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Selection of monosomic addition plants in offspring families using repetitive DNA probes in *Beta* L.

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Abstract The distribution of two repetitive DNA probes *Sat-121* and *PB6-4*, specific for the section *Procumbentes* of the genus *Beta,* was tested in 16 *B. patellaris* monosomic addition families using a dot-blot hybridization procedure. All monosomic additions were accurately distinguished from diploid sib plants with both DNA probes. The probe *PB6-4*, with the strongest signal after hybridization, was selected for rapid screening of an extensive number of putative monosomic additions in *B. patellaris* or *B. procumbens* addition families using a squash-blot hybridization procedure. The probe *PB6-4* detected 118 monosomic additions in 640 plants (18.4%) in eight different *B. procumbens* addition families. The addition family with chromosome 4 of *B. procumbens* was semi-lethal and could not be tested. The distribution of *PB6-4* in *B. patellaris* addition families was confirmed in 63 addition families using the squash-blot procedure. In 4580 plants of these addition families, 628 individual monosomic additions (13.7%) were found. The relationship of the morphological characteristics of monosomic addition plants to the results of the squash-blot hybridization (plants with signal) using probe *PB6-4* is quite rigorous but not complete. The correlation between plants with a signal and chromosome number (2n=19) is complete. These results indicate that sequences present on *PB6-4* are probably present on all chromosomes of *B. patellaris* and *B. procumbens*. The possibility of utilizing the sequence information of *Sat-121* for a PCR-based assay to screen for putative monosomic addition plants was also investigated as an alternative to chromosome counting. The DNA-amplification profiles using the primers *REP* and *REP.INV* clearly distinguished monosomic addition plants from their diploid sibs.

Key words *Beta vulgaris* · *Beta patellaris* · *Beta procumbens* · Monosomic additions · PCR · Repetitive probe

Introduction

Sugar beet is a relatively young crop, which supposedly has a narrow genetic base (Van Geyt et al. 1990). Three wild species in the section *Procumbentes* are either resistant or immune to *Heterodera schachtii* Schm., the beet cyst nematode (BCN). Transfer of nematode resistance from these wild relatives into sugar beet has been a serious concern since 1940 (Bosemark 1969; Coons 1975; Nakamura et al. 1991).

Savitsky (1975) was the first to produce monosomic additions from crosses between triploid interspecific hybrids and diploid cultivated beets. This material was then used to transfer the gene(s) for resistance to the genome of cultivated beet (Savitsky 1978). The same strategy was followed by Speckmann and De Bock (1982), Heijbroek et al. (1983), Löptien (1984), Speckmann et al. (1985); Jung and Wricke (1987); Heijbroek et al. (1988) and Lange et al. (1990).

Two full series of monosomic additions in beet have been described (Lange et al. 1988; Van Geyt et al. 1988; Reamon-Ramos and Wricke 1992). In offspring-families of monosomic additions, plants having 2n=19 occur with frequencies of about 10–25%. Several such plants can be recognized on the basis of a deviating morphology. However, this method is not 100% reliable, and can only be used in combination with the counting of chromosome numbers, which is very laborious.

Therefore, methods which lead to a rapid and reliable identification of monosomic additions are valuable. The so-called squash-dot hybridization technique (Hutchinson et al. 1985) is particularly valuable in genetics and in breeding programmes where large numbers of plants need to be assayed (Flavell 1982). For this approach, probes with high specificity, and if possible a high copy number in the orig-

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inal genome, are needed (e.g. Hutchinson et al. 1985; Schmidt et al. 1990).

The genome of *B. vulgaris* contains 60% middle and highly repeated DNA sequences. Some members of this genome fraction are organized as tandemly arranged DNA (satellite DNA and rRNA genes), while others are clusters of simple sequence repeats (microsatellites) or dispersed sequence families (Schmidt and Heslop-Harrison 1993; Schmidt et al. 1993). The first satellite DNA family in the nuclear genome of *B. vulgaris* was isolated as a *Bam*HI-sequence family (Schmidt and Metzlaff 1991). Secondly, a sugar beet satellite DNA was isolated as a *Eco*RI sequence family that showed no homology to the first one and was present in three sections (*Beta, Corollinae,* and *Nanae*) of the genus (Schmidt et al. 1991). The probes (*PTS1* and *PTS2*) with a high degree of *B. procumbens* specificity were used for the squash-blot hybridization with the aim of screening monosomic additions carrying an alien *B. procumbens* chromosome (Schmidt et al. 1990). The same strategy was used to identify individuals from monosomic addition lines carrying the fragmented chromosome. The dispersed repetitive-DNA probe (*P643*), in conjunction with a squash-blot hybridization, was chosen to identify nematode-resistant individuals carrying *Procumbentes* chromosomes (Jung and Herrman 1991).

New members of the *Procumbentes*-specific satellite DNA family *Sat-121,* which was isolated from a genome subtraction library of AN5-90 [a BCN-resistant fragment addition *(pat-1)*], were characterized by Salentijn et al. (1992). The organization of *Sat-121* in the vicinity of the beet cyst nematode resistance locus *Hs1* in *B. patellaris* and *B. procumbens* was investigated by Salentijn et al. (1994).

The present study describes the distribution of the repetitive *Procumbentes*-specific DNA probes *Sat-121* and *PB6-4* in genomes of *B. procumbens* and *B. patellaris* using both dot-blot and squash-blot hybridization methods. It reports on the identification and screening of extensive numbers of monosomic additions from offspring of *B. patellaris* and *B. procumbens* addition families using the repetitive DNA probe *PB6-4* and the squash-blot hybridization method. The correlation between expected monosomic addition morphotype in relation to the results of the squash-blot hybridization (plants with signal), is also described, as well as the correlation between plants with signals and the results of counting chromosome numbers. Finally, the application of the polymerase chain reaction (PCR) method for the identification of monosomic additions, as an alternative to chromosome counting, will be discussed.

Materials and methods

Plant material

The plant material consisted of *B. vulgaris,* the wild species *B. patellaris* (2n=36) and *B. procumbens* (2n=18), monosomic addition families (2n=19) representing the complete set of nine different chromosomes of *B. procumbens* in diploid *B. vulgaris* (Van Geyt et al. 1988), and 73 unidentified monosomic addition families (2n=19) of *B. patellaris,* the origin of which was described by Speckmann and De Bock (1982).

Preparation of repetitive DNA probes

Two highly repetitive DNA probes named *Sat-121* (Salentijn et al. 1992; referred to as *121-3*) and *PB6-4,* both specific for the section *Procumbentes* of the genus *Beta,* were used in this study. The probes were kindly donated by Dr. N.N. Sandal, University of Aarhus, Aarhus, Denmark. *Sat-121* (169 bp) was derived from chromosome 1 of *B. patellaris* and *PB6-4* was obtained from a genomic library of *B. procumbens* (Salentijn et al. 1994). *PB6-4* has a size of 1700 bp and contains several *Sat-121* core sequences interspersed with anonymous sequences (N. N. Sandal, personal communication).

Plasmid inserts (*Sat-121* was cloned in *SK+* and *PB6-4* in *pUC19*) were digested with *Kpn*I (*SK*$^+$) and *Eco*RI (*pUC19*) and separated from the vector by agarose-gel electrophoresis followed by purification from the gel by freeze-squeezing. Inserts were labelled with a randomly primed DNA labelling kit (USB) with ^{32}P α-dATP.

Dot-blot hybridization

Total genomic DNA was extracted from frozen leaves according to Van der Beek et al. (1992); 1.5 µg of DNA from each sample was denaturated by heating to 100°C for 10 min and spotted onto a dry Hybond-N$^+$ membrane, which was then dried, crosslinked with UV for 45 s and hybridized with the ^{32}P-labelled DNA probes *Sat-121* and *PB6-4.* For hybridization with the *Sat-121* probe, five monosomic addition plants and their diploid sibs, identified by chromosome counting, were used. For *PB6-4* 16 monosomic addition plants and their diploid sibs were used. Total DNA samples of *B. vulgaris, B. patellaris, B. procumbens* and the two plasmid inserts *Sat-121* and *PB6-4* served as controls.

Squash-blot hybridization

For squashing, two sheets of Whatman 3-MM paper were immersed in 0.5 M NaOH and placed on a piece of glass. Hybond-N$^+$ membrane was soaked in 0.5 M NaOH and laid on the Whatman 3-MM paper. A plastic sheet with small holes it (5 mm in diameter) was placed on the membrane. Leaf pieces from individual seedlings were squashed onto the Hybond-N$^+$ nylon membrane in two replications. In order to fix enough DNA on the filters, leaf pieces were squashed twice on the same spots. For each family, if possible, 80 individual seedlings were spotted on each membrane. *B. vulgaris, B. patellaris* and *B. procumbens* were used as controls on each membrane. After squashing of the leaf pieces, the membrane was washed in 2×SSC for 2 min, dried overnight and crosslinked with UV light for 45 s.

Southern hybridization

PB6-4 was random primed labelled (Feinberg and Vogelstein 1983) and hybridized overnight (60°C) to the membranes in 1% SDS, 1 M NaCl, 10% dextransulphate, 50 mM Tris-HCl pH=7.5, 100 ng/µl of denatured salmon sperm DNA after a 2 h pre-hybridization. Membranes were washed for 1 h in 0.5×SSC followed by 1% SDS at 60°C for 1 h. The membranes were sealed in Saran Wrap and exposed to X-ray film (Kodak) at –80°C for 1–4 days using intensifying screens.

Chromosome studies

Based either on strong signals in the squash-dot assay or on morphological characteristics (Lange et al. 1988), plants were selected as candidate monosomic additions. To verify the presence of the extra

chromosomes or chromosome fragments, root tips were pre-treated with aqueous 8-hydroxyquinoline (2 mM, 6 h), fixed in acetic-ethanol (1:3 v/v), hydrolyzed in 1 N HCl at 60°C for 6 min, squashed in 45% acetic acid, and stained by carefully lifting the cover slip and adding a drop of 1% aqueous crystal violet (Salentijn et al. 1992).

PCR

To evaluate PCR markers in different monosomic addition families and their diploid sibs, genomic DNA from 16 monosomic additions and their diploid sibs, as well as from *B. vulgaris, B. patellaris* and *B. procumbens*, was used as a template for PCR amplification. PCR was carried out using the primers REP: CGTAAGAGACTATGA and REP.INV: TGAACACCTTTCAAAT. These primers are designed to amplify the interspersed DNA between consecutive *Sat-121* monomeric units (Salentijn et al. 1994).

Results

Determination of the specificity and the distribution of the repetitive-DNA probes *PB6-4* and *Sat-121* in *B. patellaris* addition families

To investigate whether the two repetitive-DNA probes (*Sat-121* and *PB6-4*) are randomly dispersed over all chromosomes, their presence was evaluated in randomly chosen monosomic additions and their diploid sibs. This was done by dot-blot hybridization. Total DNA was extracted from pooled plants of 16 monosomic addition families carrying an extra *B. patellaris* chromosome and their diploid sibs, of which the chromosome number had already been established, and was dot blotted. *B. vulgaris*, *B. patellaris*, *B. procumbens* and the two clones (*Sat-121* and *PB6-4*) were also spotted as controls. Both the repetitive-DNA probes *PB6-4* and *Sat-121* gave an effective signal in all monosomic additions, *B. patellaris* and *B. procumbens,* but not in the diploid sibs and *B. vulgaris*. Upon cross hybridization with each of the probes separately, signal was found on the spot of the other plasmids, confirming that the two repetitive-DNA probes share homology (N. N. Sandal, personal communication). The repetitive-DNA probe *PB6-4* gave strong signals in all 16 distinct *B. patellaris* addition families (Fig. 1). This indicates that *PB6-4* is dispersed over different chromosomes, since the different addition plants carry different chromosomes as judged from their morphotypes. No addition plants without a strong signal for *PB6-4* were found, indicating that the sequence *PB6-4* is possibly present on all chromosomes.

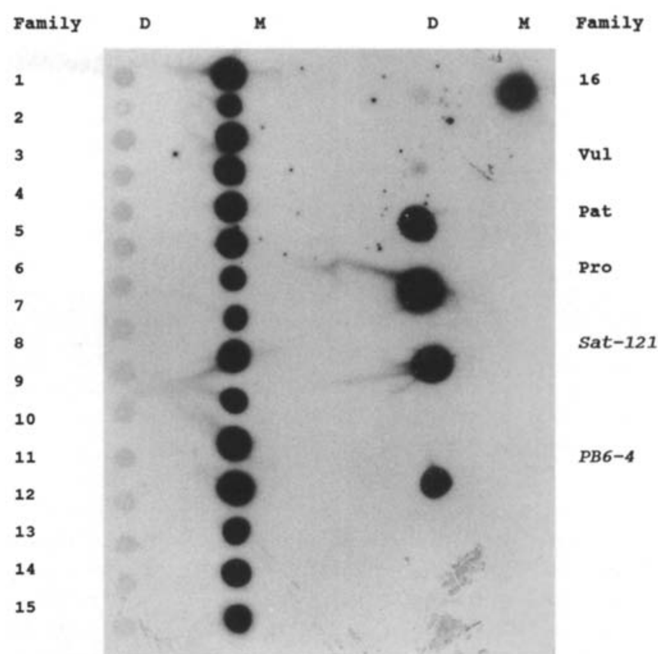

Fig. 1 Dot-blot hybridization of 16 different monosomic addition families (1–16), carrying an extra chromosome of *B. patellaris* in *B. vulgaris* (*M*) and their diploid sibs (*D*), to the *Procumbentes*-specific repetitive-DNA probe (*PB6-4*). *B. vulgaris (Vul)*, *B. patellaris (Pat)*, *B. procumbens (Pro)* as well as two clones, SK^+ with a *Sat-121* insert and *pUC19* with a *PB6-4* insert, which were spotted as controls

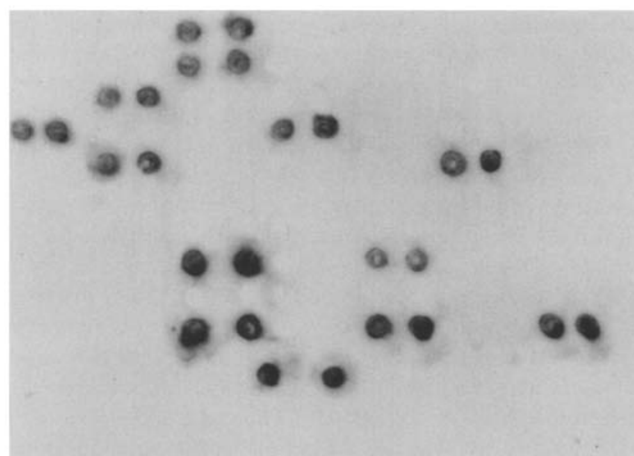

Fig. 2 A squash-blot of 80 individual seedlings from one addition family in two replications and of *B. vulgaris, B. patellaris and B. procumbens* as controls, hybridized to the repetitive-DNA probe *PB6-4*. Addition plants carrying an extra chromosome of *B. patellaris*, as well as *B. patellaris* and *B. procumbens*, gave a strong signal after autoradiography

Identification of monosomic addition plants with the DNA probe *PB6-4* and the squash-blot method

The squash-blot hybridization method (Hutchinson et al. 1985) was used to distinguish putative monosomic addition plants carrying an extra chromosome of *B. procumbens* or *B. patellaris* from diploid sibs, and for rapid screening of numerous plants. The autoradiographs (see Fig. 2 as an example) show a strong hybridization of the DNA probe to the DNA of some of the plants. Such plants are the expected monosomic additions, which contain the whole genome of *B. vulgaris* and just one chromosome of *B. patellaris* or *B. procumbens*. Squashes of *B. patellaris* and *B. procumbens*, serving as controls, also gave a strong signal. No signals were observed in the squashes of *B. vul-*

Table 1 Proportions of deviating and normal morphotypes among plants with and without a signal in the squash-blotting experiments, using *B. procumbens* addition families, and the results of a 2×2 contingency test

Chr. no.	Family name	Number of plants	With signal (2n=19)		Without signal (2n=18)		χ^2 (2×2 contingency)[a]
			Putative	Normal	Putative	Normal	
Pro-1	D1-2-13	80	15	0	0	65	73.57***
Pro-2	D2-2-27	80	14	7	9	50	17.55***
Pro-3	D3-2-17	80	8	12	2	58	15.23***
Pro-5	I3-2-24	80	17	0	1	62	68.81***
Pro-6	D3-2-35	80	4	0	3	73	32.70***
Pro-7	AU6-1-4	80	12	1	0	67	65.70***
Pro-8	D3-2-13	80	12	0	0	68	72.34***
Pro-9	C6-1-3	80	16	0	7	57	45.31***

[a] *** Significant at $P< 0.001$

garis and of many of the tested plants. The latter are thought to be the diploid sibs, not carrying the alien chromosome.

In a total of 640 squashed plants from eight *B. procumbens* addition families 118 plants (18.4%) gave signals. The addition family carrying chromosome 2 of *B. procumbens* has the highest frequency (26.3%), and the family carrying chromosome 6 of *B. procumbens* has the lowest frequency, of monosomic additions (5.0%). In the family carrying chromosome 4 of *B. procumbens,* plants with the expected morphotype died at the seedling stage and squash-blotting was not possible. The result clearly shows the random distribution of this repetitive-DNA probe on at least eight different chromosomes of *B. procumbens.* A total of 4580 candidate plants from the *B. patellaris* addition families were tested yielding 628 plants (13.7%) with a positive signal. The family named A3-1-3 has the highest frequency of monosomic addition plants (30.8%), whereas the family B1-1-285 has the lowest frequency (1.3%). The addition families A5-1-14, A5-1-24 and B1-1-10 do not have individuals giving a signal, suggesting that no monosomic addition plants are present in these families. The observation shows that there is variation between addition families in relation the presence of a signal. To test for heterogeneity between families, a chi-square test was performed on data classified by the presence or the absence of plants with a signal in the different *B. procumbens* and *B. patellaris* addition families. The χ^2 value in *B. procumbens* addition families is significant (χ^2=16.58, *df*=7) at the 5% level (*P*=0.02). The χ^2 value in *B. patellaris* addition families is also highly significant (χ^2=208.2, *df*=65) at the 0.5% level.

Comparison of putative monosomic addition plants for their morphotype per family

For the *B. procumbens* families the morphotype of the addition plants has already been described (Lange et al. 1988). Therefore the plants with such a morphotype could be identified, and the results compared with those of squash-blotting to test in how many cases the morphotype does not predict the addition phenotype, particularly at the seedling stage. In this respect two classes of morphotype (*B. procumbens* putative monosomic addition morphotype versus plants with normal morphotype) were compared with two classes of plants (the addition plants giving a signal, 2n=19, versus plants without a signal, 2n=18) in the eight families of *B. procumbens* and in ten of *B. patellaris.*

In *B. procumbens* addition families, among 118 plants giving a signal 98 had the putative morphotype and 20 had a nearly normal morphotype. Most of the plants (500) showing no signal had the *B. vulgaris* morphotype, but some plants without a signal (22) had a deviating morphotype. The distribution over the two morphotypes is shown in Table 1. A 2×2 contingency test was carried out to determine whether these two characteristics are independent. The χ^2 values were highly significant.

In the *B. patellaris* addition families most of the plants without a signal (604) again showed a normal morphotype while, as is presented in Table 2, the results of plants with a signal varied. Among 115 plants giving a signal, 66 had a deviating morphotype and 49 had a nearly normal morphotype. The contingency test showed highly significant χ^2 values, except for the families B3-1-1 and B1-1-10. Among 80 plants in family B1-1-10 12 candidate plants with the putative morphotype did not give a signal after hybridization, indicating that no correlation exists in this family. Chromosome counting in this family, however, proved that the plants with the putative morphotype had only 18 chromosomes.

Chromosome studies

After squash-blot hybridization, all addition families but three gave clear signals for at least some plants, indicating the addition of a chromosome of *B. patellaris* or *B. procumbens.* To verify the addition, the chromosome number both of putative additions on the basis of morphotype and of plants with a signal were compared in arbitrarily chosen *B. patellaris* and *B. procumbens* addition families. In 13 addition families out of 106 plants, 57 plants with an extra chromosome, a telosomic addition or a fragment addition, gave a signal, while 49 plants with 2n=18 chromo-

Table 2 Proportions of putative and normal morphotypes among plants with and without a signal in the squash-blotting experiments, using *B. patellaris* addition families, and the results of a 2×2 contingency test per family

Family name	Number of plants	With signal (2n=19)		Without signal (2n=18)		χ^2 (2× 2 contingency)[a]
		Putative	Normal	Putative	Normal	
B1-1-8	80	14	7	2	57	34.90***
B1-1-9	78	9	5	9	55	13.61***
B1-1-10	80	0	0	12	68	–
B3-1-1	80	2	10	5	63	0.25
D1-1-1	67	6	0	9	52	18.20***
D1-1-2	80	12	3	5	60	33.87***
D1-1-3	80	6	4	7	63	12.60***
D1-1-4	78	6	8	2	62	15.62***
D1-1-5	80	3	5	5	67	4.27*
D1-1-6	80	8	7	8	57	10.38***

[a] *** and * Significant at $P<0.001$ or $P<0.05$

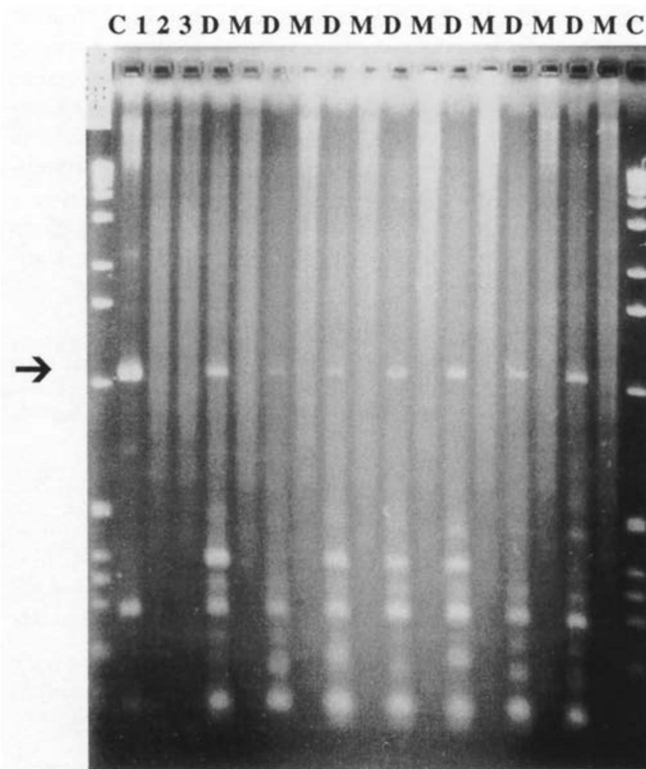

Fig. 3 PCR patterns obtained with primers *REP* and *REP.REV* using genomic DNA from monosomic additions and their diploids as a template. *Lanes 1, 2, 3* represent *B. vulgaris, B. patellaris, and B. procumbens,* respectively. Addition families and their diploid sibs from left to right are A5-1-24 to A5-1-30 (*D*=diploid and *M*=monosomic addition; *C*=1-kb ladder). Note the bright band (*arrow*) amplified in *B. vulagris* (*lane* 1)

somes lacked a signal. The outcome of chromosome counting clearly confirms the accuracy of the result of the squash-blot hybridization.

Apart from plants with the addition of a full chromosome, plants were also observed which carried only a part of the extra chromosome. All selected plants with a signal in the addition families D1-1-4 and D1-1-5 were telosomic additions. In addition family B3-1-1 one plant among 12 selected with a signal appeared to be a telosomic addition. In addition family B1-1-9 out of 14 selected plants two with a divergent morphotype were fragment additions. These types of plants with signals but with different morphotypes can be considered as a target for the detection of possible fragment additions.

DNA amplification by the polymerase chain reaction (PCR)

To assay the possibility of using the polymerase chain reaction (PCR) for the identification of monosomic additions as an alternative to chromosome counting, the PCR was carried out using primers *REP* and *REP.INV*. Genomic DNA from different monosomic additions families and their diploid sibs, whose chromosome numbers had already been established by counting, was used as a template for PCR amplification. DNA from the parents of the additions plants (*B. vulgaris*, *B. patellaris* and *B. procumbens*) served as controls. The results of these PCR amplifications are shown in Fig 3. Several bright bands were amplified in *B. vulgaris* only, clearly distinguishing *B. vulgaris* from both *B. patellaris* and *B. procumbens*. The same bright bands are also present in all diploid sibs but completely absent in *B. patellaris* or *B. procumbens* monosomic additions which, like the *B. patellaris* and *B. procumbens* control, showed a continuous smear. To test the reproducibility of the amplification patterns, more addition families were investigated and the results obtained were always the same.

Discussion

The distribution of two repetitive-DNA probes, *Sat-121* and *PB6-4*, specific for the section *Procumbentes* of the genus *Beta*, was tested with a dot-blot hybridization procedure. Both DNA probes gave sufficient signals in all monosomic additions as well as in *B. patellaris* and *B. pro-*

cumbens. No definite cross hybridizations to diploid sibs and *B. vulgaris* DNA were observed. This indicates that both probes are dispersed over different chromosomes, since the distinct addition plants carry dissimilar chromosomes as judged both from cytological studies and their diverse morphotypes. No addition plants without a strong signal for *PB6-4* were found, indicating that sequences on the probe *PB6-4* are possibly present on all chromosomes. Using a squash-blot hybridization procedure and a repetitive-DNA probe (*PB6-4*) an extensive number of putative monosomic additions in *B. patellaris* or *B. procumbens* addition families was screened rapidly. The presence of *PB6-4* sequences in the addition family carrying chromosome 4 of *B. procumbens* with small seedlings, and showing semi-lethality, is still under study. The location of these repetitive sequences is not known and it is not clear whether they are restricted to the pericentromic and distal regions only or occur all along the chromosomes. The location of these sequences may be more precisely established by fluorescence in situ hybridisation.

In the present investigation the correlation between deviating morphotype and plants with a signal is high but not complete. There is considerable variation between distinct chromosome families. The correlation between plants giving a signal after hybridization with *PB6-4* and with chromosome number (2n=19) is complete. This shows the accuracy of the results of the squash-dot hybridization. In this experiment 628 individual monosomic additions were found amongst 4580 plants (13.7%) in *B. patellaris* addition families, while 118 monosomic additions were found in 640 plants (18.4%) in *B. procumbens* addition families. The screening of these large numbers of plants, where the growing of families was the limiting factor, was achieved in a couple of weeks. This confirms that the technique is very attractive for the quick screening of large numbers of genotypes. In addition, the technique also provides the opportunity to target telosomic and fragment addition plants, which occur at low frequency and show less obvious morphological characteristics. The cytological investigation surprisingly showed that all selected plants with a signal in the addition families named D1-1-4 and D1-1-5 were actually telosomic additions. In addition family B1-1-9, two plants with a signal but with a divergent morphotype were identified. Chromosome counting confirmed that these plants were fragment additions.

The possibility of utilizing sequence information from *Sat-121* for a PCR-based assay to screen for putative monosomic addition plants was also investigated as an alternative to chromosome counting. The amplified products using the primers *REP* and *REP.INV* (Salentijn et al. 1994) clearly distinguished monosomic addition plants from their diploid sibs. In *B. patellaris*, *B. procumbens* and monosomic additions a continuous smear was produced, whereas a few bright bands were amplified in *B. vulgaris* and in all diploid sibs. The origin of the amplified sequences is not known exactly, but probably the majority of the amplified products originate from DNA sequences interspersing the *Sat-121* monomeric units. The results also made clear that repetitive-DNA sequences are dispersed strongly over all chromosomes of *B. patellaris* or *B. procumbens*, because monosomic additions carrying only one alien chromosome in the background of *B. vulgaris* yield patterns of amplified products identical to the smear like pattern seen in *B. patellaris* and *B. procumbens*. However, the finding of bright-view amplification products in *B. vulgaris* is surprising. Apparently a few *Sat-121*, or *Sat-121*, containing sequences are present in sufficient close proximity to each other to produce bands on a few loci. The presence of the bright bands in monosomic addition plants, which obviously have all *B. vulgaris* chromosomes and thus the potential to produce the bright bands, may be concealed because of the competitive amplification of numerous other loci on *B. patellaris*- or *B. procumbens*-derived chromosomes. The results suggest these primers can be used successfully in general to identify monosomic additions of chromosomes of species from the section *Procumbentes*. An advantage of the PCR technique is that amplification products can generally be detected by gel electrophoresis followed by staining with ethidium bromide, so that radioactive probing as used in the squash-blot method is no longer needed. DNA preparation from individual plants may be a time limiting factor, but simple and rapid DNA micro-extraction methods are already available (e.g. Cheung et al. 1993) enhancing the value of the polymerase chain reaction (PCR) for the identification of monosomic additions.

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