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DNA transfer by highly asymmetric somatic hybridisation in *Medicago truncatula* (+) *Medicago rugosa* and *Medicago truncatula* (+) *Medicago scutellata*

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Abstract A regenerable line of *Medicago truncatula* (Jemalong 2HA) as a recipient species, was fused with the sexually incompatible species *Medicago scutellata* or *Medicago rugosa.* The treatments described maintain the chromosome number of the recipient but enable the transfer of small amounts of DNA of the donor species, probably by intergenomic recombination. Without a chromosome number-change fusion products can readily regenerate to produce fertile plants; and potentially a library with a diverse array of new genetic material. The selection of fused cells is based on treatment of the recipient cells with iodoacetamide (IOA), a non-regenerable donor, γ-irradiation of the donor, and regeneration on a medium favouring the recipient. DNA transfer was demonstrated by amplified fragment length polymorphism (AFLP), Southern hybridisation and changed morphology.

Keywords DNA transfer · Highly asymmetric somatic hybridisation · *Medicago truncatula* · *Medicago rugosa* · *Medicago scutellata*

Introduction

The success of transformation over the last decade, including transformation within the *Medicago* genus (Rose et al. 2001), has meant that a characterised gene coding for a trait of interest can be readily incorporated into a genome. Ye et al. (2000) have successfully transferred four genes into rice. Nevertheless, when the responsible gene(s) for a trait has (have) not been isolated, particularly for polygenic traits, cell fusion-based genetic transfer is an attractive concept. Somatic hybridisation has, however, been difficult to achieve (Sigareva and Earle 1999) and has not always been as useful as expected for producing novel genotypes by introducing characters from wild relatives (Bates 1992; Vasil and Vasil 1992). This has been particularly because of regeneration and fertility difficulties which are often due to aneuploidy (Bates 1992).

Highly asymmetric hybrids that possess the complete genome of a recipient species, and only one or a few transferred chromosomes, may overcome the regeneration and fertility difficulties of aneuploidy (Dudits et al. 1987). However, elimination of donor chromosomes appears to be limited, and few highly asymmetric hybrids have been produced (Famelaer et al. 1989; Yamashita et al. 1989; Wijbrandi et al. 1990; Liu et al. 1995; Liu et al.1999).

Donor-chromosome elimination and fertile plants can be successfully obtained by cybridization (Melchers et al. 1992; Sidorov et al. 1994), indicating that complete chromosome elimination is possible. Also, intergenomic recombinations or translocations have been displayed in highly asymmetric somatic hybrids without donor chromosomes (Hinnisdaels et al. 1991; Li et al. 1993; Skarzhinskaya et al. 1996; Liu et al. 1999). Intergenomic translocations or rearrangements have been detected by genomic in-situ hybridisation in *Nicotiana tabacum* (+) *Nicotiana plumbaginifolia* and *Nicotiana sylvestris* (+) *Nicotiana plumbaginifolia* (Piastuch and Bates 1990; Parokonny et al. 1992, 1994), by genomic probes in *Medicago sativa* (+) *Onobrychus viciifolia* (Li et al. 1993) and *Brassica napus* (+) *L. fendleri* (Skarzhinskaya et al. 1996), and by cytological and/or genetic analysis (de Vries et al. 1987; Hinnisdaels et al. 1991). The translocations can be transmitted through male and female meiosis to sexual progeny and can create new recombinant chromosomes after the first backcross (Parokonny et al. 1994, 1997).

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Work carried out by us on asymmetric somatic hybridisation, using the model legume *Medicago truncatula* and the donor species *Medicago scutellata* (Tian and Rose 1999), suggested methods to achieve highly asymmetric somatic hybridisation (HASH) where there is elimination of all donor chromosomes but with intergenomic recombination to achieve nuclear DNA transfer. The current study examines HASH to achieve nuclear DNA transfer as detected by AFLP (amplified fragment length polymorphism) analysis, Southern hybridisation, cytoplasmic DNA analysis and the production of diploid fertile plants with a changed morphology. A regenerable line of *Medicago truncatula* (Jemalong 2HA) as the recipient species, was fused with the sexually incompatible species *M. scutellata* or *Medicago rugosa.*

Materials and methods

The regenerable recipient and non-regenerable donors

Leaves of a highly regenerable seed line Jemalong 2HA (Rose et al. 1999) from *M. truncatula* Gaertn. cv Jemalong, were from plants maintained in a controlled environment growth cabinet (Rose and Nolan 1995; Tian and Rose 1999). Cotyledons of the non-regenerable donors were used from *M. scutellata* (L.) Miller cv Robinson and *M. rugosa* Desr. cv Paraponto. Seeds of the donors were obtained from the *Medicago* Genetic Resource Centre SARDI, Waite campus, University of Adelaide.

Isolation, purification and fusion of protoplasts

M. truncatula mesophyll protoplasts and *M. scutellata* and *M. rugosa* seedling cotyledon protoplasts were utilised. Protoplasts were isolated, purified and fused as described previously (Rose and Nolan 1995; Tian and Rose 1999). Briefly, the youngest fully expanded *M. truncatula* leaves were used. After sterilisation and plasmolysis in 5 ml of P1 culture medium (Thomas et al. 1990), 8 ml of PES-5 enzyme medium (Rose and Nolan 1995) was added to each Petri dish which contained 0.4 g of tissue. The Petri dishes were incubated on a 60 rpm rotary shaker at 27°C for 3 h. The released protoplasts were filtered through a 40-µm nylon-mesh filter into a 15-ml centrifuge tube, and purified by centrifugation for 20 min at 80 g in 20% Percoll (containing 0.11 M mannitol). The protoplast band was then transferred to a new tube, resuspended and pelletted three times in P1 medium at 100 g for 8 min.

The cotyledons of 9–13-day sterile seedlings of *M. scutellata* and *M.rugosa* were cut into small pieces and plasmolysed for 30 min in 5 ml of P1 medium. Protoplasts were isolated and purified as described for mesophyll protoplasts but using a coarser nylon mesh of 67 or 100 µm and a final Percoll concentration of 27% rather than 20%.

The parental protoplasts were mixed at a ratio of 0.5–2: 1 (mesophyll:cotyledon) depending on the protoplast yields of both parents, and at a concentration of $6–7\times10^{6}$ ml^{-1}. A 350-ml drop of fresh PEG 1540 solution was pipetted onto a Nalgene PMP Petri dish. Then a 150-µl mixture containing approximately 1×10^{6} protoplasts was added to each drop of the PEG solution to give a final PEG concentration of 25% (w:v). Following a 5–10 min incubation, two drops of 300 µl high Ca^{2+} (pH 10) solution (according to Kao 1986) were added at 5-min intervals. After a 10-min incubation, 1.5 ml of P1 medium was added to each drop twice at 5-min intervals, and then left for 5 min. Following the fusion procedure, the fused protoplasts were pelleted at 100 g for 10 min and purified by flotation through 33% Percoll in P1 medium. After mixing thoroughly, 1 ml of P1 medium was layered over the Percoll suspension and centrifuged for 20 min at 80 g. The protoplasts from the interface were resuspended in P1 medium and pelleted for 8 min at 100 g. This was repeated twice and the protoplasts were then prepared for regeneration.

Selection strategies

Recipient mesophyll protoplasts from *M. truncatula* were inactivated by incubating with 3–6 mM of iodoacetamide (IOA) in P1 medium at 4°C for 20 min. The protoplasts were then washed three times with P1 medium (100 g, 5 min). This concentration of IOA completely inhibited the division of mesophyll protoplasts, and created metabolic complementation in the heterokaryons. In most treatments the donor cotyledon protoplasts were formed from 350–700 Gy γ-irradiation before protoplast isolation (see Table 1). This radiation dosage completely prevented colony formation in control cultures. Ionizing irradiation can induce fragmentation of the donor genome and promote donor chromosome elimination (Sidorov et al. 1987). The cotyledons from the donor species were non-regenerable and the medium favoured the growth of the recipient.

The culture procedure

Protoplasts from *M. rugosa* and *M. scutellata* were cultured in media with different hormones to test the requirements for division induction. However, the medium that was the best for recipient growth and plant regeneration was used in order to favour the production of highly asymmetric hybrids. The culture procedure has been described by Tian and Rose (1999). Briefly, fused protoplasts at a concentration of $8–10\times10^{5}$ ml^{-1} were embeded in 1% low-melting-point agarose (Sea Plaque, FMC, Rockland, USA) droplets surrounded with liquid P1 medium containing 25 µM of NAA, 4 µM of BAP and 0.45 M of glucose (P1 25:4). During incubation the liquid medium was changed at 7-day intervals with P1 and P4 medium (10 µM of NAA + 4 µM of BAP) (Thomas et al. 1990) at ratios of 2:1, 1:2, 1:5 and 0:1. After 4 or 5 weeks, the microcalli were transferred to P4 agar plates to grow in the dark. The calli were subcultured in the dark every 3–4 weeks until embryo formation, then they were transferred to light (16 h photoperiod with a light intensity of 16 µmol·m^{-2}s^{-1}). An important modification was to cycle some embryos that were not forming shoots back through P4 (10 µM of NAA+4 µM of BAP), before returning to P40 liquid medium (with 1 µM of NAA) (Thomas et al. 1990) on filter paper.

AFLP analysis and chromosome counts

For AFLP analysis the protocol of the suppliers (Gibco BRL) was followed with varying primers, as described by Tian and Rose (1999). After screening experiments, two primer combinations were used for the 22 plants analysed in this study, and 6–7 primer combinations were used for detailed analysis of selected plants. In general, the individual band intensity, the size distribution of amplified products, and the overall pattern were always the same in different repetitions with the same primer pairs and the same DNA template, the variation being between different genomic DNA samples and different primer combinations. Chromosome counts have previously been described (Schlarbaum et al. 1989; Tian and Rose 1999).

Isolation and cloning of donor AFLP bands

AFLP products run on 6% denaturing polyacrylamide gels were visualised by staining with silver (Bassam et al. 1991), rather than by isotopic labelling, to facilitate cutting out the band. DNA from the band was isolated by eluting the band into sterile distilled water (Chalhoub et al. 1997) or TE buffer and amplified by the polymerase chain reaction (PCR) employing the same primers as used in the AFLP process. As it was possible that there was more than a

single sequence in the isolated band the PCR products were in some cases cloned into pCR-Script™ Amp.

Southern hybridisation

AFLP products were run on agarose gels and blotted onto nylon membranes as described by Wang et al. (1996). The nylon membranes were probed with cloned donor bands, that as assessed by AFLP had been transferred to the recipient, after labelling with ^{32}P-dCTP. Membranes were washed twice at 42°C for 20 min in 5% SDS, 1 mM EDTA, 40 mM $NaHPO_4$ at pH 7.2 followed by two additional washes at 42°C for 20 min in 1% SDS, 1 mM EDTA, 40 mM $NaHPO_4$ at pH 7.2. In some cases polyacrylamide gels were used, electroblotted and then treated as for the agarose-gel blots.

Chloroplast and mitochondrial DNA analysis

Chloroplast DNAs were extracted using a procedure based on Liu and Rose (1993). Basically, chloroplasts were isolated from 3 to 10 g of leaves using the sucrose gradient technique described by Palmer (1982). The chloroplast pellet was resuspended in 2.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2% sarkosyl and 0.02% Proteinase K, and incubated at 37°C for 1–2 h. Chloroplast DNA was extracted from the lysate using TE buffer-saturated phenol and chloroform, repeated three times.

Mitochondria were isolated from 7 to 10 g of leaves, which were homogenized in a grinding medium consisting of 10 mM TES buffer (pH 7.2), 0.5 M mannitol, 5 mM EDTA, 0.2% bovine serum albumin, and 0.05% L-cysteine. The remainder of the extraction procedure was according to Kemble et al. (1980). After restriction digestion, cpDNA and mtDNA fragments were loaded onto 0.85%–1% agarose gels.

Results

Regeneration of fused protoplasts

The methods described for protoplast isolation, purification, fusion, culture and plant regeneration were used to investigate highly asymmetric hybridisations between *M. truncatula* and *M. scutellata*, as well as *M. truncatula* and *M. rugosa*. The donor species *M. scutellata* and *M. rugosa* are non-regenerable, while the recipient *M. truncatula* Jemalong 2HA is highly regenerable. The surviving population of embryos and plantlets became more recipient-like with time. Cells with abnormal numbers of chromosomes were less-likely to form colonies, induce embryos, or regenerate plants. Ninety five percent of plants were recipient-like diploid plants.

Heterokaryon division was initiated and sustained in the 1st week of culture. After 3–4 weeks of culture there were 0–100 colonies per agarose droplet. A close relationship between the plating efficiency and the number of embryogenic colonies was noted. No embryogenic colonies were obtained if the number of colonies per agarose drop was less than ten. About 83% of the embryogenic colonies were produced in the first 2 months of callus culture that followed a 6-week protoplast-culture period, and these were the colonies from which mature plants were obtained. A low frequency of plant regeneration from fusion products was observed with the annual *Medicagos* compared to experiments using *M. sativa*. Fifty plants were produced from 10^8 protoplasts with M. *truncatula* fusions (Table 1). In the case of *M. sativa*, a similar number of plants were produced from 10^6 protoplasts (Li et al. 1993).

M. truncatula (+) *M. scutellata*

Some products of this hybridisation have been studied previously (Tian and Rose 1999). In that study out of 20 plants regenerated from fused cells one was highly asymmetric (TS0) and one was asymmetric (TS36). In the present study, ten additional regenerants analysed by AFLP showed two highly asymmetric hybrids. Irradiation of the donor cotyledons of *M. scutellata* used for protoplast production produces a higher frequency of embryogenic colonies and a higher number of plants in soil compared to treatments without irradiation (Table 1). In the absence of γ-irradiation, there was a decrease in embryogenic colonies and plants in soil, even though non-irradiation produced a higher plating efficiency. It is likely that irradiation facilitated the elimination of chromosomes. It is important to emphasize that, in seeking to obtain highly asymmetric hybrids, it is essential that the mesophyll protoplasts of *M. truncatula* are completely inactivated by iodoacetamide and this has been shown by Tian and Rose (1999).

Table 1 Fusion experiments between *M. truncatula* (recipient) and *M. scutellata*, and between *M. truncatula* (recipient) and *M. rugosa*

Fusion combination	No. of protoplasts (for each exp.)	Inactivation		Embryogenic calli/ total calli (for each exp.)	Plants in soil/ plantlets (for each exp.)
		IOA (mM)	Gamma (Gy)		
M. truncatula + *M. scutellata*	1×10^7	3	0	6/340	3/9
	1×10^7	3	350	34/216	14/>30
	1×10^7	3	700	47/239	19/>40
M. truncatula + M. scutellata	$3–10 \times 10^7$ [a]	3–6	350	150–198/ 852–1300	40–56/>200
M. truncatula + *M. rugosa*	$3–10 \times 10^7$ [b]	3–6	350–700	0–17/5–180	0–14/0–31

[a] Data from three experiments [b] Data from two experiments

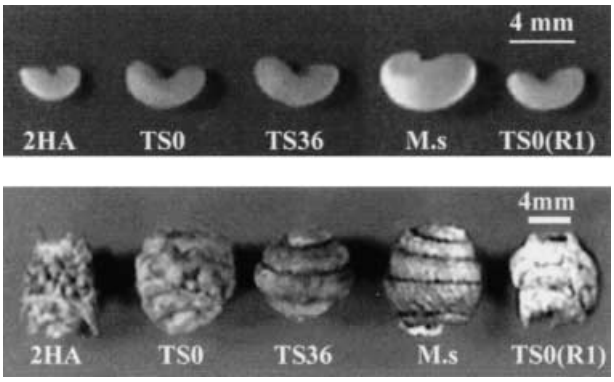

Fig. 1 Pods from *M. truncatula* Jemalong 2HA (2HA), *M. scutellata* (*M.s*), a highly asymmetric hybrid (*TS0*) and an asymmetric hybrid (*TS36*) and the seeds from them. *TS0* and *TS36* are from regenerated plants (*R0*) and the next generation is R1

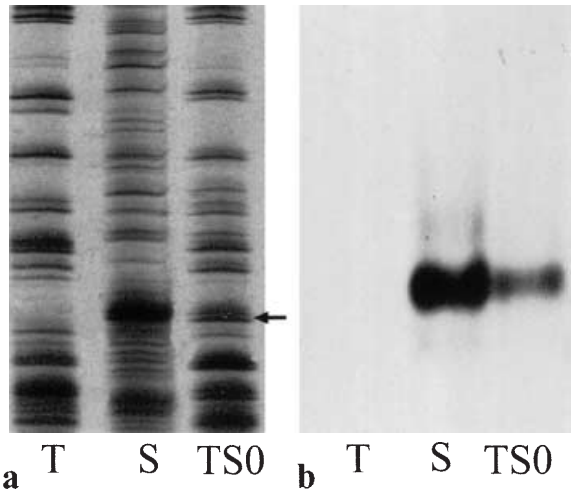

Fig. 2 **a** Part of an AFLP separation from DNA of *M. truncatula* (*T*), *M. scutellata* (*S*) and the highly asymmetric hybrid (*TS0*), using the primers E-AAG+M-CAC. **b** The *arrowed* band area from TS0 was isolated, cloned and hybridised to a Southern blot of an AFLP separation in an agarose gel

Fig. 3 Anthocyanin patterns on leaflets (folioles) from young plantlets of *M. truncatula* Jemalong 2HA (*2HA*), *M. rugosa* (*M.r*) and the highly asymmetric hybrid *TR8* of the R1 generation (see description in Fig. 1). The top row is the adaxial surface and the bottom row is the abaxial surface

TSO – the highly asymmetric *M. truncatula* (+) *M.scutellata*

In our previous study (Tian and Rose 1999), changes in leaf shape and pod morphology were noted. Shown in Fig. 1 is another difference that has not been reported, and emphasises the value of HASH. TSO shows an increase in seed size which is comparable with the increase in size evident in TS36 where ten chromosomes have been transferred. In TSO the mean increase in seed weight, compared to *M. truncatula*, is 28.5%.

TSO – AFLP and Southern-hybridisation studies

Though the AFLP data show that there are bands of the same size in the donor and the HASH plant and absent in the recipient, it is possible that the bands are not homologous. It is possible, for example, that the band in the hybrid may have been produced by variation that induces fortuitous loss or gain of *Eco*RI-*Mse*I restriction sites. In the case of TS0 this has been examined further by Southern hybridisation. A donor band present in the hybrid was isolated, amplified by PCR and then cloned. This band was then labelled and hybridised against the donor, hybrid and recipient DNA that had all been subjected to AFLP. The results in Fig. 2 show that there is a single band with hybridisation to the donor and hybrid, but not to the recipient. The relatively thick hybridisation bands are because of the agarose gel being used for the Southern blotting, rather than the polyacrylamide gel usually used in AFLP studies.

M. truncatula (+) *M. rugosa*

Fusions between *M. truncatula* and *M. rugosa* were carried out using the same procedures as for *M. truncatula* and *M. scutellata*, with protoplasts obtained from γ-irradiated seedling cotyledons and iodoacetamide-treated mesophyll protoplasts from *M. truncatula*. These fusion combinations produced plants in soil (Table 1) and 12 were examined by AFLP. Three plants showed donor bands in the hybrid and no change in chromosome number. The plant TR8 was studied most extensively as the progeny seedlings had distinctive changes in leaf anthocyanin pigmentation patterns.

TR8 – the highly asymmetric *M. truncatula* (+) *M. rugosa*

In TR8 seedlings on the adaxial side of the leaf there was a central solid block of anthocyanin, and a scattered pat-

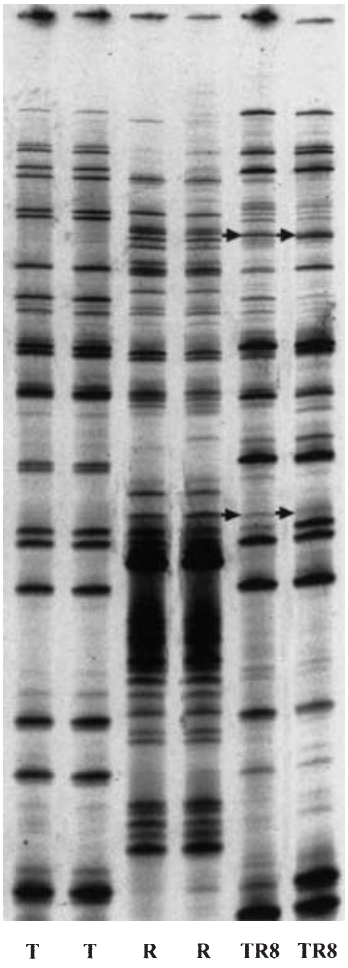

Fig. 4 AFLP band patterns using the primers E-AAG+M-CTG for *M. truncatula* (*T*), TR8 and *M. rugosa* (*R*). *Arrows* indicate bands that are specific for *M. rugosa* and TR8

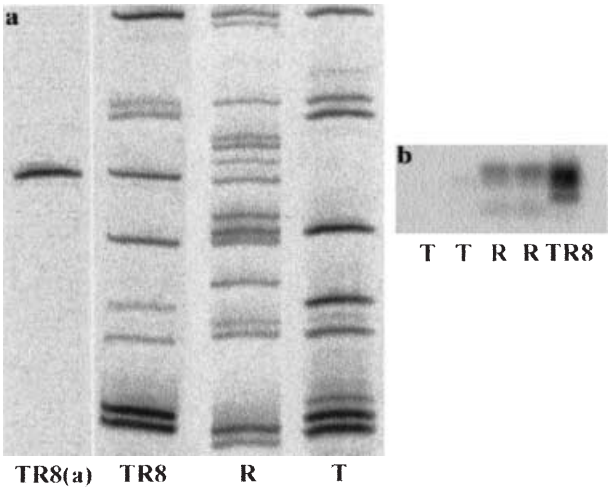

Fig. 5 **a** Part of an AFLP separation from DNA of *M. truncatula* (*T*), *M. rugosa* (*R*) and a highly asymmetric hybrid (*TR8*), using the primers E-AAG+M-CTG. **b** A donor band in TR8 was isolated, PCR-amplified and electrophoresed under the same conditions as the original AFLP separation, to check for size, TR8(a), and hybridised to a Southern blot of an AFLP separation in an agarose gel

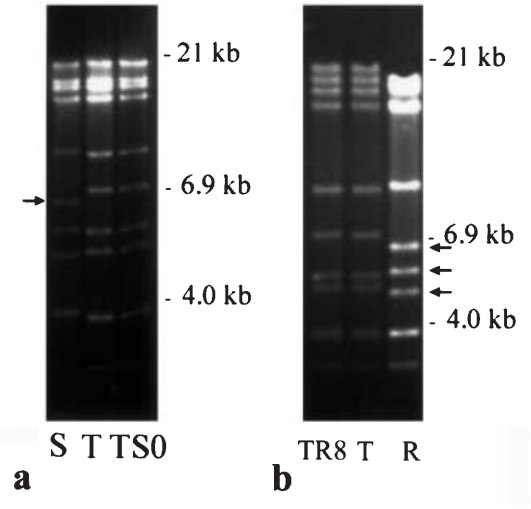

Fig. 6 **a** Restriction endonuclease digest, using *Xho*I, of cpDNA isolated from *M. truncatula* (*T*), *M. scutellata* (*S*) and the highly asymmetric hybrid (*TS0*). **b** Restriction endonuclease digest, using *Xho*I, of cpDNA isolated from *M. truncatula* (*T*), *M. rugosa* (*R*) and a highly asymmetric hybrid (*TR8*). *Arrows* indicate unique bands in donor parents. The top band in *M. rugosa* is lower in molecular-weight than *M. truncatula* and TR8

tern of anthocyanin pigment appeared on the abaxial side of the leaves (Fig. 3). This seedling anthocyanin pigmentation pattern has both *M. truncatula* and *M. rugosa* characteristics. *M. truncatula* seedling leaves have a central solid block of anthocyanin on their adaxial side and no anthocyanin on their abaxial side (Fig. 3). *M. rugosa* seedling leaves have a scattered pattern of anthocyanin on their adaxial and abaxial sides (Fig. 3).

TR8 – AFLP and Southern hybridisation studies

AFLP and Southern-hybridisation studies were also carried out on TR8. AFLP band patterns for TR8 compared to *M. rugosa* and *M. truncatula* are shown in Fig. 4. TR8 has an AFLP pattern very similar to *M. truncatula* but with a few putative donor-specific bands (arrows). Consistent with the AFLP data, TR8 and *M. truncatula* have the same chromosome number. A putative donor-specific band transferred to the hybrid TR8 was isolated and amplified by PCR for use as a Southern hybridisation probe. There was homology with what appear to be three bands in the donor and hybrid. In the case of the lowest-molecular-weight donor band homology was with a band in the hybrid that had run further down the gel (Fig. 5). There was no such distinctive hybridisation to *M. truncatula* DNA.

Chloroplast and mitochondrial DNAs of TSO and TR8

AFLP data obtained from the HASH plants TS0 and TR8 was based on the extraction of total DNA and it was pos-

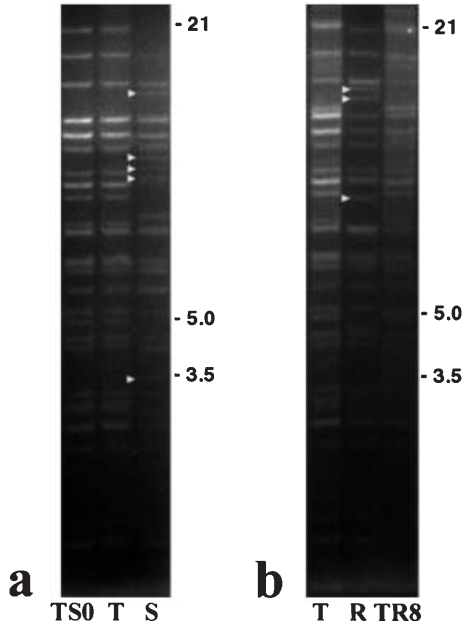

Fig. 7 **a** Restriction endonuclease digest, using *Xho*I, of mtDNA isolated from *M. truncatula* (*T*), *M. scutellata* (*S*) and a highly asymmetric hybrid (*TS0*). **b** Restriction endonuclease digest, using *Xho*I, of mtDNA isolated from *M. truncatula* (*T*), *M. rugosa* (*R*) and a highly asymmetric hybrid (*TR8*). *Arrowheads* indicate unique bands in donor parents

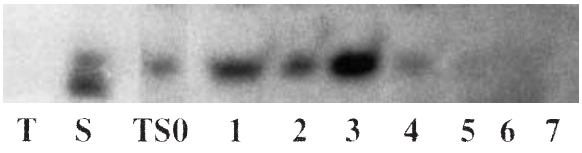

Fig. 8 Southern blot for a selected donor band in TS0 progeny. The band is present in *M. scutellata* (*S*), *TS0* and TS0 progeny *1*, *2*, *3* and *4*; but not in *M. truncatula* (*T*) and TS0 progeny *5*, *6* and *7*. Primers used were E-AAG + M-CAC

sible that, rather than the transfer of nuclear DNA, we were observing changes in organelle DNAs due to transfer of the donor cytoplasm. This proved not to be the case. Restriction digests of both chloroplast (Fig. 6) and mitochondrial DNA (Fig. 7) of TSO and TR8 indicated that the chloroplast DNA and mitochondrial DNA came from the recipient *M. truncatula* and not from the donor *M. rugosa* or *M. scutellata*. The organellar DNAs from both parents were very similar. There was also no indication that the organellar DNA was recombinant.

Progeny analysis of the R1 generation

The transferred DNA is inherited. This has been studied in TS0 by Southern hybridisation where the probe of transferred donor DNA was hybridised against R1 plants (Fig. 8). In the TSO R1 generation 14 plants had the donor band and 17 had no band. In TR8 inheritance of the anthocyanin pigment on the back of leaves from TR8, progeny were recorded as 100 anthocyanin pigmented plants: 74 no-anthocyanin pigmented plants. On the other hand with the pod type in TS0, 100% of the progeny had the new pod shape and characteristic seeds.

Discussion

This study with highly asymmetric hybrids emphasises that it is possible to obtain the transfer of small amounts of DNA by cell fusion and retain high fertility. Both HASH plants TS0 and TR8 produce similar numbers of pods and seeds as the recipient *M. truncatula*, while the asymmetric hybrid TS36 only produced a few pods. It would seem that HASH has the capacity to produce libraries of plants containing donor DNA for selection purposes that are fertile and without a requirement for backcrossing. Cell fusion remains a practicable way of transferring DNA to produce novel and interesting genotypes. Historically there has perhaps been too much emphasis on the transfer of whole genomes. Morphological change is not necessarily a prerequisite for transferring resistances that are of interest agronomically, although some variation lends credence to the effectiveness of the hybridisation protocol.

The techniques used in this study have similarities to the recent study where recipient rice cell suspension protoplasts were treated with IOA and the mitotically inactive donor *Zinzania* mesophyll cells were γ-irradiated (Liu et al. 1999). Gamma-irradiation assists regenerability, presumably because of increased chromosome elimination. Forsberg et al. (1998) have shown that higher doses of X- or UV-irradiation can produce fertile highly asymmetric hybrids in studies involving DNA transfer from *Arabidopsis thaliana* to *Brassica napus*, and probably increase intergenomic translocations. In this latter study increased asymmetry led to increased fertility.

The AFLP technique has the capacity to detect thousands of independent genetic loci without information on specific DNA sequences (Vos et al. 1995; Powell et al.1996). The AFLP study is consistent with the absence of chromosome transfer and the transfer of donor DNA by highly asymmetric somatic hybridisation due to the small number of bands involved. Southern hybridisation of donor bands to the hybrids supports this conclusion. The results indicate that intergenomic recombination probably had occurred in the process of chromosome interaction prior to elimination. Similar kinds of captured donor DNA have been reported in the fusion combinations of *B. napus* (+) *Lesquerella fendleri* (Skarzhinskaya et al. 1996), *M. sativa* (+) *Onobrychus viciifolia* (Li et al. 1993;) and *Zizania latifolia* (+) *Oryza sativa* (Liu et al. 1999).

It is not known whether or not there is an equal chance of transferring all parts of the genome, how large the DNA is that tends to transfer successfully, what minimum amount of DNA can be effectively detected by AFLP, how many independent translocations have occurred with each regenerant, and what the exact mechanism is for intergenomic translocation.

It seems from AFLP studies with other HASH plants that which donor band is transferred to a regenerant is a random event, and there is no indication that special priority is given for the transfer of some parts of the genome. Similar results were noted also in alien DNA marker translocations from somatic hybrids by McGrath et al. (1996), where the introgression does not appear to be restricted to one or a few chromosomes.

The likelihood of translocation seems to be moderately high if only the number of regenerants is taken into account. Five plants overall out of 22 have obtained a number of the donor polymorphic bands, which is 23% of the total sample. This compares with the percentage of translocation, 38%, 3 out of 8 analyzed plants (Piastuch and Bates 1990), 12.9%, 4 from 31 analyzed plants (Parokonny et al. 1992), and about 40%, 12 somatic hybrid plants with a recipient chromosome number from a total of 30 (Skarzhinskaya et al. 1996). Intergenomic translocation may occur at low frequency during the long culture process of fused cells. However, because of the large number of heterokaryons, there are enough events to make use of them for practical purposes.

Obtaining highly asymmetric hybrids is not usually an easy process when the plants are in the same genus, it being more common when wider crosses are used (Dudits et al. 1987). The factors we emphasise in obtaining HASH plants in the *Medicago* genus are the lack of donor regenerability, irradiation treatment of the donor, an unfavourable culture medium for the donor and the IOA treatment of the recipient. The natural growth and development advantage of normal diploid cells is also more likely to occur in fusion combinations with limited regenerability. Though *M. truncatula* cv Jemalong 2HA has high regenerability compared to other annual *Medicagos* it is not as great as in the highly regenerable species from the *Solanaceae* or *Cruciferae*, families where aneuploidy after cell fusion and plant regeneration is common (Wolters et al. 1991; Forsberg et al. 1998; Oberwalder et al. 1998).

In asymmetric somatic hybrids, progeny analysis in terms of fertility, chromosome behaviour, alien DNA introgression, and inheritance of intergenomic translocation has been reported (Famelaer et al. 1989; Parokonny et al. 1994; McGrath et al. 1996). Inheritance of characteristics in asymmetric somatic hybrids was investigated by Ratushnyak et al. (1991), where their sexual progeny was similar to the original somatic hybrids in their biochemical and morphological features.

Intergenomic translocation in somatic hybrids has been shown to be able to be transmitted through selfing or backcrossing to their progeny at a ratio of 2:3 for self and 13:25 for backcross plants with intergenomic translocation: plants without intergenomic translocation (Parokonny et al. 1994). In order to investigate the inheritance of AFLP markers, progeny from TS0 were analysed by the primer combination of E-AAG + M-CAC and by Southern hybridisation. These results indicated that the AFLP band(s) can transmit to their sexual progeny. Our inheritance data show that the inheritance of some characters (pigmentation) and DNA bands (AFLP bands) approaches a 1:1 ratio. However there are some characters that show 100% transmission. This appears to us a reflection of the nature of HASH, built on the selection of the interaction of two genomes and the subsequent loss of one during the culture phase. The important point is that the putative recombinations, rearrangements and translocations are a way of accessing new characters based on hybridisation and subsequent selection pressures.

Highly asymmetric somatic hybridisation does seem a practical breeding technique provided enough plants are produced to capture the DNA of interest. In the case of *M. truncatula* this is a generic technique potentially capable of transferring DNA from any member of the genus.

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