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Transfer and segregation of triazine tolerant chloroplasts in *Brassica napus* L.

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Summary. Hypocotyl protoplasts of 45 different genotypes of German winter oilseed rape *Brassica napus* L. (double zero quality: high in yield, seeds low in erucic acid and glucosinolate content) were regenerated to plants. Triazine/triazinone (tri)-tolerant chloroplasts of the Canadian spring oilseed rape variety OAC Triton were introduced into some winter oilseed rapes by means of protoplast fusion. X-ray irradiation was used to limit the transfer of nuclear DNA of Triton protoplasts and to promote the selective transfer of tri-tolerant chloroplasts. Regenerated "cybrid" plants survived a treatment rate of 1000 g/ha metribuzin. The presence and segregation of the tri-tolerant chloroplasts in winter oilseed rape plants, regenerated from fusion products and their progeny, was investigated by restriction fragment length polymorphism (RFLP). Our results indicate that chloroplast segregation was not completed in plants regnerated from fusion products derived from X-irradiated OAC Triton mesophyll protoplasts and German winter oilseed rape hypocotyl protoplasts. In regenerants and their progeny both chloroplast types can still be present. Chloroplasts derived from wintertype protoplasts can outcompete tri-tolerant chloroplasts during plant development. In some instances, even progeny plants not kept under selective conditions (metribuzin) lost tri-tolerant chloroplasts. A homogenous population of tri-tolerant chloroplasts was necessary to obtain stable tri-tolerant winter oilseed rape plants.

Key words: *Brassica napus* – Somatic hybridization – Triazine tolerance – Chloroplast segregation – RFLP-analysis

Introduction

The introduction of winter oilseed rape varieties with "double zero" quality is connected with the problem of volunteer plants containing high levels of glucosinolate. A possible way to eliminate these volunteer plants is the use of "double zero" oilseed rape varieties resistant to a herbicide not selective in oilseed rape. Certain forms of triazine/triazinone (tri)-tolerance are cytoplasmatically inherited (Souza Machado 1982) and encoded by chloroplast DNA (Arntzen and Duesing 1983; Hirschberg and McIntosh 1983; Reith and Straus 1987). Spring oilseed rape varieties with tri- tolerance are already available in Canada where tri-tolerance was transferred from *B. campestris* to *B. napus* by cross breeding (Beversdorf et al. 1980). Protoplast fusion techniques have made it feasible to produce cells with mixed chloroplast populations in species where chloroplasts are maternally inherited. Genetic traits located in chloroplasts or mitochondria can be combined with a desired genome of a recipient plant by irradiating the unwanted nucleus of the donor plant before fusion (Menczel et al. 1982; Aviv and Galun 1985; Kumashiro and Kubo 1986; Imamura et al. 1987).

An important prerequisite for the use of somatic hybridization in oilseed rape is an efficient and reproducible regeneration procedure for protoplasts of a wide range of genotypes. The advantage of hypocotyl protoplasts and their utilization for fusion experiments has been described (Glimelius 1984; Menczel and Wolfe 1984; Sundberg and Glimelius 1986). Efficient regeneration media for hypocotyl protoplasts of a number of oilseed rape varieties have been published (Glimelius 1984; Chuong et al. 1985; Barsby et al. 1986).

In this paper, we describe a regeneration procedure for hypocotyl protoplasts of 45 genetically different winter oilseed rape lines and the transfer of tri-tolerant chloro-

plasts of the Canadian spring oilseed rape variety OAC Triton (Beversdorf and Hume 1984) into some high-yielding German winter oilseed rape lines by means of protoplast fusion. The tri-tolerant chloroplasts of the spring oilseed rape variety OAC Triton are mutated in the psbA gene coding for the 32 kD herbicide binding protein. The Triton psbA gene contains at position +790 an A to G change resulting in a serine to glycine substitution at position 264 of the amino acid sequence (Reith and Straus 1987). In addition, this mutation introduces a new restriction enzyme target for the enzyme BstXI, providing an ideal opportunity for the identification and differentiation of tri-tolerant chloroplasts and tri-sensitive chloroplasts by restriction fragment length polymorphism (RFLP). Results demonstrating segregation of chloroplasts in "cybrid" plants and their progeny are presented.

Materials and methods

Plant material

Seeds of 50 different winter oilseed rape *B. napus* (L.) lines were kindly supplied by Norddeutsche Pflanzenzucht Lembke KG, Holtsee; Gebr. Dippe Saatzucht GmbH, Bad Salzuflen; Kleinwanzlebener Saatzucht AG, Einbeck; Kartoffelzucht Boehm KG, Langquaid; and Deutsche Saatveredelung GmbH, Lippstadt; all FRG. The seeds (high in yield, double zero quality) represented the best breeding material of each plant breeder. Tri-tolerant OAC Triton seeds were obtained from the University of Guelph, Ontario, Canada.

Seeds were surface-sterilized in 70% EtOH for 1 min followed by continuous shaking in a 6% solution of NaOCl with a drop of Tween 80 for 20 min and rinsed 4 times in deionized water. Seeds were germinated on a hormone-free half strength MS-medium (1/2 MS) (Murashige and Skoog 1962), containing 15 g/l sucrose and 0.25% (v/w) of Gelrite (Scott Laboratories, Fiskeville RI, USA) in the dark or light at 26 °C. Shoot cultures were established from seedlings 5–7 days after germination in the light. Seedlings were grown in jars on 1/2 MS at 26 °C with a 16 h photoperiod of 3,500 lux intensity.

Isolation and regeneration of protoplasts

Hypocotyls from 3 to 4-day-old seedlings grown in the dark were cut into 0.5–1.0 mm segments, preplasmolysed in a CPW salt solution (Frearson et al. 1973) with 13% mannitol (CPW 13M), pH 5.5 for 1 h. The salt solution was replaced by an enzyme solution consisting of 1% cellulase R-10, 0.3% macerozyme R-10 (Yakult Honsha, Tokyo, Japan) and 0.05% pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan) dissolved in CPW 13M, pH 5.5. The mixture was placed on a shaker (40 rpm) for 15 h in the dark at 26 °C. The enzyme-protoplasts mixture was sieved (50 µm) and the filtrate was centrifuged at 100 × g for 8 min. The pellet was suspended in CPW containing 22% sucrose (CPW 22S) and spun at 100 × g for 8 min. Floating protoplasts were collected and washed by centrifugation at 100 × g for 8 min in a W5 salt solution (Menczel and Wolfe 1984). The protoplast pellet was suspended in culture medium or in W5 for protoplast fusion or X-irradiation.

Leaves for protoplast isolation were taken from 4-week-old shoot cultures and protoplasts were isolated as described for hypocotyls. Protoplasts were cultured in petri dishes with 1.5 ml protoplast culture medium (PC-medium) according to Glimelius (1984) at a density of 1 to 2×10^5 protoplasts/ml. The dishes were kept in dim light for 2 days at 26 °C and then in a 16 h photoperiod of 1,500 lux intensity. For reduction of the osmoticum aliquots of 0.5 ml of dilution medium (PC-medium with 30 g/l sucrose instead of glucose) was added after 7, 9 and 11 days. For the last dilution step 1 ml callus medium (CC-medium), a MS supplemented with 0.1 mg/l 2.4D, 0.1 mg/l NAA, 0.5 mg/l BAP and 20 g/l sucrose, pH 5.8 was added after 14 days. When most cells of the microcolonies had formed extended cells after approximately 16 days they were plated onto CC-medium solidified with 0.6% agarose (type I, Sigma). After 7 days microcalli of 0.5–1.0 mm were separated and placed close together on fresh CC-medium for further growth.

Protoplasts of rapeseed lines which tended to produce an extensive amount of brown exudate were embedded in drops (100 µl) of PC-medium (2×10^5/ml) solidified with 0.6% agarose (Sigma, Type VII) within 48 h after protoplast isolation. Twelve of these droplets were pipetted into 9 cm diameter petri dishes and 5 ml of liquid PC-medium was added after gelling of the agarose.

Calli of 1–3 mm were placed onto a shoot inducing SR-medium (MS with 15 g/l sucrose, 0.1 mg/l IAA, 1.00 mg/l BAP, 2.00 mg/l Zeatin and 0.25% Gelrite, pH 5.8) and transferred to fresh media after a growth period of 3–4 weeks. Shoots were removed, together with a tiny part of callus at the basal cut-end, and transferred to 100 ml jars onto SMI medium (consisting of MS with 15 g/l sucrose, 0.5 mg/l BAP, 0.1 mg/l NAA and 0.25% Gelrite, pH 5.8) for further growth and multiplication. After 10 days on this medium, shoots were transferred to R-medium (1/2 MS supplemented with 0.5 mg/l NAA solidified with Gelrite) for root formation. When first roots appeared, plantlets were transferred to peat pellets (Jiffi-7) in 1 liter preserving jars.

X-ray irradiation

Leaf mesophyll protoplasts of OAC Triton were suspended in W5 (5×10^5 protoplasts/ml) and irradiated by an X-ray source (Philips G301, 300 kV, 5 mA, 15 min) with a dose of 10 kR or 15 kR. After irradiation, protoplasts were washed with W5 by centrifugation at 80 × g for 8 min.

Protoplast fusion

Hypocotyl protoplasts and X-irradiated mesophyll protoplasts were mixed in a 1:1 ratio in W5, centrifuged at 80 × g for 8 min, and immediately fused (2×10^6 protoplasts/ml) according to Menczel and Wolfe (1984) with slight modifications of the fusion solution. Fusion solution: (A) 1.5 g polyethylene glycol (PEG 6,000), 88 mg $CaCl_2 \times H_2O$ and 180 mg mannitol (Serva, FRG) were dissolved in 8.0 ml H_2O; (B) 468 mg glycine was dissolved in 25 ml H_2O, adjusting the pH to 10.0 with 10 M NaOH. Prior to fusion, 800 µl of solution A and 100 µl of solution B were thoroughly mixed with 100 µl dimethylsulfoxide (DMSO) and immediately used (final conc. 15% PEG 6,000, 60 m*M* $CaCl_2 \times 2H_2O$, 90 m*M* mannitol, 25 m*M* glycine and 10% DMSO).

Eight equally sized drops of the fusion solution were arranged in pairs in a 5 cm diameter plastic petri dish. One drop of the protoplast mixture was gently added to each pair of drops so that the three drops coalesced. Two further drops of the fusion solution were added to both sides of each coalesced drop. After 10 min, 2 ml of W5 supplemented with 50 m*M* morpholinoethane sulfonic acid (MES), pH 5.5 was gradually added over a period of 5 min. Subsequently, the whole petri dish was filled and stored at room temperature for 1.5 h.

Isolation of fusion products

Fusion products were isolated within 24 h after fusion using a microcapillary connected with a 10 µl syringe integrated in a programmable microprocessor controlled pipette (Micro lab P, Hamilton, Switzerland). Additionally, a 5 ml syringe was connected to the system via a 3-port valve for flushing the system with oil to remove entrapped air bubbles. An external speed control and a foot pedal for starting each sucking step were connected to the microprocessor. Each fusion product was isolated in a volume of 50–80 nl. Approximately 50 collected fusion products were injected into drops of 20 µl of PC-medium supplemented with 0.6% agarose (Sigma, type VII) placed in a 3.5 cm diameter petri dish. After solidification of the droplets a 2-day-old hypocotyl protoplast nurse culture was added and cultured as described below. Alternatively, the collected fusion products were transferred onto a transparent Biopore membrane of a minicell insert (Millipore, FRG), which was placed into a petri dish containing a nurse culture.

Selection of tri-tolerant plants

Regenerated plants derived from fusion experiments and their progeny were tested for tri-tolerance under greenhouse conditions (20 °C, 16 h photoperiod) by leaf application of the triazinon metribuzin (Sencor®, Bayer AG, FRG) in the range of 25–1000 g/ha (pure substance). Plants surviving a treatment rate of 300 g/ha were vernalized for 8–10 weeks at 4 °C and later selfpollinated. "Cybrids" were identified on the basis of their tolerance to metribuzin and the need to be vernalized in order to flower.

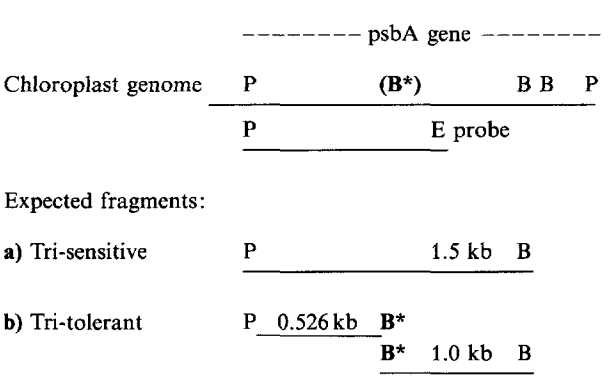

Fig. 1. Schematic map of the psbA gene on the chloroplast genome of *B. napus*, homology of the probe and expected restriction fragment sizes: *a* for tri-sensitive chloroplasts and *b* for tri-tolerant chloroplasts; (**B***) = BstXI only present on DNA of tri-tolerant chloroplasts; P = PstI; B = BstXI; E = EcoRV

Isolation of plant DNA and RFLP analysis

Plant DNA was isolated according to Taylor and Powell (1983). For RFLP analysis of the chloroplast DNA by Southern blotting (Southern 1975), 2 µg of total plant DNA was cleaved for 3 h in 300 µl of the appropriate restriction buffer using approximately 40 units of the restriction enzymes indicated. The mixture was extracted with phenol and chloroform and the DNA precipitated with EtOH. The DNA was dissolved in LTE buffer (0.1 m*M* EDTA; 10 m*M* Tris, pH 7.5) and applied to an 1% agarose gel containing TEA buffer (40 m*M* Tris; 20 m*M* sodium acetate; 18 m*M* sodium chloride and 2 m*M* EDTA, pH 7.8) and, after electrophoresis, transferred to nitrocellulose filters. Hybridization probes (a subcloned PstI/EcoRV fragment, see Fig. 1; from the psbA gene of *Solanum nigrum*; supplied by Dr. P. H. Schreier) were synthesized by nick translation. The probes were labeled to give approximately $>10^8$ cpm/µg. Hybridization was for 24 h at 42 °C and filters were washed at 50 °C as described by Hughes et. al. (1978). Films were exposed at −70 °C for 1–3 h using intensifying screens.

Results

Plant regeneration

Most of the media developed for the regeneration of *B. napus* protoplasts are suitable for only a limited number of oilseed rape genotypes. We chose some winter oilseed lines from different plant breeders (R26, R38, R41, R69, R72) and assessed several culture media (Glimelius 1984; Barsby et al. 1986; M. McLellan, personal communication; Chuong et al. 1985; Pelletier et al. 1983) for plant regeneration capability. Shoot regeneration was insufficient on most of these media. Best plant regeneration frequencies were obtained by using a combination of PC-medium for protoplast development (Glimelius 1984), CC-medium for callus formation and SR-medium for shoot regeneration. This media combination turned out to be best for plant regeneration of hypocotyl protoplasts derived from all winter oilseed rape lines tested. Using this media combination, plant regeneration was obtained from protoplasts of 45 different lines. However, differences were found among the lines in the capability of calli to regenerate shoots. Shoot induction in the regenerated calli was still the bottleneck. The regeneration frequency for most lines was between 10%–20%, depending on the genotype. Best frequencies were obtained with line R109 (40%), R72 (22%) and R38 (18%). Only a few lines tested failed to regenerate shoots or formed shoots at a very low frequency (<3%).

After 7–10 days in culture medium protoplasts of some winter oilseed rape lines released a brown exudate that caused a drastic decrease of the further development of microcolonies (Schenck and Roebbelen 1982; Glimelius 1984). Protoplasts of those lines could be regenerated, when they were embedded into agarose droplets, where browning did not severely affect normal growing cells. Hypocotyl protoplasts in liquid medium formed microcolonies with elongated cells after 14–18 days in culture. These microcolonies were plated onto CC-medium, where they developed within 7 days to microcalli of 0.5 mm in diameter. Calli of 1–3 mm in diameter were transferred to SR-medium for shoot induction. Shoots with a morphologically abnormal appearance often formed normal plantlets when they were transferred with a piece of remaining callus tissue at the cut-end onto SMI for shoot growth and multiplication. Following root development, plantlets were transferred to peat pellets contained in preserving jars where rapid root growth occurred. A gradual hardening-off of the plants was achieved by a stepwise removal of the lids.

The fusion method as described by Menczel and Wolfe (1984) turned out to be most convenient for the isolation of individual fusion products by means of a microcapillary. Fusion products consisted mainly of 2–3 protoplasts. Multifusion bodies were rarely observed. Fusion frequencies of more than 20% (mesophyll × hypocotyl) were achieved by an increase of PEG and the addition of Ca^{++} and small amounts of mannitol. The use of W5 as washing solution prior to fusion was an essential prerequisite for a high fusion frequency. Washing solutions containing high concentrations of mannitol or sucrose reduced the fusion rate to less than 3%, confirming the results obtained by Negrutiu et al. (1986). More than 50% of the fusion products divided after 3 days in PC-medium. Mesophyll protoplasts of almost all winter lines tested did not further develop in PC-medium. However, mesophyll protoplasts were able to regenerate when mixed with hypocotyl protoplasts. The isolation and ejection of fusion products in a definite volume of culture medium was possible using micropipettes controlled by a micromanipulator connected to the "Micro lab P". The injection of collected fusion products into agarose medium droplets before solidification made it possible to nurse fusion products by adding hypocotyl protoplasts to the solidified droplets. The alternative method of culturing fusion products in minicell inserts showed that it was also possible to regenerate fusion products when the inserts were placed in a protoplast nurse culture. However, it was difficult to regenerate fusion products when browning appeared. In this case, better results were obtained by using agarose droplets.

A dose of 10 kR was found to be effective to inactivate nuclear function without damage to the cytoplasmic function in *Nicotiana debneyi* (Kumashiro and Kubo 1986). After X-irradiation with a dose of 10 kR, single *Brassica* protoplasts still underwent first cell division but no microcolonies were formed. A dose of 15 kR prevented hypocotyl and mesophyll protoplast completely from cell division and promoted "cybrid" formation.

Selection of "cybrids"

All plants regenerated from fusion experiments were treated routinely with 300 g/ha of the triazinone metribuzin. Tri-tolerant plants survived a treatment rate up to 1000 g/ha metribuzin, whereas sensitive plants were killed within 10 days by rates of less than 60 g/ha. Among the first 100 regenerants, 8 plants survived a treatment with metribuzin and possessed the winter habit (r26a, r26b, r38, r41, r69, r72a, r72b and r72c) (Table 1). These plants required vernalization to induce flowering. Six of these regenerants were morphologically normal in appearance, developed flowers and set viable seeds. Regenerant r72c was female sterile and r26a formed no normal racemes. Both plants possessed wrinkled leaves.

Table 1. Proportion of tri-tolerant chloroplasts estimated by RFLP (*pv* = prior to vernalization, *ds* = during seed setting) in regenerants and progenies derived from fusion experiments between protoplasts of tri-tolerant spring oilseed rape OAC Triton and protoplasts of German winter oilseed rape lines (double zero quality). Percental proportion of tri-tolerant and tri-sensitive chloroplasts was estimated by counting the filterbound radioactivity

% proportion of tri-tolerant chloroplasts							
Regenerant	pv	ds	Progeny S1	pv	ds	Progeny S2	ds
r26a	>99	–					
r26b	–	0					
r38	10	>10					
r41	–	0	r41-S1/1	>99[a]	>99	r41-S2	100
			r41-S1/2	>99	>99		
r69	–	0					
r72a	–	0					
r72b	–	0	r72b-S1/1	>99[a]	>99	r72b-S2	100
			r72b-S1/2	>99	0		
r72c	>99	–					

[a] Plants were watered with 10^{-7} M metribuzin until first racemes occurred

Molecular and genetic analysis of "cybrids"

For genetic analysis, plants derived from the first 100 seeds from regenerants r41, r72a and r72b were treated with the herbicide. Surprisingly, no progeny plants derived from regenerant r72a, only 3 progeny plants of r41 and 6 plants of r72b survived the herbicide application. Because of these unexpected results, the regenerants and 4 surviving progeny plants (r41-S1/1, r41-S1/2 and r72b-S1/1, r72b-S1/2) were investigated by RFPL analysis (Fig. 1) to obtain information concerning the chloroplast composition of these plants.

For RFLP analysis, total plant DNA was isolated from leaves of Triton, line R26, R38 (controls), all 8 regenerants (r26a, r26b, r38, r41, r69, r72a, R72b and r72c) and the progeny of r41 and r72b. DNA was isolated from plants at the vegetative stage (prior to vernalization) or at the flowering stage. Digestion of the genomic DNA with PstI generated a 2.3 kb fragment that hybridized to the probes (Fig. 2). PstI/BstXI digestion of DNA isolated from the tri-tolerant OAC Triton generated two fragments of 1.0 kb and 0.526 kb (Fig. 2a). In contrast, DNA isolated from the tri-sensitive winter rapeseed lines (R26 and R38) generated only one fragment of 1.5 kb hybridizing to the probe (Fig. 2a).

Analysis of the 8 regenerants (Table 1) showed that 3 regenerants (r26a, r38 and r72c) possessed tri-tolerant chloroplasts, indicated by the 2 hybridizing fragments (Fig. 2a). These regenerants were analysed prior to vernalization. Regenerants r26a and r72c possessed almost 100% tri-tolerant chloroplasts. Only a very weak signal

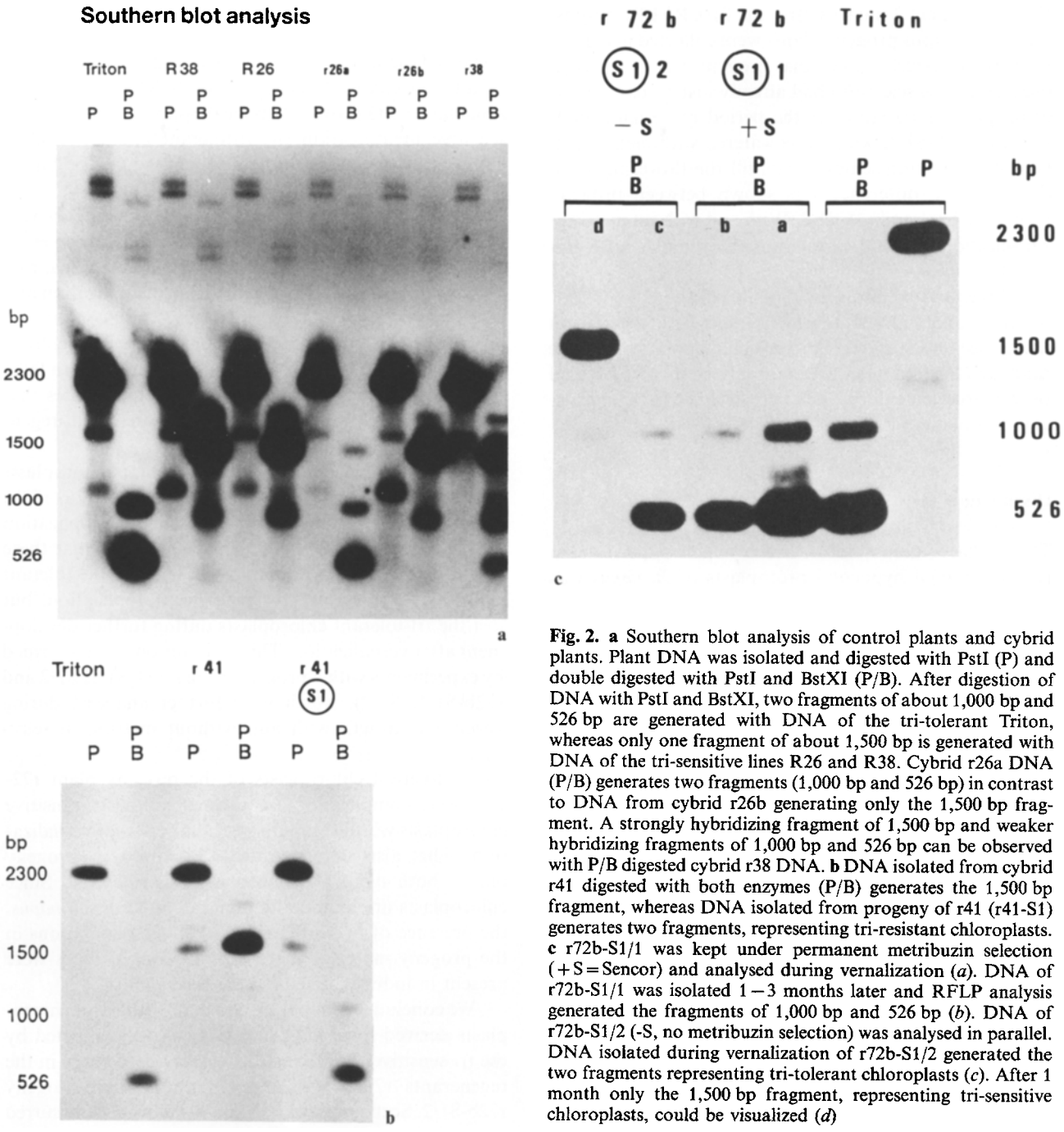

Fig. 2. **a** Southern blot analysis of control plants and cybrid plants. Plant DNA was isolated and digested with PstI (P) and double digested with PstI and BstXI (P/B). After digestion of DNA with PstI and BstXI, two fragments of about 1,000 bp and 526 bp are generated with DNA of the tri-tolerant Triton, whereas only one fragment of about 1,500 bp is generated with DNA of the tri-sensitive lines R26 and R38. Cybrid r26a DNA (P/B) generates two fragments (1,000 bp and 526 bp) in contrast to DNA from cybrid r26b generating only the 1,500 bp fragment. A strongly hybridizing fragment of 1,500 bp and weaker hybridizing fragments of 1,000 bp and 526 bp can be observed with P/B digested cybrid r38 DNA. **b** DNA isolated from cybrid r41 digested with both enzymes (P/B) generates the 1,500 bp fragment, whereas DNA isolated from progeny of r41 (r41-S1) generates two fragments, representing tri-resistant chloroplasts. **c** r72b-S1/1 was kept under permanent metribuzin selection (+S = Sencor) and analysed during vernalization (*a*). DNA of r72b-S1/1 was isolated 1 − 3 months later and RFLP analysis generated the fragments of 1,000 bp and 526 bp (*b*). DNA of r72b-S1/2 (-S, no metribuzin selection) was analysed in parallel. DNA isolated during vernalization of r72b-S1/2 generated the two fragments representing tri-tolerant chloroplasts (*c*). After 1 month only the 1,500 bp fragment, representing tri-sensitive chloroplasts, could be visualized (*d*)

could be detected at 1.5 kb, representing less than 1% of tri-sensitive chloroplasts. On the other hand, plant r38 contained about 10% of tri-tolerant chloroplasts and 90% sensitive chloroplasts. Plant r38 was then watered with 10^{-7} M metribuzin to investigate the influence of metribuzin selection on the proportion of both chloroplast types. This plant was analysed 3 months later and a slight shift toward the tri-tolerant chloroplasts was observed (data not shown). Five of the regenerants (r26b, r41, r69, r72a and r72b) were analysed during late seed setting and all of them contained only tri-sensitive winter oilseed rape chloroplasts.

However, progeny plants of the regenerants r41 (Fig. 2b) and r72b (Table 1) that survived a herbicide application contained about 99% tri-tolerant chloroplasts when analysed before vernalization. Four of theses progeny plants (r41-S1/1, S1/2 and r72b-S1/1, S1/2) were further analysed. One progeny plant of r41 and r72b (r41-S1/1 and r72b-S1/1) was kept under permanent metribuzin selection (watered with 5×10^{-7} M metribuzin).

During the next 3 months after the first RFLP analysis, leaves of the four progeny plants were collected and analysed. It was clearly shown that progeny plant r72b-S1/2 (no metribuzin selection) had already lost all tri-tolerant chloroplasts 1 month after the period of vernalization, whereas r72b-S1/1, which was watered with metribuzin, kept its tri-tolerant chloroplasts till the flowering stage (Fig. 2c). No difference was shown between progeny plants r41-S1/1 and r41-S1/2. With and without metribuzin selection pressure tri-tolerant chloroplasts were stably maintained.

Threehundred progeny plants (S2) of r41-S1/1, r41-S1/2 and r72b-S1/1 were treated with metribuzin (300 g/ha) and survived the herbicide application. Nine randomly chosen plants were analysed by RFLP and only tri-tolerant chloroplasts were detected in these progeny (S2) plants (data not shown).

Discussion

The media combination described in this paper for the regeneration of hypocotyl protoplasts of *B. napus* was the best for the regeneration of a wide range of winter oilseed rape lines. Tri-tolerance has already been introduced into *B. napus* varieties by protoplast fusion (Pelletier et al. 1983; Robertson et al. 1987; Yarrow et al; 1986; Barsby et al. 1987). We succeeded in transferring tri-tolerance from the spring variety OAC Triton into some German winter oilseed rape lines of double zero quality. In order to promote a selective transfer of organelles in the production of cybrids, X-irradiation has been successfully used to significantly reduce the amount of unwanted nuclear DNA of the irradiated fusion partner (Aviv et al. 1980; Imamura et al. 1987). In our experiments a dose of 15 kR promoted cybrid formation and effectively prevented hypocotyl protoplasts of winter oilseed rape from forming microcolonies. Nevertheless, some of the genomic material of the X-irradiated protoplasts can still be expected to be found in fusion products (Imamura et al. 1987). Chloroplast segregation following protoplast fusion has been studied intensively in *Nicotiana* (Chen et al. 1977; Sidorov et al. 1981; Flick et al. 1983, 1985). Usually a mixed population of chloroplasts does not persist in hybrid or cybrid plants. In most plants regenerated from fusion products, chloroplasts sort out and only one type of chloroplast remains (Aviv et al. 1980). Morgan and Maliga (1987) reported that chloroplast segregation was complete already after 20 cell divisions in *B. napus* cybrids. Only chloroplasts of iodoacetate-treated hypocotyl protoplasts maintained after double-inactivation experiments. Sidorov et al. (1981) found a preferential maintenance of chloroplasts form the X-irradiated fusion partner after double-inactivation experiments in *Nicotiana*.

Thus, we expected for our experiments that all regenerated plants that survived the metribuzin treatment and possessed the winter type nucleus would contain only the tri-tolerant chloroplasts. The new BstXI restriction enzyme target site provided an ideal opportunity for a very sensitive identification of tri-tolerant and tri-sensitive chloroplasts by restriction fragment length polymorphism (RFLP).

RFLP-analysis of the 8 regenerants (r26a, r26b, r38, r41, r69, r72a, r72b, R72c) demonstrated that tri-tolerant chloroplasts were only present in regenerants (r26a, r38, r72c) when analysed between metribuzin application and vernalization (Table 1). Regenerant r38 still contained a mixture of 10% tri-tolerant and 90% tri-sensitive chloroplasts, whereas r26a and r72c possessed more than 99% tri-tolerant chloroplasts. This clearly demonstrates that both types of chloroplasts can still be present in regenerants.

No detectable amounts of tri-tolerant chloroplasts were found in regenerants (r26b, r41, r69, r72a, r72b) when analysed 6–8 months after metribuzin application during the late flowering stage. We assumed that these five regenerants initially contained enough tri-tolerant chloroplasts to survive the metribuzin application, but lost the tri-tolerant chloroplasts during further development after vernalization. This assumption was confirmed by experiments with 4 progeny plants (r41-S1/1, S1/2 and r72b-S1/1, S1/2), which were further analysed during vegetative growth with and without metribuzin selection.

Tri-tolerant chloroplasts of the progeny plant r72-S1/2 were completely out-competed by the tri-sensitive chloroplasts within 1 month after the period of vernalization. This also demonstrated that, even in progeny plants, both types of chloroplasts can still exist. Since chloroplasts are maternally inherited in *Brassica napus*, the presence of two different chloroplast populations in the progeny indicates that both chloroplast types were present in individual cells of the regenerants.

We conclude from our results that tri-tolerant chloroplasts derived form OAC Triton were out-competed by the tri-sensitive chloroplasts of winter oilseed rape in the regenerants r26b, r41, r69, r72a, r72b and progeny r72b-S1/2. Sorting-out of tri-tolerant chloroplast occurred after the metribuzin treatment during the further development of these plants as shown for the progeny plant r72b-S1/2. The source of protoplasts seems to have an influence on chloroplast segregation. The tri-tolerant chloroplasts originally derived from an atrazine-resistant biotype of *B. campestris* (Beversdorf et al. 1980) might be less compatible to the nucleus of *B. napus*. A certain disadvantage of the tri-tolerant spring type chloroplasts compared to the winter type chloroplasts during vernalization cannot be excluded. One reason for r41-S1/1 maintaining tri-tolerant chloroplasts over a period of

3 months without herbicide selection might be due to a better compatibility of Triton chloroplasts to the particular nucleus of line R41 than to the nucleus of line R72. Yarrow et al. (1986) report that sorting of chloroplasts may have been non-random in their experiments and that the native *B. napus* chloroplast might have been favoured.

Our data demonstrate that both parental chloroplasts can persist in regenerants and progeny plants. In progeny plants few tri-sensitive chloroplasts can still out-compete tri-tolerant chloroplasts when no selection pressure is applied. However, we finally succeded in obtaining stable tri-tolerant winter oilseed rape lines derived from "cybrids" r41 and r72b in the S2 generation.

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