

Received 25 March 2002

Accepted 17 May 2002

### **MORPHOLOGICAL, CYTOLOGICAL AND BSA-BASED TESTING ON LIMITED SEGREGATION POPULATION AFLPS**

PIOTR TOMASZ BEDNAREK\*, HELENA KUBICKA  
and MAŁGORZATA ZAWADA

The Botanical Garden – the Centre for the Conservation of Biological Diversity  
of the Polish Academy of Sciences, 02-973 Warsaw, ul. Prawdziwka 2, Poland;

**Abstract:** Cytoplasmic male sterility (*cms*) in rye (*Secale cereale* L.), especially cytoplasm *PAMPA*, is used commercially in hybrid breeding programmes. The development of molecular markers that are tightly linked to the numerous genes coding for pollen fertility is expected to have great impact in the field.

Morphological and cytological analyses of plants from a three-way cross C394: [(S67P/94 x S38/94) x CHD296] indicated the presence of at least several genes acting at different stages of pollen grain development, and proved the concurrence of both approaches in plant classification. The AFLP technique combined with the Bulk Segregant Analysis (BSA) were applied to identify DNA fragments linked to the genes of interest. All the 256 possible primer pair combinations based on the *Mse*I and *Eco*RI restriction sites generated distinct band patterns allowing the identification of 31143 DNA fragments, visualised using the isotopic method. On average, any given primer combination generated 122 fragments. Among 1111 and 431 potential genetic markers respectively identified in the restorer form and the maternal lines, 775 and 295 were present in the F2 population. These numbers were then reduced to 109 and 51. The identified DNA fragments were tested on a limited segregating population, C394-F2, in order to eliminate false signals and to select markers for a future marker-assisted selection programme. Twenty-five markers were selected. Four of these markers were not identified via the BSA approach, indicating that if a highly polymorphic component is used for a cross, or a polygenic trait is studied, then the use of a limited population may be required.

**Key Words:** AFLPs, BSA, *cms* *PAMPA*, Cytology, Morphology, *Secale cereale* L.

---

\* Corresponding author : E-mail: [tmol.ob@ihar.edu.pl](mailto:tmol.ob@ihar.edu.pl), Fax:+48 22 757 66 45

## INTRODUCTION

The first data on the male sterility phenomenon in rye [1] stimulated research on the identification of its sources and its classification [2-10]. Due to the additional heterosis effect, it was expected that systems based on male sterility would be more effective in breeding programs. Nowadays, most commercially important *cms* hybrids are based on *PAMPA* cytoplasm which was found in primitive Argentinian rye [2, 11].

The development of modern rye hybrids is based on maternally inherited pollen sterility, which is exhibited as easily-detected alterations in pollen and anther development and tapetal tissue behaviour [12], which seem to be the result of changes identified in the mitochondrial genome [13]. This type of sterility could be overcome by a pollinator carrying the appropriate nuclear genes to restore pollen fertility. Thanks to its high stability and the absence of negative side effects on important agricultural traits, *cms PAMPA* proved its utility in the development of almost all of the commercially available rye hybrids occupying nearly 60% of the total rye acreage in Germany in 1999 [14].

Although it was demonstrated that in some cases it is possible to reduce the time required for rye hybrid evaluation by anther culture [15], up till now this approach has had no practical applications, probably due to problems with its application to other accessions or to the high costs involved. The breeding scheme for the production of hybrid components developed in the early 70's [11, 16] is still in use. Briefly, a male sterile inbred line on *cms PAMPA* is created during a time-consuming breeding process involving the transfer of the desired genotype onto the sterile cytoplasm. After several backcrosses, two nearly isogenic lines can be obtained. The sterile male one is called maternal, and its fertile analogue (on normal cytoplasm) is referred to as the maintainer. The latter, lacking the genes responsible for pollen fertility restoration, cannot restore fertility and is thus usually used to maintain the sterile line. Since crosses between these highly inbred lines do not guarantee effective seed production, another line with a slightly different genotype, but still lacking the restorer genes, is used. Crosses between these two lines result in a seed-producing offspring (a single cross). The commercial hybrid is formed after the pollination of the plants grown from seeds of the single cross with a restorer line which is pre-selected on *cms*-inducing cytoplasm. Thus, the hybrid is created via a three-way cross.

Other than the time needed to obtain inbred lines forming a seed-producing component, the most challenging problem in hybrid rye breeding is the development of effective restorers [11, 14, 17-19]. A high standard of restoration is a prerequisite for the prevention of ergot fungus (*Claviceps purpurea*) infections of the pistils under adverse weather conditions [19, 20] and thus, is a prerequisite for increasing total seed yield. To achieve that, the appropriate genes should be combined to give an effective restorer.

Both genetic [5, 11, 16, 21] and molecular investigations [14, 18, 19] clearly demonstrated that the restoration of pollen fertility to *cms PAMPA* is encoded by at least several genes depending on the respective restorer sources (two major genes on the 1R and 4R chromosomes, and additional genes of less importance on 3R, 5R and 6R [14, 18, 19]). Although successful, the breeding approach does not guarantee that all the genes important for the expression of this trait are included in the hybrid. This makes further improvement of a given variety by e.g. gene pyramiding a rather complicated task. Thus, more sophisticated tools should be involved.

The first attempt to rationalise the breeding process leading to the development of an inbred rye line with a gene of interest was performed by means of the *Prx-7* isozyme locus located on 1RS [18]. The marker had a recombination value of about 20%, and thus could not be effectively applied in marker assisted selection. It was recently demonstrated [19] that the Restriction Fragment Length Polymorphism (RFLP) technique could be successfully applied for such a purpose. Further analysis [14] resulted in the identification of RFLPs tightly linked to pollen fertility genes. These markers could be potentially applied in marker-assisted selection programmes. However, the RFLP technique is time-consuming and requires a large amount of DNA, making plant material screening ineffective. More recently, [22, 23] the Amplified Fragment Length Polymorphism (AFLP) [24] technique was applied for the development of molecular markers for the pollen fertility trait in hybrids on *cms PAMPA*. This technique could easily be combined with the Bulked Segregant Analysis (BSA) [25], a method used for preliminary identification of the DNA fragments linked to a trait of interest. It was also mentioned [23] that, in the case of rye, bulks should be formed on a secondary template in order to amplify even those restriction fragments that are underrepresented in the reaction mixture.

The aim of this study was to both morphologically and cytologically characterise the components forming the three-way C394 cross and the individual plants of the cross, and at the same time to test their molecular purity and level of polymorphism, to search for AFLPs that differentiate bulked samples consisting of plants with sterile and fertile phenotypes originating from C394-F2 (*cms-P*), and finally, by means of a limited C394-F2 segregating population and  $\chi^2$  statistics, to assess which of the BSA-based DNA fragments should be selected as molecular markers for further research.

## MATERIAL AND METHODS

### Plants

Plant materials were supplied by “Danko”, and consisted of the sterile S67P/94 inbred line (S18), its fertile analogue S67N/94 (S18) – the maintainer, the fertile S38/94 line (S18), the single sterile cross CSIN393: S67P/94 x S38/94, the self-incompatible restorer population CHD296 (pollinator) and seeds of the C394-F1: [(S67P/94 x S38/94) x CHD296] population. The C394-F2 and F3 populations

were planted under field conditions during the 1999-2001 seasons at the Botanical Garden – the Centre for the Conservation of Biological Diversity of the Polish Academy of Sciences. C394-F2 originated from selfs or sibs of individual C394-F1 plants. Similarly, C394-F3 plants were created.

### **Morphological and cytological analysis**

Individual plants representing both the inbred lines and the C394-F2/F3 populations were classified by rating the proportion of pollen-shedding anthers and the degree of degradation on a scale of 1-9 [6, 20] and with the aceto-carminic smear technique. Both approaches allowed the grouping of the C394 populations into three categories: sterile plants (SP /1-3 in visual scale/), partly sterile plants (PSP-/4-6/) and normally fertile plants (NFP-/7-9/).

### **DNA isolation**

Total genomic DNA was isolated according to the procedure included in the Dnease MiniPrep kit (purchased from Qiagen). DNA quantity was evaluated spectrophotometrically ( $\lambda_{260/320\text{nm}}$ ). Its integrity and purity was verified on 1.4% agarose gels.

### **Oligonucleotides**

The oligonucleotides for the AFLP technique were either of our own synthesis or purchased from various suppliers. Synthesis was performed on an Applied Biosystems 392 DNA/RNA synthesizer, and the products were purified by HPLC-RP on C18 columns and, if necessary, by PAAG. Rough oligonucleotides, purchased from EpiCentre Technology, underwent a HPLC-RP (C-18) purification step. The oligonucleotides purchased from the Centre of Molecular and Macromolecular Studies of the Polish Academy of Sciences (Łódź) were ready for use.

### **Amplified Fragment Length Polymorphism (AFLP)**

The AFLP procedure was performed according to the standard procedure [24] with slight modifications [26]. Briefly, 0.5  $\mu\text{g}$  of the total genomic DNAs were simultaneously digested with *MseI* and *EcoRI* endonucleases. The reactions were carried out in a final volume of 15  $\mu\text{l}$  (4h at 37°C followed by 15 minutes at 70°C in a Perkin Elmer 9600 thermocycler. The appropriate adapters were ligated in a final volume of 25  $\mu\text{l}$  at 20°C for 12h and diluted in a TE buffer (1:6). Afterwards, pre-selective PCR was performed with the standard profile, in a total volume of 25  $\mu\text{l}$ . If necessary, bulked samples were combined. To test the enzymatic reactions, 15  $\mu\text{l}$  of each sample was run on 1.4% agarose gel and the remaining part was diluted in TE (1:20). The samples were processed according to a selective PCR cycling programme. Reactions were run in 10  $\mu\text{l}$  of the final volume and in the presence of 5'-end hot labelled ( $^{32}\text{P}$ ) *EcoRI* and unlabelled *MseI* selective primers, and were followed by the addition of 6  $\mu\text{l}$  of 80% formamide loading buffer in presence of bromophenol blue and xylene cyanol

dyes. After a denaturation step (95°C for 10 minutes followed by immediate cooling to 5°C), six microliters of each mixture were loaded on 5% denaturing PAAG (50cm long and 0.2mm wide) and, after electrophoretic separation (which ended after the elution of the upper dye), was exposed to FOTON XC film or similar. Autoradiograms were analysed visually.

### **Bulked Segregant Analysis**

The bulked samples representing a given inbred line, the phenotypic classes of the C394-F2 population (sterile (SP) and normally fertile plants (NFP)) and the restorer form were combined after pre-selective PCR. Equal volumes of 1:6 dilutions from at least 12 samples were used in each case, and a secondary template was formed and applied for selective amplification with 256 primer combinations. The bulked samples forming normally fertile plants were combined from self-compatible and incompatible C394-F2 plants.

### **Data evaluation**

To test concurrence of the morphological data based on a visual scale of rating pollen fertility. Cytological observations of pollen viability regression analyses were also used. The hypothesis was tested with the regression coefficient at  $p_{val}=0.05$ .

A similarity chi-square test, with one degree of freedom at a significance level of 0.05 or lower, was used to differentiate between the AFLP bands. AFLPs with a chi-square greater or equal to the critical value were considered as markers.

### **Molecular markers**

A DNA fragment was considered as prospective if it was present both in one phenotypic group and one parent, or if its band intensity in a parent and in the appropriate offspring were greater than in the another. All the primer pairs that generated signals classified as prospective based on BSA underwent further testing on the C394-F2 population, consisting of 15 fully fertile and 21 sterile plants that were classified as 7-9 and 1-3 according to the visual scale. To identify molecular markers, the similarity of the sterile (SP) and fertile (NFP) phenotypes of the C394-F2 population with respect to the AFLP band frequency distribution was checked with the chi-square test.

## **RESULTS**

### **Morphological and cytological data**

The morphological and cytological analyses were performed on individual plants representing inbred lines (S67N/94, S67P/94, S38/94) and a single hybrid CSIN393: S67P/94 x S38/94, used in the creation of the heterozygous form C394. They demonstrated that the plants originating from S67P/94 were sterile, while their analogues on normal cytoplasm, S67N/94 and line S38/94, were fully fertile. Plants representing the single hybrid CSIN393 were also found to be

sterile. In the case of the sterile line S67P/94 and CSIN393, no seed formation was observed after selfing.

After the crossing of CSIN393 with the pollinator, CHD296, the three-way hybrid, C394-F1, was obtained. The C394-F2 population was derived by self-pollination of the C394-F1 plants. Individuals with varied viability of pollen grains, classified morphologically (Fig. 1a, 1b) and cytologically (Fig. 2a, 2b), were identified. The population of the F2 generation consisted of 1065 plants, in which fertile pollen was observed in 105, partly fertile in 144 and sterile in 816 cases. The ratio of plants with fertile to sterile pollen was approximately 1:10. In the C394-F3 generation, the ratio of fertile plants to sterile increased to 1:6. Only 3.7% of plants were fertile (as characterized by a pollen grain viability of over 80%). The use of this assessment method of pollen grain fertility (morphological and microscopic) gave convergent results (regression coefficient  $r=0.91$  at  $p_{val}=0.05$ ).

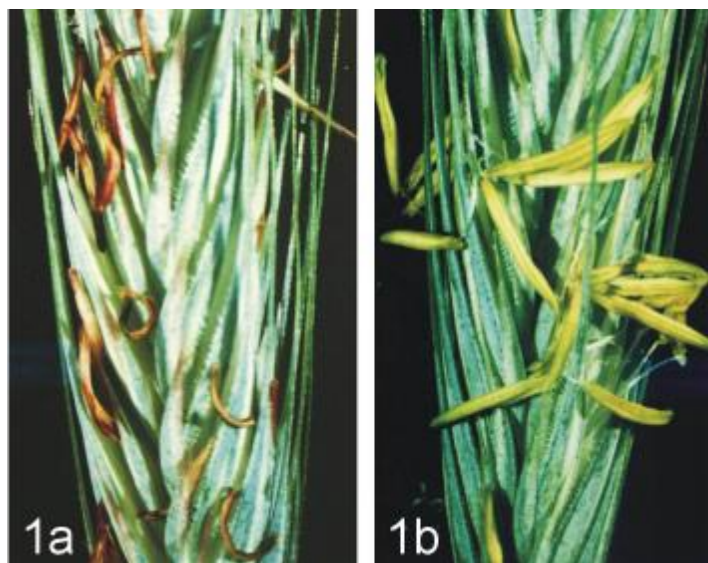


Fig. 1. Photos of the sterile (a) and fertile (b) anthers of the C394-F2 segregating population.

The viability of pollen grains among partially and fully male fertile plants varied from 5.8 to 98.6%. Differences in the size, shape and wall thickness of the exine of pollen grains were observed. In total, 14.5% of the plants forming the C394-F2 population set seeds after self-pollination, while 2.2% of the originated from sterile plants.

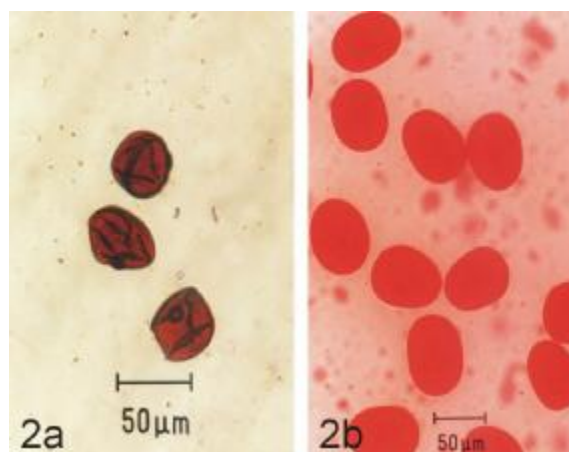


Fig. 2. Microscopic images of the sterile (a) and fertile (b) pollen grains of the C394-F2 segregating population visualized with aceto-carmin dye.

#### **Inbred lines, single cross, C394-F2 and CHD296 – variability evaluation**

The genetic uniformity of the plant material was confirmed by AFLP data. Twenty selective primer pairs generated individual fingerprints with more than 20,000 DNA fragments, which were identified by specialized software. Analyses of each inbred line was performed on at least 12 genomic DNAs, isolated from a single leaf of individual plants. DNA profiling demonstrated a lack of any polymorphisms among the plants of a given inbred line or the single cross CSIN393. Moreover, no DNA fragments were identified as differentiating the S67N/94 fertile inbred line and its sterile S67P/94 analogue. Although, nearly all the bands were identified in any given parent of the CSIN393, in S67P/94 or S38/94, some of them, usually of low intensity, were hardly seen or even not visible.

A high level of polymorphisms was detected among plants representing the restorer form CHD296, which had the greatest contribution to genetic variation within all the C394 populations. The molecular profiling of the C394-F2 plants manifested a high level of polymorphisms which mainly originated from the pollinator. It should also be stressed that, among the fertile plants, there were both compatible and self-incompatible plants, the latter making up about 10% of the total.

#### **Bulked Segregant Analysis**

The segregating C394-F2 population was divided into two phenotypic groups consisting of sterile (SP) and normally fertile plants (NFP). After pre-selective PCR, DNA, previously isolated from individual plants, were combined within each phenotypic group to form bulks. Similar bulks were obtained from inbred

lines, single cross and parental plants. All 256 primer combinations available for the *EcoRI/MseI* system were tested to identify DNA fragments potentially linked to the pollen fertility restoration trait. All primer pairs generated easily detected, reproducible and individual molecular patterns even if only one selective nucleotide was changed among primer pairs. In total, 31143 DNA fragments, visualised via the isotopic method, were identified. On average, any given primer combination generated 122 fragments ranging from 5 to as many as 182 clearly visible signals. The smallest and the greatest number of identified bands were in the case of the EAAT/MCAG and EATG/MCCC primer pairs, respectively. The molecular weight of the analysed bands was below 400 base pairs.

Among the 1111 and 431 signals respectively identified in the restorer form and maternal line, 775 and 308 were present in the bulks. The restorer form and maternal component made up 2.49% and 0.99% of the polymorphisms, respectively. Thus, the influence of the restorer was 2.5 times higher than the seed producing component. 209 DNA fragments seem to be potential markers for plants with fertile pollen, and 81 for plants with sterile phenotypes. These bands were generated by 174 primer combinations. Considering the band intensity (only strong signals were counted), the number of bands that needed to be analysed was reduced to 109 and 51, respectively (64 primer combinations).

#### Limited segregating population and marker identification

The molecular profiling of the limited C394-F2 segregating population performed with 64 primer combinations enabled the testing of 160 polymorphic bands and verification of their linkage with the trait by  $\chi^2$  statistics. Not all the DNA fragments proved useful. Most of those identified by BSA signals were

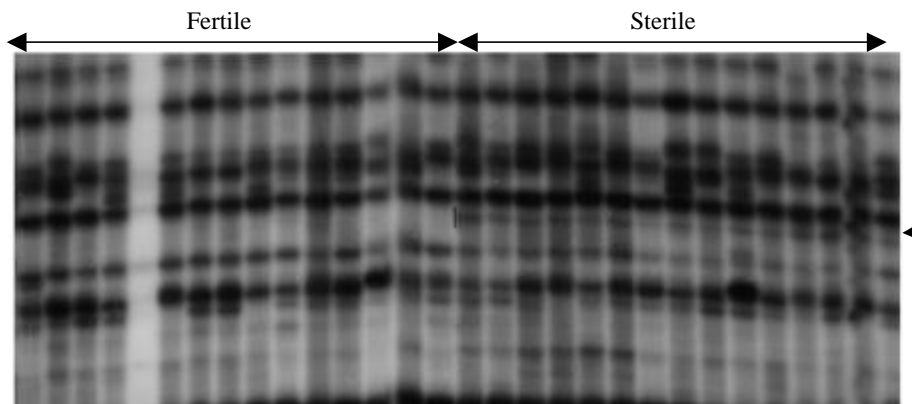


Fig. 3. Fragment of the AFLP profiling of the C394-F2 segregating population. Starting from the left-hand side: first 15 lines represent fertile plants, the next 15 lines represent sterile plants. An arrow indicates marker level. Small arrow indicated marker.

either false bands or were of little importance. Moreover, the BSA approach failed to identify three out of the six most prospective markers for the non-restorer (maintainer) gene, which were identified independently on the limited population. To be sure that we did not lose any important markers, ten profiles generated by additional primer combinations were analysed. As a result one marker for male sterility and two for pollen fertility were identified. Finally, fourteen and ten markers for pollen fertility and male sterility (non-restorer) genes were selected. Six out of ten markers in the last case nearly completely distinguished the plants of the analysed population (Fig. 3).

## DISCUSSION

To describe pollen fertility restoration in *cms PAMPA*, a visual scale [6], based on rating the proportion of pollen-shedding anthers and the degree of degradation, was described. Rarely performed [27] microscope analysis of pollen viability via the aceto-carmin smear technique could also have been applied. Previously, a concurrence between microscopy analysis of pollen vitality and the visual method of pollen fertility scoring was demonstrated [27, 28]. Our results, obtained by both methods in analyses performed on C394-F2 plants, confirmed those results, but visual scoring seems to be more effective, at least in our hands. Moreover, microscopic observations revealed alterations in pollen grain development, indicating that the C394-F2 population must be rich in genes acting at different stages of its development influencing the fertility and sterility of pollen to a varied level.

Both morphological, cytological and AFLP analyses performed on the components of the three-way cross C394 and its offspring clearly demonstrate that all the inbred lines used in the study, in contrast to the pollinator population, were highly uniform. Except for the phenotypic alterations of the highly sterile maternal line S67P/94 and its fertile analogue S67N/94 these lines did not exhibit any differences at the molecular level indicating that, although theoretically possible, the co-extraction of mitochondrial genome is negligible, and that all the bands identified by AFLP are of nuclear origin. On the other hand, as could be expected, a high level of variation was detected among plants from the CHD296 population restorer form. This population has genes responsible for pollen fertility restoration of *cms-PAMPA*, proved by the fact that the segregating C394-F2 population contained 3.7% normally fertile and 19.6% partially fertile plants. Pollen fertility genotypes usually occur at a frequency of 1 to 5% in other European rye populations [27]. Thus, the presence of such genotypes in C394-F2 at a frequency of 23% bears promise for the detection of markers for a wide range of genes for the trait. This may to some extent explain the presence of seeds in the case of the few self-pollinated plants classified as sterile. It looks as though even weak genes coding for pollen fertility may overcome the highly sterile *PAMPA* cytoplasm, leading to its partial "leakage".

There are many approaches that could be applied to identify molecular markers. When markers for genes conferring or controlling resistance-susceptibility-tolerance to extrinsic factors, pests, etc. are needed, then nearly-isogenic lines (NILs) [29] are the choice to make. Unfortunately, this approach may not be efficient in the case of a trait coded by several genes, or in the case of plants like wheat, due to their large genome size [30]. Additionally, the appropriate NILs should be available or developed. Another opportunity is to use the bulked segregant analysis (BSA) [25]. Although the approach was designed for the identification of markers for monogenic traits, it was shown at least theoretically that it could be also applied for QTL typing [31]. It was also stated that further reduction of the efficiency of the method should be expected when heterozygous or non-inbred lines are used to detect linked dominant loci [32], which is especially true in our case, where AFLPs for a oligogenic trait are needed, and the C394-F2 population is heterozygous due to the parental component CHD296. Fortunately, at least in the case of inbred lines, the number of polymorphisms identified by AFLP in combination with BSA could be reduced if bulks are formed on secondary templates [26].

Our results are comparable to those obtained on the complex autotetraploid *Solanum tuberosum* [33], where BSA in combination with AFLP techniques were applied in the identification of markers for resistance to *Phytophthora infestans* coded by the *R2* allele. In that study, even after the reduction of polymorphisms based on intensive signals, their number, reaching 160 and shared by 64 primer combinations, was still 15 times greater than in the case of the *R2* allele. This could be explained by the unknown number of genes coding the trait of interest in C394-F2 or the application of the population restorer. We also cannot exclude the possibility that some of the polymorphisms could be linked to self-fertility or self-incompatibility (SI) genes which were mapped to 1RS, 2RL and 5R [34-36]. Probably the one causing the most hinderance will be the SI allele on 1RS, since this locus, like that for pollen fertility restoration in one source (L18), is linked to the same isozyme *Prx-7* [18].

To further eliminate false signals and reduce the number of primer combinations, a limited population (C394-F2) consisting of individual samples representing distinct phenotypes (15 fertile and 21 sterile) was studied with all 64 chosen primer pairs. Finally, only twenty-one DNA fragments proved their worth. Moreover, since BSA identified many additional signals that were not confirmed with the limited population, we could not exclude the possibility that some important markers might be lost. To investigate such a possibility, 10 randomly chosen primer combinations were used. We found that bulk samples did not allow the identification of three markers for non-restorer and one for pollen fertility restoration genes. Our results suggest that when markers to polygenic traits are needed, or when one of the parents is a population, then bulked analysis should be followed by studies on a population of a limited size. If its size is large enough, then  $\chi^2$  statistics could be applied to eliminate unlinked AFLPs.

Although analysis of the limited population allowed the reduction of the AFLPs to 25 markers, their number is still too high. This may reflect the action of multiple genes with different effects, since pollen fertility restoration has complex inheritance. Our morphological observations seem to confirm this statement and the segregation ratio 1:10 (fertile to sterile) might be the result of the action of at least two genes that may differ in strength. Considering the heterogenous and heterozygous nature of the restorer form (CHD296), there are many possible parental genotypes. One of them could be AaBb. Moreover, the maternal component, namely the single cross CSIN393, may be heterozygous in a weak locus (aaBb). Previously it was suggested by Ruebenbauer *et al.* [37] that for the restoration of pollen fertility, a prevailing number of the dominant pollen fertility genes should be present. In our case, this may mean that the fertile phenotype will only be expressed when there is an AaBB genotype resulting in segregation ratio equal to 1:7. However, this does not fit our observations. The model with four genes resulting in a 1:9 segregation ratio of fertile to sterile phenotypes seems more probable. It should be stressed that at the moment we cannot differentiate between these models, but in both cases the existing deviation from morphological observations could be explained by the nature of the restorer form and/or by the possible action of some additional genes of unknown number.

Whatever the explanation for our results is, it is evident that the number of markers (or primer pair combinations) should be reduced if they are to be applied to the screening restorer population to identify plants carrying the desired genes. This could be achieved by tests on an enlarged population in order to prove the marker ability to differentiate genotypes and by linkage group identification. Moreover, identifying the location of both individual markers and linkage groups on rye chromosomes, by means of e.g. co-migrating markers [38, 39], might also be useful in defining the exact number of genes responsible for the trait in the C394-F2 population.

## REFERENCES

1. Putt, E.D. Cytogenetic studies of sterility in rye. **Can. J. Agricultural Sci.** 34 (1954) 81-119.
2. Geiger, H.H. Cytoplasmatisch-genische Pollensterilitat in Roggenformen iranischer Abstammung. **Die Naturwissenschaften** 2 (1971) 98-99.
3. Kobyljanski, V.D. The production of sterile analogues of winter rye, sterility maintainers and fertility restorers. **Tr. Prikl. Bot. Genet. Sel.** 44 (1971) 76-84.
4. Kluchko, P.F. and Belousov, A.A. Genetic study of male sterility in winter rye. **Genetika** 8 (1972) 9-15.
5. Madej, L. Research on male sterility in rye. **Hod. Rośl. Aklim. Nasienn. Warszawa** 19 (1975) 421-422.

6. Geiger, H.H. and K. Morgenstern. Angewandt-genetische Studien zur cytoplasmatischen Pollensterilität bei Winterroggen. **Theor. Appl. Genet.** 46 (1975) 269-276.
7. Madej, L. The genetical characteristics of three sources of male sterility in rye (*Secale cereale* L.). **Hod. Rośl. Aklim. Nasienn. Warszawa** 20 (1976) 157-174.
8. Adolf, K. and Winkel, A. A new source of spontaneous sterility in winter rye. Preliminary results. In: **Proc. Eucaria Meet. Cereal Sect. Rye**. Svalov, Sweeden, (1985) 293-307.
9. Warzecha, R. and Salak-Warzecha, K. Comparitive studies on CMS sources in rye. **Vortr. Pflanzenzuchtung** 35 (1996) 39-49.
10. Łapiński, M. and Stojałowski, S. The C-source of sterility-inducing cytoplasm in rye: origin, identity, and occurrence. **Vort. Pflanzenzuchtung** 35 (1996) 51-60.
11. Geiger, H.H. and Schnell, F.W. Cytoplasmic male sterility in rye (*Secale cereale* L.). **Crop Sci.** 10 (1970) 590-593.
12. Garlocka, W. and Madej, L. Microsporogenesis and anther development in male-sterile and male-fertile forms of rye. **Hod. Rośl. Aklim. Nasien.** 19 (1975) 423-428.
13. Tudzynski, P., Rogmann, P. and Geiger, H.H. Molecular analysis of mitochondrial DNA from rye (*Secale cereale* L.). **Theor. Appl. Genet.** 72 (1986) 695-699.
14. Miedaner, T., Glass, C., Dreyer, F., Wilde, P., Wortmann, H. and Geiger, H.H. Mapping of genes for male-fertility restoration in 'Pampa' CMS winter rye (*Secale cereale* L.). **Theor. Appl. Genet.** 101 (2000) 1226-1233.
15. Bicar, E.H. and Darvey, N.L. Development of the components of a cytoplasmic male sterility hybrid system in rye through anther culture. **Euphytica** 97 (1997) 151-160.
16. Geiger, H.H. Breeding methods in diploid rye (*Secale cereale* L.). **Tag.-Ber., Akad. Landwirtsch.-Wiss. DDR, Berlin** 198 (1982) 305-332.
17. Geiger, H.H. Wiederschtellung der Pollenfertilität in Roggenformen iranischer Herkunft. **Theor. Appl. Genet.** 42 (1972) 32-33.
18. Wricke, G., Wilde, P., Wehling, P. and Gieselmann, C. An isozyme marker for pollen fertility restoration to Pampa cms system of rye (*Secale cereale* L.). **Plant Breed.** 111 (1993) 290-294.
19. Miedaner, T., Dreyer, F., Glass, C., Reinbold, H. and Geiger, H.H. Mapping of genes for pollen fertility restoration in rye (*Secale cereale* L.). **Vortr. Pflanzenzuechtg** 38 (1997) 303-314.
20. Dreyer, F., Miedaner, T. and Geiger, H.H. Chromosomal localization of male-fertility restorer genes from Argentinian and Iranian rye. **Vortr. Pflanzenzuchtung** 35 (1996) 280-281.
21. Scoles, G.J. and Evans, L.E. The genetics of fertility restoration in cytoplasmic male-sterile rye. **Can. J. Genet. Cytol.** 21 (1979) 417-422.

22. Bednarek, P.T., Kubicka, H., Zawada, M. and Brukwiński, W. Application of the AFLP markers for genetic variability testing within parental form of a rye hybrid based on the PAMPA cytoplasm. **Biul. Inst. Hod. Aklim. Rośl.** 211 (1999) 229-237.
23. Bednarek, P.T., Kubicka, H., Zawada, M. and Brukwiński, W. Bulk analysis of pseudo segregating F1 plants of rye hybrid based on cms PAMPA by the AFLP technique. **Biul. Inst. Hod. Aklim. Rośl.** 216 (2000) 101-110.
24. Vos, P., Hogers, R., Bleeker, M., Lee, T. van, Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zauber, M. AFLP: a new technique for DNA fingerprinting. **Nucleic Acids Res.** 23 (1995) 4407-4414.
25. Michelmore, R.W., Paran, I. and Kesseli, R.V. Identification of markers linked to disease resistance genes by bulk segregant analysis: a rapid method to detect markers in specific genomic region by using segregating populations. **Proc. Natl. Acad. Sci. USA** 88 (1991) 9828-9832.
26. Bednarek, P.T., Chwedorzewska, K., Króliczak, J., Puchalski, J. and Zawada, M. AFLP molecular markers as a tool for genetic variability studies of rye inbred lines. **Biul. Inst. Hod. Aklim. Rośl.** 211 (1999) 219-227.
27. Yuan, Y. Umweltsensibilität der cytoplasmisch-genisch vererbten männlichen Sterilität (CMS) bei Roggen (*Secale cereale* L.). **Dissertation**, Univ. (1996) Hohenheim.
28. Morgenstern, K. Ausprägung der cytoplasmatisch-genischen Pollensterilität (CMS) bei Roggen in Abhängigkeit von Plasmotyp and Genotyp. **Dissertation**, Univ. (1983) Hohenheim.
29. Paran, I., Kesseli, R.V. and Michelmore, R.W. Identification of RFLP and RAPD markers linked to downy mildew resistance genes in lettuce, using nearly-isogenic lines. **Genome** 34 (1991) 1021-1027.
30. Ranade, S.A., Farooqui, N., Bhattacharya, E. and Verma, A. Gene tagging with random amplified polymorphic DNA (RAPD) markers for molecular breeding in plants. **Crit. Rev. Plant Sci.** 20 (2002) 251-275.
31. Wang, G.L. and Peterson, A.H. Assessment of DNA pooling strategies for mapping QTLs. **Theor. Appl. Genet.** 93 (1994) 920-925.
32. Lehner, A., Campbell, M.A., Wheeler, N.C., Poykko, T., Glossl, J., Kreike, J. and Neale, D.B. Identification of a RAPD marker linked to pendula gene in Norway spruce (*Picea abies* (L.) Karst. f. pendula). **Theor. Appl. Genet.** 91 (1995) 1092-1094.
33. Li, X., Eck, H.J. van, Voort, J.N.A.M.R. van, Huigen, D.-J., Stam, P., Jacobsen, E. Autotetraploids and genetic mapping using common AFLP markers: the R2 allele conferring resistance to *Phytophthora infestans* mapped on potato chromosome 4. **Theor. Appl. Genet.** 96 (1998) 1121-1128.
34. Senft, P. and Wricke, G. An extended genetic map of rye (*Secale cereale* L.). **Plant Breed.** 115 (1996) 508-510.

35. Voylokov, A.V., Korzun, V. and Börner, A. Mapping of three self-fertility mutations in rye (*Secale cereale* L.) using RFLP, isozyme and morphological markers. **Theor. Appl. Genet.** 97 (1998) 147-153.
36. Börner, A. and Korzun, V. A consensus linkage map of rye (*Secale cereale* L.) including 374 RFLPs, 24 isozymes and 15 gene loci. **Theor. Appl. Genet.** 97 (1998) 1279-1288.
37. Ruebenbauer, T., Kubara-Szpunar, L and Pająk, K. Testing of the hypothesis concerning interaction of genes with mutated cytoplasm controlling male sterility of the Pampa type in rye (*Secale cereale* L.). **Genetica Polonica** 25 (1984) 1-15.
38. Voort, J.R. van, Wolters, P., Folkertsma, R., Hutten, R., Zandvoort, P. van, Vinke, H., Kanyuka, K., Bendahmane, A., Jacobsen, E., Janssen, R. and Bakker, J. Mapping of the cyst nematode resistance locus *Gpa2* in potato using a strategy based on comigrating AFLP markers. **Theor. Appl. Genet.** 95 (1997) 874-880.
39. Voort, J.R. van, Zandvoort, P. van, Eck, H.J. van, Folkertsma, R.T., Hutten, R.C.B., Draaistra, J., Gommers, F.J., Jacobsen, E., Helder, J. and Bakker, J. Use of allele specificity of comigrating AFLP markers to align genetic maps from different potato genotypes. **Mol. Gen. Genet.** 255 (1997) 438-447.