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PCR detection of *Hordeum bulbosum* introgressions in an *H. vulgare* background using a retrotransposon-like sequence

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Abstract Retrotransposon-like sequences are ideal tools for initial screening assays to distinguish between closely related species because of their ubiquitous presence, high copy number, chromosome coverage and rapid sequence evolution. A retrotransposon-like sequence, pSc119.1, cloned from *Secale cereale* (rye) has been used to obtain PCR primers that are capable of detecting small introgressions of *Hordeum bulbosum* (bulbous barley grass) chromatin in a *Hordeum vulgare* (cultivated barley) background. Combining this PCR-based assay with a crude but effective high-throughput DNA extraction has enabled the rapid identification of plants possessing *H. bulbosum* introgressions from large numbers of progeny from *H. vulgare*×*H. bulbosum* crosses. These plants are then further characterized by more-refined cytological, molecular and pathological techniques to locate and map the introgressed chromatin and to evaluate their disease resistance.

Keywords *Hordeum vulgare* · *Hordeum bulbosum* · Molecular marker · Retrotransposons · Introgressions

Introduction

Improving the disease resistance of barley cultivars through the use of wide hybridization with wild species of the same genus has concentrated mainly on crosses between *Hordeum vulgare* L. (cultivated barley) and *H. vulgare* ssp. *spontaneum* C. Koch (Thell), both of which comprise the primary barley genepool (Lehmann 1991; Nevo 1992; Eglinton et al. 1999). This limits the availability of germplasm for broadening the genetic base of barley. Further, only one species (*Hordeum bulbosum* L.; bulbous barley grass) in the secondary genepool may be exploited. *H. bulbosum* is a promising source of pest and disease resistance genes for barley breeders (Shearer et al. 1977; Brown et al. 1993; Zeller 1998) but access to it has been limited by fertility barriers in *H. vulgare*× *H. bulbosum* crosses (Pickering 2000). Substitution lines (SLs) involving the substitution of one or more *H. vulgare* chromosomes by their *H. bulbosum* homoeologues, and recombinant lines (RLs) possessing introgressions of *H. bulbosum* chromatin in barley chromosomes have been produced by backcrossing partially fertile triploid hybrids (VBB) to barley (Xu and Kasha 1992; Pickering et al. 1994, 2000a) or by screening selfed progeny from tetraploid hybrids (VVBB) (Michel 1995; Pickering et al. 1995; Walther et al. 2000), where V and B denote the *H. vulgare* and *H. bulbosum* genomes, respectively.

One problem that remains is identifying rare RLs, obtained through the low level of inter-genomic crossing-over (Pickering 1991; Zhang et al. 1999), amongst large populations of backcrossed or selfed progenies. These are currently screened by comparing their morphological traits with the recurrent barley parent and by evaluating their disease response to a small range of important pathogens virulent on barley. Further analyses are then performed with genomic and fluorescent in situ hybridizations (GISH and FISH, respectively) and restriction fragment length polymorphism (RFLP) using distal molecular markers to locate and map the site of the introgression (Pickering et al. 2000b). A consequence of this strategy is that lines possessing introgressions may be discarded if they closely resemble barley or if the introgressions are too small to be observed with GISH. Furthermore, RLs with rare interstitial introgressions may be overlooked during molecular screening with distal RFLP markers. Hence, a method was sought to improve preliminary identification of plants containing *H. bulbosum* chromatin.

A prerequisite was to find a molecular sequence that hybridized preferentially to *H. bulbosum* DNA. One such sequence appeared to be pSc119, a genomic clone that was isolated from *Secale cereale* by Bedbrook et al.

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(1980) and classified as a repetitive sequence in the 120-bp repeat family. Initial studies using pSc119 to probe Southern blots revealed a lack of hybridization to *H. vulgare* DNA but strong homology with *H. bulbosum* DNA (Gupta et al. 1989). McIntyre et al. (1990) reported that pSc119 was actually a mixture of three unique and unrelated genomic *Hin*dIII fragments, which they subcloned, sequenced and designated pSc119.1, pSc119.2 and pSc119.3. The sub-clone pSc119.2 was found to exhibit the 120-bp repetitive DNA characteristics described by Bedbrook et al. (1980), and hybridized to subtelomeric and intercalary sites on rye chromosomes (Vershinin et al. 1995) as well as distally on some *H. bulbosum* chromosome arms (Xu et al. 1990; Taketa et al. 2000). It has been used with some success to characterize RLs from *H. vulgare*×*H. bulbosum* hybrids with introgressions on chromosomes 4HL and 6HS (Pickering et al. 1994, and unpublished data). pSc119.1 also hybridizes very strongly to *H. bulbosum* DNA and only weakly to *H. vulgare* DNA (Gupta et al. 1989; Pickering et al. 1994). FISH with pSc119.1 revealed a dispersed distribution across all *S. cereale* chromosomes except for most telomeres and secondary constrictions (McIntyre et al. 1990). Xu et al. (1990) observed a similar pattern in *H. bulbosum* (mostly centromeric with some interstitial and telomeric sites of hybridization) but detected no signals in barley chromosome spreads. Because of its dispersed distribution, pSc119.1 was considered more-suitable than pSc119.2 as a candidate for identifying RLs among populations of non-recombinants. In this paper, we report the use of pSc119.1 in a PCR-based assay for identifying plants containing *H. bulbosum* chromatin and its incorporation as the first step in our strategy for recombinant detection.

Materials and methods

DNA extraction

Total DNA for Southern and PCR analyses was extracted from *H. bulbosum*, *H. vulgare*, SLs and RLs using 1 g of fresh young leaves ground in liquid nitrogen and then incubated with 5 ml of CTAB buffer (Murray and Thompson 1980) at 65°C for 90 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed and centrifuged before removing the aqueous phase. DNA was precipitated with an equal volume of isopropanol, pelleted, then washed with 0.2 *M* sodium acetate in 76% ethanol and 10 m*M* ammonium acetate in 76% ethanol. The pellet was then dried and dissolved in Tris-HCl (10 m*M*) EDTA (1 m*M*) pH 8.0.

Crude "high throughput" DNA extractions for PCR assays were performed on characterized RLs and untested BC_1F_2 progenies (derived from backcrosses of VBBs to barley) by collecting the fresh leaf tips (0.5 cm) from a bulk of five seedlings (10-days old) into a single well of a 96-well microtiter plate with a lid (NUNC, Life Technologies). A magnetic stainless-steel dowel pin (McMaster-Carr) and 150 µl of 0.5 *M* NaOH were added to each well and the plate placed into the "Matrix Mill" device [Weeden, Celeste and Loomis (Rumsey-Loomis)]. This device generates a pre-selected series of electromagnetic pulses to agitate the dowel pins and release small amounts of DNA from the tissue over 2× 1-min cycles. An aliquot (20 µl) of the extract (pale green in colour) was transferred immediately into 100 µl of Tris-HCl (50 m*M*), pH 7.0, EDTA (1 m*M*) buffer to create a dilute DNA template suitable for PCR with pSc119.1 diagnostic primers. Five-plant bulks that amplified the diagnostic product for the presence of *H. bulbosum* chromatin were then re-analyzed as individual plants using DNA prepared from the "Matrix Mill" to identify which plants within the bulk possess *H. bulbosum* introgressions.

Southern analysis

All standard molecular techniques were carried out following the protocols of Sambrook et al. (1989). Southern blots of a range of SLs and RLs and their parents were made by digesting 5 µg of genomic DNA overnight with *Eco*RV at 37°C. Digested samples were separated overnight on 0.8% agarose gels and transferred by alkaline capillary blotting to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions and fixed by baking at 80°C for 2 h. Southern hybridizations were carried out in 25 ml of hybridization solution (containing 10×Denhardt's, 5% dextran sulphate, 5×SSPE, 0.5% SDS) with 10 mg of denatured and degraded herring sperm DNA (Sigma) as a blocking agent. Blots were pre-hybridized at 65°C for 5 h in hybridization solution. Probe DNA (25 ng) was labeled with [α-^{32}P]dCTP by the random prime method (Megaprime kit, Amersham Pharmacia Biotech), separated from unincorporated nucleotides using Sephadex G50 spin columns, alkaline denatured, neutralized and added to the pre-hybridization solution to hybridize overnight at 65°C. Blots were washed at 65°C in 2×SSC (where 1×SSC=0.15 *M* NaCl, 0.015 *M* trisodium citrate, pH 7), 0.1% SDS/1×SSC, 0.5% SDS/0.5×SSC, 0.1% SDS for 2×15 min each, exposed to Kodak X-OMAT film at –80°C with two intensifying screens and developed automatically using an Agfa Curix 60.

PCR analysis

DNA templates for PCR analysis were diluted in ddH_2O to approximately 25 ng/µl. All primers were designed using the Primer3 online primer design software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi) and were synthesized by Life Technologies (www.lifetech.com). pSc119.1 For1 and Rev1 primers were designed from the published sequences of pSc119.1 (McIntyre et al. 1990). For3 and Rev2 primers were designed from the sequencing of the PCR products amplified from *H. bulbosum* using For1 and Rev1 primers. Positive control primers were designed from a consensus sequence of 5.8S ribosomal RNA genes retrieved from Genbank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) across several cereal species [*H. vulgare* (Z11759), *Secale montanum* (Z11760), *Triticum aestivum* (Z11761), and *Triticum monococcum* (L11759)].

PCR primers;

pSc119.1 For1:	5′ CTC ATG CCT TTA GTC CTT GCT GT 3′
pSc119.1 Rev1:	5′ CAA CAG AAG CGA AAC AGA CCT T 3′
pSc119.1 For3:	5′ ATG GGC GCA GCC GAT AGT 3′
pSc119.1 Rev2:	5′ ATG CCT CCA TGT ATC TCT GCC 3′
5.8S rDNA For:	5′ TCG TGA CCC TGA CCA AAA CAG 3′
5.8S rDNA Rev:	5′ GCG TTC AAA GAC TCG ATG GTT C 3′.

PCR reactions were performed in a Mastercycler Gradient PCR machine (Eppendorf) with each 25 µl-reaction containing: 1×PCR buffer (Roche), 200 µ*M* of dNTPs (Amersham Pharmacia Biotech), 3.5% Glycerol (w/v), 0.7 µ*M* of each Primer, 0.5 U of *Taq* DNA polymerase (Roche) and 2 µl of template DNA. Cycling conditions were as follows.

For1, Rev1 primers:
94°C for 2 min; 36 cycles of (94°C for 60 s, 55.8°C for 60 s, 72°C for 60 s); 72°C for 8 min.

For3, Rev2 Primers with 5.8 S rDNA primers (Multiplex reaction):
94°C for 2 min; 40 cycles of (94°C for 30 s, 57.4°C for 30 s, 72°C for 30 s); 72°C for 8 min.

After cycling was completed 10 µl of sucrose loading buffer (containing xylene cyanol and bromophenol blue) was added to

each PCR reaction and 10 µl of the mixture was separated by 2% agarose (FMC)-gel electrophoresis in 0.5×TBE running buffer. Products were visualized by staining with ethidium bromide, destained in water and photographed on an image computer.

Sequencing

The two pSc119.1 PCR products amplified using For1 and Rev1 primers from *H. vulgare* and *H. bulbosum* (designated 1191Hv and 1191Hb respectively) were cloned into pGEM-T (Promega). Three clones from each product were then sequenced in both forward and reverse directions using the Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems Inc.) and sequencing gels run by the Waikato DNA Sequencing Facility (University of Waikato, New Zealand). The resulting sequences were assembled into contigs using the DNAMAN software package (Lynnon BioSoft).

FISH

Chromosome preparations were made from actively dividing root tip cells as described by Anamthawat-Jónsson et al. (1993) and Pickering et al. (1997). Air-dried slide preparations were rinsed twice (5 min) in 2×SSC and then incubated in 50 µg/ml of DNase-free RNase in 2×SSC for 40 min at 37°C followed by three washes (5 min each) in 2×SSC. The slides were fixed in freshly de-polymerised paraformaldehyde (3 g/75 ml of 2×phosphate buffered saline) for 10 min at RT and washed three times in 2×SSC before dehydrating in an ethanol series and air drying.

The 201-bp fragment of interest (1191F3R2) was amplified from *H. bulbosum* genotype HB2032 using PCR Primers For3, Rev2 (conditions described above) and purified using the High Pure PCR purification kit (Roche). This fragment was then diluted and used as the template for a second PCR reaction (using the same primers) in order to prepare a biotin-16-dUTP labeled probe for FISH [standard PCR conditions but including biotin-16-dUTP (Roche)].

The probe was mixed to a final concentration of 6 ng/µl in a solution of 40% formamide, 1×SSC, 10% dextran sulphate, 0.1% SDS and 250 µg/ml of sonicated salmon sperm DNA. The probe mixture was denatured for 6.5 min at 95°C, chilled on ice for 3 min and 20 µl applied to each slide preparation and covered with a plastic coverslip. The chromosomes and probe mixture were denatured for 6.5 min at 80°C and hybridized overnight at 37°C.

After hybridization, slides were washed in 2×SSC (2×5 min at 42°C) followed by a stringency wash (20% formamide in 0.1×SSC) at 42°C for 10 min, and then washed in 2×SSC twice for 5 min at 42°C. Subsequent signal amplification, detection and epi-fluorescence microscopy followed the method of Pickering et al. (2000b) except that counterstaining was carried out with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (1 µg/mL of Vectashield antifade). Chromosome preparations were photographed using a Kodak Elitechrome (ISO 400) colour slide film and photographic slides were scanned for final reproduction.

Disease resistance

Disease assessments in the field (natural infection) or glasshouse (artificial inoculation) for leaf rust (*Puccinia hordei* Otth), powdery mildew (*Erysiphe graminis* DC. f. sp. *hordei* Em. Marchal), scald [*Rhynchosporium secalis* (Oud.) J.J. Davis] and net blotch (*Pyrenophora teres* Drechs. f. *teres*) were carried out as previously described (Pickering 1991, 2000b).

Results

Southern analyses

When used to probe Southern blots, pSc119.1 hybridized very strongly to *H. bulbosum* with some hybridization to *H. vulgare*. *H. bulbosum*-specific bands were present in all SLs and RLs tested, making it possible to detect even very small and interstitial introgressions in an *H. vulgare* background (Fig. 1).

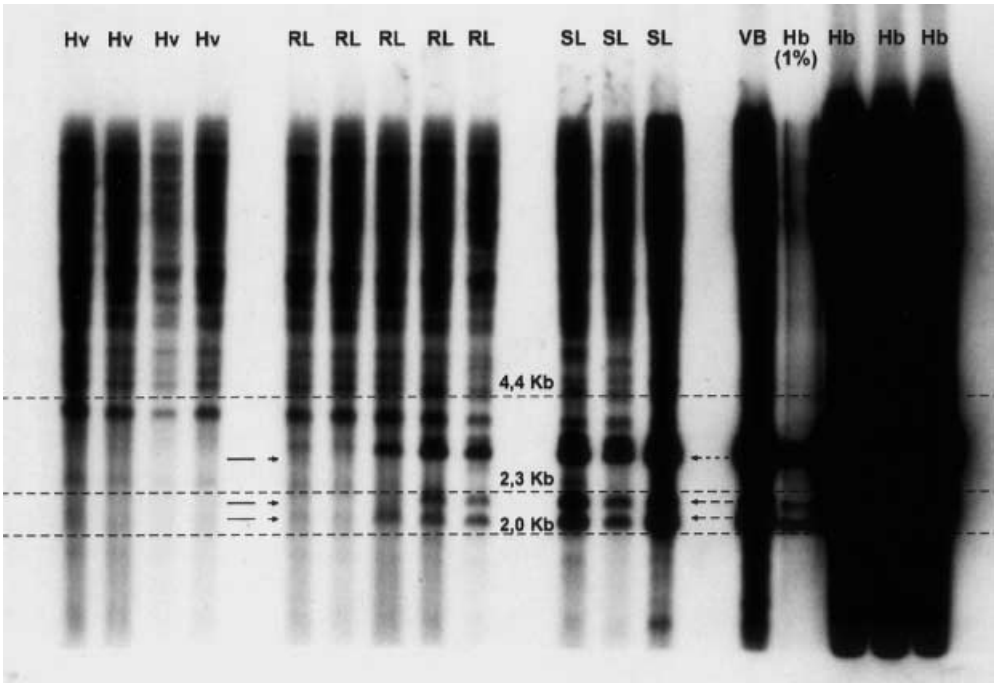

Fig. 1 pSc119.1 hybridization to a Southern blot containing 5 µg of *Eco*RV-digested DNA from a range of recombinant lines (*RL*s), substitution lines (*SL*s), one diploid hybrid (*VB*) and parental lines [*Hv=H. vulgare*; *Hb=H. bulbosum*; *Hb (1%)*=1% of *H. bulbosum* digest loaded]. RLs and SLs are aligned with increasing *H. bulbosum* content from left to right. *Arrows* indicate *H. bulbosum*-specific pSc119.1 hybridizing restriction fragments present in RLs and SLs

PCR analyses using For1 and Rev1 primers

The initial primer pair (pSc119.1 For1 and Rev1) amplified two polymorphic bands specific to *H. bulbosum* (1191Hb=703 bp) or *H. vulgare* (1191Hv=664 bp) genotypes. Both bands were amplified in RLs whilst 1191Hb was preferentially amplified in RLs, with large introgressions, and SLs, resulting in the presence of little or no 1191Hv product. The optimized assay using For1 and Rev1 primers amplified varying amounts of 1191Hb product from *H. bulbosum* chromatin, using templates ranging from *H. vulgare H. bulbosum* VB hybrids and SLs down to lines with very small distal and interstitial introgressions (Fig. 2). The effectiveness of this assay was significantly reduced by the inability, consistently and correctly, to assign 1191Hb presence due to a DNA smear above the 1191Hv product after gel electrophoresis (evident in Fig. 2). This prompted the sequencing of 1191Hv and 1191Hb and the subsequent design of new primers to produce a more robust assay for screening recombinant material.

Sequencing

The consensus sequences (con1191Hv and con1191Hb were compiled from the three clones of each PCR product and were submitted to GenBank accessions #AF396663 and #AF396664, respectively) showed 73% similarity to each other at the nucleotide level with the main difference located as a 36-bp insertion present only in the con1191Hb sequence (Fig. 3). The *H. bulbosum* sequence (con1191Hb) more closely resembled the original rye clone, showing an 87% sequence similarity, whilst the *H. vulgare* sequence (con1191Hv) showed a 70% sequence similarity. BLASTX searches using the published rye pSc119.1 sequence (McIntyre et al. 1990) and the consensus sequences, con1191Hb and con1191Hv, all revealed very strong similarity at the amino-acid level to putative retrotransposon polyproteins from *Oryza sativa*, *Sorghum bicolor*, *Zea mays* and *Arabidopsis thaliana*.

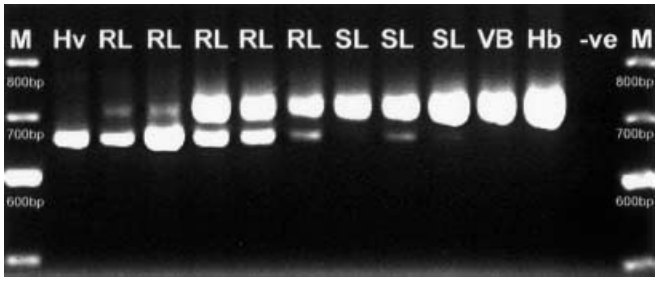

Fig. 2 PCR using the original pSc119.1 primers (For1, Rev1) showing two genotype-specific amplification products 1191Hv= 664 bp for *H. vulgare* and 1191Hb=703 bp for *H. bulbosum* with *RLs* possessing both bands and *SLs* showing preference for amplification of 1191Hb. Template abbreviations as described in Fig. 1

PCR analysis using For3 and Rev2 primers

To obtain better differentiation of amplification between the species, a new PCR primer (For3) was designed to target the 36-bp *H. bulbosum*-specific insertion, which was identified by a comparison of con1191Hb and con1191Hv sequences. A new reverse primer Rev2, was designed to target a short sequence present in both con1191Hv and con1191Hb, thus creating a PCR-assay specific for pSc119.1 sequences possessing the 36-bp insertion.

Using For3 and Rev2 primers in PCR resulted in the amplification of a 201-bp product (designated 1191F3R2) that was specific to *H. bulbosum* chromatin. All previously characterized SLs and RLs known to contain *H. bulbosum* chromatin across a range of chromosomal locations, including two lines with interstitial introgressions (GISH and RFLP, data not shown), were then tested using this PCR assay. The 1191F3R2 PCR product was not amplified using template DNA from eight barley cultivars and *H. vulgare* ssp. s*pontaneum* but was present in all previously characterized recombinant material. As a consequence of failed reactions being incorrectly scored as negative, a pair of internal positive control primers amplifying a highly conserved 329-bp segment of the 5.8S ribosomal RNA gene were subsequently included in the PCR assays. This positive control product was amplified from both *H. vulgare* and *H. bulbosum* templates and the assay was optimized to ensure no amplification interference against the diagnostic 1191F3R2 product. The multiplex assay was then retested against all fully characterized material resulting in

Fig. 3 Alignment of consensus sequences con1191Hv and con1191Hb, cloned from the original pSc119.1 PCR products using primers: For1 and Rev1 (Fig. 2). The major sequence difference can be seen as a 36-bp insertion present only in the *H. bulbosum*-derived sequence

H. vulgare cultivars and non-recombinants amplifying only the 329-bp control band and *H. bulbosum* parents; with RLs and SLs amplifying both the control band and the 1191F3R2 product (Fig. 4).

Sensitivity of the PCR assay was tested by serial dilutions of high quality *H. bulbosum* DNA with *H. vulgare* DNA whilst maintaining the template concentration at approximately 25 ng/µl. Multiplex pSc119.1 PCR (including the positive control primers) clearly amplified the 201-bp product (1191F3R2) from as little as 1 part of *H. bulbosum* DNA in 80,000 parts of *H. vulgare* DNA (0.00125%), and a faint band was still detectable at a 1/160,000 dilution (data not shown).

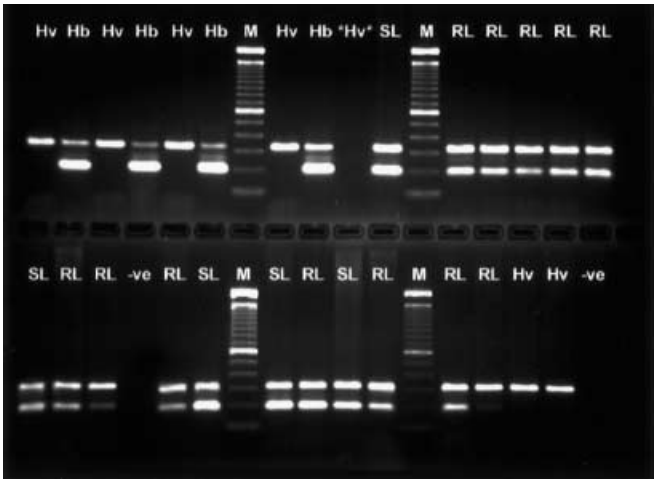

Fig. 4 pSc119.1 PCR using For3, Rev2 primers amplified a 201-bp *H. bulbosum*-specific product from a range of RLs containing introgressions of a variety of sizes and locations and SLs with various *H. vulgare* chromosomes substituted by their *H. bulbosum* homeologues. Each PCR included a 329-bp conserved *Hordeum* 5.8S rDNA positive control band amplified by all templates. The empty lane marked **Hv** indicates the value of the positive control PCR product in identifying failed reactions. Template abbreviations as described in Fig. 1. The lane marked *M* is the 100-bp DNA ladder (Life Technologies); the smallest band=100 bp, then increasing by 100 bp increments

The practical effectiveness of the "Matrix Mill" DNA template preparation was also tested using known RLs to ensure the ability to detect small amounts of recombinant leaf tissue in bulks of non-recombinant material. Using the multiplex pSc119.1 assay, 1191F3R2 was succesfully amplified from "Matrix Mill" templates containing only one recombinant plant in a bulk of five (four non-recombinants), indicating that the use of five plant bulks was an effective and convenient way to screen uncharacterized material. The value of the positive control primers was also demonstrated by the presence of 13 failed PCRs from the 446 "Matrix Mill" samples tested (2.9%), which would have otherwise been characterised as non-recombinants.

Fluorescent in situ hybridization with 1191F3R2

FISH with 1191F3R2 on *H. bulbosum* mitotic chromosome spreads confirmed previously published data using pSc119.1 (Xu et al. 1990; Taketa et al. 2000), revealing a genomic distribution that is consistent with a highly dispersed retrotransposon element (Kumar and Bennetzen 1999). Probe 1191F3R2 generated hybridization signals dispersed over the length of every *H. bulbosum* chromosome with some centromeric clustering but with fewer, less intense signals on the barley chromosomes (Fig. 5). This allowed *H. bulbosum* and *H. vulgare* chromosomes in SLs and hybrid lines to be differentiated from each other under fluorescence microscopy, but it was not possible to detect introgressions of *H. bulbosum* chromatin in an *H. vulgare* background (Pickering, unpublished).

Evaluation of the new pSc119.1 PCR assay

To compare the new pSc119.1 PCR-assay with the traditional screening approach currently used, a collection of 100 different BC_1F_2 progeny lines was analyzed, resulting in the amplification of the 1191F3R2 *H. bulbosum*-

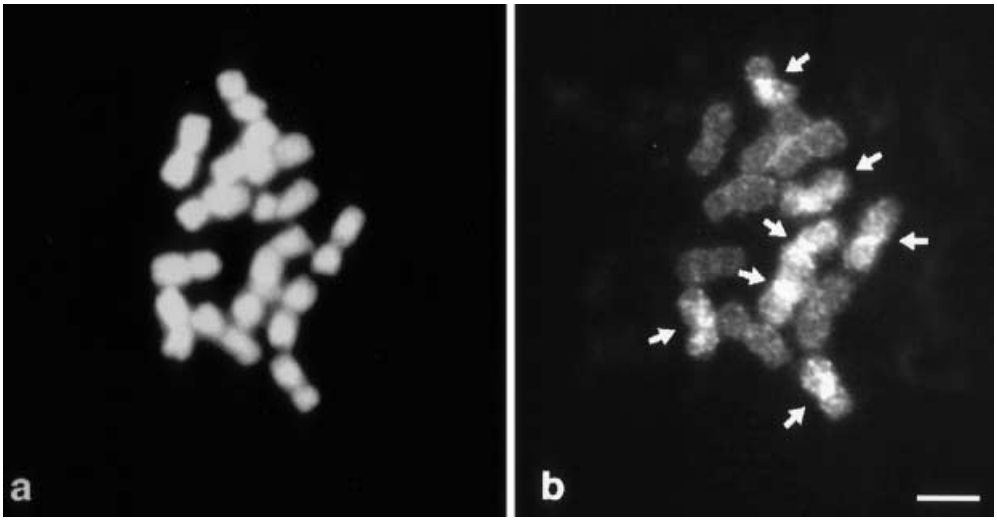

Fig. 5a, b Mitotic chromosome preparation of a *H. vulgare*×*H. bulbosum* hybrid comprising seven chromosomes from each species. **a** DAPI-stained chromosomes and **b** the same preparation after probing with pSc119.1 (1191F3R2 – see text) using fluorescent in situ hybridization. Hybridization signals show a dispersed distribution on all *H. bulbosum* chromosomes (*arrowed*) with some clustering about the centromeric regions. Some weak cross-hybridization is also evident on the *H. vulgare* chromosomes. Bar=5 µm

specific product in 32 of the 100 five-plant bulks. Of these 32 lines, one segregated for partial resistance to scald, four to net blotch, two to powdery mildew and five to leaf rust. Leaf rust-resistant plants from one of the lines also had glossy spikes and leaf sheaths; both traits are present in the *H. bulbosum* parent but not in the barley parent cv. 'Emir' and located on chromosome 2HS (Pickering 2000). Four more of the lines segregated for short vs normal stature at maturity and one line for differences in growth habit. The remaining 15 lines identified by pSc119.1 PCR as containing *H. bulbosum* chromatin were indistinguishable morphologically or pathologically from *H. vulgare* and will be investigated further. All the plants that gave negative results with the pSc119.1 assay were similar to the backcross parent 'Emir'. One hundred and fifty five plants from the 32 'pSc119.1-positive' BC_1F_2 bulks were subsequently analyzed by the "Matrix Mill" and pSc119.1 PCR, and a total of 112 plants (ranging from 1 to 5 per bulk) were identified as containing *H. bulbosum* chromatin.

Discussion

Plant genomes contain a very high proportion of retrotransposons, contributing up to 50% of the nuclear DNA content (Kumar and Bennetzen 1999). Individual elements can proliferate to account for large proportions of a species' genome. Barley, for instance, possesses as many as 30,000 copies of the BARE-1 retrotransposon, equivalent to 6.7% of its genome (Suoniemi et al. 1996). The relative abilities of plant species during evolution to inactivate or remove these elements, or to tolerate their presence, have created unique populations of retrotransposon elements in each plant genome (Bennetzen 2000). In this paper we have shown that by identifying retrotransposons or retrotransposon-like fragments with a high copy number, dispersed genome coverage and species specificity, it is possible to develop a molecular marker that can differentiate between the chromatin of two closely related species and thus identify the presence of chromatin introgression from one species into another.

The pSc119.1 sequence cloned from rye meets these criteria, resulting in its successful implementation in our introgression detection program. Southern and FISH analyses have indicated a difference in hybridization of pSc119.1 to the DNA from *H. vulgare* and *H. bulbosum* under stringent conditions (Fig. 1 and Gupta et al. 1989). This may be attributable to a considerable difference in pSc119.1 copy number between barley and *H. bulbosum* or, more probably, is due to sequence differences at the nucleotide level (77% sequence similarity between 1191Hv and 1191Hb), resulting in reduced hydridization to *H. vulgare* DNA.

The use of the "Matrix Mill" has improved the screening efficiency by providing a high-throughput DNA extraction of PCR quality that enables the pSc119.1 assay to be effectively applied at an early stage in the screening program. Up to 96 plant samples (including controls) undergoing "Matrix Mill" DNA extraction, pSc119.1 PCR and gel electrophoresis can be processed in a single working day. Individual plants can then be grown in the field and glasshouse for morphological examination and pathological screening. GISH/FISH and RFLP analyses can subsequently be performed to locate and map the introgressed DNA segment (Pickering et al. 2000b). The identification of *H. bulbosum* chromatin in 15 BC_1F_2 progenies that resemble the backcross parent ensures that this new strategy will offer a greater ability to identify RLs currently eliminated by morphological or pathological screening.

In summary, the combined improvements in screening by means of the pSc119.1 assay and the "Matrix Mill" DNA extraction will allow us more accurately to select RLs and increase the numbers of progenies analysed. By manipulating the plant growth-conditions and genotypes used for the hybrid backcross, and monitoring the frequency of introgressions amongst the BC_1F_2 plants by pSc119.1 PCR, we can then identify the best parental combinations and environments for obtaining a consistent supply of RLs and SLs. The pSc119.1 PCR should also permit the tracking of introgression transfer into more-adapted barley germplasm and allow the subsequent screening of larger backcross populations as a means to break linkage-drag associated with introgressions of *H. bulbosum* into *H. vulgare*. However, the true value of this assay for these extended purposes will be determined by the nature of the pSc119.1 sequence, particularly the copy number and coverage at each introgression location.

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