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RFLP analysis in chrysanthemum. I. Probe and primer development

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Abstract In order to study genetic variability at the DNA level in chrysanthemum (*Dendranthema grandiflora* Tzvelev) *Pst*I and *Hin*dIII genomic libraries were constructed. Probes from both libraries were tested for the presence of restriction fragment length polymorphisms (RFLPs). Of the probes from the *Pst*I library 91% appeared to hybridize to low-copy genes, while only 35% of those from the *Hin*dIII library appeared to do so. The *Pst*I probes were used in further analyses as 79% of them showed RFLPs, whereas the *Hin*dIII low-copy number probes gave only 14% polymorphic patterns. Because of the hexaploid character of chrysanthemum, complex patterns generally consisting of 6–12 fragments were visible on a Southern blot after hybridization. To simplify the genetic analysis, locus-specific polymerase chain reaction (PCR) primers were developed that gave simple polymorphic patterns in a number of cases. The RFLP probes and primers developed will be used in future marker-assisted selection in this polyploid crop.

Key words RFLP · PCR · Chrysanthemum · Genetic diversity · Polyploidy

Introduction

Chrysanthemum [*Dendranthema grandiflora* Tzvelev or *Chrysanthemum morifolium* Ramat (synonym) Anderson 1987], whose breeding started 3000 years ago in China and Japan, is a major horticultural crop in the Netherlands. The species forms part of a hexaploid species complex comprising six or seven species (Dowrick 1953). On average, 54 chromosomes are present, although counts of 36–75 are possible (Langton 1989). The genomic make-up varies among the aneuploid chrysanthemum cultivars (Endo and Inada 1992), and the exact origin of the genomes in this hexaploid species is unknown. Whether the species is an allohexaploid or an autohexaploid is not clear, although regular bivalents are formed during meiosis (Watanabe 1977; Dowrick 1953), indicating an allopolyploid origin. The presence of carotenoid pigmentation, however, appears to be inherited in a hexasomic way (Langton 1989). Chrysanthemum has a strong sporophytic self-incompatibility system, the genetics of which has not yet been completely resolved (Zagorski et al. 1983; Stephens et al. 1984). Selfing is generally not possible, although some plants are pseudo-self-compatible (Anderson et al. 1992). The success rate of crosses between related and unrelated cultivars is low, and usually only between 5% and 50% of crosses between sibs in an F_1 are compatible (Drewlow et al. 1973; Ronald and Ascher 1975; Zagorski et al. 1983; Stephens et al. 1984). Due to its unknown origin, polyploidy and self-incompatibility the genetics of chrysanthemum are difficult to study.

The breeding of chrysanthemum has usually been accomplished without the use of modern molecular techniques and without any knowledge of the genetics of the desirable characters. In many agricultural crops, traditional breeding techniques have been supplemented with the use of molecular markers in marker-assisted selection (MAS) and in guiding the introgression of desirable genes from related species. However, restriction fragment length polymorphism (RFLP) studies in horticultural crops are scarce so far.

In the last 10 years RFLPs have been the markers most often used for MAS. However, their use is labour intensive, and radioactivity is needed when there is a demand for high sensitivity. Therefore, an attractive option is to convert RFLP assays into polymerase chain reaction (PCR)-based techniques. In the case of chrysanthemum, this is particularly tempting because the three

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original genomes that compose the present genome may be homologous to such an extent that a probe originating from one contributing progenitor species may hybridize to homologous sequences of two or three genomes contributing to the total chrysanthemum genome. A PCR reaction with primers based on the probe may be more specific and amplify a locus from only one of the contributing progenitor species. The conversion of an RFLP assay into a PCR method has been successfully applied by Livneh et al. (1992) in pepper and by Williams et al. (1991) in rice.

As a part of a large project to develop insect-resistant chrysanthemum varieties, we have investigated the presence of molecular variability using RFLPs in chrysanthemum. The results of this investigation are presented here.

Materials and methods

Isolation of genomic plant DNA

Fresh leaf tissue was supplied by three Dutch chrysanthemum breeders. The leaves were ground in liquid nitrogen and freeze dried under vacuum. DNA was extracted from the dry powder according to Saghai-Maroof et al. (1984) with several modifications. These were a double chloroform/isoamylalcohol (24:1) treatment, the use of cold absolute ethanol to precipitate the DNA, two washes in 76% ethanol/0.2 *M* NaAc prior to the wash in 76% ethanol/10 m*M* NH_4OAc and the dissolving of the DNA in 10 m*M* Tris/1 m*M* EDTA (pH 8.0). The DNA concentration was estimated on an agarose gel stained with ethidium bromide. The yield was approximately 100 µg DNA from 400 mg dried powder.

DNA digestion and blotting

Six individuals, namely the parents of a testcross, two F_1 individuals and two commercially available cultivars, were used for making test blots. From each individual 10 µg DNA was digested with the enzymes *Eco*RI, *Hin*dIII or *Eco*RV (Gibco-BRL). The fragments were separated by electrophoresis on a 0.7% agarose gel in 1 × TBE (45 m*M* Tris-borate, 1 m*M* EDTA) at 1 V/cm for 20 h. Southern blots were made onto Genescreen Plus membranes (Du Pont) according to the instructions of the manufacturer. To fix the DNA onto the membrane we incubated it for 1 min in 0.4 *M* NaOH and then for 1 min in 0.2 *M* Tris-HCl, pH 7.5/1 × SSC. After the membrane was dried for 15 min at room temperature it was UV-crosslinked in the Stratalinker (Stratagene) and baked for 2 h at 80 °C.

Construction of genomic libraries and screening

The enzymes *Hin*dIII and *Pst*I (Gibco-BRL) were used to digest 20 µg genomic chrysanthemum DNA. After gel electrophoresis of the digested DNA the 600- to 7000-bp fraction was cut out of the gel and purified using the Gene Clean Kit (BIO 101). The genomic fragments were ligated in a de-phosphorylated pEMBL 9 *Hin*dIII-digested vector or in a pEMBL 8 *Pst*I-digested vector using T4 DNA ligase (Boehringer). The pEMBL plasmids are the pBR322-derived plasmids described by Cesareni and Murray (1987).

The ligation mixtures were used to transform competent *E.coli* SURE cells (Stratagene) according to the manufacturer's instructions. Plasmid DNA was isolated using the alkaline-lysis method of Birnboim (Sambrook et al. 1989). Restriction enzyme analysis and gel electrophoresis was used to estimate the size of inserts. To investigate the copy number of the cloned inserts we screened the recombinant plasmids by dot-blot hybridization to labelled genomic DNA.

Probe isolation

For probe isolation the recombinant plasmids were first digested with *Pst*I or *Hin*dIII (Gibco-BRL), and after gel electrophoresis the probe was purified with the Gene Clean Kit. Several probes, with a maximum length of 2000 bp, were amplified by the PCR. For this purpose the plasmids were diluted 200 times. The primers used to amplify the probes, positioned on the pEMBL plasmids just outside the multiple cloning site, were 5′-GTATGTTGTGTGGAATTGTGAGCGG-3′ and 5′-GATGTGCTGCAAGGCGATTAAGTTG-3′. The PCR mixture was composed of 100 pg plasmid DNA, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 20 m*M* Tris-HCl, pH 8.3, 0.01% gelatin, 0.1 m*M* of each d*NTP*, 50 pmol of each primer and 1 unit AmpliTaq (Perkin Elmer) in 50 µl. Mineral oil was used to cover the reaction. The PCR programme was run on a Perkin Elmer DNA Thermal cycler 480: 7 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 65 °C and 2 min at 72 °C. A last step of 5 min at 72 °C completed the reaction. The reaction products were loaded onto a preparative gel to isolate the amplified probes. The Gene Clean kit was used to purify the DNA.

Probe labelling and hybridization

Aliquots of 10 ng of the probes were labelled with α-[^{32}P]-dCTP according to the method of random hexamer priming (Feinberg and Vogelstein 1983). After labelling, the probe was purified using a push column (Stratagene). Prehybridization of the blots was at 65 °C in 0.2 ml buffer/cm^2 (1 *M* NaCl, 10% dextran-sulfate, 1% SDS and 0.1 mg/ml denaturated herring sperm DNA). The blots were hybridized overnight at 65 °C in 0.1 ml buffer/cm^2 as mentioned above with 10^6–10^7 cpm of denatured labelled probe/ml buffer. The blots were washed as follows: 5 min at room temperature with 2 × SSC/0.1% SDS, 5 min at 65 °C with 2 × SSC/0.1% SDS and twice for 30 min at 65 °C with 0.5 × SSC/0.1% SDS. The membranes were exposed to a Kodak X-Omat film using one intensifying screen at −80 °C for 1–5 days. For reprobing, the membranes were stripped by boiling for 5 min in 0.1 × SSC/1% SDS.

Probe sequencing and primer testing

The probes that were to be sequenced were cloned in M13 mp18 and/or M13 mp19. Single-strand DNA was isolated according to Sambrook et al. (1989). Sequencing reactions were performed with the T7-Sequencing Kit (Pharmacia) according to the manufacturer's directions. Gels were made and run according to the manufacturer's instructions (BRL-Sequencing gel electrophoresis system). In the case of a small probe (about 700 bp) the complete sequence was determined; otherwise, only the ends of a probe were sequenced. The computer programmes PC-Gene and OLIGO were used to analyse the resulting sequences and to find suitable obligonucleotides for PCR. Oligo's were synthesized with a DNA synthesizer type 381 A (Applied Biosystems).

A PCR test was performed to test each primer set derived from the sequences of a probe. The PCR was the same as that used for probe isolation, except for some small changes. Samples of 100 ng of genomic DNA were used, and the $MgCl_2$ concentration was varied between 1.0 and 3.5 m*M*. The annealing temperature depended on the primer set used and was chosen to be 5 °C below the melting temperature of the primers, which was calculated with the following formula: T_m = (number of $G + C$ bases) × 4 °C + (number of $A + T$ bases) × 2 °C. After PCR amplification 25 µl of the reaction was analysed on a 1.5% agarose gel. When no polymorphism was detected after PCR, the DNA was precipitated and several restriction enzymes were tested.

Results

Construction of genomic library

A total of 612 recombinant plasmids were screened on dot-blots to determine their copy number; of these 245 were *Hin*dIII derived and 367 carried a *Pst*I fragment. The probes were divided visually into five classes on the basis of the signal strength of the dot on the autoradiogram. Class 1 gave a weak signal, comparable with or only slightly stronger than the signal of the negative control, which was the plasmid without an insert, and class 5 gave a strong signal, comparable to the signal of the positive control, which was 100 ng chrysanthemum genomic DNA used for cloning. A majority of the *Pst*I probes (91%) appeared to fall into class 1, whereas only 35% of the *Hin*dIII probes appeared to be in this class. We subsequently used the clones from signal class 1 as probes on Southern test blots to test whether these probes hybridized to polymorphic fragments.

Analysis of the genomic probes

Twenty-one *Hin*dIII probes were tested on test blots as just 3 of them showed polymorphisms. Of the 131 *Pst*I fragments tested 103 showed variation between the cultivars tested with the restriction enzymes *Eco*RI, *Eco*RV and *Hin*dIII, and for this reason we decided to proceed further with the *Pst*I probes. Not all of them were suitable for RFLP linkage analysis because some of them gave too many bands, a relatively strong background signal and/or a dark smear. Probes giving a smear had an average length of 3300 bp, which is high compared to the length distribution of all *Pst*I probes with signal strength 1 (Table 1). A high percentage (50%) of the non-polymorphic probes had a length of more than 3000 bp. The use of short probes (shorter than 2500 bp) is, therefore, preferred over longer probes because of the higher success rate (good RFLP patterns and no smears). Figure 1 shows an example of an autoradiogram of a genomic blot after hybridization with a *Pst*I probe.

Of the 121 polymorphic *Pst*I probes 41 were selected that were useful for linkage analysis (Table 2). For each probe the restriction enzyme selected was the one that gave the best scorable RFLP pattern on the blot. Table 2 shows the length of the probes and the restriction enzymes selected. More than 70% of the 41 probes selected gave useful RFLP patterns with *Eco*RI and *Eco*RV. We did not find any probes that showed variation with *Hin*dIII and not with *Eco*RI or *Eco*RV. One probe was polymorphic with *Eco*RV only. In most cases all three enzymes produced polymorphisms with the same probe, suggesting insertion/deletions and not point mutations.

There was no relationship between the length of a probe and the number of bands on the Southern blot, nor was there a relationship between the length of the probe and the length of the fragments on the Southern blots.

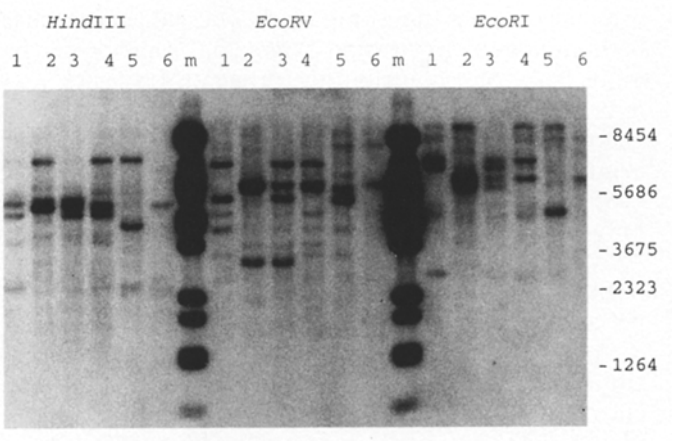

Fig. 1 Autoradiogram of chrysanthemum test filter hybridized to *Pst*I probe WR339. *Lanes 1, 2* parents of the testcross (CH53 and CH54), *lanes 3, 4* two of their offspring, *lanes 5, 6* two different cultivars (CH1 and CH6). The size marker (*m*) is lambda cut with *Bst*EII

Table 2 Numbers of selected *Pst*I probes, their lengths, and their partitioning according to the restriction enzyme that gave the best RFLP pattern on the test blot

Length of the probes	Total number of tested probes	Number of selected probes	Enzyme with best RFLP pattern		
			*Hin*dIII	*Eco*RI	*Eco*RV
< 1000 bp	33	18	2	8	8
1000–2000 bp	34	13	2	5	6
2000–3000 bp	33	5	2	1	2
> 3000 bp	30	5	3	1	1
Total	131	41	9	15	17

Table 1 Length distribution of the *Pst*I probes with signal strength 1

Length of the probe (bp)	Number	%
< 1000	63	19
1000–2000	131	39
2000–3000	67	20
> 3000	73	22
Total	334	100

Probe sequencing and primer testing

We selected 6 RFLP probes of varying lengths that gave good RFLP patterns. Either the total sequence or only the ends of the sequence were determined. We used the computer programme OLIGO to search for optimal primers. The length of the primers varied between 19 and 23 bases, depending on the composition of the DNA

Table 3 Review of the probes and the sequence and composition of the derived primers

Probe	Length (bp)	Primers	Sequence of the primers 5′-3′	Length of the primer (b)	%GC	T_m (°C)
325	1000	325P1	GGGCTTTTATTTGTCCACCG	20	50	60
		325P2	GGCAAAAAACAACTTCCCG	19	45	56
339	750	339P1	GACCTCCACGCCGCAACTAC	20	65	66
		339P2	ATTGTGACCTAACACCCCGC	20	55	62
427	1900	427P1	CCATAAATCCGCATACTCCAA	21	43	60
		427P2	ACGACGGCCAGTGCCAAGC	19	68	64
428	2800	428P1	CGGTGCATTTGTTGTTGATGTTG	23	43	66
		428P2	GGGGAAGAGAAACAGGTGGTGGC	23	58	74
432	800	432P1	ATGGGATTACTTTTATGCGAAT	22	32	58
		432P2	CGAATAAGAGGTGGAAAAATG	21	38	58
450	700	450P1	CACCGCTTTTCCTATTGCC	19	53	58
		450P2	TGCAGTTGAGTGGGGAATT	19	47	56

sequence. Primers with a GC content of at least 50% could not always be found because of the abnormal composition (high AT%) of some of the sequences of the chrysanthemum DNA. Therefore, some of the primers have a GC content lower than 50%. Table 3 shows the primer sequences as well as the GC content of the selected primers.

All primer sets were tested by PCR with a positive control (recombinant plasmid) and a negative control (water instead of DNA). The cultivar used for the construction of the genomic library, three cultivars from different breeders and the two parents of the testcross were chosen as genomic chrysanthemum DNA to see whether polymorphisms could be detected. All of the primer sets showed the correct band after reaction with the positive control, and no signal was detected in the negative control. Table 4 shows the results of the primer sets after PCR and the optimal concentration of $MgCl_2$, which varied between 1.5 m*M* and 2.5 m*M*.

Table 4 PCR results with the derived primer sets and their optimal $MgCl_2$ concentration

Probe	Primer set	Optimal $MgCl_2$ concentration (m*M*)	PCR amplification[a]	Results of the digestion after PCR[a]
325	325P1/325P2	2.0	–	n.d.
339	339P1/339P2	1.5	+	+[b]
427	427P1/427P2	2.0	–	n.d.
	427P1/427P4	2.0	–	n.d.
	427P3/427P2	2.0	–	n.d.
	427P3/427P4	2.0	–	n.d.
428	428P1/428P2	2.5	–	n.d.
432	432P1/432P2	2.0	+ n.v.	+[c] n.v.
450	450P1/450P2	2.5	+	n.d.

[a] +, Amplification product; –, no product; n.v., no variation; n.d., not done

[b] Variation after digestion with *Rsa*I, *Hae*III and *Taq*I.

[c] *Alu*I, *Hin*PI, *Mse*I, *Taq*I and *Sau*3A digested the PCR products, but no variation was detected; *Rma*I, *Rsa*I, *Hin*fI and *Hae*III were tested and did not digest the PCR products.

Three of the six primer pairs were successful in amplifying the positive control but did not amplify any of the genomic DNAs, or if they did so, the yields were very low. The other three primer pairs amplified genomic DNA, although primer pairs 339P1/P2 and 432P1/P2 did this to different extents for different cultivars. Primer pairs 339P1/P2 and 450P1/P2 gave polymorphic patterns (Fig. 2), whereas primer pair 432P1/P2 did not show polymorphism, not even after digestion with several restriction enzymes (results not shown). Primer pair 339P1/P2 not only gave polymorphic patterns after amplification, but showed additional polymorphisms after digestion with restriction enzymes.

Because of the negative results with primer set 427P1/427P2 on the genomic DNAs and because of the length of the probe (1900 bp), we searched for new primers. Primer 427P3 (= 5′ AACAATCTTCATC-TCACGACG 3′) could replace primer 427P1 in reaction with 427P2, and we synthesized 427P4 (= 5′ AAAAT-TCGTCTGAGTTCCTGG 3′), which could replace primer 427P2 in reaction with 427P1. In addition, 427P3 and 427P4 could be used together in a reaction, and the fragment then produced was about 400 bp shorter than the fragment produced with 427P1/427P2. These additional primers were also not able to amplify the fragment from genomic DNA. We searched for new primers to replace 428P1 and 428P2 but because of the abnormal structure of the DNA sequence of probe 428, with only 35% GC and long AT-stretches, we did not find suitable primers that could produce a band of 1500–2000 bp in PCR.

Discussion

This study is the first to show the presence of RFLPs in chrysanthemum. Up to now there have been only a few reports on the use of RFLPs in horticultural crops, and most of these are concerned with cultivar identification, as in the rose cultivar identification by Rajapakse (1992).

In hexaploid *Triticum aestivum* only 20% of the probes revealed polymorphisms when two to four re-

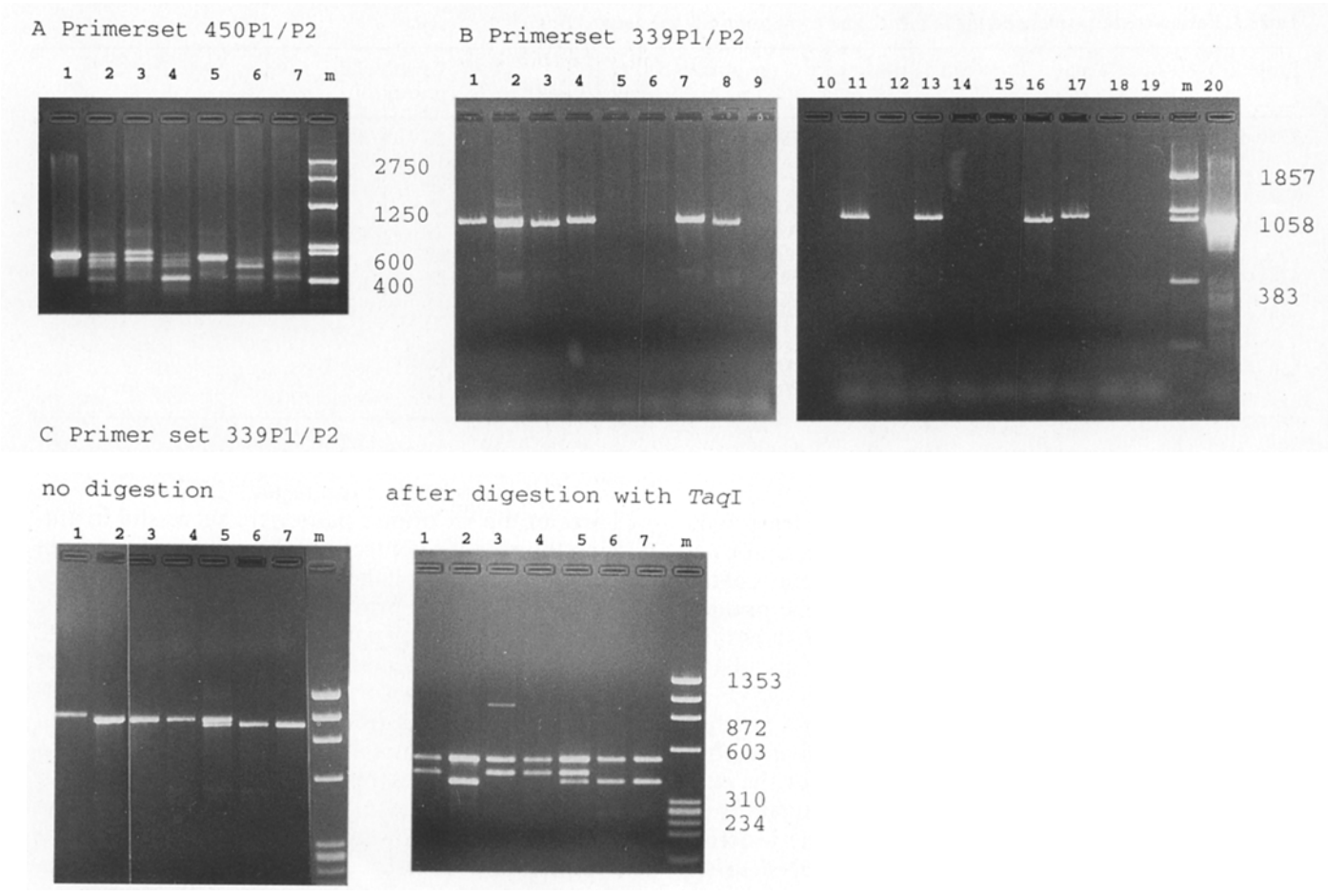

Fig. 2 PCR-generated fragments of chrysanthemum as obtained with primers derived from RFLP probes. **A** Primer set 450P1/P2: *lane 1* postive control (clone 450: plasmid with insert), *lanes 2–7* different chrysanthemum cultivars (CH11, A3, HR04, FR06, CH53, CH54), *lane 8* marker (*m*). **B** Primer set 339P1/P2: *lanes 1–18* different chrysanthemum cultivars (A1, A3, A5, B1, B4, B7, HR1, HR4, HR6, HV1, HV2, HV4, FR5, FR6, FR7, FV3, FV14, FV15), *lane 20* marker (*m*), *lane 21* positive control (p, clone 339: plasmid with insert). **C** Primer set 339P1/P2: *lanes 1,2* the parents of the testcross (CH53, CH54), *lanes 3–7* five of their offspring, after amplification and after amplification and digestion with *Taq*I, *m* marker (Registered names of cultivars can be obtained from the breeders)

striction enzymes were used (Liu et al. 1990). In tetraploid peanut it also appears that polymorphisms between cultivars are rare, although diploid and tetraploid wild progenitors show considerable variation (Kochert et al. 1991). In chrysanthemum, on the other hand, there is an extremely high level of polymorphism, although the species is hexaploid and has been bred for many centuries. This is probably due to the complex composition of the species and the strong self-incompatibility system. In a previous study it was shown that chrysanthemum is highly polymorphic at the DNA level (Wolff and Peters-Van Rijn 1993). In that preliminary study we used the technique of random amplified polymorphic DNAs (RAPD, Williams et al. 1990) and were able to show that the variation within an F_1 generation and between cultivars was high: 18 randomly chosen cultivars could be distinguished from each other using two random primers. Preliminary results from the screening of RFLP probes here has revealed even higher levels of variation between cultivars.

In chrysanthemum we were much more successful using genomic probes than using cDNA probes (K. Wolff unpublished results). In other crops various results have been found with respect to the comparative usefulness of cDNA and genomic DNA cloning. Helentjaris et al. (1986) reported that in maize cDNA clones were less useful than genomic probes, whereas in tomato cDNA clones were very useful. In *Pinus taeda* as well as in citrus, genomic probes were strongly preferred over cDNA clones (Devey et al. 1991; Durham et al. 1992). The preference for either a genomic library or a cDNA library is species dependent and possibly also dependent on the specific cloning technique used.

It was clear from the dot-blot hybridization with genomic DNA that most *Pst*I probes are low-copy sequences (91%), whereas *Hin*dIII probes have a high percentage of middle- to high-copy sequences. This phenomenon has also been found with many other plant species (e.g. Figdore et al. 1988; Nodari et al. 1992). This can be explained by the fact that *Pst*I is a methylation-sensitive enzyme, and because many repeated sequences are methylated they will be underrepresented in a genomic library that is made with size-selected fragments (600–7000 bp) after digestion with a methylation-sensitive restriction enzyme.

The three restriction enzymes used (*Eco*RI, *Eco*RV and *Hin*dIII) were shown to be satisfactory for screening probes. *Eco*RI and *Eco*RV generally gave the best polymorphic patterns. In subsequent research we will use only one enzyme in combination with each probe, namely the one that gives the best scorable RFLP pattern. Our results show that if variation is found with one enzyme, other enzymes will also give polymorphic patterns. This points at addition/deletions or rearrangements as the cause of this variation. The high frequency of rearrangements as a cause of variation is a general phenomenon in plant species (Figdore et al. 1988). For this reason it would be unnecessary to use more than one enzyme per probe.

The development of sequence-specific primers was successful for three out of six primer sets. The reaction was optimized by using a range of Mg^{2+} and DNA concentrations. The positive control was amplified with all six primer pairs. The reason why genomic DNA sequences could not be amplified may be because of structures in the DNA, either folding or loop formation. Alternatively, the products may be too long in some instances, e.g. probes 427 (1.9 kb) or 428 (2 kb), for successful amplification from genomic DNA. Those primer pairs that amplified genomic DNA showed polymorphisms after restriction digestion in two out of the three cases. The resulting patterns were reliable and much more simple than the RFLP patterns. Therefore, we believe that designing primers on probe sequences is a valuable strategy for detecting polymorphisms in chrysanthemum.

Anderson et al. (1992) studied inbreeding depression in inbred lines of several chrysanthemum individuals that were selfed or inbred for up to five generations. It appeared that inbreeding depression, expressed as germination and survivorship, was more severe in more advanced inbreds than in inbred progeny of F_1 or F_2 individuals. Therefore, inbred lines are probably not useful as starting material for MAS in chrysanthemum. As chrysanthemum is highly heterozygous because of its self-incompatibility system and generations of outcrossing it is useful to study markers and characters in F_1 sibs of a suitable biparental cross. This strategy has been succesfully applied in linkage mapping with diploid potato lines (Gebhardt et al. 1989, Ritter et al. 1990).

Most of our probes gave relatively complex RFLP patterns relative to what is generally seen in diploid species. Often 6–12 bands per lane were visible. The fact that no real single-copy sequences seem to have been cloned means that there are many gene duplications and/or that there is strong homology between homologous sequences of the different progenitor genomes of chrysanthemum. For some polyploid species the best strategy is to make maps using a diploid progenitor of the speices, as this is far less complicated. In potato, maps have been made using such diploid individuals (Gebhardt et al. 1989). For some other polyploid species, like chrysanthemum, this is not possible as closely related diploid species are not known. Recently, Wu et al. (1992) and Sorrells (1992) have shown theoretically that it is generally possible to use the same method in polyploid species as that used in diploids to make a map and localize quantitative trait loci (QTL) with DNA markers. It is based not on the genotyping of individuals of a segregating population but by scoring the absence and presence of single-dose restriction fragments (SDRF).

In our preliminary study into RFLPs in chrysanthemum we have shown that RFLP analysis is feasible in chrysanthemum, a hexaploid with a large genome size. The construction of a genomic library and the development of a PCR test from genomic probes has been described. In a subsequent study the levels of variability among related species, cultivars and clonally derived cultivars will be described, as well as the segregation of polymorphic markers and QTLs in an F_1 population.

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