Implementation of Molecular Techniques (RAPDs, AFLPs) on Camomile (*Chamomilla recutita* (L.) Rausch.) for Genotyping and Marker Development

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Abstract

Camomile varieties of different provenience can be distinguished by morphological and physiological traits. However, these traits vary under different environmental conditions leading to difficulties in the discrimination of genotypes. PCR-based molecular techniques like RAPDs and AFLPs are useful tools to characterise genotypes rapidly and reliably on the DNA-level independently from ecological factors. Besides this, these techniques facilitate the development of molecular markers for ingredients of camomile oil, e.g. (-)-α-bisabolol, thereby enabling pre-flowering selection.

Therefore, attempts were carried out to establish these molecular techniques on camomile. In a first step genetic similarity (Jaccard, 1908) was estimated in a set of released cultivars, breeding populations and twice self-pollinated lines. Based on data obtained by 20 RAPD primers and 16 AFLP *Eco*RI+3/*Mse*I+3 primer combinations genetic similarity was estimated between 0.52 and 0.91 (RAPDs) and 0.58 to 0.79 (AFLPs). By cluster analysis as well as by principle co-ordinate analysis genotypes were grouped according to their different proveniences. Regarding the self-pollinated lines which differ in their (-)-α-bisabolol content a high degree of homogeneity within lines (0.81-0.94) and a relatively low genetic similarity between them (0.46-0.56) was determined by RAPDs. By crosses between these lines a segregating F_2-population was created for developing molecular markers corresponding to the (-)-α-bisabolol content. Using bulked segregant analysis (Michelmore et al., 1991) a set of 200 RAPD-primers and 256 AFLP-primer combinations were tested up to now and two AFLP-markers linked to the (-)-α-bisabolol content were detected. Future work will aim at the development of additional more closely linked markers.

INTRODUCTION

The identification of the main active chemical ingredients of the essential oil like matricin, (-)-α-bisabolol and the flavonoids led to the development of new camomile varieties, which besides a higher oil content carry higher percentages of these active components. Discrimination between different genotypes was generally performed on the basis of morphological and physiological traits which are in general influenced by environmental conditions. Molecular PCR-based markers like Random Amplified Polymorphic DNAs (RAPDs)(Williams et al., 1990) and Amplified Fragments Length Polymorphisms (AFLPs)(Zabeau and Vos, 1993) provide opportunities for assessing genetic diversity within and between different varieties and germplasms. Thus, molecular techniques were established on camomile for determining the intra- and intergenetic diversity of different camomile genotypes and cultivars (Wagner et al., 2001).

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Besides this, molecular techniques are useful tools for the developing of markers closely linked to traits of interest following a simple or complex mode of inheritance (for review cf. Ordon et al., 1998, Friedt et al., 2002). Therefore, attempts were carried out to develop such markers for the (-)-α-bisabolol content following a monogenic recessive mode of inheritance (Horn et al., 1988). Identification of closely linked molecular markers for this gene could facilitate pre-flowering marker assisted selection procedures thereby enhancing the process of breeding camomile varieties with a high content of (-)-α-bisabolol. For this purpose bulked segregant analysis (BSA) (Michelmore et al., 1991) was performed on a segregating F_2-population (842 individuals) of a cross between camomile genotypes with a high and a low (-)-α-bisabolol content using the RAPD and AFLP methods by bulking DNA samples from 10 individuals at the phenotypic distribution extremes.

MATERIALS AND METHODS

Plant Material

Genetic diversity based on RAPDs and AFLPs was estimated on six camomile cultivars, i.e. ´Bodegold´, ´Bona´, ´Lutea´, ´Mabamille´, ´Margaritar´, ´Soroksari40´ (Anonymos, 1996) as well as on four twice self-pollinated lines (C3, C9, B8 and B20) and two populations (Akk34, D46) developed for an increased (-)-α-bisabolol content of the essential oil. Furthermore, genetic diversity of three and 10 plants respectively derived from the tetraploid ($2n = 4x = 36$) lines B8, B20, C3 and C9 used as crossing parents because of their different (-)-α-bisabolol content, were analysed by RAPDs to determine intra- and interpopulation genetic variability. Out of these lines crosses between single plants with a high and low (-)-α-bisabolol content (C9/2 x B20/3) were carried out to generate a segregating F_2-population for marker development using BSA (Michelmore et al., 1991). Self-pollination of four F_1-plants originated from the same cross combination generated a F_2-population of 842 individuals. Out of this, one sub-population (25/2) was selected for marker development using RAPDs and AFLPs. The bulks consist each of 10 individual plants differing in the contents of bisaboloides. Polymorphisms between the respective bulks were mapped on a sub-population of 92 F_2-individuals up to now.

DNA Isolation

Young leaves (0.1 g) were ground in nitrogen to fine powder. Thereof, DNA was isolated following the DNA-mini-extraction method described by Doyle & Doyle (1987). All DNAs were diluted to concentrations of 5 ng/µl for RAPDs and 25 ng/µl for AFLPs, respectively.

RAPD Analysis

RAPD analysis (Williams et al., 1990) was performed according to Özdemir et al. (1999). Fragments were separated on a 2 % agarose gel at 140 V about 4.5 h, stained in ethidium bromide, visualised with UV-ligth (254 nm) and photographed.

AFLP Analysis

For AFLP analysis (Zabeau and Vos, 1993) DNA restriction and ligation was performed using the AFLP Core Reagent Kit (Gibco Life Technologies). 150 ng genomic DNA were digested with *Eco*RI and *Mse*I. Ligation was followed by two pre-amplification steps, one non-selective (+0-pre-amplification) followed by a selective step with one additional nucleotide (+1-pre-amplification) (Schiemann et al., 1999). After this, selective amplification with three additional nucleotides (+3-amplification) was performed. PCR conditions were set according to Vos et al. (1995) with minor modifications. Electrophoresis was conducted on a 8 % polyacrylamide gel at 1500 V, 40 W, 40 mA and 48°C about 4 h using the LI-COR DNA GeneReadir 4200 (MWG Biotech).

Genotyping and Data Analysis for Estimating Genetic Diversity

An initial screening of seven sets of decamer-primers (Operon Technologies) on camomile had been carried out by Özdemir et al. (1999) to examine amplification profiles for polymorphism, readability and reproducibility. From this assortment 18 primers (OPC01, OPC04, OPC05, OPC11, OPF01-OPF03, OPM01, OPM02, OPM04, OPM14, OPN06, OPN07, OPN14-OPN16, OPQ16) were selected for determining genetic diversity of the different cultivars/varieties and four additional primers (OPM03, OPM05, OPM06, OPN05) were used to determine the intra-population variability of the four parental lines. Regarding AFLP-analysis 16 *Eco*RI+3/*Mse*I+3 primer combinations (E31/M48, E32/m49, EE34/M48, E35/M50, E35/M61, E35/M62, E36/M48, E36/M50, E36/M62, E39/M48, E40/M48, E40/M62, E41/M48, E41/M50, E42/M48, E47/M63) were used for determining genetic diversity of the different cultivars/varieties.

RAPD and AFLP patterns were scored using the software package RFLPscan 2.0. Pairwise genetic similarity was estimated applying Jaccard's similarity index (1908). A dendrogram was generated using the unweighted pairgroup method average (UPGMA) clustering procedure and in addition, principal co-ordinate analysis (PCoA) was performed to visualise the dispersion of the genotypes in relation to the first three principal axes of variation. The goodness of fit of the dendrograms was assayed by the Mantel test statistic (Mantel, 1967). For all these computations the software program NTSYS-pc-2.01 (Rohlf, 2000) was used. Correlation between the similarity matrices of RAPDs and AFLPs was computed by Spearman rank correlation coefficient using the software package SPSS 10.0.

Marker Development Using Bulked Segregant Analysis

Ten single plants of the F_2-sub-population 25/2 were grouped according to their phenotypic extremes in four bulks differing in the contents of the bisaboloides, i.e. (-)-α-bisabolol, biosabololoxide A, bisabololoxide B and bisabolonoxide A. A screening was carried out with up to now 200 decamer-primers (sets OPK01-OPK20 to OPT01-OPT20) and 256 *Eco*RI+3/*Mse*I+3-AFLP primer combinations (all of the combination E01/M02). Polymorphisms detected were tested on the 40 individual plants included in the bulks first and next potential markers were analysed on a sub-population of 92 individuals and mapped by using the software MAPMAKER 3.0b applying the Kosambi function (Kosambi, 1944).

RESULTS

Genetic Diversity of Cultivars in Comparison to Advanced Populations

Based on evaluation of 445 RAPD- and 1507 AFLP-fragments corresponding to 342 and 1097 polymorphic fragments, respectively, pairwise genetic similarity was estimated between 0.52 (´Margaritar´ *vs*. ´C3´ and ´B8´; ´Bona´ *vs*. ´B8´) and 0.91 (´Lutea01´ *vs*. ´Lutea02´) regarding RAPDs and between 0.58 (´Mabamille´ *vs*. ´C9´) and 0.79 (´D46´ *vs*. ´Akk34´) for AFLPs. The number of amplified fragments varied in the range of 12 to 32 (RAPDs) and 55 to 130 (AFLPs) with an average of 24.7 and 94.2 fragments/primer and primer combination. Accordingly, the number of polymorphic fragments/primer varied between 9 and 30 (RAPDs) and 34 and 100 polymorphisms/primer combination (AFLPs) with an average of 19.0 and 68.6, respectively. Thus, the level of polymorphism (polymorphic fragments/total fragments) detected with RAPDs varied in a range of 59-94 % (76 %) and with AFLPs in a range of 52-84 % (72 %), respectively.

UPGMA analysis of the genetic similarity values gave similar results for both marker systems (Fig. 1), showing a separation in two distinct groups comprising the populations and lines of our institute on the one hand and the released cultivars on the other hand. Regarding those, ´Mabamille´ is separated from the others. ´Bona´ and ´Lutea´ and ´Bodegold´ and ´Soroksari40´, respectively, are grouped together in subclusters. Furthermore, the two seed samples of ´Lutea´ are clearly separated by both

techniques. The cultivar ´Margaritar´ could not bee clearly allocated in one of these subgroups. With regard to the PCoA-analysis similar results were obtained. Mantel statistics revealed a good fit of the dendrograms to the similarity matrices, i.e r=0.91 for RAPDs and r=0.89 for AFLPs. The correlation between genetic similarity matrices of RAPDs and AFLPs was estimated at r=0.466 using Spearman rank correlation coefficient.

Genetic Diversity of the Parental Lines

Evaluation of 22 RAPD primers generated 537 fragments of which 430 were polymorphic (19.5 polymorphisms/primer). Looking at the dendrogram single plants of all lines show close intra-genetic relationships with similarity indices in the range of 0.81-0.83 (B8), 0.82-0.84 (B20), 0.82-0.92 (C9) and 0.83-0.94 (C3), respectively, and a wide inter-genetic relationship (0.46 to 0.56, Fig. 2A). Besides this, the PCoA-analysis clearly separates these four lines, too (Fig. 2B). The goodness of fit computed by Mantel statistics yields a correlation coefficient of r=0.99 revealing a very good fit.

Segregating F_2-Generation and First Results of Marker Development

Each offspring of the four selected F_1-individuals as well as summarised data showed a good fit to a segregation ratio of 1:3 regarding a high (-)-α-bisabolol content and a high bisabololoxide B content, respectively (Tab. 1). In the case of a tetraploid population this segregation ratio hints to the presence of only one dominant allele in the respective parents. Based on these phenotypic data bulked segregant analysis was carried out on bulks of the following composition: I. high (-)-α-bisabolol content, free of the bisabololoxides; II. low (-)-α-bisabolol content, high bisabololoxide B content, free of bisabololoxide A and bisabolonoxide A; III. low (-)-α-bisabolol content, bisabololoxide A and B in equal contents, traces of bisabolonoxide A; IV. low (-)-α-bisabolol and bisabololoxide B content, high bisabololoxide A content, traces of bisabolonoxide A. By screening these bulks with 200 decamer primers and 256 *Eco*RI+3/*Mse*I+3-primer combinations 30 polymorphic RAPD primers and 97 AFLP-primer combinations are detected. By testing these primers on the basis of the 40 single plants included in the bulks two RAPD- and 23 AFLP-markers revealed linkage to the (-)-α-bisabolol content. However, out of these only two AFLP markers showed relatively close linkage to the (-)-α-bisabolol locus within a distances of 11.9 and 15.2 cM, respectively, by analysing the sub-population of 92 F_2-individuals.

DISCUSSION

RAPD and AFLP markers were chosen to examine the inter- and intra-specific genetic diversity because both can quickly and cost-effectively generate markers in species where sequence information does not exist, a condition that precludes the use of other techniques, such as SSRs. According to Vos et al. (1995) the ideal fingerprinting assay should require no prior sequence knowledge like RAPDs and AFLPs. These techniques have been successfully used in many taxonomic and genetic diversity studies on different crops e.g. on barley, Ordon et al., 1997, Russel et al., 1997. Concerning medicinal plants these techniques are considerably rarely used, like e.g. on *Echinacea* species (Kapteyn et al., 2002), *Mentha* species (Khanuja et al., 2000) and *Digitalis obscura* L. (Nebauer et al., 1999).

Regarding genetic analysis on different camomile varieties an exceptional but comparable high level of polymorphism (mean percentage of polymorphic fragments per primer/primer combination, respectively) was obtained using RAPDs and AFLPs while in other studies a lower level of polymorphism for AFLPs was observed (Russel et al., 1997, Yee et al., 1999, Garcia-Mas et al., 2000). In comparison to RAPDs, AFLPs produced about 3.5 times as many discrete fragments per reaction. Thus AFLPs can be considered as the more efficient technique. However, according to Yee et al. (1999) the ability to resolve genetic variation among accessions may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed.

The dendrograms derived from UPGMA clustering of RAPD and AFLP fingerprints by using Jaccard's coefficient revealed two distinct clusters (Fig. 1). The correlation between clusters and the original similarity matrices are good to very good. Besides this, the correlation between RAPD and AFLP similarity matrices was higher than e.g. for barley (Russel et al., 1997), but lower than for pea (Simioniuc et al., 2002).

Regarding the four local lines UPGMA clustering as well as PCoA analysis clearly separates four groups demonstrating a close relationships within and clear distinction between lines. Thus, it may be concluded that an adequate homogeneity within and a sufficient heterogeneity between the lines is present. Therefore, these lines are well suited for the construction of a segregating population.

The relatively low genetic similarities of camomile genotypes and cultivars could be an indicator that up to now no stringent selection has been carried out in camomile (cf. Fuentes et al., 1999) preserving a high level of genetic diversity. In summary, these results elucidate that PCR-based molecular marker systems, i.e. RAPDs and AFLPs can be efficiently used in camomile for genotyping and for the development of molecular markers, which have proven their efficiency already in many crop plants e.g. on barley (Ordon et al., 1999, Scheurer et al., 2001).

Furthermore, a segregating F_2-population has been developed based on genetic similarity and phenotypic data. Phenotypic data confirmed a monogenic recessive mode of inheritance (Horn et al., 1988) of the high (-)-α-bisabolol content. In the case of a tetraploid population the observed segregation ratio of 1:3 can only be explained by the presence of only one dominant allele in the parents. This was a happenstance as in comparison to four dominant alleles (1:35) the number of informative genotypes is higher. Furthermore, the segregation ratio of 4:3:9 for high(-)-α-bisabolol content : high bisabololoxide B content : high bisabololoxide A, A/B content (Tab.1) confirms assumptions on the genetics of the bisaboloide synthesis (Horn et al., 1988). Based on these phenotypic data first markers have been developed using bulked segregant analysis which in comparison to the development of near isogenic lines (NILs) can be considered as an effective and time saving method in camomile.

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Tables

Table 1. Segregation ratios of the F_2-generation(s) regarding bisaboloides and χ^2-values.

F_2	N plants	(-)-α-bisabolol high	(-)-α-bisabolol low	χ^2 1:3	bisabolol-oxide B high	bisabolol-oxide A high	χ^2-1:3	χ^2-4:3:9
25/1	307	81	226	0.31	54	172	0.15	0.46
25/2	183	47	136	0.05	35	101	0.04	0.08
59/1	190	38	152	2.53	26	126	5.05	7.92
59/3	161	37	124	0.35	35	89	0.69	1.06
Σ	841	203	638	0.33	150	488	0.75	1.65

Figures

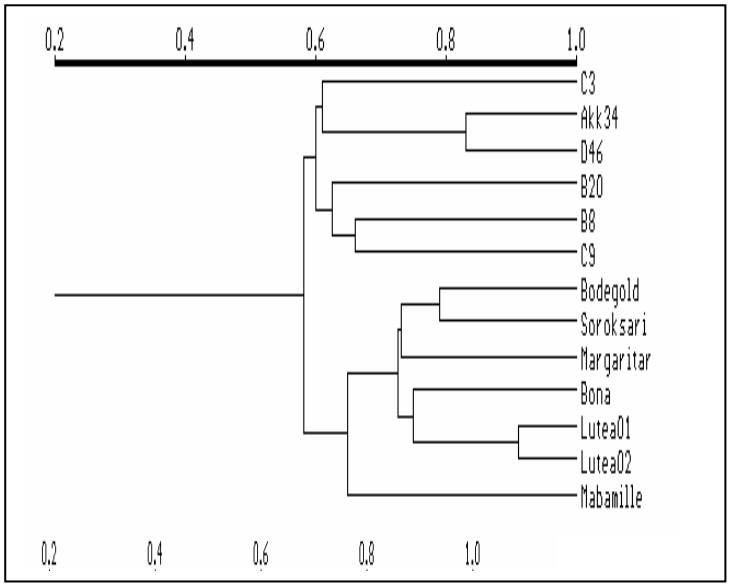

Fig. 1. Genetic relationships between camomile genotypes based on an UPGMA-cluster analysis (A: RAPDs, B: AFLPs) of the genetic similarity (Jaccard 1908) estimated by 18 RAPD-primers (corresponding to 445 bands) and 16 AFLP-primer combinations (corresponding to 1507 bands), respectively.

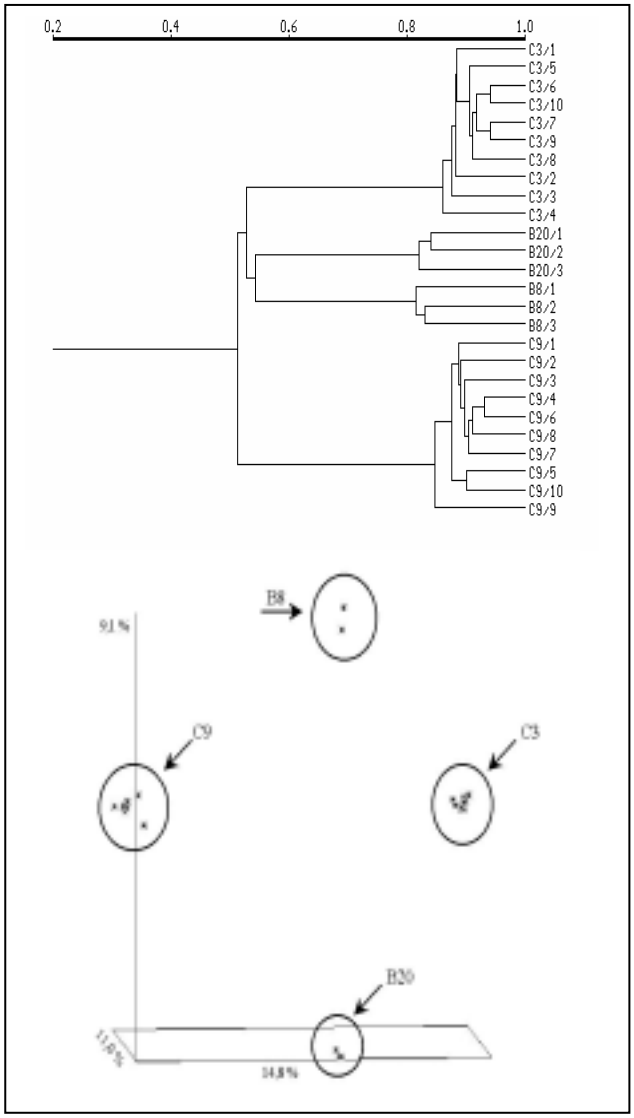

Fig. 2. Genetic relationships between four self-pollinated lines based on an UPGMA-cluster analysis (A) and a principle co-ordinate analysis (B) of the genetic similarity (Jaccard 1908) determined with 22 RAPD-primers (corresponding to 537 bands).