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MAP-BASED CLONING STRATEGY FOR ISOLATING THE RESTORER GENE *Rf1* OF THE PET1 CYTOPLASM IN SUNFLOWER (*Helianthus annuus* L.)

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SUMMARY

Map-based cloning of the restorer gene *Rf1*, responsible for fertility restoration of the PET1 cytoplasm in sunflower, requires tightly linked markers to the gene of interest. Screening 1200 decamer primers by bulked segregant analyses identified seven RAPD markers mapping on the same linkage group as the restorer gene *Rf1*. In the F_2 population of the cross RHA325(cms) × HA342 (183 individuals) one of the RAPD markers, OPK13_454, mapped 0.9 cM from *Rf1*, followed by OPY10_740 with 2.2 cM. Bulked segregant analyses using 1024 AFLP primer combinations identified 282 polymorphisms in 203 primer combinations. Selected single plants from the bulks and recombinant plants were screened to further reduce the number of primer combinations to be mapped in the F_2 population. The map for the linkage group carrying the restorer gene now consists of 43 markers (7 RAPD-, 1 SSR-, and 35 AFLP-markers) and covers 191.9 cM. E32M36_155 and E44M70_275 were mapped 0.7 cM and 0.1 cM from the restorer gene, respectively. Two of the RAPD markers, OPK13_454 and OPY10_740, were successfully converted into SCAR markers, HRG01 and HRG02, respectively. Amplification of the markers OPK13_454/ HRG01 and OPY10_740/HRG02 was investigated for a set of 11 restorer and nine maintainer lines of PET1. For cloning the restorer gene *Rf1*, colony hybridizations against high-density BAC filters of our sunflower BAC library and 3D-PCR pooling strategies were used to identify positive BAC clones. BAC fingerprinting using different restriction enzymes in combination with hybridizations was performed to develop a contig around the restorer locus *Rf1*.

Key words: BAC, CMS, fertility restoration, *Helianthus*, sunflower

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INTRODUCTION

Fertility restoration represents an essential part of hybrid breeding based on cytoplasmic male sterility when the seeds are harvested as in sunflower. For the PET1 cytoplasm, the only CMS source used in commercial hybrid breeding, a number of restorer lines with known pedigree (Korell *et al.*, 1992) have been identified. Depending on the cross combination, one to four dominant restorer genes have been described to be necessary for fertility restoration of the PET1 cytoplasm (Serieys, 1996). However, most cultivated sunflower lines seem to have two dominant nuclear genes, *Rf1* and *Rf2* (Leclercq, 1984). *Rf2* was described to be present in nearly all inbred lines, including maintainers of CMS, and only the second gene is introduced by male restorer lines to produce fertile sunflower hybrids. The *Rf1* gene was mapped on linkage group 13 in the SSR-based consensus map (Tang *et al.*, 2002).

A 16-kDa protein (Horn *et al.*, 1991; Laver *et al.*, 1991) encoded by the mitochondrial *orfH522* seems to be responsible for causing cytoplasmic male sterility in the PET1 cytoplasm (Köhler *et al.*, 1991; Laver *et al.*, 1991). The 16-kDa protein, which is also expressed in nine other CMS sources in sunflower, is membrane-bound (Horn *et al.*, 1996). The PET1-cytoplasm causes premature programmed cell death (PCD) of the tapetum cells (Balk and Leaver, 2001). Fertility restoration involves the anther-specific reduction of the co-transcript of *orfH522* and the *atpA* gene as well as of the CMS-associated protein (Monéger *et al.*, 1994). The tissue-specific increase in the level of polyadenylated *atpA-orfH522* transcripts was associated with the tissue-specific instability of *atpA-orfH522* mRNAs in the anthers of the fertility-restored hybrids (Gagliardi and Leaver *et al.*, 1999). To study the function of the dominant nuclear restorer gene, the gene needs to be isolated. This can be performed by a map-based cloning strategy or by a candidate gene approach if corresponding genes have been isolated.

In this study, we developed a map-based cloning strategy to isolate the restorer gene *Rf1*. We constructed a linkage map around the restorer gene *Rf1* using the AFLP and RAPD techniques and could identify markers tightly linked to the *Rf1* gene in sunflower. These PCR-based markers were converted into easy-to-handle SCAR markers that are now available as useful tools for marker-assisted backcross programs. In addition, the markers were used to identify positive BAC clones and to develop a putatively closed contig around the restorer gene *Rf1*.

Material and Methods

Plant materials

F_2, F_3 and F_2 BC_1 populations (backcrossed on male-sterile HA89 carrying PET1 cytoplasm) were derived from the cross RHA325 × HA342. RHA325 is a restorer line carrying the PET1 cytoplasm, HA342 is a high-oleic maintainer line (Table 1). F_2, F_3 and F_2 BC_1 populations were grown in the field of Groß-Gerau near

Frankfurt/Main and evaluated for male fertility/sterility. Leaf material from F_2 individuals for DNA analyses was immediately frozen in liquid nitrogen and stored at -20°C. The F_2 population used to map the restorer gene *Rf1* consisted of 183 individuals.

In addition to the restorer line RHA325, seven open American lines, RHA265, RHA348, CM587, CM592, CM596, CM610 and Gio55, and three inbred lines developed from interspecific hybrids were included in the investigations (Horn *et al.*, 2003). On the maintainer side, DNA analyses were performed with the lines HA342, HA89, HA291, HA323, HA350, HA850, CM594, CM603 and CM611.

Isolation of genomic DNA

Genomic DNA was isolated according to Doyle and Doyle (1990). In liquid nitrogen grounded powder of 2.5 g leaf material was incubated with 15 ml extraction buffer (100 mM Tris/HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% $Na_2S_2O_3$) at 65°C for 30 min. After chloroform extraction, the aqueous phase was obtained by centrifugation. The procedure was repeated and the DNA was finally precipitated in the aqueous phase by adding 1 ml ammonium acetate (10 M) and 1 ml sodium acetate (3 M pH 5.5) in addition to two-thirds volume 2-propanol at 4°C. High molecular weight DNA was transferred by a glass hook to a new tube and washed once with wash alcohol (70% ethanol, 10 mM ammonium acetate). DNA that was shortly dried was then dissolved in 1 ml TE (10 mM Tris/HCl pH=8.0, 1 mM EDTA).

AFLP analyses

AFLP analyses using *Eco*RI primers E31 to E46 and *Mse*I primers M31 to M94 were performed according to Vos *et al.* (1995). For the selective amplification *Eco*RI primers (500 ng) with three selective nucleotides were labeled using T4 polynucleotide kinase and [γ-^{33}P]-ATP. Labeling was performed for 1 h at 37°C in 50 µl kinase buffer (70 mM Tris/HCl pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 1 mM 2-mercaptoethanol). The enzyme was inactivated by heating the reaction mix to 70°C for 10 min. For the selective amplification 5 ng labeled *Eco*RI primer and 30.2 ng *Mse*I primer were used. Alternatively, IRD-labeled *Eco*RI primers from MWG Biotech (Ebersberg, Germany) were used for non-radioactive labeling of the selective amplification products and run on a LICOR (MWG Biotech).

The primer combinations E32M64, E35M57, E36M70, E38M49, E38M59, E39M48, E42M76, E44M70, E46M60, and E46M60 were used for the recombinant screening.

RAPD analyses

From the segregating F_2 population of RHA324 × HA342 one bulk of male sterile and one of fertility restored plants consisting of 10 individuals each were formed. In addition the parental lines RHA325 and HA342 were included in the

investigations. PCR reactions were performed as described in Horn *et al.* (2003). A total of 1200 arbitrary decamer primers (Operon Technologies, kits A to Z and kits AA to AZ) was analyzed for the ability to produce polymorphic bands between the bulks.

Conversion of RAPD and AFLP markers into SCAR markers

Bands of RAPD markers were excised from the agarose gel, extracted by the gel extraction kit and used in the TOPO A Cloning Kit according to the manufacturer's recommendations.

For AFLP markers, a piece of the dried gel corresponding to the marker of interest was cut out and 100 µl H_2O were added. After 10 min at room temperature, samples were boiled for 10 min, centrifuged and 5 µl of the supernatant were used for PCR according to the AFLP conditions. The PCR program was elongated by an additional final elongation step for 10 min at 72°C and cloning was performed as described by the manufacturer of the TOPO A Cloning kit (InVitrogen). White clones were picked for overnight culture and minipreparation of plasmid DNA using the Quiagen kit. The sequence of the cloned PCR amplification product was used to design SCAR primers. PCR amplification using the sequence-specific primers was performed as described in Horn *et al.* (2003).

Linkage analyses

Linkage analyses were performed using the program MapMaker Version 3.0b (Lander *et al.*, 1987). The linkage map was constructed on a minimum LOD score of 3.0. The ripple function was used to confirm the linkage map. The Kosambi function was used to obtain the genetic distances in centiMorgan (cM) (Kosambi, 1944).

Construction of a BAC library

High molecular weight DNA was isolated from sunflower nuclei preparations as described in Özdemir *et al.* (2002). A *Hind*III BAC library was constructed using pBeloBac11 as vector. The BAC library, which comprises 104,736 BAC clones, was double spotted on four high density filter in a 5x5 pattern (Özdemir *et al.*, 2003). In addition, BAC pools were prepared for units of 48 plates to allow 3D-PCR screening.

RESULTS

Segregation of fertility restoration in the enlarged mapping population

For fine-mapping, the mapping population based on the cross RHA325 (cms) × HA342 was enlarged to 1571 F_2 individuals. Apart from the F_2 plants, F3 families and backcrosses of the fertile plants to HA89 (cms) were evaluated to determine whether the F_2 plants were homozygous or heterozygous. Two phenotypes were observed: male fertile plants with normal developed anthers producing much yellow pollen and male-sterile plants with rudimentary anthers and no pollen pro-

duction. Segregation analysis (Table 1) showed that the investigated cross segregated for a single dominant gene, corresponding to the *Rf1* gene, that is responsible for fertility restoration.

Table 1: Segregation of homozygous fertile (*Rf1Rf1*), heterozygous fertile (*Rf1rf1*) and homozygous male sterile (*rf1rf1*) plants in the mapping population. The evaluation was performed on the F_2 individuals as well as on the derived F_3 and F_2BC_1 populations

Cross	Generation	Observed segregation *Rf1Rf1* : *Rf1rf1* : *rf1rf1*	S	Expected segregation	χ^2	P (df=2)
RHA325(*cms*)×HA342	F_2	384 : 785 : 402	1571	1 : 2 : 1	0.41	0.8

Identification of markers linked to the restorer gene *Rf1*

In total, 1200 RAPD-primers were screened for polymorphism between homozygous fertility-restored and homozygous male-sterile bulks. Eighteen polymorphisms were identified, seven were mapped in the same linkage group as the restorer gene *Rf1*. In addition, 1024 AFLP primer combinations were investigated by bulked-segregant analyses.

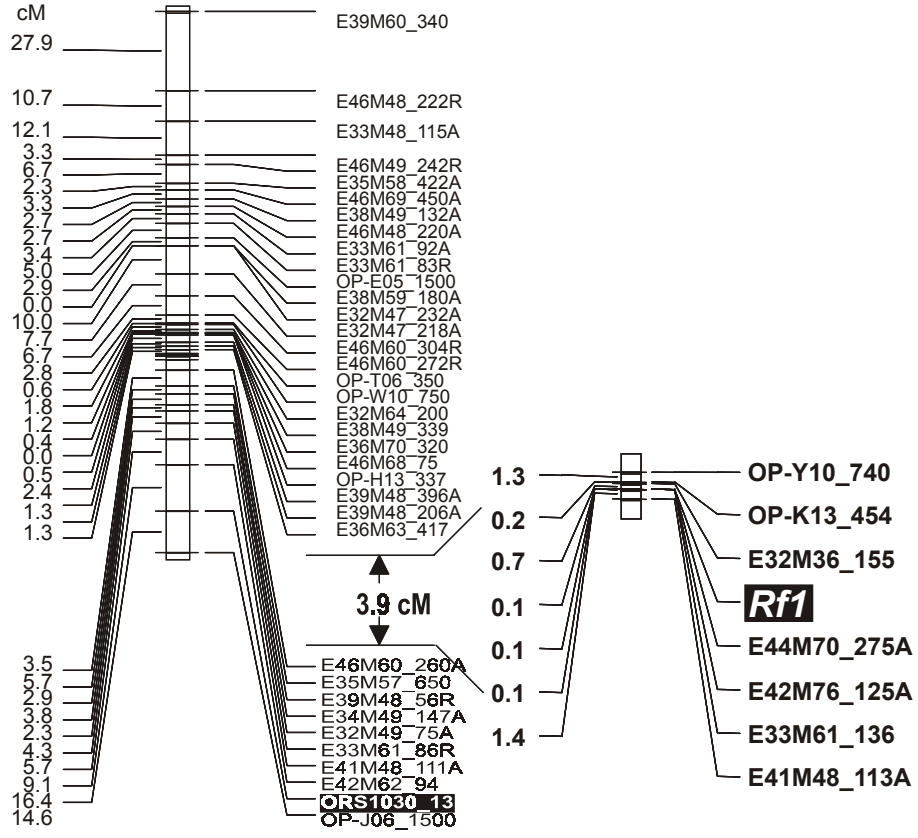

Figure 1: Linkage group of the restorer gene Rf1 of sunflower (43 markers)

A total of 203 polymorphic primer combinations could be identified. Polymorphisms between male fertile and male sterile bulks ranged from 1 to 4 per primer combination. To reduce the number of polymorphisms (282 markers) that needed to be mapped in the F_2 population, a recombinant screening was initiated. Ten AFLP-Primer combinations corresponding to 14 markers in the marker interval E38M59-180A and E39M48-58R around the restorer gene *Rf1* were used to identify recombinants. For the recombinant screening, the male-sterile F_2 plants had to be crossed with RHA325 (cms) and only in the selfings of these backcrosses could homozygous recombinant plants be obtained. Three male-sterile homozygous recombinant plants were identified. On the fertile side, homozygous recombinant plants can be recognized in the F_3 generation. Testing 200 fertile plants, six homozygous recombinants could be identified. To reduce the number of AFLP primer combinations that need to be mapped in the F_2 population, three selected plants from the fertile and the male-sterile bulks as well as the recombinants were used for prescreening. Only primer combinations that did not show recombination within the individuals of the bulks were finally mapped in the F_2 population. The linkage group for the restorer gene *Rf1* is shown in Figure 1. The map consists of 43 markers (7 RAPD, 1 SSR and 35 AFLP markers) and covers 191.9 cM. The enlarged marker interval of 3.9 cM around the restorer gene *Rf1* contains 7 markers and has a marker density of 1 marker/0.6 cM. The AFLP-markers E32M36_155, E44M70_275A and E42M76_125A, which map 0.7 cM, 0.1 cM and 0.2 cM from the restorer gene, respectively, were identified in addition to the previously mapped markers.

Conversion of markers into STS markers and overgo probes

To screen our BAC library, RAPD and AFLP markers tightly linked to the restorer gene *Rf1* were converted into sequence specific markers. The RAPD markers OP-K13_426 and OP-Y10_740 were successfully converted to the dominant STS-markers HRG01 and HRG02 (Horn *et al.*, 2003). The marker OP-H13_337 was used to develop a CAPS marker, which allowed differentiation of the parental lines by digesting the PCR product with *HinfI*. For marker selected breeding, codominant markers would be preferred as dominant marker system does not allow the differentiation between heterozygous and homozygous plants and false negatives can occur due the failure of the PCR reaction. To address the latter problem, internal controls using primers for mitochondrial genes were tested. Primers were derived from *atp9* and *coxII* (Horn, 2002). In both combinations, HRG01/*coxII* and HRG02/*atp9*, the mitochondrial genes could be successfully applied as internal PCR controls. The dominant STS-markers HRG01 and HRG02 as well as the CAPS marker are now available for marker assisted breeding in sunflower hybrid breeding.

Converting the AFLP markers E41M48_113A and E33M61_136R into sequence specific markers resulted in a monomorphic pattern because the polymorphism

between the parental lines probably lies within the recognition sites of the restriction enzymes (Horn *et al.*, 2003). These AFLP markers are nevertheless interesting for the map-based cloning approach as these can be used to develop overgo probes for colony hybridizations against the high-density BAC filters.

Screening of maintainer and restorer lines with the identified markers

To investigate the utility of the identified markers for marker-assisted selection in other cross combinations, a set of 20 maintainer and restorer lines was investigated (Table 2). The marker OP-K13_454/ HRG01 did not show an amplification product in any of the maintainer lines, but the marker was amplified in all restorer lines, except for the high-oleic line RHA348 and the line GIO55 (dwarf). The investigated restorer lines also included three lines, which were developed from interspecific hybrids *H. annuus* x *H. mollis* (sf1616, sf1636) and *H. annuus* x *H. rigidus* (sf1578). The marker OP-Y10_740/ HRG02 gave the same pattern as the marker OP-K13_454/ HRG01. This demonstrated that both markers tightly linked to the restorer gene *Rf1* can also be used for the marker-assisted development of new lines from interspecific lines. The amplification products of the primer combinations E33M61 and E39M48 are shown in Table 2. The primer combinations E42M76 and E46M68 are shown in Figure 2.

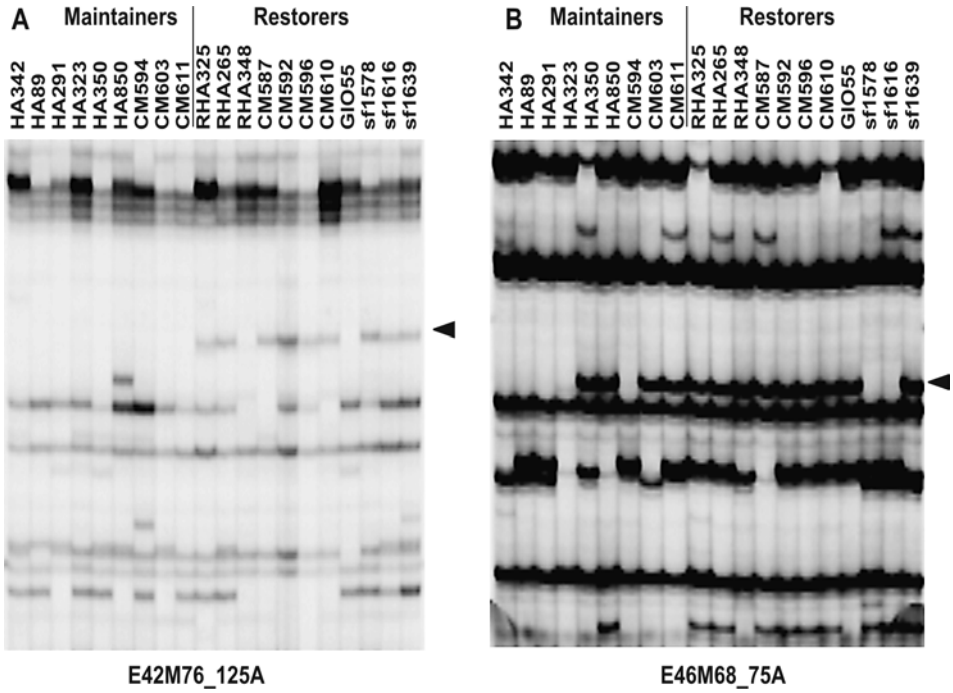

Figure 2: Amplification products of AFLP primer combinations in a set of 20 maintainer and restorer lines. ***A.*** *AFLP marker E42M76_125A,* ***B.*** *AFLP marker E46M68_75A. The markers are indicated by arrow heads.*

The primer combination E39M48 (Table 2) is of special interest because it gives a marker in attraction, E39M48_210, and a marker in repulsion, E39M48_58, to the restorer gene *Rf1*. Therefore, this AFLP primer combination also allows the differentiation of homozygous and heterozygous plants using a single primer combination.

Table 2: Investigation of 20 maintainer and restorer lines of the PET1 cytoplasm with markers closely linked to the restorer gene *Rf1*. Maintainer lines: HA342, HA89, HA291, HA323, HA350, HA850, CM594, CM603, CM611; restorer lines: RHA325, RHA265, RHA348, CM587, CM592, CM596, CM610, Gio55, sf1578, sf1616, sf1636.

Line	HRG01	HRG02	E33M61_136	E33M61_86	E33M61_83	E39M48_58	E39M48_210
HA342	-	-	+	+	+	+	-
HA89	-	-	-	+	-	+	-
HA291	-	-	-	-	+	+	-
HA323	-	-	-	-	+	+	-
HA350	-	-	+	+	-	+	-
HA850	-	-	-	-	-	+	-
CM594	-	-	-	-	-	+	-
CM603	-	-	nd*	nd	nd	+	-
CM611	-	-	-	-	-	+	-
RHA325	+	+	-	-	-	-	+
RHA265	+	+	-	-	-	-	+
RHA348	-	-	+	+	+	+	-
CM587	+	+	nd	nd	nd	-	+
CM592	+	+	nd	nd	nd	-	+
CM596	+	+	-	-	-	-	+
CM610	+	+	+	-	-	-	+
Gio55	-	-	-	-	-	+	-
sf1578	+	+	-	-	-	-	+
sf1616	+	+	-	-	-	-	+
sf1636	+	+	-	-	-	-	+

* nd not determined

Investigating the set of 20 maintainer and restorer lines demonstrated, it was found that all of them, especially the RAPD markers that had been converted into sequence-specific markers, are well suited for marker-assisted breeding programs (Horn *et al.*, 2003). The markers coming from the AFLP primer combination E33M61 (markers in repulsion, Table 2) and E46M68 (marker in attraction, Figure 2) are less suitable for marker-assisted selection, as in the case of E33M61 only few of the maintainer lines amplify the marker and the marker E46M48_75A is not amplified in all restorer lines but four out of nine maintainer lines also showed the PCR product.

Identification of positive BAC clones and development of a contig around the restorer gene

Our BAC library was spotted on four high-density BAC filters, which allowed colony hybridization using our markers as probes. The BAC library represents a 2-3 fold haploid genome equivalent (sunflower genome 3000 Mb, Arumuganathan and Earle, 1991). For colony hybridization we used the sequence-specific markers HRG01 and HRG02 or the overgo probes developed from the AFLP markers E33M61_136R and E41M48_113A. All positive BAC clones were verified by additional hybridization to exclude false positives.

Figure 3 shows the colony hybridization for HRG01 against the high-density BAC filters and the verification and characterization of the three identified clones by hybridization of the BAC fingerprints obtained by digestion with *Hind*III, *Dra*I and *Bam*HI.

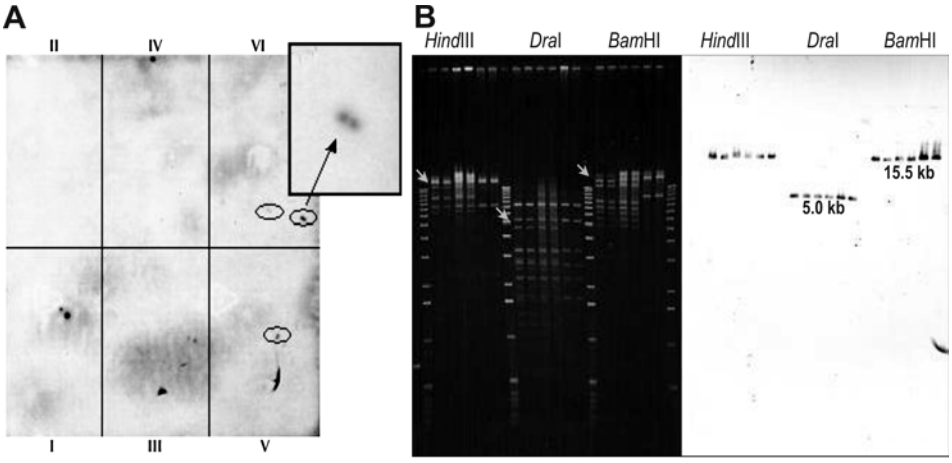

Figure 3: Colony hybridization of HRG01 against the high-density BAC filters and characterization of positive clones. ***A.*** *Autoradiogram of one filter: the three positive signals on the filter are encircled and one of the double spotted positive clones is shown enlarged. High-density BAC filter are divided into six fields, each of it representing 12 microtiter plates (384 well format).* ***B.*** *BAC-fingerprinting of the three positive BAC clones and verification by hybridization against HRG01.*

In addition, BAC pools (273 plate, 144 column, and 96 row pools) were made allowing 3D-PCR screening. These BAC pools were used to identify BAC clones for the markers OP-H13_337, E39M48_396A and OP-K13_454. The 3D-PCR screening verified the results obtained for OP-K13_454 by colony hybridization.

Table 3 summarizes the positive BAC clones by the different markers. The colony hybridizations, using the sequence-specific markers or overgos as probes, proved to be more efficient in detecting positive BAC clones than the 3D-PCR screening. The PCR screening would have required a fourth dimension in the case that more than 1 plate pool had showed an amplification product. For each investi-

gated marker, 1 to 3 BAC clones could be identified by colony hybridization or 3D-PCR screening.

Table 3: Overview of positive BAC clones identified with markers tightly linked to the restorer gene *Rf1*

Marker	Distance to restorer gene *Rf1*	Identification by (probe)	BAC-clone
OP-K13_454	0.9 cM	Colony hybridization (STS-marker) and 3D-PCR BAC Pools	59J13; 67N4; 67I5
E33M61_136R	0.3 cM	Colony hybridization (Overgo)	224O7; 224O8
E41M48_113A	1.7 cM	Colony hybridization (Overgo)	40L18; 94F15
OP-H13_337	7.7 cM	3D-PCR BAC Pools	92E22
E39M48_396A	7.2 cM	3D-PCR BAC Pools	62L14; 219A7; 236P7

To develop a contig around the restorer gene *Rf1*, BAC fingerprinting was performed using *Hind*III as restriction enzyme (Figure 4). The banding pattern was confirmed by hybridization. This procedure allowed the development of a preliminary contig around the restorer gene *Rf1*. The BAC clones 67I5 and 67N4 showed an identical banding pattern, which overlapped with the smaller BAC clone 59J13.

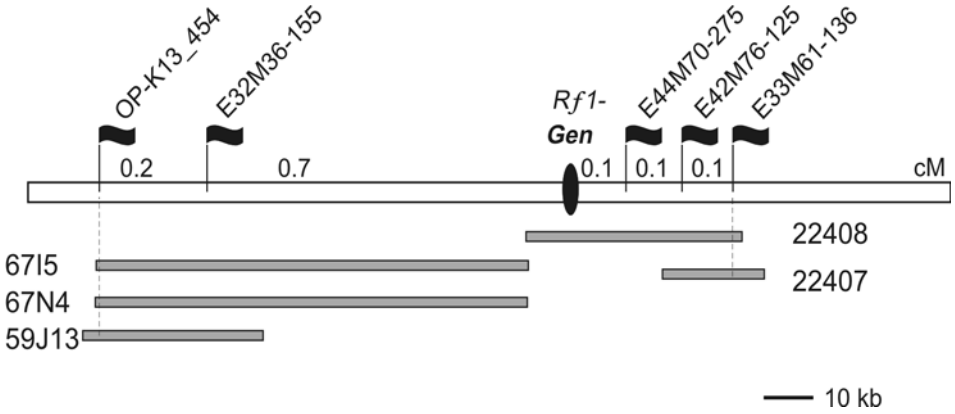

Figure 4: Preliminary contig around the restorer gene Rf1. The BAC clones were identified by using HRG01 and overgos for the markers OP-K13_454 and E33M61_136.

To clone and sequence the BAC end of 59J13, the BAC clone was digested with *Bam*HI, cloned in pUC18 and hybridization with pBeloBAC11 as probe identified subclones that contained part of the vector pBeloBAC11 in addition to the insert (Figure 5). The end of the BAC clone 59J13 was sequenced to develop markers and to search for homology to the sequence databases. In total, 1608 bp of the BAC clone 59J13 were sequenced. The BAC clones 224O8 and 224O7 also share *Hind*III fragments, but represent different clones. Hybridization using 67I5 as a probe identified a 3.7-kb-fragment shared between clone 224O8 and the clones 67N4 and 67I5. This indicates that the contig around the restorer gene *Rf1* might have already been closed. The newly identified AFLP markers E32M36_155, E44M70_275 and

E42M76_125 can now be used to verify the identified BAC clones and might allow the identification of additional BAC clones in this region. In addition, the ends of the BAC clones 67I5 and 67N4 on one side and 224O8 and 224O7 on the other need to be cloned and sequenced to verify that the ends are really overlapping.

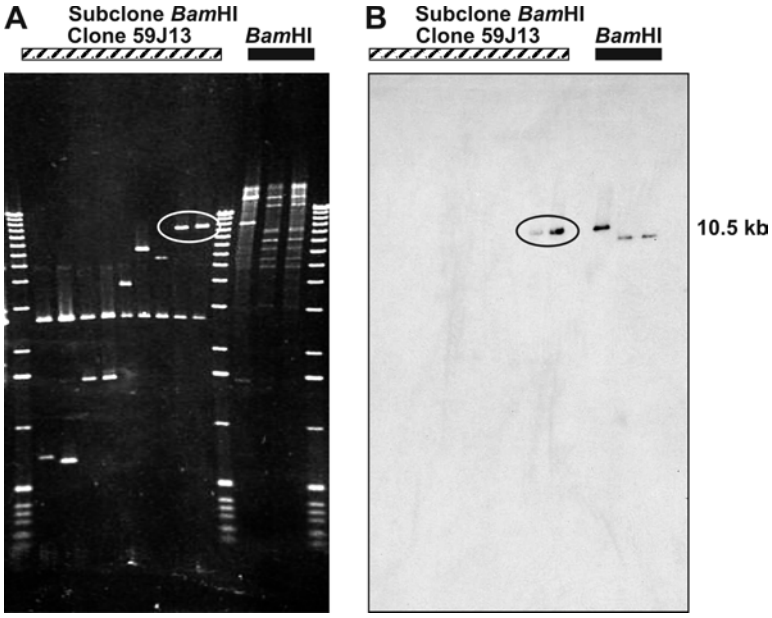

Figure 5: Identification of the end of BAC clone 59J13 and the corresponding BamHI-subclone. ***A.*** *BamHI-subclones of BAC clone 59J13 and the BAC clones 59J13, 67I5 and 67N4 after restriction digestion with BamHI.* ***B.*** *Hybridization using pBeloBac11 as probe.*

DISCUSSION

A map-based cloning strategy was applied to isolate the restorer gene *Rf1*. Using bulked segregant analyses, closely linked AFLP and RAPD markers could be identified and mapped in the F_2 population. To reduce the number of polymorphic AFLP primer combinations that need to be mapped in the segregating population, a recombinant screening as well as a screening of selected individual plants from the bulks were performed. The development of recombinants proved to be time- and labor-consuming. Two generations (backcross with RHA325 and selfing) were necessary on the male-sterile side as well as one additional generation on the fertile side. These generations needed to be screened to identify recombinants. Comparing the recombinant approach with the use of individuals from the bulks it can be said that the screening of the individuals allowed considerable reduction of AFLP primer combinations to be mapped in the F_2 population without requiring extra breeding generations. Therefore, this approach seems to be more advisable than the recom-

binant approach in the case of investigating markers for restorer genes. Three markers closer linked to the restorer gene *Rf1* than the previously published markers (Horn *et al*., 2003, Kusterer *et al*., 2002) could be identified. Markers were successfully used to identify BAC clones by colony hybridization and 3D-PCR screening. The development of overgo probes proved to be very efficient in probing the high-density BAC filter. Characterization of the BAC fingerprints using different restriction enzymes showed that a putatively closed contig around the restorer gene *Rf1* could be established. However, BAC end cloning and sequencing is needed to verify this. In addition, the newly identified markers will be converted into sequence-specific markers and used to identify new BAC clones and/or verify the already identified BACs.

So far, restorer genes have been isolated for the T-cytoplasm in maize (Cui *et al*., 1996), the RM cytoplasm in petunia (Bentolila *et al*., 2002), the Ogura CMS cytoplasm in rapeseed (Brown *et al*., 2003) and the radish Kosena CMS cytoplasm (Koizuka *et al*., 2003). The last three of these represent pentatricopeptide repeat-containing genes that are supposed to play a role in RNA processing. However, a candidate gene approach to identify the restorer gene in sunflower will be difficult as the pentatricopeptide repeat-containing gene is a member of a very large and heterogeneous group of genes, but hybridizing with a pentatricopeptide repeat-specific probe against the already identified BACs might reveal the presence or absence of such sequences at the restorer locus in sunflower.

The map-based cloning strategy of the restorer gene also provided markers that can be used in marker-assisted development of new restorer and maintainer lines for sunflower hybrid breeding. These are especially valuable as they were converted into sequence-specific markers and are closely linked to the restorer gene *Rf1*.

REFERENCES

Arumuganathan, K. and Earle, E.D., 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. 9: 208-218.

Balk, J., and Leaver, C.J., 2001. The PET1-CMS mitochondrial mutation in sunflower is associated with premature programmed cell death and cytochrome c release. Plant Cell 13: 1803-1818.

Bentolila, S., Alfonso, A.A. and Hanson M.R., 2002. A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Proc. Nat. Acad. Sci. USA 99: 10887-10892.

Brown, G.G., Formanova, N., Jin, H., Wargaschuk, R., Dendy, C., Patil, P., Laforest, M., Zhang, J., Cheung, W.Y. and Landry, B.S. 2003. The radish *Rfo* restorer gene of Ogura cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. Plant J. 35: 262-272

Cui, X., Wise, R.P. and Schnable P.S., 1996. The *rf2* nuclear restorer gene of the male-sterile T-cytoplasm. Science 272: 1334-1336.

Doyle, J.L. and Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Gagliardi, D. and Leaver, C.J., 1999. Polyadenylation accelerates the degradation of the mitochondrial mRNA associated with cytoplasmic male sterility in sunflower. EMBO J. 18: 3757-3766.

Horn, R., Köhler, R.H. and Zetsche, K., 1991. A mitochondrial 16 kDa protein is associated with cytoplasmic male sterility in sunflower. Plant Mol. Biol. 7: 26-33.

Horn, R., Hustedt, J.E.G., Horstmeyer, A., Hahnen, J., Zetsche, K. and Friedt, W., 1996. The CMS-associated 16 kDa protein encoded by *orfH522* is also present in other male sterile cytoplasms of sunflower. Plant Mol. Biol. 30: 523-538.

Horn, R., 2002. Molecular diversity of male sterility inducing and male-fertile cytoplasms in the genus *Helianthus*. Theor. Appl. Genet. 104: 562-570.

Horn, R., Kusterer, B., Lazarescu, E., Prüfe, M., Özdemir, N. and Friedt, W., 2002. Molecular diversity of CMS sources and fertility restoration in the genus *Helianthus*. Helia 25: 29-40.

Horn, R., Kusterer, B., Lazarescu, E., Prüfe, M. and Friedt, W., 2003. Molecular mapping of the *Rf1* gene restoring pollen fertility in PET1-based F_1 hybrids in sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 106: 599-606.

Köhler, R.H., Horn, R., Lössl, A. and Zetsche, K., 1991. Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the *atpA* gene. Mol. Gen. Genet. 227: 369-376.

Koizuka, N., Imai, R., Fuijimoto, H., Hayakawa, T., Kimura, Y., Kohno-Murase, J., Sakai, T., Kawasaki, S. and Imamura, J., 2003. Genetic characterization of a pentatricopeptide repeat protein gene, orf687, that restores fertility in the cytoplasmic male-sterile Kosena radish. Plant J. 34: 407-415.

Korell, M., Mösges, G. and Friedt, W., 1992. Construction of a sunflower pedigree map. Helia 15: 7-16.

Kosambi, D.D., 1944. The estimation of map distances from recombination values. Ann. Eugen 112: 172-175.

Kusterer, B., Prüfe, M., Lazarescu, E., Özdemir, N., Friedt, W. and Horn, R., 2002. Mapping of the restorer gene *Rf1* in sunflower (*Helianthus annuus*). Helia 25: 41-46.

Laver, H.K., Reynolds, S.J., Moneger, F. and Leaver, C.J., 1991. Mitochondrial genome organization and expression associated with cytoplasmic male sterility in sunflower (*Helianthus annuus*) Plant J. 1:185-193.

Lander, E.S., Green, J., Abrahamson, J., Batlow, A., Daly, M.J., Lincoln, S.E. and Newburg, L., 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174-181.

Leclercq, P., 1984. Identification de gčnes de restauration de fertilité sur cytoplasmes stérilisants chez le tournesol. Agronomie 4: 573-576.

Monéger, F., Smart, C.J. and Leaver C.J., 1994. Nuclear restoration of cytoplasmic male sterility in sunflower is associated with tissue-specific regulation of a novel mitochondrial gene. EMBO J. 13: 8-17.

Özdemir, N., Horn, R. and Friedt, W., 2002. Isolation of HMW DNA from sunflower (*Helianthus annuus* L.) for BAC cloning. Plant Mol. Biol. Rep. 20: 239-249.

Özdemir, N., Horn, R. and Friedt W., 2003. Construction and characterization of a BAC library for sunflower (*Helianthus annuus* L.). Canadian J. Plant Science (submitted)

Serieys, H., 1996. Identification, study and utilisation in breeding programs of new CMS sources. FAO progress report. Helia 19 (Special issue):144-158.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23: 4407-4414.

Tang, S., Yu, J.K., Slabaugh, M.B., Shintami, D.K. and Knapp, S.J., 2002. Simple sequence repeat map of the sunflower genome. Theor. Appl. Genet. 105: 1124-1136.

ESTRATEGIA DE CLONACIÓN PARA EL AISLAMIENTO DEL GEN RESTAURADOR *Rf1* EN EL CITOPLASMA PET1 EN GIRASOL (*Helianthus annuus* L.) A BASE DE LOS MAPAS CONSTRUIDOS

RESUMEN

La clonación mediante el mapa construido del gen restaurador *Rf1*, que es responsable para la restauración de fertilidad del citoplasma PET1 en girasol, exige marcadores que sean estrechamente ligados con el gen en referencia. Mediante el scrining 1200 del primer decamerio, mediante el análisis de segregación de masa, fueron identificados siete marcadores RAPD, mapeados en el

mismo grupo de genes ligados como el gen restaurador en *Rf1*. En la población F_2 de cruzamiento RHA325 (*cms*) × HA342 (183 plantas), uno de los marcadores RAPD, OPK13_454, mapeó 0.9 cM de *Rf1*, e inmediatamente tras él, fue OPY10_740 con 2.2 cM. El análisis de segregación de masa, mediante la combinación de primers 1024 AFLP, identificó 282 polimorfismos en 203 combinaciones de primers. Las plantas particulares, seleccionadas de masa y de las plantas recombinantes, fueron sometidas a skrining, para que se disminuyera adicionalmente el número de combinaciones de primers, los cuales deben ser mapeados en la población F_2. El mapa para el grupo de genes ligados, que contiene dicho gen restaurador, ahora consta de 43 marcadores (7 RAPD-, 1 SSR-, y 35 marcadores AFLP) y cubre 191.9 cM. E32M36_155 y E44M70_275 son mapeados en 0.7 cM y 0.1 cM respectivamente, de los genes restauradores. Dos de los marcadores RAPD, OPK13_454 y OPY10_740, fueron convertidos con éxito en marcadores SCAR, HRG01 y HRG02 respectivamente. La amplificación de los marcadores OPK13_454/ HRG01 y OPY10_740/HRG02 fue investigada para el conjunto de 11 restauradores y líneas mantenedoras del citoplasma PET1. En clonación del gen restaurador *Rf1*, para la identificación de los clones positivos BAC, se utilizaron las hibridaciones coloniales, frente a los filtros BAC de alta densidad de nuestra colección de girasoles BAC y de la estrategia de agrupación 3D-PCR. Se hizo el fingerprint de BAC, mediante diferentes enzimas restrictivos, en combinación con hibridación, para que se creara el contig alrededor del locus del restaurador *Rf1*.

STRATÉGIE DE CLONAGE POUR ISOLER LE GÈNE RESTAURATEUR *Rf1* DANS LE CYTOPLASME PET1 DU TOURNESOL (*Helianthus annuus* L.) À LA BASE DES CARTES CONSTRUITES

RÉSUMÉ

Le clonage au moyen des cartes construites du gène restaurateur *Rf1*, qui est responsable de restauration de fertilité de cytoplasme PET1 du tournesol, exige des marqueurs étroitement liés au gène concerné. En examinant ("genetic screening") 1200 "decamer primers" à l'aide de l'analyse "Bulked segregant" (BSA) sept marqueurs RAPD ont été identifiés et classifiés dans une carte pour le même groupe de gènes liés comme gène restaurateur *Rf1*. Dans la population F_2 de croisement RHA325(*cms*) × HA342 (183 plantes), un des marqueurs RAPD, OPK13_454, est classifié dans la carte 0,9 cM du gène *Rf1*, et ensuite OPY10_740 de 2,2 cM. L'analyse "Bulked segregant" (BSA) utilisant 1024 combinaisons de "primers" a identifié 282 polymorphismes dans 203 combinaisons de "primers". Les plantes isolées et sélectionnées de "bulk" et les plantes en recombinaison subissent le procédé de "genetic screening" afin de diminuer le nombre de combinaison de "primers" qui doit être classifié dans la carte de la population F_2. La carte du groupe de gènes liés qui possède le gène restaurateur en question et qui contient maintenant 43 marqueurs (7 RAPD-, 1 SSR-, et 35 AFLP) couvrant 191,9 cM E32M36_155 et E44M70_275 classifiés dans la carte à 0,7cM c'est-à-dire à 0,1 cM du gène restaurateur. Deux des marqueurs RAPD, OPK13-454 et OPY10_740, sont convertis avec succès en marqueurs SCAR, HRG01 et HRGO2 respectivement. La multiplication des marqueurs OPK13_454/ HRG01et OPY10_740/HRG02 a été examiné pour un groupe de 11 restaurateurs et de 9 lignes qui maintiennent le cytoplasme PET1. Pendant le clonage du gène restaurateur *Rf1*, les hybridations coloniales opposées aux filtres BAC de haute densité de notre collection BAC de tournesols et la stratégie 3D-PCR "pooling" ont été utilisées pour identification des clones positifs BAC. L'empreinte génétique BAC "finger printing" à l'aide de différents enzymes restreints en combinaison d'hybridation a été effectuée pour développer un "contig" autour du locus restaurateur *Rf1*.