriction polymorphisms. The *Hin*dIII digested mtDNA fragments were cloned into pUC18 vector and the resulting recombinant plasmids were used to prepare a mitochondrial DNA library. Positive clones were selected by *Hin*dIII hybridization pattern with *atp9* as probe and sequenced.

Analysis and use of BAC-end sequences

Eleven positive bacterial clones (BAC-end sequences) were included in the investigations, which had been identified by hybridizations with markers linked to the restorer gene *Rf1* (Hamrit 2009) on the basis of the two available BAC-libraries in sunflower (Özdemir et al. 2004; Clemson University Genomics Institute-http://www.genome.clemson.edu). The BAC ends had been sequenced using SP6 and T7 primers. The BAC-end sequences of 100L22, 94F15, 147A3, 67N4, 261F19, 126N19, 447N6, 59J13, 450J13, 450B06, 480G04 and 139A17 were included in this study. The software BioEdit 7 was used for processing and analysis of the BAC-end sequences with the aim to develop STS (Sequence-Tagged-Site)-markers for back-mapping of these BAC clones in the population. The design of STS-primers was carried out by the program Web primer (http://www.yeastgenome.org/cgi-bin/web-primer). The design of CAPS (Cleaved Amplified Polymorphic Sequence) markers was realized with the help of the program NEB cutter V2.0 (http://tools.neb.com/NEBcutter2/).

PCR amplification of STS- and CAPS-markers

PCR amplification with STS-primers was performed with 15 ng DNA in the PCR cycler 2700 under the following conditions: 3 min denaturation at 94°C, followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing using different temperatures depending on the primers (range between 55°C and 64°C), 30 sec polymerization at 72°C of polymerization; followed by a final 7 min period of elongation at 72°C. For CAPS-marker 480G04_BsrGI, the PCR products were directly digested with the restriction endonuclease BsrGI. The amplified products were separated by 2 % agarose gel electrophoresis at 100 V for 35 minutes. The PCR-products were visualized with ethidium bromide solution.

Cloning and sequencing of PCR products

PCR products were cloned using the pGEM®-T Easy vector (Promega). After minipreparation, plasmids were sequenced using T7 and SP6 primers.

AFLP and SSR analyses

Amplified fragment length polymorphism (AFLP) analyses were done as described by Vos et al. (1995). For the *Rf1* gene, three new preamplifications were used: (1) E02 and M01, (2) E02 and M02 and (3) E02 and M04. For the selective amplifications, 16 *Eco*RI primers were combined with 48 *MseI* primers. For the *Rf_PET2* gene, AFLP analyses were performed based on the preamplification of E01 and M02 as primers. For the selective amplification, 16 *Eco*RI primers (E31 to E46) and 16 *Mse*I primers (M47 to M62) were combined.

SSR analyses were performed as described in Sajer et al. (2013). Using the SSR primer combinations for ORS317, ORS630 and ORS1030 of linkage group 13 (Tang et al. 2005) and

the M13tailing procedure (Oetting et al. 1995) PCR products were labelled with IRD800 and separated on the DNA Analyzer 4300 (LI-COR, Biosciences).

RESULTS AND DISCUSSION

Molecular characterization of the PET2 cytoplasm

Comparing the Southern hybridization pattern of mitochondrial DNA (*HindIII* digested) from the PET2 cytoplasm and the male fertile cytoplasm a restriction polymorphism was detected using the *atp9* gene as probe. One fragment of 3.4 kb was identical in the male fertile cytoplasm and CMS PET2, but an additional fragment of 4.1 kb was only present in CMS PET2. Cloning and sequencing of the *HindIII* fragments showed that the 3.4-kb-fragment contained the regular copy of the *atp9* gene, whereas the PET2-specific 4.1-kb-fragment contained a split second copy of *atp9*, which resulted in two new open reading frames of 228 bp and 231 bp (Figure 1). Both *orf*s show partial homology to *atp9*. The insertion of 271 bp splitting the *atp9* represents sequences of unknown origin. The new *orf*s encode proteins of 11.1 kDa and 7.9 kDa, respectively. RT-PCR analyses showed that the two PET2-specific *orfs* are co-transcribed and that the co-transcript is specifically reduced in the anthers of fertility-restored hybrids. Mitochondrial genes of the F1F0 ATP synthase are the most frequent genes involved in creating cytoplasmic male sterility (Horn et al. 2014). In sunflower, one other CMS source, the PEF1 cytoplasm, which originates from an interspecific cross of *H. petiolaris* ssp. *fallax* and *H. annuus* (Serieys & Vincourt 1987), also showed changes in the *atp9* gene, here a 0.5-kb-insertion in the 3'-UTR, associated with the male sterility phenotype (de la Canal et al. 2001).

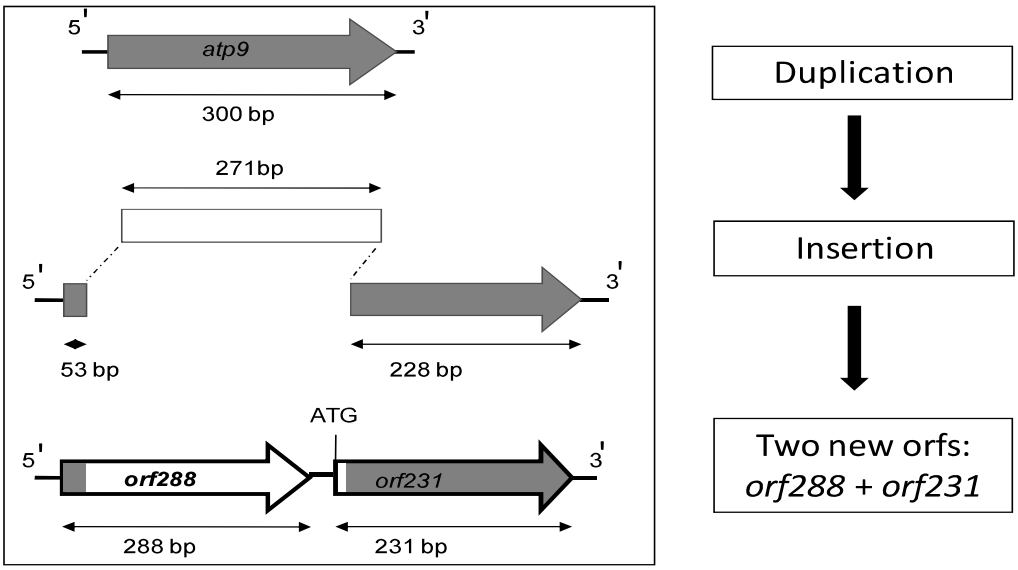


Figure 1: Model of the recombination events

Even though both CMS cytoplasm PET1 and PET2 were derived from an interspecific cross between *H. petiolaris* and *H. annuus*, the mechanism behind the male sterility of CMS PET2 is totally different than in the CMS PET1 cytoplasm, which makes it interesting to use the CMS PET2 cytoplasm for commercial sunflower hybrids. Now that the mechanism is known primers specifically differentiating the two CMS sources can be developed.

First round of developing of STS-markers for *Rf1* from BAC-end sequences

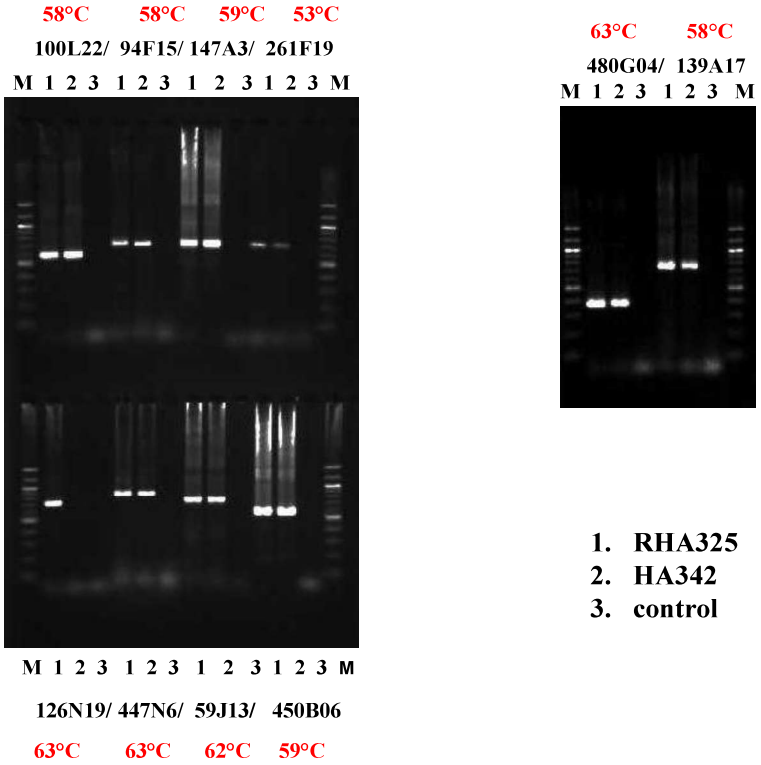
Based on the positive BAC-clones identified during the process of preliminary chromosome walking at the *Rf1* locus (Hamrit 2009), 11 STS-primer combinations were derived from the BAC-end sequences (Table 1). With the aim to improve the quality of the amplified products with the respective primers, a series of PCR-reactions with different annealing temperatures were tested. The optimal parameters for PCR-amplification for each of the investigated primers are given in Table 1.

Table 1: Design of STS-primers from the BAC-end sequences for back mapping the identified positive BACs

BAC-end	Primer name	Primer sequences 5' - 3'	T_A	Expected size of PCR product	Observed size of PCR product	Mono-/ Polymorph
100L22	100L22_for	GAACTTGCTAAATGTTAACGAG	58	573 bp	578 bp	monomorph
	100L22_rev	ATGCAAAAACCGCCTAAG				
94F15	94F15_for	TTAGTCGCCATGTGTACCGAT	58	712 bp	716 bp	monomorph
	94F15_rev	CCACTTTCGATGATGGAGTTG				
147A3	147A3_for	GTTATGCCCGATATCGTAAT	59	702 bp	709 bp	monomorph
	147A3_rev	ACCATTTTAAGTCCCGTAAG				
67N4	67N4_for	TTTCTTGTGTTTTACGATGCC	52-55	714 bp	-	-
	67N4_rev	TGTAACCGTCCGGAACAAAA				
261F19	261F19_for	ACCAAAAGGATCTAGAACTG	53	708 bp	717 bp	monomorph
	261F19_rev	CATTTTAAGGTCATATGGGC				
126N19	126N19_for	ACGCTGTGGCAATAAGACACA	63	701 bp	707 bp	monomorph
	126N19_rev	ACTTTGCAATTGTCACCAAAA				
447N6	447N6_for	TTCATGCTTTTAGCTGCCTGT	63	879 bp	884 bp	monomorph
	447N6_rev	TGCAGTTTAACTGCCCAAGA				
59J13	59J13_for	GCTTCTTGTGCTTCTTTTAAC	62	726 bp	726 bp	monomorph
	59J13_rev	TATCATGACGCTATCGGTTG				
450B06	450B06_for	AGCAGATTGTCAATCGGACAG	59	560 bp	564 bp	monomorph
	450B06_rev	GCTGAAAGATGAGCATCCAA				
480G04	480G04_for	GGTTCACATGGTGTGGATAA	63	361 bp	365 bp	monomorph
	480G04_rev	CTTCAATCAGACATCTATAGAGA				
139A17	139A17_for	GTAACGACTAGCAGGCAATAACA	58	717 bp	720 bp	monomorph
	139A17_rev	TGCGGACGTGAAATAGG				

The primers were tested with the parental lines RHA325 and HA342 to detect polymorphisms that would allow using them directly as markers. The PCR products showed the expected sizes (Table 1), but all showed monomorphic patterns between RHA325 and HA342 (Figure 2). The primers derived from 67N4 and 126N19 did not result in good PCR amplification products.

Figure 2: PCR products using the STS-primers derived from the BAC-end sequences for amplification from the lines RHA325 and HA342



Development of STS- and CAPS-markers for *Rf1* from cloned PCR-products

As the PCR amplification products with the derived STS-primers were monomorph for the parental lines (RHA325 and HA342) the PCR-products were cloned into the pGEM-T vector and sequenced to detect single nucleotide polymorphism (SNP) between the two lines that can be used to develop markers. Positive clones were obtained from the PCR products of the primers derived from the BAC-end sequences of 100L22, 147A3, 480G04, 450B06, 139A17, 59J13, 94F15. On the basis of the sequences analyses, variations in the nucleotide sequence of the investigated DNA fragments from RHA325 and HA342 were found (data not shown); these will be used to design specific polymorphic STS-markers and CAPS-markers. First results are shown for 480G04 (Table 2).

For the BAC clone 480G04, both types of markers could be designed. Based on variations in the 480G04 sequences of the two parental lines RHA325 and HA342, the specific STS-primers 480G04_RH325 and 480G04_HA342 were developed, which combined with the 480G04_rev primer specifically amplified a PCR product from RHA325 or HA342, respectively. Testing the primers in the bulks confirmed the association of the polymorphisms with fertility restoration (data not shown). However, these markers are again dominant.

More interesting was the development of a co-dominant marker based on a SNP in the restriction site of *BsrGI* between RHA325 and HA342. Whereas the PCR product of 361 bp of RHA325 (480G04_for/480G04_rev primer combination) is cut into a 138-bp-fragment and a 223-bp-fragment, the PCR product of HA342 remains uncut (Figure 3). The use of this CAPS-marker in the bulks showed its linkage to the fertility restorer gene. However, this marker still needs to be mapped to the restorer gene *Rf1* in the F2-population. This co-dominant marker will be very helpful in distinguishing plants, heterozygous or homozygous for the restorer gene *Rf1*.

Table 2: Development of STS- and CAPS-markers from cloned sequences

BAC-clone	STS-marker				
	Primer name	Forward primer sequences 5'-3' (reverse as before)	Expected size (Line)	Observed size (Line)	Monomorph/ Polymorph
480G04	480G04_ HA342	TTTGTGGGCTTCGTTTAATCGG	278 bp (HA342)	278 bp (HA342)	Polymorph (dominant)
480G04	480G04_ RHA325	TTTGTGGGCTTCGTTTAATAAC	277 bp (RHA325)	277 bp (RHA325)	Polymorph (dominant)
BAC-clone	CAPS-marker				
	Restriction enzyme	Cutting sequence	Expected size (Line)	Observed size (Line)	Monomorph/ Polymorph
480G04	BsrGI	5′…T˘GTACA…3′ 3′…ACATG˘T…5′	138 bp/223 bp (RHA 325) 361 bp (HA342)	138 bp/223 bp (RHA 325) 361 bp (HA342)	Polymorph (co-dominant)

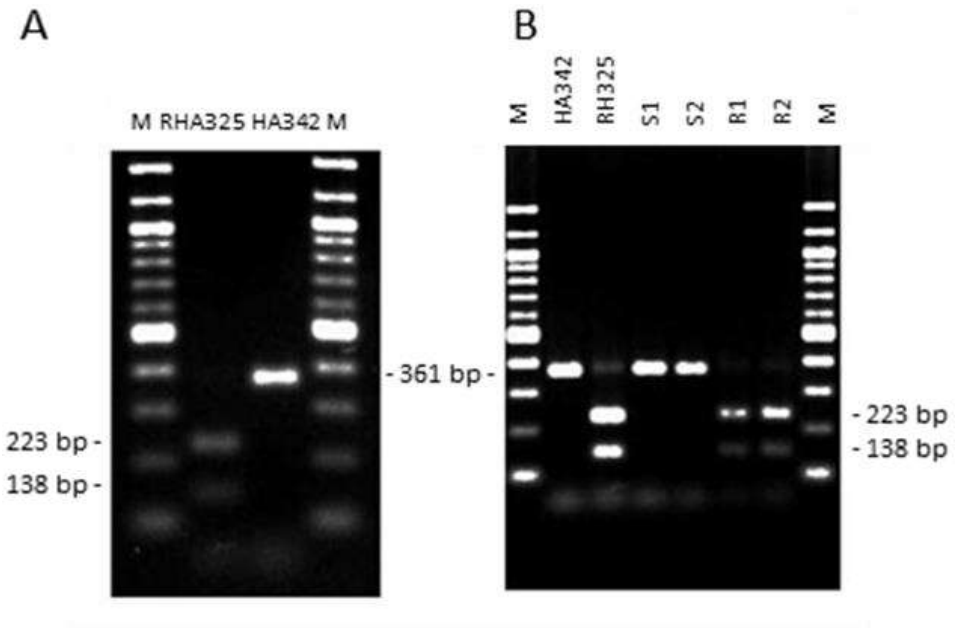


Figure 3: CAPS-marker 480G04_BsrGI. DNA was amplified using the primer combination 480G04_for/480G04_rev and digested with the restriction endonuclease *BsrGI*. A. parental lines (RHA325 and HA342) and B. RHA352 and HA342 as well as bulks (S1, S2, R1, R2). M: 100 bp marker

Comparative mapping of the *Rf1* and *Rf_PET2* gene

New AFLP-markers linked to the restorer gene *Rf1* have been identified as well as first AFLP-markers closely mapping to the *Rf_PET2* gene. Some of these have been cloned and sequenced.

The restorer gene *Rf1* had been mapped to the linkage group 13 (Kusterer et al. 2005) using SSR-markers of the general genetic map of sunflower (Tang et al. 2003). In order to see if the *Rf_PET2* restorer gene might be also located on the same linkage group, three SSR markers (ORS317, ORS630 and ORS1030) bordering the *Rf1* gene were mapped in the F2 population RHA265(PET2) x IH-51 segregating for the *Rf_PET2* gene. Comparative mapping showed that *Rf_PET2* maps less than 10 cM from the *Rf1* gene. This will facilitate cloning of the two restorer genes. Comparing the mechanism of fertility restoration from *Rf1* and *Rf_PET2* will give a better understanding about the processes behind fertility restoration in sunflower.

Conclusions

The molecular basis of the new CMS source PET2 was revealed, which now allows the development of markers differentiating between CMS PET1, CMS PET2 and the fertile cytoplasm. In combination with the identification of AFLP-markers linked to the restorer gene *Rf_PET2* this new CMS source would be now ready to be used for the development of commercial sunflower hybrids.

In addition, a step forward has been made in chromosome walking at the fertility restorer locus *Rf1*. The BAC-end sequences can be used to obtain new overgo probes for subsequent hybridizations against BAC-filters for identification of new positive BAC-clones. However, BAC clones have still to be back-mapped by markers. Their arrangement in contigs will ultimately result in a complete contig around the *Rf1* gene. The newly identified AFLP-markers for the *Rf1* gene will be helpful for this work as well. All markers described up to now have been dominant markers, which do not allow differentiation between homozygous and heterozygous fertile plants. Therefore, the development of the co-dominant CAPS-marker 480G04_BsrG I for the fertility restorer gene *Rf1* is especially interesting. Both, the two new STS-markers as well as the CAPS-marker will be used to back-map the BAC-clone 480G04 in the F2-population and to confirm its position in the region of gene *Rf1*. Interestingly, the restorer gene *Rf_PET2* seems to be located less than 10 cM from the *Rf1* gene.

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