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Asymmetric somatic hybrid plants between *Medicago sativa* L. (alfalfa, lucerne) and *Onobrychis viciifolia* Scop. (sainfoin)

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Abstract. This paper reports on the production of intergeneric somatic hybrid plants between two sexually incompatible legume species. *Medicago sativa* (alfalfa, lucerne) leaf protoplasts were inactivated by lethal doses of iodoacetamide. *Onobrychis viciifolia* (sainfoin) suspension-cell protoplasts were gamma-irradiated at lethal doses. Following electrofusion under optimized conditions about 50,000 viable heterokaryons were produced in each test. The fusion products were cultured with the help of alfalfa nurse protoplasts. Functional complementation permitted only the heterokaryons to survive. A total of 706 putative heterokaryon-derived plantlets were regenerated and 570 survived transplantation to soil. Experimentation was aimed at the introduction of proanthocyanidins (condensed tannins) from sainfoin, a bloat-safe plant, to alfalfa, a bloat-causing forage crop; however, no tannin-positive regenerant plants were detected. Most regenerant plants have shown morphological differences from the fusion parents, although, as expected, all resembled the "recipient" parent, alfalfa. Southern analysis using an improved total-genomic probing technique has shown low levels of sainfoin-specific DNA in 43 out of 158 tested regenerants. Cytogenetic analysis of these asymmetric hybrids has confirmed the existence of euploid (2n = 32; 17%) as well as aneuploid (2n = 30, 33–78; 83%) plants. Pollen germination tests have indicated that the majority of the hybrids were fertile, while 35% had either reduced fertility or were completely sterile.

Key words: Intergeneric hybrid – Protoplast fusion – *Leguminosae* – Total-genomic probing – Bloat

Introduction

Alfalfa has been regarded as the world's most important forage crop and has been heralded as having the highest feeding value and producing more protein per ha than any other forage crop (Duke et al. 1981).

Bloat is a serious problem for ruminants, particularly for beef cattle and dairy cows grazing on pasture of bloat-causing forage species such as alfalfa and white clover. It is caused by the formation of stable proteinaceous foams in the rumen which prevents gas escape and eventually results in mortality and morbidity (Clarke and Reid 1974).

Experimental evidence both in vitro and in vivo has indicated that condensed tannins play a substantial role in preventing bloat. Tannins can suppress and destabilize foam formation by protein precipitation (Jones and Mangan 1977) and microbial inhibition (Lees 1984). Foliar condensed tannin is a common trait in bloat-safe legume species such as sainfoin and birdsfoot trefoil (Goplen et al. 1985). Extensive surveys within the genus *Medicago* have not identified any plant possessing foliar tannins (e.g., Rumbaugh 1979; Goplen et al. 1980; Marshall et al. 1981). Consequently, gene transfer from tannin-positive species to tannin-negative species, such as alfalfa and white clover, can be expected to confer bloat-safety to the latter. Moreover, this protein-tannin interaction may also increase the fraction of dietary protein which escapes degradation by rumen bacteria. Up to 30% of dietary protein may

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be wasted due to degradation in the rumen (Barry and Manley 1984).

To-date little is known about the biosynthesis of condensed tannins in legume species. Cloned-gene transfer is therefore presently out of the question. On the other hand, conventional hybridization is not possible due to sexual incompatibility between the target species. An alternative method, cell-mediated gene transfer by protoplast fusion, is expected to bypass intergeneric incompatibility barriers. Since the goal is a tannin-positive alfalfa rather than a full hybrid, asymmetric rather than symmetric hybridization has been adopted to increase the genetic content of the 'recipient', alfalfa.

There have been only two reports on hybrid calli produced by intergeneric somatic hybridization of *Leguminosae* species, involving *Lotus corniculatus* with *Medicago sativa* (Niizeki and Saito 1989) or with *Glycine max* (Kihara et al. 1992). The former failed to regenerate plants; the latter regenerated a few plants but failed to confirm hybridity and the plants all died before flowering. Successful production of intergeneric asymmetric hybrid or cybrid plants has so far been reported in the families *Umbelliferae* (e.g., Dudits et al. 1980), *Solanaceae* (e.g., Gupta et al. 1984), *Cruciferae* (e.g., Chatterjee et al. 1988) and *Rutaceae* (e.g., Vardi et al. 1989). The application of a total-genomic probing technique has only been reported in species of the *Gramineae* (e.g., Li et al. 1989; Schwarzacher et al. 1989).

Materials and methods

Plant materials

The alfalfa (*M. sativa* cv 'Rangelander') protoplast-regenerable genotype R15 (Davies et al. 1989) was maintained in vitro as a shoot culture on RL medium (Phillips and Collins 1984).

The leaf callus of the sainfoin genotype Ot4 (*Onobrychis viciifolia* cv 'Othello') was developed on UMCN202. UMCN202 is a modified MS202 medium (Pupilli et al. 1989), in which UM vitamins (Uchimiya and Murashige 1974) substitute for MS vitamins, with the addition of 2.5% coconut water (Gibco® Life Technologies, Inc.) and 200 mg/l of casein hydrolysate (N-Z-Amine® Type A, Kraft Inc.). A finely-divided suspension of rapidly-growing Ot4 cells was achieved after repeated subculture in liquid UMCN202.

Protoplast isolation

R15 shoot-culture leaves were cut into strips and plasmolyzed in a non-enzyme washing solution (NEWS), a non-enzyme version of the enzyme solution for alfalfa protoplasts (Davies et al. 1989), for 1 h under vacuum. The strip were then digested in the above enzyme solution for about 16 h at 25 °C in the dark. The protoplasts were purified by sieving through 328-, 110- and 50-μm meshes consecutively and removed from enzyme solution by sedimenting (100 *g*, 5 min) and resuspending in an electrofusion buffer (EFB) consisting of 530 mM glucose, 1 mM $CaCl_2$ and 0.5 mM MES adjusted to pH 5.9 with KOH. The protoplasts were further purified by centrifuging (100 *g*, 5 min) through Percoll stepwise density gradients composed of 5%, 15% and 25% (v/v) pure Percol® (Pharmacia) plus glucose as an osmoticum made up to 700 m Osm/Kg. Purified R15 protoplasts were enriched between the 5% and 15% layers.

Ot4 suspension cells were removed from UMCN202 medium by sedimenting (100 *g*, 5 min) and resuspending three times in NEWS and then digesting in the same enzyme solution as above, but with half the concentration of each enzyme, for about 16 h with shaking (30 rpm, 25 °C, dark). The purification steps were the same as described above, but the purified Ot4 protoplasts were enriched between the 15% and 25% Percoll layers.

Fusion treatments

Ot4 protoplasts were diluted to 2×10^5/ml with EFB and gamma-irradiated with 200–1,000 Gy from a ^{60}Co source before final dilution to $0.5–3.5 \times 10^5$/ml with EFB.

R15 protoplasts were also diluted to 2×10^5/ml with EFB, treated with 0.3–10 mM iodoacetamide (IOA) for 30 min (4 °C, dark), removed from IOA by sedimenting (100 *g*, 5 min) and resuspending three times before final dilution as for Ot4.

The two protoplast suspensions were mixed (1:1) and 0.5-ml aliquots dispensed into a dish consisting of 2 cm × 2 cm square wells (Sterilin). The protoplasts were aligned with a sinusoidal AC wave (0.5–2.3 MHz. 30–360 V_{p-p}/cm, 30–120 s) and fused with 1–5 DC pulses (750–2100 V/cm, 10–1,000 μs, 1–100 ms interval between pulses) from a 900 μf capacitor. A transferable type of electrode (Watts and King 1984) was used in the fusion. The optimized electrofusion conditions are listed in the Results.

Total protoplasts, heterokaryons, and dividing cells were estimated by counting on a haemocytometer. Three samples were taken from each treatment. The proportion of total protoplasts which were viable was estimated using fluorescein diacetate (Larkin 1976).

Plant regeneration

The fusion products were cultured in KWM medium (Davies et al. 1989) at 25 °C in the dark. They were nursed using either calcium alginate beads (Larkin et al. 1988) or Millicell™-HA inserts (Millipore). In the latter case the fusion products were cultured on the insert membranes in a dish and without shaking. Alfalfa nurse protoplasts in KWM medium were added to the dish so that they were level with the insert membrane. The fusion products were subcultured as in Larkin et al. (1988). Well-rooted plantlets were transplanted to sand on a misting-bench and finally to soil in a glasshouse.

DNA extraction

Leaves of R15 or the putative hybrids were ground to a fine powder in liquid nitrogen. An extraction buffer containing 0.3 M EDTA, 1% (w/v) SDS, 0.1 mg/ml Proteinase K and 50 mM Tris-HCl, pH 8.0, was added to the powder, mixed, and kept at 37 °C for 2 h. Twenty percent (w/v) $NaClO_4 \cdot H_2O$ was then added, mixed and centrifuged. The supernatant was mixed with 2.5 vol of ethanol perchlorate solution containing 2.86 M $NaClO_4$ and 80% (v/v) ethanol and centrifuged to precipitate the DNA. The pellet was rinsed with 80% (v/v) ethanol before dissolving in a buffer containing 10 mM EDTA and 10 mM Tris-HCl, pH 8.0. When the DNA mixture dissolved completely 1% (v/v) Triton X-100, 0.2 M NaCl, and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) were added and mixed until an emulsion formed. The emulsion was centrifuged and the DNA was precipitated from the aqueous phase with 2.5 vol of ethanol, hooked up, rinsed with 70% (v/v) ethanol

and air-dried before dissolving in TE buffer containing 1 mM Tris-HCl, pH 8.0.

The DNA solution was further purified by incubating with 0.8 mg/ml RNase A at 37 °C for 30 min. Ammonium acetate was added to a final concentration of 2 M and the DNA extracted with phenol-chloroform-isoamyl alcohol, precipitated, and dissolved in TE buffer as above.

Ot4 suspension cells were washed with H_2O until the filtrate was clear and then ground to a fine powder in liquid nitrogen. The extraction buffer was added and DNA extracted as for the leaves.

Total-genomic probing

DNA concentration was estimated by ethidium bromide fluorescence. The sample DNA and standard DNA were electrophoresed on an 0.8% (w/v) agarose gel with 0.2 µg/ml of ethidium bromide in a TAE buffer (Loening 1967) before comparison of the fluorescence intensity.

The DNA was then restriction-cleaved with *Hinc* II. The DNA fragments were size-separated by electrophoresis on an agarose gel, as above, and transferred to a Hybond N^+-charged nylon membrane (Amersham) using the LKB 2016 VacuGene™ XL vacuum blotting system (Pharmacia) and following the manufacturer's alkaline vacuum transfer protocol except that 0.4 M NaOH was used as the transfer buffer and 6 × SSC was used to wash away agarose from the membrane.

R15-blocking DNA was sheared to 100–500 bp fragments by sonication using a Labsonic 1510 unit (30 W, 6 min) and denatured by boiling in water for 7 min before use.

Ot4-probing DNA was labelled with (α-^{32}P) dATP using the nick translation kit N5500 (Amersham) following the manufacturer's instruction except that a 1.4 × enzyme solution was used. The probe with a specific activity of (3.0–6.2) × 10^8 dpm/µg was purified by centrifuging (500 *g*, 4 min) through a 3-cm column of Sephadex G-50 and denatured by boiling as above.

The transferred membrane was prehybridized in a hybridization solution (Khandjian 1987), containing high contents of formamide (50% v/v), dextran sulphate (10% w/v), NaCl (1 M) and Denhardt's reagent (10 ×), at 42–65 °C for 1 h. Then 90% of the denatured blocking DNA (2–10 times the filter-bound DNA amount) was added to the solution. The remaining 10% blocking DNA and the probe (1/50–1/200 of the filter-bound DNA amount) were added to the solution 12 h following the primary blocking. The probing and secondary blocking continued for 24 h at 42–65 °C.

Hybridization stringency control was completed by subsequent stepwise washings up to 0.1 × SSC at 42–74 °C for 1 h. The optimized total-genomic probing conditions are listed in Figs. 4 and 5. The hybridized membrane was exposed to X-ray film at −70 °C for 1–7 days and then developed with a Kodak™ automatic developer machine.

The signals were also quantitated with a Molecular Dynamics Image Quant™ (V. 3.0) and PhosphorImager. The background was set so that the alfalfa-sainfoin DNA control mixtures gave signals of volume integration proportional to the content of sainfoin DNA.

Chromosome preparation

Newly-rooted cuttings with roots about 1.2 cm long were pretreated in a saturated solution of paradichlorobenzene and bromonaphthalene for 2 h. The roots were collected, fixed for 1 h at 4 °C in Carnoy's solution containing glacial acetic acid, chloroform and ethanol (1:3:6 v/v/v), hydrolyzed in 1 N HCl at 60 °C for 8 min and washed with 70% ethanol. The root tips were stained with Carbol fuchsin solution (Carr and Walker 1961) modified so that 33 ml of Stock solution B was mixed with 94 ml of 47% acetic acid, 3.6 ml of 37% formaldehyde and 1.8 g of sorbitol (Xu Shujun, personal communication) and immediately squashed.

Fast-growing Ot4 suspension cell were pretreated in the saturated solution as above but with shaking. The cells were hydrolyzed in 5 N HCl at room temperature for 45 min and then transferred to 70% ethanol. The cell samples were stained and squashed as for the root tips.

Tannin assay

Green leaves were squashed in a roller between chromatography paper. The leaf residue was removed and the paper was treated with a vanillin-HCl solution consisting of 3.1% (w/v) vanillin, 62.5% (v/v) methanol and 37.5% (v/v) concentrated HCl (modified from Jones et al. 1973). A nondiffusing red colour indicated the presence of condensed tannins.

Male-fertility determination

Newly-opened flowers, prior to anthesis, were collected around 9 am. Fresh pollen grains were dusted into a dish containing a thin layer of liquid germination medium (Brewbaker and Kwack 1963) and incubated at 30 °C in the dark for about 2 h. One percent (w/v) acetocarmine was then added. Pollen germination rate was estimated by counting the proportion of total viable pollen which had germinated. Pollen was scored as viable if it stained with acetocarmine. It was scored as germinated if the length of the pollen tube was greater than the pollen grain diameter. Six to nine inflorescences were collected from each plant and tested independently. Only the inflorescence with maximum germinating pollen was scored in order to avoid any underestimation caused by the different physiological status of the pollen.

Results

Prefusion treatments and electrofusion

The effects of gamma dosage on viability and division of Ot4 protoplasts are shown in Fig. 1. As expected,

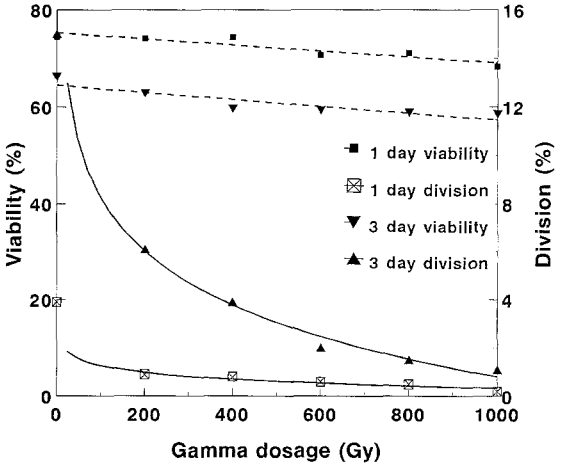

Fig. 1. The effect of gamma dosage on viability and division of Ot4 protoplasts cultured in KWM medium (Davies et al. 1989). The means of three independent experiments are shown

gamma irradiation did arrest cell division as reported previously (e.g., Hall et al. 1992) and this was in a dose-dependent manner. However, it did not seriously affect viability in the first 3 days. Ideally an inactivation treatment should arrest division completely but keep the protoplasts otherwise intact. Doses of 600, 800 and 1,000 Gy efficiently arrested cell division to a similar extent. These doses were therefore adopted in the following fusion experiments.

The effect of the concentration of IOA, an irreversible metabolic inhibitor (Wright 1978), on the viability of R15 protoplasts is shown in Fig. 2. The extent of inactivation was also concentration-dependent. No dividing cells were observed in any of the tested concentration levels from 0.3 mM to 10 mM during the tested time course. IOA concentrations of 0.3, 2.5 and 10 mM were adopted in the following fusion experiments.

After extensive experimentation over a wide range of electrofusion conditions, as list in the Materials and methods, the optimum conditions were determined as follows: AC: 150 V_{p-p}/cm, 2.3 MHz, 30 s; DC: 1,400 V/cm, one pulse, 10 µs; protoplast density: 3.5×10^5/ml;

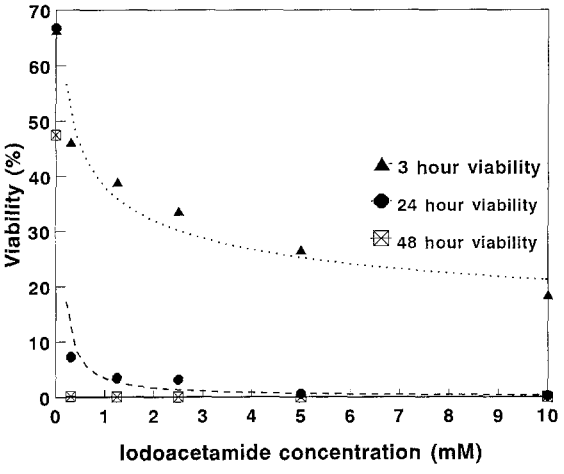

Fig. 2. The effect of IOA concentration on viability of R15 protoplasts cultured in KWM medium (Davies et al. 1989). The means of three independent experiments are shown

fusion buffer: 1 mM $CaCl_2$, 530 mM glucose, 0.5 mM MES, pH 5.9.

Initially heterokaryons were readily distinguishable because their cell contents consisted of unmixed clusters of green R15 chloroplasts and colourless Ot4 cytoplasm. There was a low frequency of multiple heterokaryons (≥ 5 protoplasts) which could be recognized by their larger sizes and these were excluded from the counting.

The heterokaryon frequency was $4.8 \pm 1.2\%$ (mean $\pm$ SE, n = 5), which stands for the heterokaryon number 1 h *after* fusion versus the parental protoplast number *before* fusion. Similarly the viable heterokaryon frequency was $1.6 \pm 0.3\%$ as estimated using fluorescein diacetate 24 h after fusion. Since an average of 3.1×10^6 protoplasts were fused in each fusion experiment, about 50,000 viable heterokaryons were produced, which were predominantly from binary fusions.

Plant regeneration

A summary of three successful electrofusion experiments between IOA-treated R15 leaf protoplasts and gamma-irradiated Ot4 suspension-cell protoplasts is given in Table 1.

No regenerated plantlets were found in 5×10^6 pretreated, but unfused, control protoplasts indicating that the prefusion treatments had successfully prevented any regenerants from parental escape, while the functional complementation did allow heterokaryons to survive.

In Experiment I, the ratio of regenerated plantlets to heterokaryons was higher (0.25%) and more hybrid plants (32 confirmed) were produced when the parental protoplasts were treated less severely and the fusion products were nursed in alginate beads. It appears that the more stringent conditions in Experiment III did not substantially improve the proportion of confirmed asymmetric hybrids relative to tested regenerants but substantially reduced the number of regenerated plants.

In total, 706 putative heterokaryon-derived plantlets were regenerated, of which 570 survived transplantation into soil. Most of these regenerant plants have

Table 1. Summary of the asymmetric somatic hybridization experiments between R15 and Ot4 protoplasts

Experiment	Parental protoplasts ($\times 10^6$)	IOA to R15 (mM)	Gamma rays to Ot4 (Gy)	Nurse method[a]	Heterokaryons ($\times 10^6$) A	Regenerated plantlets B	DNA-tested plants C	Asymmetric hybrids D	B/A (%)	D/C (%)
I	4.3	0.3	600	b	0.23	564	129	32	0.25	25
II	2.4	2.5	800	b, i	0.077	97	20	7	0.13	35
III	3.2	10	1,000	i	0.13	45	9	4	0.035	44
Total	9.9				0.44	706	158	43	0.16	27
Controls	5.0	0.3–10	600–1,000	b, i	0	0				

[a] b, alginate beads; i, Millicell™ HA insert

shown various degrees of morphological differences from the fusion parents. However, as expected from the gamma treatment of the sainfoin protoplasts, all the plants resembled the fusion "recipient", alfalfa, which has pinnately trifoliolate leaves. Figure 3 shows examples of the confirmed asymmetric hybrids and the morphological differences from their parental plants. The leaves of Ot4 (I) are elliptical and the leaves of R15 (II) are heart-shaped. The hybrid plants each had a characteristic leaf shape and size [e.g., varying from very round (VII), elongated (V), to almost triangular (III)] and thickness [e.g., very thick (III) to very thin (IV)]. The leaves of hybrid #313 (VI) are tetra- and penta-foliolate as well as trifoliolate.

In many hundreds of R15 protoplast regenerants, we have observed no variations in leaf number and shape of the type seen in the hybrids. We consider it likely that the morphological variation observed in the hybrids was due to fusion-induced genetic changes and not culture-induced somaclonal variation.

DNA analysis

A series of Southern hybridization experiments were carried out to improve the total-genomic probing technique before it could be applied to the legume species. Fig 4 I-II shows the detection sensitivity and blocking efficiency achieved using the improved total-genomic probing. The very high stringency control (65 °C with 50% formamide in blocking and probing; 0.1 × SSC at 68 °C for 1 h in washing) and the very high ratios of blocking-DNA to probe (400 ×) and to filter-bound DNA (approximately 10 ×), have enhanced the detection sensitivity to about 0.001. That is, one part (2 ng) of sainfoin total-genomic DNA is discernible among one thousand parts (2 µg) of alfalfa total-genomic background (M2 of Fig. 4 I). Under lower stringency control the detection sensitivity decreased remarkably.

To avoid overloading the hybridization signal in the pure sainfoin-DNA lanes (S in Fig. 4), these lanes were each loaded with 100 ng of sainfoin DNA which was only 5% of the the loading in the other lanes. The total genomic DNAs of sainfoin and alfalfa were also mixed and used as simulated hybrid controls.

Figure 4 shows that alfalfa and sainfoin do have some DNA sequences in common. Without the action of the blocking-DNA (Fig. 4 II), not only were the cross-hybridization signals much stronger, but additionally the alfalfa-DNA control (Fig. 4 II, lane A)

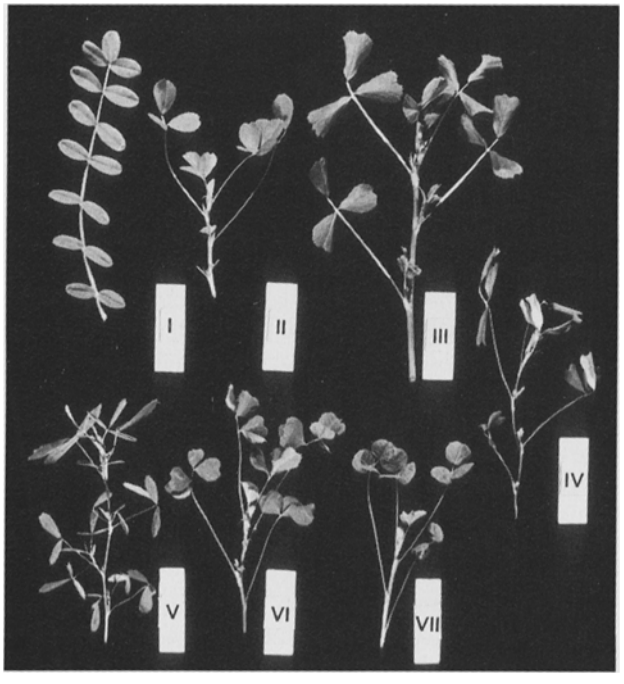

Fig. 3. Morphological comparison between vegetative shoots of the asymmetric somatic hybrids and parental plants. *I* fusion donor, sainfoin Ot4; *II* fusion recipient, alfalfa R15; *III–VII* asymmetric somatic hybrids: *III* #138, *IV* #652; *V* #643; *VI* #313 and *VII* #416

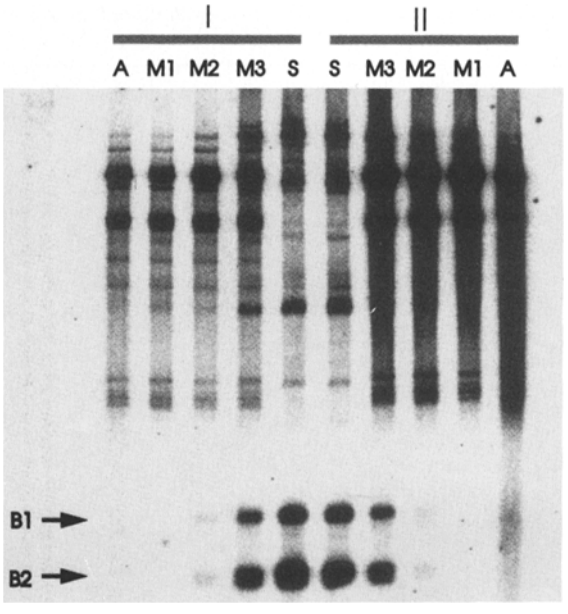

Fig. 4 I–II. Detection sensitivity and blocking efficiency of the optimized total-genomic probing in discrimination of *Hinc* II digests of R15 and R15-Ot4 DNA mixtures on Hybond N^+-charged nylon membrane (hybridized at 65 °C with 50% formamide and washed with 0.1 × SSC at 68 °C for 1 h, probe concentration 20 ng/ml). *Lanes A*, R15 total genomic DNA 2 µg; *M1, M2 and M3*, mixtures of R15 total genomic DNA 2 µg plus 0.2, 2 and 20 ng Ot4 total genomic DNA respectively; *S*, Ot4 total genomic DNA 100 ng (Note: 5% loading of the other lanes). *I*, blocked with R15-blocking DNA at a concentration of 8 µg/ml; *II*, without blocking. *Arrows B1–2*, Ot4-specific DNA bands 1–2

hybridized strongly to the sainfoin probe. With blocking (Fig. 4 I), the cross-hybridization was reduced so much that the signal ratio of lanes A to M3 was decreased by a factor of 13 (Table 2a). In Fig. 4 I, nothing was discernible from the alfalfa-DNA control in the lower part of lane A; however, that part of the lanes containing sainfoin DNA (lane S) and the sainfoin-alfalfa DNA mixtures (lanes M1, M2) consistently showed two bands between 300 and 1,000 base pairs in length (arrows B1 and B2). Moreover, the amount of label bound to these areas was proportional to sainfoin-DNA loads (Table 2a). The region of the gels corresponding to those two bands (B1 and B2) was therefore chosen as the *the diagnostic area* for hybrid detection in the following DNA analysis of the putative hybrid plants.

Of the 570 regenerated putative hybrids, 158 showing more unusual morphological variations were analyzed using total-genomic probing. An example of such a DNA analysis is shown in Fig. 5, in which two hybrids were confirmed. R1 and R5 showed detectable levels of at least one sainfoin-specific DNA band (B1) and perhaps a novel band (B3) that is not found in either of the fusion parental species. The amount of the Ot4 genomic probe detected with the PhosphorImager in the diagnostic area of Fig. 5 was proportional to the amount of Ot4 genomic DNA loaded on the gel (lanes S, M1, M2; Table 2b). Levels of probe corresponding to approximately 6 ng and 9 ng of Ot4 genomic DNA were detected by the PhosphorImager in hybrids R1 and R5 respectively (Table 2b). In Table 2b some signal was also detected in R2, R3, R4, and R6 with the PhosphorImager, but this was not discernible on X-ray film and was not significantly above the background noise (lane A). Signals of Ot4 genomic DNA of one nanogram or so were also not significantly above background noise (e.g., M2 compared to A, Table 2b).

Table 3 is a summary of the Southern analysis of the putative asymmetric hybrid plants using the optimized total-genomic probing. One hundred and fifty three putative hybrids were tested and 43 were confirmed asymmetric hybrids containing low levels of sainfoin-specific DNA.

Extrachromosomal DNA generally accounts for only 0.1–0.01% of the total DNA of higher plants and is highly conserved between species. Therefore, the excess R15 total-genomic DNA was expected to have blocked most of the extrachromosomal DNA. The remaining extrachromosomal DNA which is specific to Ot4 would not bind detectable amounts of probe using the total-genomic probing method.

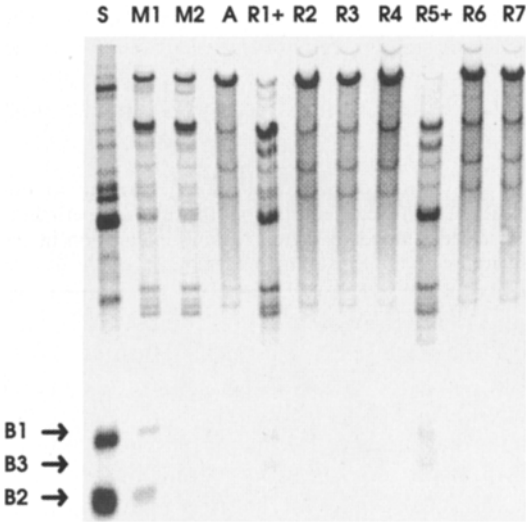

Fig. 5. Southern analysis using the total-genomic probing method to confirm hybridity of the putative hybrid plants regenerated following fusion of R15 and Ot4 protoplasts. All the conditions were the same as in Fig. 4 I except that the membrane was washed up to 72 °C for 1 h in 0.1 × SSC. *Lanes S and A* as in Fig. 4. *M1 and M2*, R15 total genomic DNA 2 μg plus 10 ng and 1 ng of Ot4 total genomic DNA respectively; *R1–R7*; Regenerants tested (+ confirmed asymmetric hybrid), 2 μg total genomic DNA each. *Arrows B1–2*, as in Fig. 4 I; *B3*, a novel DNA band

Table 2a. The amount of Ot4 total-genomic probe hybridizing to the diagnostic area of Fig. 4 I, II as quantitated by signal volume integration with the PhosphorImager

Lane	Ot4 DNA loaded (ng)	Signals relative to S lane (%)	
		Blocked	Unblocked
S	100	100	100
M3	20	26	36
M2	2	2.5	6.6
M1	0.2	1.6	6.2
A	0	1.2	21

Table 2b. The amount of Ot4 total-genomic probe hybridizing to the diagnostic area of Fig. 5 as quantitated by signal volume integration with the PhosphorImager

Lane	Ot4 DNA loaded (ng)	Signals relative to S lane (%)
S	100	100
M1	10	10.4
M2	1	0.9
A	0	0.8
R1+	–	6.2
R2	–	2.2
R3	–	1.7
R4	–	1.7
R5+	–	9.6
R6	–	1.4
R7	–	0.6

+, confirmed asymmetric hybrids

The presence of two strongly-hybridizing low-molecular-weight bands unique to Ot4 has been used to identify hybrid plants which contain introgressed DNA. Because of the extremely stringent hybridization conditions it is likely that these bands used for hybrid confirmation represent highly-repeated sequences.

Table 3. Southern analysis using total-genomic probing[a] to confirm hybridity of some regenerants from the fusion experiments of Table 1 as quantitated by signal volume integration in the diagnostic area with the PhosphorImager

DNA type[b]	Ot4 DNA loaded (ng)	Signal relative to S lane (mean %)	Number of replicates	Number of plants
S	50	100	12	–
M1	5	9.7	2	–
M2	0.5	0.5	2	–
A	0	0.4	8	–
HG1	–	7.3	–	8
HG2	–	9.0	–	12
HG3	–	12	–	7
HG4	–	15	–	8
HG5	–	22	–	5
HG6	–	33	–	3
NH	–	0.6	–	110

[a] All the conditions were the same as in Fig. 5 except that 1 μg of DNA was loaded in each lane and run about 1/3 of the distance
[b] S, M and A, as in Fig. 5; HG1–6, hybrid groups classified according to intensity of the hybridization signals; NH, non-hybrids

Table 4. Chromosome analysis of R15, Ot4 and some confirmed hybrids

	Mitotic chromosome number (2n)	Number of hybrids
R15	32	–
Ot4	48	–
Group 1	32	6
Group 2	30, 33–44	7
Group 3	54–78	23

Other analyses of the regenerants

Of the 43 hybrid plants obtained, 36 were analyzed for their mitotic chromosome numbers. A dramatic variation in the chromosome number was observed (Table 4).

The chromosomes of R15 and Ot4 are not readily distinguishable morphologically (Fig. 6a–b). However, the amount of DNA introduced into the hybrids may be estimated using the following two assumptions:

(1) an even distribution of the sainfoin-diagnostic DNA sequences throughout the sainfoin genome and,
(2) a typical sainfoin chromosome is equivalent to an average alfalfa chromosome in terms of the amount of DNA.

With these assumptions one typical sainfoin chromosome in the presence of 32 average alfalfa chromosomes would be the equivalent of about 30 ng of sainfoin genomic DNA in 1 μg of an alfalfa genomic DNA sample. This would give a signal 60% as strong as lane S (Table 3). The minimum signal that could be confidently detected in the hybrids was about 7% of lane S (HG1, Table 3), whereas the strongest signal (HG6, Table 3) was about 33% of lane S. These signals would be generated by the inclusion of about 0.1 to 0.6 of an average Ot4 chromosome in an alfalfa genome (2n = 32). Table 2 and Fig. 5 have shown that signals of Ot4 DNA of 1–2 nanograms are not distinguishable from background noise, indicating a detection limit of about 0.03 for an average Ot4 chromosome in the alfalfa genome. Based on the above estimation, most of the extra-chromosomes, e.g., in Group 3 (Table 4), should be of alfalfa origin. This is consistent with the alfalfa-like morphology of all the hybrid plants. Therefore, hybrids with 32 chromosomes might be due to a

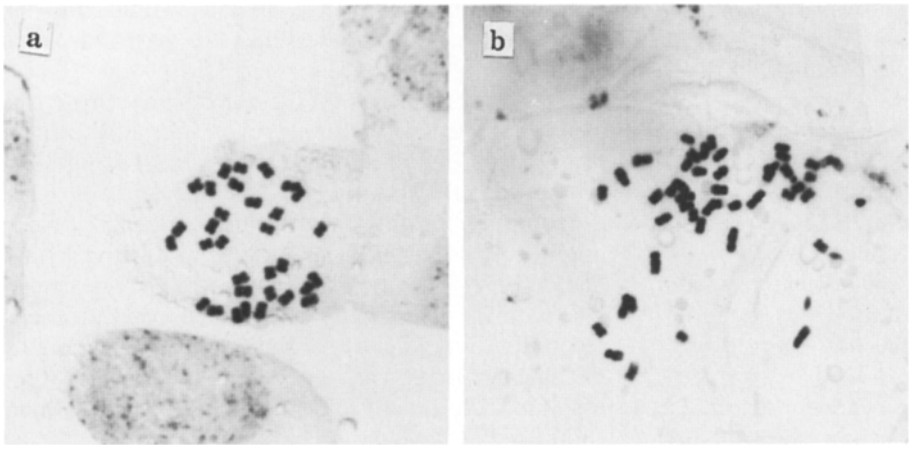

Fig. 6a, b. Mitotic chromosomes of R15 root tips and Ot4 suspension cells. **a** R15 (2n = 32); **b** Ot4 (2n = 48)

translocation(s) between alfalfa and sainfoin chromosomes. The hybrids with 30 to 44 chromosomes might result from the fusion of one or two R15 protoplasts with one Ot4 protoplast followed by the random deletion of R15 and Ot4 chromosomes. The hybrids with 54 to 78 chromosomes might result from the fusion of two or three R15 protoplasts with one Ot4 protoplast followed by extensive loss of R15, as well as Ot4, chromosomes. Quite a few plants in the last group also resembled octoploid alfalfa in general morphology. It is noteworthy that the chromosome number, as well as morphology, of some hybrid plants changed during growth in the glasshouse. For example, the chromosome number of hybrid #354 changed from 78 to 72 in 7 months.

No regenerated plants showed condensed tannins in the leaves. Since the tannin assay using vanillin-HCl is sensitive enough to detect several micrograms of condensed tannins per leaf, no hybrid plants contained tannins at a useful level.

Thirty-seven of the forty-three confirmed hybrid plants were analyzed for male fertility. The control R15 plants had a pollen germination of above 65%, which is regarded as normal fertility. Twenty four plants were of normal fertility; seven were of low fertility ($20 \pm 15\%$ pollen germination) and six were effectively male sterile (below 5% germination).

Discussion

The asymmetric somatic hybridization described above has produced a number of hybrid plants which resemble alfalfa in general morphology but carry small amounts of sainfoin DNA. The majority of these plants were male fertile, with the potential to introduce novel germplasm into a breeding population.

The experimental protocol, from protoplast isolation to the analyses of regenerants, has been reproducible and robust. It can also be extrapolated from our results that the entire sainfoin haploid genome could be covered by only 200 asymmetric hybrids if each hybrid retained on average 0.004 of the introduced sainfoin genome and were non-overlapping. Therefore, it is expected that producing an alfalfa genotype with condensed tannins might depend on producing a large number of heterokaryon-derived plants. To enhance the throughput of hybrid plants however, it is better to use lethal but lower levels of the prefusion treatments combined with alginate bead nursing.

This breeding strategy has three distinctive advantages: firstly, the protoplasts of donor species are not required to be regenerable, and this non-regenerability may even favour the production of highly-asymmetric hybrids; secondly, selective markers are not required in the fusion parents for hybrid selection, since the pre-treatments using IOA and gamma rays efficiently prevented parental escapes while functional complementation allowed survival of heterokaryons; and thirdly, cloned species-specific probes are not required for hybrid confirmation, since Southern analysis using total-genomic probing is versatile and sensitive enough to detect asymmetric hybrids containing a small amount of donor DNA.

Compared with the cloning and screening for species-specific probes, total-genomic probing is simpler and more economical in terms of time and labour. Moreover, a cloned probe may be only homologous to a few chromosomes or chromosome segments (Anamthawat-Jónsson et al. 1990). A total-genomic probe involves all the species-specific sequences, and using such a probe is similar to using a mixture of all the possible cloned probes. The detection sensitivity of total-genomic probing was greatly improved under stringent hybridization as well as stringent washing. This is quite different from conventional stringency control which involves a permissive hybridization and a stringent washing (Anderson and Young 1985). However, the sensitivity needs to be further improved if detection of extremely asymmetric hybrids is required.

While many of the hybrids have more than 32 chromosomes, of particular interest are those asymmetric hybrids possessing 32 chromosomes (Group 1 of Table 4). The results of DNA analysis suggested that they were unlikely to represent substitutions of whole sainfoin chromosomes for alfalfa chromosomes. Instead they are assumed to result from the translocation(s) of sainfoin chromatin into an otherwise-normal alfalfa genome. In-situ hybridization using total-genomic probing may be a valuable tool to clarify the occurrence of chromosome translocation, addition, or substitution, as in cereals (Le et al. 1989; Schwarzacher et al. 1989; Anamthawat-Jónsson et al. 1990). In a practical plant improvement context, however, large numbers of regenerated plants need to be screened for the donor character of interest; rapid screening for the desired character is thus of vital importance and Southern and in-situ analyses would only be used to confirm and understand the origin of plants of interest.

Despite the apparent success of the prefusion treatments in preventing parental escapes in 5×10^6 control protoplasts, 73% of the DNA-tested regenerants were not confirmed as asymmetric hybrids. Three possible explanations for this can be envisaged: firstly, some might be extremely asymmetric hybrids which have retained too-little sainfoin-specific DNA to be detectable with the sensitivity limit of the current protocol; secondly, some might contain sainfoin-specific DNA but not the diagnostic sequences; and thirdly, some might have been derived from heterokaryons but have eliminated all the sainfoin chromatin.

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