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Exploiting plant somatic radiation hybrids for physical mapping of expressed sequence tags

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Abstract Methods are described for the optimisation of the generation of radiation hybrids suitable for physical mapping of a plant (barley) genome. A combination of PCR-based technologies, involving the use of whole genome, mixed primer and hemi-nested primer amplifications, can greatly extend their utility for the physical mapping of expressed sequence tags (ESTs). Using panels of hybrids and ESTs, donor DNA retention and individual marker retention frequencies for the expressed portion of the barley genome in the hybrids were estimated.

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Introduction

Somatic hybridisation bypasses barriers of sexual reproductive isolation and allows interspecies hybrids to be generated directly through the fusion of protoplasts. This technology facilitates the transfer of desirable traits, for which the physical identification of genes may not be easy, which may be polygenic in origin, or are expressed through the cytoplasmic genome. Transfer of only a portion of the donor DNA (asymmetric hybridisation) rather than the entire genome (symmetric hybridisation) is also possible. Induction of asymmetry is facilitated by the treatment of donor protoplasts with either γ- or X-ionising irradiation prior to fusion. This treatment fragments the chromosomes and increases the rate of integration of donor chromosome fragments into the recipient genome, resulting in intergenomic translocations (Liu et al. 1999).

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While somatic hybridisation techniques have been exploited to produce novel hybrids for incorporation into plant breeding programmes, there is also potential for utilising this technology to facilitate the physical mapping of plant genomes. Mammalian genomics studies have developed similar technologies (Goss and Harris 1975) to produce radiation hybrid (RH) panels, which can be used in genome mapping procedures (Cox et al. 1990). As with plant asymmetric somatic hybridisation, RH production is based on irradiation and fusion gene transfer (IFGT) within somatic cells (Walter et al. 1994). Rather than producing a single hybrid organism, the aim of this technology is to produce a panel of hybrid cell lines that can be exploited for the construction of whole genome maps as well as for high resolution mapping of single chromosomes. Characterisation of radiation hybrid cell lines and subsequent mapping is achieved by assessment of co-retention frequencies for molecular marker alleles originating from the donor material and using statistical methods to calculate the order and distance between marker loci (Boehnke et al. 1991). This technology has been successfully applied to a number of mammalian species (McCarthy et al. 1997; Rexroad et al. 2000; Watanabe et al. 1999), including humans (Gyapay et al. 1996; Olivier et al. 2001), and the application of this approach to plant systems will greatly aid physical mapping of plant genomes and complement other novel techniques currently being developed for use in the production of physical maps of large crop plant genomes. In barley, for instance, the use of irradiated addition lines (Riera-Lizerazu et al. 2000), microdissected translocation chromosomes (Künzel et al. 2000) and deletions (Serizawa et al. 2001) has been documented.

Recently we described a protocol for the production of asymmetric radiation hybrids that could be used to produce physical maps of the barley genome (Wardrop et al. 2002). The utility of such hybrids was compromised by difficulties in their generation and continued culture, resulting in substrate DNA limitations during marker analysis for map construction. Here we describe the optimisation of a reliable asymmetric somatic hybridisa-

tion protocol for the generation of radiation hybrid cell lines. We show that a combination of PCR amplification protocols can be applied to the limited DNA quantities obtained from these hybrids, both facilitating their identification and extending their use in the physical mapping of expressed sequence tags (ESTs). The retention frequencies of these markers, originating from the expressed portion of the barley genome, are compared to that previously determined for simple sequence repeat (SSR) markers across the radiation hybrid panel.

Materials and methods

Plant material and protoplast isolation

Transgenic *Hordeum vulgare* cv. Golden Promise (supplied by the John Innes Centre, Norwich, UK) was used as a source of barley donor protoplast material. Embryogenic callus was initiated by culture (14–21 days) of immature embryos on MS medium (Murashige and Skoog 1962) supplemented with 0.3% (w/v) maltose and 2.5 mgl^{-1} 3,6-dichloro-*o*-anisic acid (DICAMBA) made semi-solid with 0.35% (w/v) Gelryte. Primary callus after 14 days of culture was assessed for suitability as a protoplast source, and also as a starting material for the initiation of both short and long-term cell suspension cultures of barley. For protoplasts, callus was incubated overnight (16 h) in the dark on a rotary shaker (40 rpm) in an enzyme solution (Stöldt et al. 1996) containing 1% (w/v) cellulase RS (Yakult Honsha, Tokyo, Japan) and 0.05% (w/v) Pectolyase Y23 (Kikkoman, Tokyo, Japan) in CPW salt solution (Frearson et al.1973) containing 13% (w/v) mannitol (CPW 13 M). Cell suspensions were initiated by transfer of embryogenic callus to liquid L1 medium (Lazzeri et al. 1991) in 100 ml conical flasks. These cell suspensions were cultured on a rotary shaker (120 rpm) and maintained by routine subculture every 7 days. Protoplasts were isolated from cell suspension cultures in the exponential growth phase (4 days post-sub-culture) at 4, 8, 12 and 16 weeks. Cells were incubated overnight (16 h) in the dark on a rotary shaker (40 rpm) in an enzyme solution (Lazzeri et al. 1991) containing 1% (w/v) cellulase RS (Yakult Honsha), 0.1% (w/v) Pectolyase Y23 (Kikkoman) in CPW salt solution (Frearson et al. 1973) containing 13% (w/v) mannitol (CPW 13 M). In some experiments this medium was supplemented with 0.5% macerozyme R10 (Yakult Honsha). Following enzymatic digestion, suspensions were passed through sieves of pore size 100 μm and 50 μm. Protoplasts were then layered onto 5 ml of 16% (w/v) sucrose solution. Following centrifugation (100 *g*; 5 min), the protoplast layer was transferred from the surface of the sucrose solution to a measured volume of CPW 13 M solution. The protoplasts were then exposed to heat shock treatment, which consisted of incubation at 45°C for 5 min followed by plunging into ice for 10 s. Protoplasts were then resuspended in buffer (13% (w/v) mannitol, 0.5 mM $CaCl_2$) for fusion.

Young, fully expanded leaves of tobacco (*Nicotiana tabacum* cv. Xanthi) were harvested from plants maintained in a controlled environment growth cabinet (16:8 h day:night cycle at 22°C) and sterilised in 0.75% (v/v) sodium hypochlorite solution for 20 min. The leaves were washed in sterile distilled water and the lower leaf epidermis removed by peeling. Peeled leaves were transferred to an enzyme solution that consisted of 0.1% (w/v) cellulase RS, 0.05% (w/v) driselase (Sigma, Poole, UK) and 0.02% (w/v) macerozyme R10 (Yakult Honsha) dissolved in To medium (Caboche 1980), lacking sucrose, and incubated in the dark (22±1ºC) for 16 h. Following digestion, the resulting protoplasts were filtered through a sieve of 100 μm pore size. Protoplasts were layered onto 5 ml of 16% (w/v) sucrose solution and, following centrifugation at 100 *g* for 5 min, the protoplast layer was transferred to electrofusion buffer.

X-irradiation and protoplast fusion

Prior to commencement of fusion experiments, assessments were carried out to evaluate the levels of bialaphos required in hybrid selection medium to ensure elimination of unfused tobacco protoplasts. Protoplasts were cultured in the presence of various concentrations of bialaphos (Meiji Seika Kaisha, Tokyo, Japan) ranging from 1 to 5 mgl^{-1}. The protoplast plating efficiency was defined as the number of protoplasts undergoing cell division following 7 day culture.

Irradiation treatment of barley protoplasts was conducted in a linear accelerator (ABB CH20 Linear Accelerator) that produces an X-ray beam of 6 MV energy, delivering a 50 Gy radiation dose.

Equal amounts of donor (barley) and recipient (tobacco) protoplasts were mixed and resuspended at a density of 1.0×10^5 ml^{-1} for fusion. Protoplasts were aligned in an AC field (900 KHz; 300 Vcm^{-1}), where fusion was facilitated by application of varying DC pulse intensities (160–240 Vcm^{-1}) and duration (0.5–1.0 ms) for electrofusion. For polyethylene glycol (PEG)-mediated fusion, the protoplast mixture was centrifuged (100 *g*; 5 min) and 2 ml of PEG fusion solution [30% (w/v) PEG 6000 or 8000, 4% (w/v) sucrose and 0.01 M $CaCl_2$] were added to each tube and the protoplasts were incubated at room temperature (RT) for 5–20 min.

Fusion solutions were then replaced with To medium supplemented with 3% (w/v) sucrose and the heterokaryon formation frequency determined. Protoplasts were maintained in To solution (24 h) in the dark followed by culture at a density of 1×10^5 ml^{-1} in MS liquid culture medium supplemented with 3% (w/v) sucrose, 9% (w/v) mannitol, 2 mgl^{-1} naphthalene acetic acid (NAA) and 0.5 mgl^{-1} 6-benzylaminopurine (BAP). Protoplast plating efficiency was defined as the percentage of protoplasts undergoing mitotic division following 14 days of culture. At this stage, protoplasts were transferred to MS liquid medium supplemented with 6% (w/v) mannitol, 0.1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. Following 4–6 weeks of culture, visible microcalli had formed. Calli were then subjected to selection on MS medium supplemented with 1 mgl^{-1} 6-BAP, 0.1 mgl^{-1} NAA, 3 mgl^{-1} bialaphos and made semi-solid with 0.8% (w/v) agar. All herbicide-resistant calli were sacrificed after maintenance in vitro for up to 12 weeks for use as part of a whole genome radiation hybrid panel.

Characterisation of radiation hybrid cell lines

DNA was isolated from the radiation hybrid lines and parental species using the DNeasy Plant Mini Kit (Qiagen, Crawley, West Sussex, UK). Pre-amplification of hybrid DNA was carried out using PEP (primer extension pre-amplification), essentially as described by Zhang et al. (1992). Ten microlitre reactions contained 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM each dNTP, 32 μM primer (Operon SP180–1, a polyN mixture of 15-mers), and 1 U Taq DNA polymerase (Sigma or Roche, Welwyn Garden City, Hertfordshire, UK). These reactions were cycled with an initial step of 5 min at 93°C followed by 50 cycles each of 92°C for 1 min, 37°C for 2 min, 37°–55°C ramp over 3 min, and 55°C for 4 min. PEP products were stored at 80°C until use as templates for marker typing.

Primer design and PCR

Thirty-six sequences, representing a set of genes expressed during malting of the barley grain, were used for primer design. Before use, primers were tested on genomic DNA from both parental species: on barley to identify primer sets that either failed to amplify or that clearly represented multicopy sequences, and on tobacco to eliminate primers which amplified products from this genome. A two-phase hemi-nested PCR protocol was applied, in which the external primer pairs (10 pairs) were multiplexed in the first phase; primers were designed to operate at uniform PCR conditions with calculated melting temperatures of between 53°and 60°C. First-phase reactions (10 μl) consisted of 0.5 μl of PEP

product, 0.125 μM of each external primer, 1× Taq reaction buffer, 1 U Taq polymerase (Sigma or Roche), 2.5 mM $MgCl_2$ and 250 μM each dNTP. Touchdown cycling conditions involved a denaturation step of 5 min at 94°C followed by 37 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. The annealing temperature was decreased by 1°C for the first 7 cycles to a final temperature of 58°C. From these complex PCR product solutions, 0.5 μl was used as template for the second-phase amplification, where specific PCR products were obtained by using one nested internal primer for each EST. Second-phase reactions (10 μl) consisted of 1× Taq reaction buffer, 1 U Taq polymerase (Sigma or Roche), 250 μM each dNTP, 2.5 mM $MgCl_2$, and 0.125 μM each primer. The samples were cycled as for the mixed primer reactions. PCR products were supplemented with a half volume of loading dye (60% w/v glycerol, 4.2 mg/ml bromophenol blue, 4.2 mg/ml xylene cyanol, 1× TBE), run on a 2% agarose/0.5× TBE gel and stained with SYBR Gold (Molecular Probes, Leiden, The Netherlands). Primer sequences are supplied as electronic supplementary material. Mapping utilised RHMAP software (Lange et al.1995).

Results

Establishment of a protocol for isolation of viable barley protoplasts

A number of protocols were assessed to determine the most reliable and efficient method for isolating viable protoplasts from transgenic barley for asymmetric somatic hybridisation studies. Yield was assessed following protoplast isolation from primary callus and both short and long-term cell suspension cultures. Primary callus produced a yield of protoplasts too low ($3.15\pm0.93\times10^5$) to be suitable for use in somatic hybridisation studies and long-term cell suspensions proved difficult to establish, releasing viable protoplasts only for the first 3 months, after which time yields decreased and cell suspensions became brown and eventually died. Further experiments compared the effect of differing enzymatic digestion conditions on young embryogenic cell suspensions (4–8 weeks), initiated from embryogenic callus derived from immature zygotic embryos. A protocol omitting macerozyme yielded a higher number ($4.01\pm1.07\times10^6$) of barley protoplasts than in its presence ($5.13\pm1.16\times10^5$). This protocol reproducibly yielded protoplasts in numbers of this order of magnitude (up to 2×10^7 was achievable) and, after irradiation (conditions for which have been described previously; Wardrop et al. 2002), these protoplasts were routinely used for somatic hybridisation.

Assessment of bialaphos tolerance in tobacco protoplasts

A dicotyledon fusion partner was necessary to mitigate against the molecular marker analysis of putative hybrid material resulting in false positives due to homology between the donor and recipient genomes. Tobacco was chosen as a fusion partner because this species has well established protoplast isolation and culture protocols. It was also necessary to ensure that the system used for selection of putative hybrid material was efficient in eliminating unfused tobacco protoplasts prior to the commencement of fusion procedures. Results confirmed that no tobacco protoplasts survived 7 days of culture in the presence of bialaphos at the lowest concentrations (1 mgl^{-1}) tested, while in the absence of bialaphos, 31.55±5.65% (mean± SEM, n=5) survived over this time period. Cultures routinely contained 3 mgl^{-1} bialaphos for selection of putative hybrid cell lines.

Establishment of fusion parameters

Heterokaryons were identified as spheroplasts possessing chloroplasts in a colourless, densely cytoplasmic background. A comparison of electrofusion and PEG-mediated fusion protocols (Table 1) revealed that, while a significant percentage of heterokaryons could be generated using both protocols, the use of PEG was associated with a greater degree of cell lysis post-fusion. Cell lysis was minimal in electrofusion protocols and the conditions that resulted in the highest heterokaryon frequency, with the lowest

Table 1 Assessment of optimum protoplast fusion parameters[a]

DC voltage (Vcm^{-1})	PEG (mw)	Pulse duration (ms)	Incubation period (min)	Heterokaryon frequency (%)	Extent of lysis[b]
160		0.5		0	–
160		0.75		2.25±0.63	–
160		1.0		1.75±0.85	–
200		0.5		4±0.91	–
200		0.75		7.75±0.75	*
200		1.0		8±1.47	*
240		0.5		5.75±1.18	*
240		0.75		6.25±1.44	**
240		1.0		0	***
	6,000		5	5.62±2.53	*
	6,000		10	2.81±0.2	**
	6,000		15	3.99±0.78	**
	6,000		20	0	***
	8,000		5	3.72±0.41	*
	8,000		10	2.93±0.1	**
	8,000		15	0.47±0.47	***
	8,000		20	0.36±0.36	***

[a] n=4; values represent mean±SD
[b] *=40–60% lysis, **=60–80% lysis, ***=>80% lysis

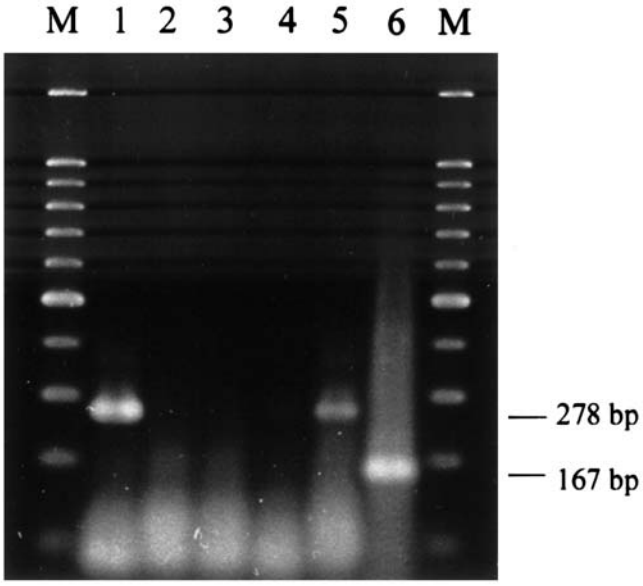

Fig. 1 Analysis of PCR products obtained using substrate DNA prepared from barley (cv. Golden Promise; *lane 1*), water (*lane 2*), tobacco (cv. Xanthi; *lane 3*), a 1:10 dilution of RH line 137 (*lane 4*), after PEP of a 1:10 dilution of RH line 137 (*lane 5*), after PEP of a 1:10 dilution of RH line 137 followed by mixed and hemi-nested primer amplifications (*lane 6*), using external (*lanes 1–5*) and hemi-nested (*lane 6*) primer pairs designed to detect EST485. Expected sizes of products as indicated relative to marker DNA (*lanes M*)

amount of lysis, were 200 Vcm^{-1} for 1 ms. Experiments conducted under these conditions generated a total of 200 bialaphos-resistant calli that were used for PCR analysis.

PCR analysis

Analysis of the products obtained after various stages in the whole genome and nested primer PCR protocols using primers derived from one EST sequence is shown in Fig. 1. Direct PCR using the external primer pair yielded no visible product when a 1:10 dilution of the hybrid DNA was used as substrate. After the PEP reaction, this primer pair generated a product of a size (278 bp) consistent with that expected. Completion of the whole protocol, including mixed followed by hemi-nested PCR, generated a strong PCR product of a size (167 bp) consistent with that expected from the use of a nested primer PCR. In both cases, the identity of the PCR product was confirmed by DNA sequencing. Application of the complete protocol for this EST across a sample of 28 radiation hybrid lines is shown in Fig. 2. The presence of this marker was detected in 7 of the 28 RH lines. This analysis was extended to a further 46 EST-derived markers and across a further 93 RH lines. There was wide variation in the retention frequency of individual markers across the panel (Fig. 3), with a mean of 12.46% and standard deviation of ±17.20% (n=47). Less variation was observed in the number of markers retained in each hybrid line (Fig. 3), with a mean of 13.01% and standard deviation of ±8.78% (n=121).

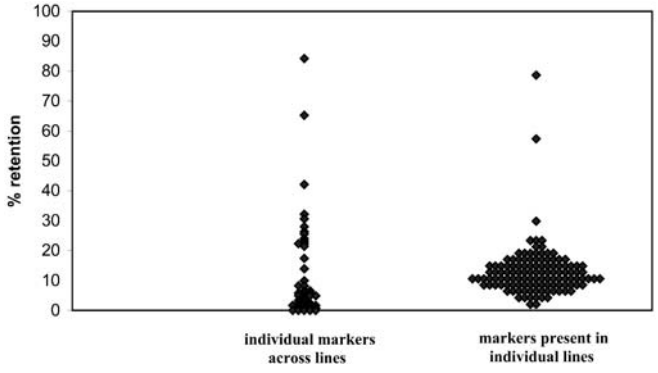

Fig. 3 Retention frequency of individual markers across the radiation hybrid panel and of markers in individual hybrid lines

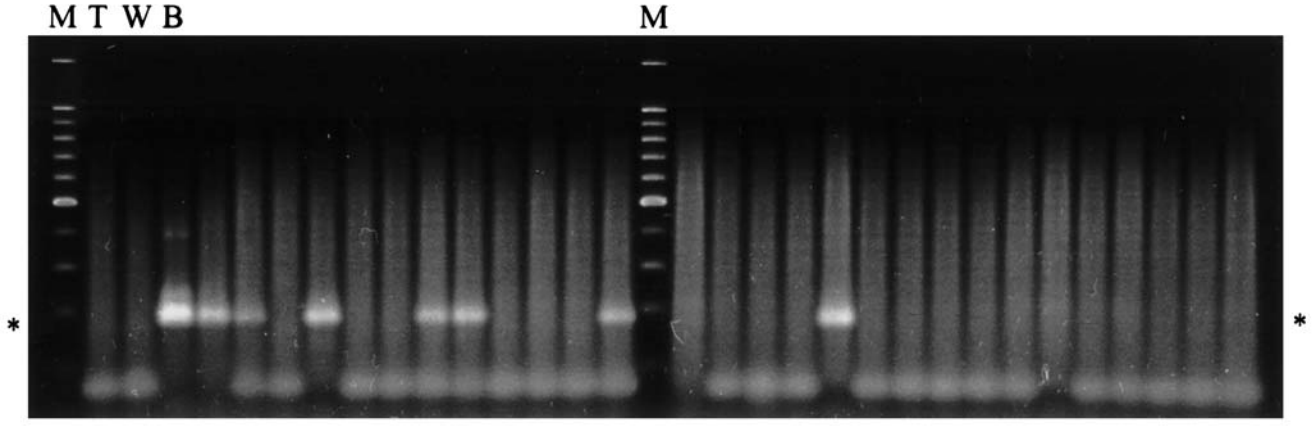

Fig. 2 Analysis of PCR products obtained from the combined PEP, mixed and hemi-nested primers protocol using substrate DNA from 28 RH lines (*undesignated lanes*), tobacco (cv. Xanthi; *lane T*), water (*lane W*), barley (cv. Golden Promise; *lane B*) and primer pairs designed to detect EST485. Asterisks indicate expected sizes of product (167 bp) as indicated relative to marker DNA (*lanes M*)

Discussion

Efficient cell fusion requires a ready source of protoplasts from both parental species. Like many monocotyledonous species, barley is relatively recalcitrant to many plant tissue culture manipulations including protoplast isolation and manipulation. A reliable protocol for the preparation of protoplasts, utilising young embryogenic barley cell suspensions as the donor tissue, was established. This allowed the isolation of adequate numbers of barley protoplasts for fusion procedures, after irradiation treatment (Wardrop et al.2002). Tobacco leaf mesophyll was chosen as the recipient tissue. Procedures for protoplast preparation from this material are facile and reliable and we demonstrated that unfused tobacco cells are highly sensitive to bialaphos, permitting their counterselection in culture. With these parental species, fusions were successful using both electrofusion and PEG-mediated procedures, but the highest yield of fusion products with minimal lysis was given by electrofusion. Fusion products were cultured under selection for up to 12 weeks, at which time they were sacrificed for DNA preparation. DNA yield was maximal at this point; extended culture resulted in tissue necrosis and reduced yield. Optimisation of protoplast isolation, fusion and culture protocols facilitated a fast and efficient throughput of hybrid cell lines in the numbers required for marker analysis.

Previous screening for hybridity involved PCR detection of the *bar* gene, originating from the transgenic barley donor and conferring bialaphos-resistance, and donor DNA retention frequency was subsequently determined using a genome-wide set of microsatellite markers (Wardrop et al.2002). It became apparent during this study that the amounts of hybrid DNA available would not support extensive marker analysis by such direct methods. We have therefore sought to extend the utility of the hybrid DNA by employing PCR technology for whole genome amplification followed by amplifications using (hemi)-nested primers. The overall protocol devised works well and reproducibly for a range of primer sequences determined from EST information, yielding an unequivocal presence/absence result using a simple gel system permitting unambiguous scoring. In combination, the PEP and hemi-nested amplification protocols extend the number of assays achievable by a factor of 2000, greatly improving the utility of the RH lines for the physical mapping of ESTs.

Both the average number of markers retained in any hybrid line, and the average retention frequency of individual EST markers across a panel of hybrid lines, were lower than those previously observed for a set of SSR markers (Wardrop et al.2002). In the case of EST-derived markers, in addition, a larger variation in marker retention frequency across the panel of lines was observed, although in both cases a number of individual loci were retained at both very low and very high frequencies. It has been suggested that this might result from the effects of biological functions, expressed from the retained barley chromatin, which could be associated with the differential survival of hybrid calli post-fusion. Potential functions are ascribable to some of the barley genes represented by particular ESTs, and mechanisms by which these might disrupt cellular function in a heterologous physiological background can be envisioned. It remains possible, however, that such markers might be detected in a panel of larger size, since introgression into transcriptionally quiescent segments of the tobacco genome is also possible. An expansion of the size of the radiation hybrid panel was required to further address this issue. For this expansion, a re-examination using the improved PCR protocols outlined here has indicated the presence of barley DNA in samples from calli which survived selection but in which the presence of a *bar* gene was initially not detected (Wardrop et al. 2002). The process of donor chromosome loss is dynamic throughout cell division and callus formation, and these samples may represent instances where an initially retained *bar* gene has been lost while other barley sequences are retained. Such samples can still be incorporated into the radiation hybrid panel, however, and will be informative in the generation of a whole-genome physical map for barley. Using the expanded panel there remain a few EST-derived markers whose presence has not been detected and a further panel or alternative procedures might be required for their physical mapping.

The expansion of the panel size has also, for the first time, allowed the meaningful application of statistics to detect co-retention of markers in the RH panel, although the number of markers placed on the panel remains modest, as yet. One preliminary linkage group is represented by three EST-derived markers (ESTs 111, 348 and 2,949) linked at a LOD score >3. These ESTs represent barley genes that have orthologues at the distal end of the short arm of rice chromosome 1 in a 1.8 Mb region (spanning ~8 cM on rice genetic maps). With the addition of further markers to the expanded panel, accelerated construction of linkage groups, referenced to existing rice and barley physical and genetic maps, represents the next phase in the exploitation of this physical mapping resource.

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