FERTILITY RESTORATION OF THE PET1 CYTOPLASM AND OTHER CMS SOURCES IN THE GENUS *HELIANTHUS*

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Abstract

Restorer genes involved in fertility restoration of the PET1, PEF1 and PET2 cytoplasms were investigated. In a map-based cloning approach to isolate the restorer gene *Rf1* of the PET1 cytoplasm in sunflower tightly linked markers were identified. Bulked segregant analyses using 1200 decamer primers identified seven RAPD markers mapping in the same linkage group as the restorer gene *Rf1*. In our segregating population of the cross RHA 325 (CMS) x HA342 (F2 population,183 individuals) one of the RAPD markers, OP-K13_454, mapped 0.9 cM from *Rf1*, followed by OP-Y10_740 with 2.2 cM. Screening 1024 AFLP primer combinations by bulked segregant analyses detected 282 polymorphisms in 203 primer combinations. On the linkage group carrying the restorer gene *Rf1*, 43 markers (7 RAPD-, 1 SSR-, and 35 AFLP-markers) were mapped that covered 250.3 cM. Three new AFLP markers, E32M36_155, E44M70_275 and E42M76_125 were mapped with 0.7 cM, 0.1 cM and 0.2 cM from the restorer gene. Two of the RAPD markers, OP-K13_454 and OP-Y10_740, were successfully converted into SCAR markers. A CAPS marker was developed from the RAPD marker OP-H13_337. For analysis of the genome organization around the restorer gene *Rf1*, BAC clones were identified by colony hybridization and 3D-PCR pooling strategies. BAC fingerprinting using different restriction enzymes in combination with hybridization was performed to develop a contig around the restorer locus *Rf1*. AFLP polymorphic primer combinations in the cross RHA 325 (CMS) x HA 342 were also tested in bulked segregant analyses of populations segregating for fertility restoration of PEF1 and PET2.

Introduction

Stable fertility restoration represents an essential part of sunflower (*Helianthus annuus* L.) hybrid breeding based on cytoplasmic male sterility (CMS). However, little is known about the function and genome organization of restorer genes 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 as 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 as 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) encoded by the mitochondrial *orfH522* seems to be responsible for causing cytoplasmic male sterility in the PET1 cytoplasm (Köhler et al., 1991). The PET-1 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, 1999). To study the function of the dominant nuclear restorer gene, the gene needs to be isolated.

In this study, we identified markers linked to the restorer gene *Rf1* of the PET1 cytoplasm. In addition, we investigated these markers for polymorphisms in bulks of segregating populations of the PEF1 and PET2 cytoplasms. PCR-based markers (AFLP and RAPD) closely linked to the restorer gene *Rf1* of PET1 were converted into SCAR or CAPS markers which can now be used for marker-assisted breeding. To analyse the genome organization around the *Rf1* gene and to compare it with restorer genes for PEF1 and PET2 cytoplasms BAC clones were identified by colony hybridisation and 3D-PCR screening of our sunflower BAC library.

Materials and Methods

Plant Materials. F2, F3 and F2BC1 populations (backcrossed on male-sterile HA 89 carrying PET1 cytoplasm) were derived from the cross RHA 325 x HA 342. RHA 325 is a restorer line carrying the PET1 cytoplasm, and HA 342 is a high-oleic maintainer line. F2, F3 and F2BC1 populations were grown in the field of Gross-Gerau near Frankfurt/Main and evaluated for male fertility/sterility. In addition to the restorer line RHA 325, seven American lines RHA 265, RHA 348, CM587, CM592, CM596, CM610, 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 HA 342, HA 89, HA 291, HA 323, HA 350, HA 850, CM594, CM603 and CM611.

AFLP Analyses. AFLP analyses using *Eco*RI and *Mse*I primers were performed according to Vos et al. (1995). *Eco*RI primers were either radioactively labeled or IRD-labeled by MWG Biotech (Ebersberg, Germany). The primer combinations E32M64, E35M57, E36M70, E38M49, E38M59, E39M48, E42M76, E44M70, E46M60, and E46M60 were used for the recombinant screening.

RAPD Analyses. 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. RAPD and AFLP marker were converted into SCAR markers as described in Horn et al. (2003). Conditions for PCR amplification using the sequence-specific primers are given in the same publication.

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 centiMorgans (cM) (Kosambi, 1994).

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 a vector. The BAC library, which comprises 104,736 BAC clones, was double spotted on four high density filters in a 5x5 pattern (Özdemir et al., 2004). In addition, BAC pools were prepared for units of 48 plates to allow 3D-PCR screening.

Results and Discussion

Fertility Restoration in the Enlarged Mapping Population. For fine-mapping of the *Rf1* gene, the segregating population based on the cross RHA 325 (CMS) x HA 342 was enlarged to 1571 F2 individuals. F2 plants, F3 families and backcrosses of the fertile plants to HA 89 (CMS) were evaluated. Two phenotypes were observed: male-fertile plants with normally developed anthers producing a lot of yellow pollen and male-sterile plants with rudimentary anthers and no pollen production. Segregation analysis (384 Rf1Rf1: 785 Rf1rf1: 402 rf1rf1, Chi square=0.41) showed that the investigated cross segregated for a single dominant gene, corresponding to the *Rf1* gene, that is responsible for fertility restoration.

Markers Linked to the Restorer Gene Rf1. For the RAPD analyses, 1200 decamer primers were investigated by bulked-segregant analyses. Eighteen polymorphisms were identified, and seven were mapped in the same linkage group as the restorer gene *Rf1*. In addition, 1024 AFLP primer combinations were screened for polymorphisms between homozygous fertility-restored and homozygous male-sterile bulks. As polymorphisms between male-fertile and male-sterile bulks ranged from 1 to 4 per AFLP primer combination, the detected 203 polymorphic primer combinations represent 282 markers. To reduce the number of polymorphisms to be analysed in the mapping population, a recombinant screening was performed. 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. Three male-sterile homozygous recombinant plants and nine homozygous fertile recombinants were identified. Three individuals from the fertile and the male-sterile bulks, and three sterile and six fertile homozygous recombinants were used for a prescreening to reduce the number of interesting AFLP primer combinations. Only primer combinations that did not show a recombination within the individuals of the bulks were finally mapped in the F2 population. The linkage group for the restorer gene *Rf1* now consists of 43 markers (7 RAPD, 1 SSR and 35 AFLP markers) and covers 250.3 cM. In addition to the previously mapped markers, three AFLP-markers E32M36_155, E44M70_275A and E42M76_125A, which mapped 0.7 cM, 0.1 cM and 0.2 cM from the restorer gene, were identified.

Development of Sequence-Specific Markers and Overgo Probes. To obtain sequence-specific markers for marker-assisted breeding and screening of our BAC library, RAPD and AFLP markers tightly linked to the restorer gene *Rf1* were converted into sequence-specific markers. Dominant STS-markers HRG01 and HRG02 were successfully developed from the two RAPD markers OP-K13_426 and OP-Y10_740 (Horn et al., 2003). The marker OP-H13_337 was converted into a CAPS marker, which allowed differentiation of the parental lines by digesting the PCR product with *Hinf*I. For marker-selected breeding, codominant markers are preferred as dominant marker systems do not allow differentiation between

heterozygous and homozygous plants and false negatives can occur due the failure of the PCR reaction. To address the latter problem, duplex PCRs were performed including internal controls, which were obtained by using primers for mitochondrial genes (*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-marker HRG01 and HRG02 as well as the CAPS marker are now available for marker-assisted selection in sunflower hybrid breeding.

The sequence-derived primers of the AFLP markers E41M48_113A and E33M61_136R gave monomorphic PCR amplification products. Probably the polymorphisms between the parental lines are located within the recognition sites of the restriction enzymes (Horn et al., 2003). However, overgo probes could be designed from the sequences that proved to be useful in colony hybridizations against the high-density BAC filters.

Evaluation of the Identified Markers for Other Cross Combinations. Investigating a set of 20 maintainer and restorer lines demonstrated that 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 marker OP-K13_454/ HRG01 showed the amplification product in all restorer lines, except for the high-oleic line RHA 348 and the line GIO55 (dwarf), but not in any of the investigated maintainer lines. Since the restorer lines also included three lines developed from interspecific hybrids, this marker can also be used for the marker-assisted development of new lines from crosses with wild species of the genus *Helianthus*. The marker OP-Y10_740/ HRG02 gave the same pattern as the marker OP-K13_454/ HRG01.

The AFLP primer combinations E33M61, E39M48, E42M76, E46M68 and E39M48 were also tested in the set of 20 maintainer and restorer lines. The primer combination E39M48 proved to be interesting because it resulted in a marker in attraction, E39M48_210, and a marker in repulsion, E39M48_58, to the restorer gene *Rf1*. Therefore, this AFLP primer combination allows differentiation of homozygous and heterozygous plants.

Marker application in the set of 20 maintainer and restorer lines demonstrated that especially the sequence-specific markers developed from the RAPD markers are interesting for marker-assisted breeding programs (Horn et al., 2003). The markers from the AFLP primer combinations E33M61 and E46M68 are less useful for other cross combinations, because the marker in repulsion to *Rf1* from E33M61 is only amplified in few of the maintainer lines and the marker E46M48_75A in attraction did not show an amplification product in all restorer lines but four of nine maintainer lines also amplified the marker.

Markers linked to the restorer gene *Rf1* were also investigated for their presence in bulks of populations segregating for fertility restoration of PEF1 and PET2 cytoplasms. The bulks developed from the crosses PEF1 x NS and PEF1 x LC showed hardly any of the polymorphisms present in RHA 325(CMS) x HA 342. Investigation of the parental lines will show whether these lines are much less polymorphic or whether the absence of polymorphisms for these markers of *Rf1* indicates that the restorer gene for PEF1 is localized somewhere else in the genome. Bulks developed from the cross PET2 x IH showed most of the polymorphisms present in the bulks of RHA 325 (CMS) x HA 342. However, markers in attraction to the restorer gene *Rf1* seem to be in repulsion to *Rf-PET2*. This might indicate that *Rf-PET2* is an allele of *Rf1* or a member of a restorer gene cluster at the *Rf1* locus.

Colony Hybridization and 3D-PCR of the BAC Library. For colony hybridizations against the four high-density BAC filters, the sequence-specific markers HRG01 and HRG02

or the overgo probes developed from the AFLP markers E33M61_136R and E41M48_113A were used. All positive BAC clones were verified again by an additional hybridization to exclude false positives.

In addition, BAC pools (273 plate, 144 column, and 96 row pools) were prepared for a 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. For each investigated marker, 1 to 3 BAC clones could be identified by colony hybridization or 3D-PCR screening.

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 one plate pool showed an amplification product.

BAC fingerprinting was performed using *Hind*III to develop a contig around the restorer gene *Rf1*. Restriction fragments were hybridized using *Hind*III digested BAC clones to verify fragment identities. A preliminary contig was developed around the restorer gene *Rf1*. The BAC clones 67I5 and 67N4 showed an identical banding pattern, which overlapped with the smaller BAC clone 59J13. One BAC end of 59J13 was cloned into pUC18 as a *Bam*HI fragment. The BAC end 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 shared *Hind*III fragments, but represent different clones. Hybridization using BAC clone 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 be closed. The newly identified AFLP markers E32M36_155, E44M70_275 and E42M76_125 can help to verify the identified BAC clones and might allow the identification of additional BAC clones in this region. The 3.7-kb fragment shared between 67I5, 67N4 and 224O8 needs to be cloned and sequenced to verify that the ends are really overlapping.

Conclusions

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 three latter ones represent pentatricopeptide repeat-containing genes that are supposed to play a role in RNA processing. It is not known whether the restorer genes in sunflower also belong to this class of PPR genes. 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.

Markers identified for the restorer gene *Rf1* were also tested for their presence in populations segregating for fertility restoration of PEF1 and PET2 cytoplasms. This will allow information to be obtained about the distribution of restorer genes in the sunflower genome. Restorer genes might be present in clusters as some resistance genes or distributed over the whole genome.

References

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

Brown, G.G., N. Formanova, H. Jin, R. Wargaschuk, C. Dendy, P. Patil, M. Laforest, J. Zhang, W.Y. Cheung, and B.S. Landry. 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., R.P. Wise, and P.S. Schnable. 1996. The *rf2* nuclear restorer gene of the male-sterile T-cytoplasm. Science. 272: 334-1336.

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

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

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

Horn, R., B. Kusterer, E. Lazarescu, M. Prüfe, and W. Friedt. 2003. Molecular mapping of the *Rf1* gene restoring pollen fertility in PET1-based F1 hybrids in sunflower (*Helianthus annuus* L). Theor. Appl. Genet. 106:599-606.

Köhler, R.H., R. Horn, A. Lössl, and K. Zetsche. 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., R. Imai, H. Fuijimoto, T. Hayakawa, Y. Kimura, J. Kohno-Murase, T. Sakai, S. Kawasaki, and J. Imamura. 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., G. Mösges, and W. Friedt. 1992. Construction of a sunflower pedigree map. Helia. 15:7-16.

Kosambi, D.D. 1994. The estimation of map distances from recombination values. Ann. Eugen. 112:172-175.

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

Lander, E.S., J. Green, J. Abrahamson, A. Batlow, M.J. Daly, S.E. Lincoln, and L. Newburg. 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 gènes de restauration de fertilité sur cytoplasmes stérilisants chez le tournesol. Agronomie. 4:573-576.

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

Özdemir, N., R. Horn, and W. Friedt. 2004. Construction and characterization of a BAC library for sunflower (*Helianthus annuus* L.). Euphytica. (submitted).

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

Tang, S., J.K. Yu, M.B. Slabaugh, D.K. Shintami, and S.J. Knapp. 2002. Simple sequence repeat map of the sunflower genome. Theor. Appl. Genet. 105:124-1136.

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