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Identification and molecular analysis of transgenic potato chromosomes transferred to tomato through microprotoplast fusion

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Abstract Results are reported on the integration sites and copy number of alien marker genes neomycin phosphotransferase II (*nptII*) and β-glucuronidase (*uid*A), introduced into diploid potato *Solanum tuberosum* through transformation by *Agrobacterium tumefaciens*. Also, the transgenic potato chromosomes 3 and 5 harbouring the *nptII* and *uid*A genes, which were transferred to tomato (wild species *Lycopersicon peruvianum*) by microprotoplast fusion, as revealed by genomic in situ hybridization (GISH), were identified by RFLP analysis using chromosome-specific markers. The data revealed three integration sites in the donor potato genome, each containing the *uid*A gene, and two also harbouring the *nptII* gene. Analysis of monosomic-addition hybrid plants obtained after microprotoplast fusion showed that each of these three integration sites is located on a different potato chromosome. The microprotoplast hybrid plants contained only the chromosomes that carried the selectable gene *nptII*. The data on sexual transmission of the donor potato chromosome carrying the *uid*A and *nptII* genes were obtained by analysing the first backcross progeny (BC_1) derived from crossing a monosomic-addition hybrid plant to tomato (*L. peruvianum*). The glucuronidase (GUS) assay and PCR analysis using primers for the *uid*A gene indicated the presence of the potato chromosome in GUS-positive and its absence in GUS-negative BC_1 plants. RFLP analysis confirmed sexual transmission of the potato chromosome carrying the *nptII* and *uid*A genes to the BC_1 plants. A few BC_1 plants contained the *nptII* and *uid*A genes in the absence of the potato additional chromosome, indicating that the marker genes were integrated into the tomato genome. The potential applications of the transfer of alien chromosomes and genes by microprotoplast fusion technique are discussed.

Key words Microprotoplast fusion · Chromosome transfer · Alien gene integration · Chromosome identification · Sexual transmission

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Introduction

Advances in recent years in the field of plant cell and molecular biology have led to important breakthroughs which are potentially relevant with respect to plant breeding. The developments in these disciplines are especially significant for broadening the genetic base in crop plants by creating new genetic variation and for transferring economically important traits between sexually non-hybridizing plant species. Genetic manipulation and parasexual techniques are applicable to somatic cells, thus bypassing crossing barriers or taxonomic boundaries (Gleba and Sytnik 1984; Sybenga 1989, 1992; Potrykus et al. 1995; Waara and Glimelius 1995). Distant hybrids that can not be produced by conventional sexual crosses, can now be obtained through somatic hybridization. For example, while sexual hybridization between potato and tomato has never been reported, such hybrids have been successfully produced through somatic hybridization (Melchers et al. 1978; Shepard et al. 1983; Jacobsen et al. 1992). Melchers (1984) pointed out some of the potential applications of somatic hybridization for crop improvement. In most of these applications, an exchange (meiotic) of genetic material or introgression is required. In this regard, the availability and introduction of alien marker genes, such as resistance to

kanamycin, hygromycin and herbicides, together with genes coding for β-glucuronidase are useful for the selection of fusion hybrids. Further, they can be used for the analysis of integration and expression of genes in the hybrids as well as in the backcross progenies when they are linked to known chromosomes and genes.

Partial genome transfer has been achieved by carying out asymmetric somatic hybridization using irradiated donor protoplasts and normal recipient protoplasts. However, several studies have shown that the hybrids obtained were genetically complex in containing several donor chromosomes, often carrying unwanted genes, and were frequently sterile (Famelaer et al. 1989; Wijbrandi et al. 1990; Puite and Schaart 1993; Wolters et al. 1994). Recently, we found that microprotoplast-mediated chromosome transfer (MMCT) is an efficient parasexual technique for transferring single, intact chromosomes from one species to the other (Ramulu et al. 1996a,b). The establishment of procedures for the isolation of small subdiploid microprotoplasts and the fusion of these with the recipient protoplasts enabled the development of MMCT technology (Verhoeven and Ramulu 1991; Ramulu et al. 1993). The transfer of single, specific chromosomes to directly produce monosomic addition plants in sexually incongruent plant species offers new opportunities for the transfer of economically important genes, introgressive breeding and for analysing plant genome evolution (Bennett 1988; Sybenga 1989; Fedak 1992; Tanksley et al. 1992; Heslop-Harrison et al. 1993).

Using the MMCT technique, Ramulu et al. (1996a,b) transferred single chromosomes of potato to tomato (*L. peruvianum*) or tobacco. This article presents data firstly on the molecular analysis of the integration of alien marker genes neomycin phosphotransferase II) (*npt-II*) and β-glucuronidase (*uid*A) in the chromosomes of the diploid potato (*S. tuberosum*) used as the donor source for microprotoplast fusion. Secondly, microprotoplast fusion hybrids and backcross progeny have been analysed to identify the potato chromosomes present using restriction fragment length polymorphism (RFLP) analysis and exploiting chromosome-specific markers.

Materials and methods

Plant materials and their origin

Suspension cells of transformed potato (*Solanum tuberosum*) line 413 were used as the source of microprotoplasts (Ramulu et al. 1996a). The alien marker genes *nptII* and *uid*A (Fig. 1) were introduced into the potato genome through *Agrobacterium*-mediated transformation (Gilissen et al. 1991). The wild tomato *Lycopersicon peruvianum*, line PI128650 (Koornneef et al. 1987), was the source of recipient protoplasts. Microprotoplast hybrid plants were produced by the fusion of potato microprotoplasts with recipient tomato protoplasts (Ramulu et al. 1996a). Several microprotoplast hybrid plants derived from different fusion products, i.e. 5-1, 5-3 and 5-5, were monosomic additions with 24 or 48 *L. peruvianum* chromosomes and one potato chromosome (Ramulu et al. 1996a). Backcross progeny plants (BC_1) were obtained after crossing a monosomic addition plant (plant 5-3-20; $2n = 24 + 1$) as a female with the cross-compatible diploid genotypes LA6 and LA7 of *L. peruvianum* ($2n = 2x = 24$).

Isolation of plant DNA and Southern analysis

Southern hybridization was carried out to determine the copy number and distribution of the marker genes *nptII* and *uid*A in the potato line 413 genome ($2n = 2x = 24$) and in microprotoplast hybrid plants. Genomic DNA was isolated from leaves of greenhouse-grown plants according to Bonierbale et al. (1988). After digestion, DNA was separated on a 0.7% agarose gel and blotted onto Gene ScreenPlus. Probes were random primed-labelled with α-[^{32}P]-dATP (according to Life Technologies RadPrime Labelling System). For probe DNA, the 1.0-kb *Bam*HI fragment from pNG1 containing the *nptII* coding region (Klein et al. 1990) and the 1.8-kb *Bam*HI-*Sst*I fragment from pGUS1 containing the *uid*A coding region of pBI121 (Jefferson et al. 1987) were used. Hybridization and washing were performed according to the Du Pont hybridization protocol prior to exposure.

GUS assay and polymerase chain reaction (PCR) analysis

The first backcross (BC_1) progeny plants were analysed by GUS assay to determine the number of plants harbouring the *uid*A gene marker. The GUS assay was performed as described previously (Ramulu et al. 1996a). The GUS negative plants were subsequently analysed by PCR to determine the presence of the *uid*A gene sequence. The rapid method described by Wang et al. (1993) was used with several modifications. The method was tested for potato and tomato as well as for Petunia, and the results were reproducible. From each plant, a small leaf was harvested in a 1.5-ml eppendorf tube and stored on ice. The following steps, which took about 15 min, were performed on ice: 200 µl 0.4 N NaOH was added, and grinding was performed with a disposable pipette tip. The mixture was centrifuged for 5 min, and 20 µl of the supernatant was transferred to a new 1.5-ml eppendorf tube. This was subsequently closed and placed for 35 s in a 700-W microwave at maximum power to degradate the RNA and fragment the DNA. The liquid was spun down and 30 µl 1 *M* TRIS-HCl pH 7.5 was added to reach a final pH of 8.2. For a 100-µl PCR reaction 0.3 µl of the mixture was used. The PCR reaction buffer consisted of 20 m*M* TRIS-HCl pH 8.4, 50 m*M* KCl, 200 µ*M* dNTPs, 1.5 m*M* $MgCl_2$, 0.5 µ*M* of each

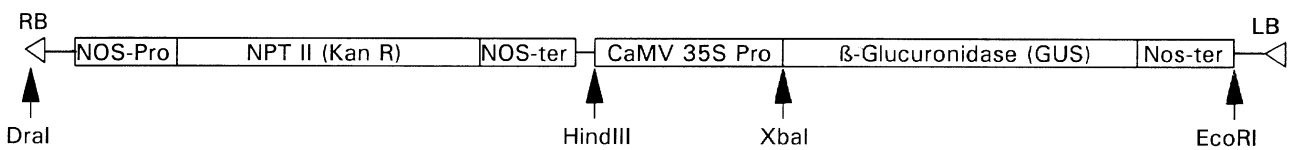

Fig. 1 Diagramatic presentation of the introduced T-DNA part with the location of the restriction sites *Dra*I (in RB), *Hin*dIII, *Xba*I and *Eco*RI into potato donor line 413

oligonucleotide primer and 0.5–1 unit *Taq* DNA polymerase (GIBCO BRL) per reaction. The oligonucleotides 5′-CTG TAG AAA CCC CAA CCC GTG-3′ and 5′-CAT TAC GCT GCG ATG GAT CCC-3′ were used, which produce a 514-base pair (bp)reaction product in the presence of the *uid*A gene (Van Wordragen et al. 1991). The programme for the amplification reaction was 2 min at 940°C, followed by 30 cycles of 50 s at 94°C (melting), 45 s at 60°C (annealing) and 2 min at 72°C (elongation). Finally, the reaction products were brought to full length by using an extra 5 min elongation cycle at 72°C. The PCR products were analysed on a 1.2% (w/v) agarose gel.

RFLP analysis to identify potato addition chromosomes in microprotoplast hybrids

For the identification, potato chromosome-specific markers were checked for RFLPs between potato line 413 and *L. peruvianum*. Tomato and potato DNA were digested with the restriction enzymes *Dra*I, *Eco*RV and *Hin*dIII, and Southern blots were made as described above. These were hybridized with 12 chromosome-specific markers, 1 for each chromosome (Table 1). The markers have been previously described (Tanksley et al. 1992; Jacobs 1995). The potato chromosome-specific marker was used when the hybridization bands of potato line 413 and *L. peruvianum* were distinctly different in size for restriction enzymes *Dra*I, *Eco*RV or *Hin*dIII. A sample of plants were chosen which were either *uid*A-positive and *nptII*-positive, or which hybridized with potato DNA as probe in genomic in situ hybridizations (GISH) (Ramulu et al. 1996a). Southern hybridization was performed as described above but using chromosome-specific markers. The hybridization patterns of the DNA from microprotoplast hybrid plants and backcross progeny plants were compared to that of *L. peruvianum*. Patterns differing from *L. peruvianum*, were compared using the RFLPs between potato and tomato to determine the presence of specific potato chromosomes.

Results

Analysis of the copy number of *nptII* and *uidA* genes and integration sites in donor potato line 413

Figure 1 is a diagramatic presentation of the T-DNA part from pBI121 with the locations of various sites of restriction enzymes that was used to determine the number of T-DNA insertion sites and copy number of the *nptII* and *uid*A genes in transgenic potato line 413. The data on Southern blot hybridization with the *nptII* and *uid*A probes are shown in Fig. 2. The restriction enzymes *Bgl*II and *Nde*I, which have no recognition site in the T-DNA, revealed two T-DNA insertion sites with the *nptII* probe (Fig. 2A) and three with the *uid*A probe (Fig. 2B, lane e). The restriction patterns with enzymes *Hin*dIII, *Xba*I, *Eco*RI and *Dra*I (Fig. 2B), which have one recognition site between the right and left borders of the T-DNA, clearly showed that three copies of the *uid*A gene have integrated in the potato genome, indicating three integration sites, each containing one copy of the *uid*A gene.

With respect to the *nptII* gene, the restriction enzymes *Xba*I, *Eco*RI, *Dra*I, *Bgl*II, and *Nde*I (Fig. 2A) showed two copies, whereas *Hin*dIII revealed three copies, suggesting that the two copies shown by the former restriction enzymes masked the third copy. The two *nptII* hybridization bands are located at the same position as two of the *uid*A bands, confirming that both genes are within the same DNA fragment. The combined data on *uid*A and *nptII* suggested that one *uid*A copy is separated from *nptII*.

Thus, the *nptII* and *uid*A copies were distributed in three integration sites in transgenic potato line 413. These consisted of (1) one copy each of *uid*A and *nptII*, (2) one *uid*A copy and two *nptII* copies and (3) one *uid*A copy.

Table 1 Details of the chromosome-specific markers used as probes for analysing the polymorphisms between potato and tomato species *Lycopersicon peruvianum* and identification of potato addition chromosomes in microprotoplast hybrid plants

Potato/tomato chromosome	Chromosome-specific markers
1	TG27
2	TG48
3	TDs369
4	TG123
5	TG23
6	TDs389* (near TG193)
7	TDs39
8	TDs300
9	TDs116
10	TDs152
11	Ssp75
12	TG491

* Not described earlier

Microprotoplast hybrid plants

Figure 3 shows the Southern hybridization patterns of *nptII* and *uid*A in microprotoplast hybrid plants. Three different hybridization patterns, i.e. 1–3 (Fig. 3B, C) were observed. The 7.3 kb *nptII* band observed in the microprotoplast hybrid 5-3-20 (Fig. 3B, lane 1), which did not occur in potato line 413 (lane N), was probably due to a change in size of the 6.1-kb fragment size

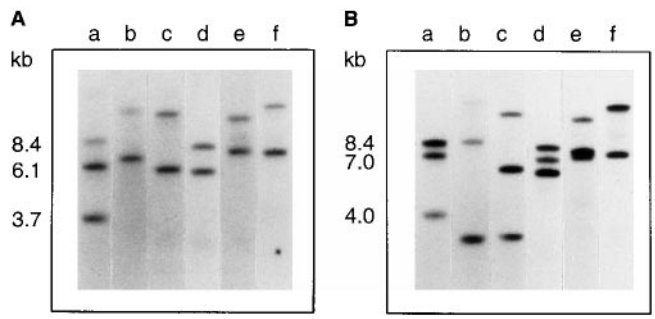

Fig. 2A, B Southern hybridization with *nptII* (**A**) and *uid*A (**B**) probes to genomic DNA of potato donor line 413 digested with *Hin*dIII (lane a) and *Xba*I (lane b), *Eco*RI (lane c), *Dra*I (lane d), *Bgl*II (lane e) and *Nde*I (*lane f*). Band sizes are given for *lane a* only

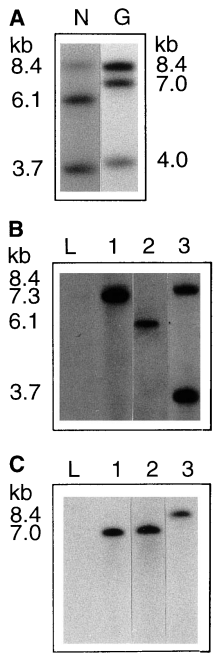

Fig. 3A Southern hybridization with *nptII* (*N*) and *uid*A (*G*) probes to *Hin*dIII-digested DNA of potato donor line 413. **B**, **C** Hybridization with *nptII* probe (B) and with the *uid*A probe (C), respectively, in microprotoplast hybrid plants. *L* Recipient line of *L. peruvianum* containing no *nptII* or *uid*A copies. Lanes 1–3 are examples of the three different hybridization patterns observed in microprotoplast hybrid plants 5-3-20 (*lane 1*), 5-2-9 (*lane 2*) and 5-5-5 (*lane 3*)

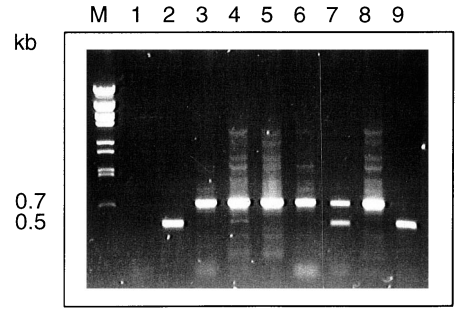

Fig. 4 The results from PCR analysis using primers for the *uid*A gene. With no template DNA (*lane 1*); with control template DNA of pGUS1 (*lane 2*); separate DNA samples from four representative examples of BC_1 progeny plants, C7-38 (*lane 3*), C7-39 (*lane 4*), C7-41 (*lane 5*), C7-42 (*lane 6*); microprotoplast hybrid plant 5-3-20 (*lane 7*); *L. peruvianum* (lane 8); potato line 413 (lane 9). Marker: lambda DNA digested with *Bst*EII (*lane M*)

because the 7.0-kb *uid*A band (present in 5-3-20) was linked to the 6.1-kb *nptII* band. Thus, these three hybridization patterns represent the first two integration sites described in the above section. The data also revealed that the pattern representing the third integration site, containing the 4.0 kb band of *uid*A (Fig. 3A, lane G) gene was not observed in microprotoplast hybrid plants (Fig. 3C) because the fusion products were selected on the basis of kanamycin resistance. These results revealed that the three integration sites are located on three different chromosomes.

Glucuronidase (GUS) assay and PCR analysis of the backcross progeny

The BC_1 progeny plants derived from crosses of the microprotoplast monosomic hybrid plant 5-3-20 with the recipient *L. peruvianum* (genotypes LA6 and LA7) were assayed for *uid*A expression. The GUS assay of leaves revealed that 14 of the 80 progeny plants expressed *uid*A. From the remaining 66 plants, 60 were further tested by PCR for the presence of the *uid*A gene. The results shown in Fig. 4 revealed that these plants, did not contain the 514 bp reaction product (*uid*A gene sequence), as shown with the control DNA of pGUS1 (lane 2), microprotoplast hybrid plant 5-3-20 (lane 7) and potato line 413 (lane 9). Furthermore, by comparing lanes 7, 8 and 9, it can be seen that the DNA of *L. peruvianum* (lane 8) or the DNA of microprotoplast hybrid plant 5-3-20 (lane 7) as template gave a PCR product approximately 700 bp in size. However, the nature of this product, originated in the presence of tomato DNA, is not known.

Identification of the potato addition chromosome using chromosome-specific RFLP markers

A total of 12 chromosome-specific probes were used for analysing the polymorphisms between potato and *L. peruvianum*, and for the identification of potato addition chromosomes in microprotoplast hybrids and BC_1 plants (Table 1). Figure 5 shows three RFLP hybridization patterns from the potato addition chromosomes identified in this study. The best results were obtained with *Eco*RV (Fig. 5A, lane 2) for the chromosome-specific marker for chromosome 3 (TDs369) and *Hin*dIII (Fig. 5B,C, lane 3) for chromosomes 4 (TG123) and 5 (TG23).

The data in Fig. 6A showed that the microprotoplast hybrid plant 5-5-5 (lane 5) contained the band (arrow marked) representing the chromosome 3 from potato and that this was absent in microprotoplast hybrid 5-5-20 (lane 6). Both these plants were derived from the same fusion product and contained 24 *L. peruvianum* and one potato addition chromosome. However, the microprotoplast hybrid plant 5-5-5 was *uid*A and *nptII* positive, whereas 5-5-20 was negative for both marker genes. Both plants shared the hybridization band (marked with arrow) (Fig. 6B, lanes 5 and 6) which belongs to chromosome 4, as shown by the RFLP data on potato and *L. peruvianum* (Fig. 5B, lane 3). Thus, these data show that 5-5-20 contained chromosome 4, whereas 5-5-5 had chromosomes 3 and 4. Also, the results of genomic in situ hybridization (GISH) on 5-5-5 showed that it contained a large potato chromosome with two centromeres (dicentric), indicating the presence of two fused chromosomes (data not shown). The results that 5-5-5 contained two copies of *nptII* and one copy of *uid*A (Fig. 3B and C) as well as the bands representing chromosomes 3 and 4, whereas 5-5-20

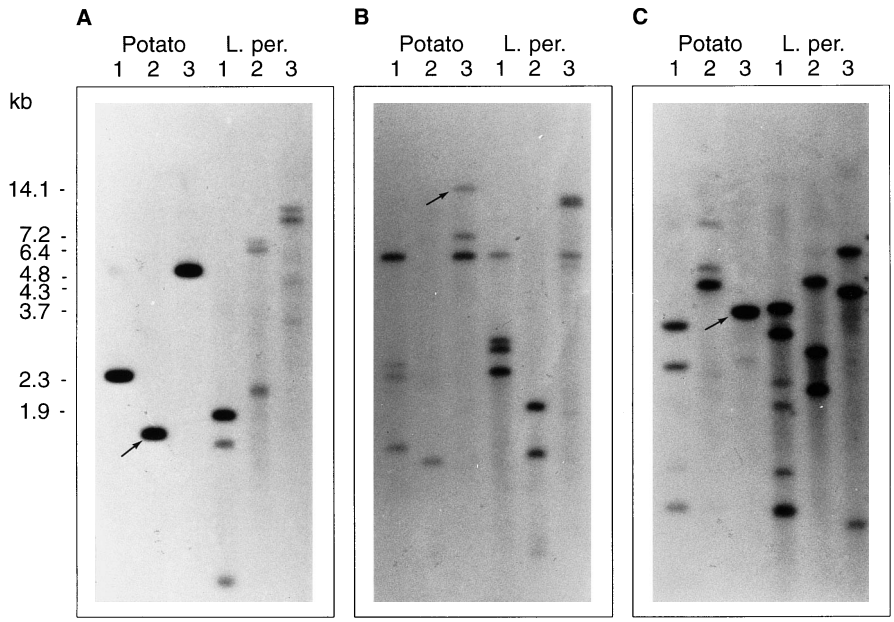

Fig. 5A–C Southern hybridization with probes for potato/tomato chromosome 3 (TDs369) (**A**), chromosome 4 (TG123) (**B**) and chromosome 5 (TG23) (**C**) to DNA of potato line 413 and *L. peruvianum* (*L. per.*) digested with *Dra*I (*lane 1*), *Eco*RV (*lane 2*) and *Hin*dIII (*lane 3*). The potato hybridization bands used for identification of the addition chromosomes are marked with *arrows*

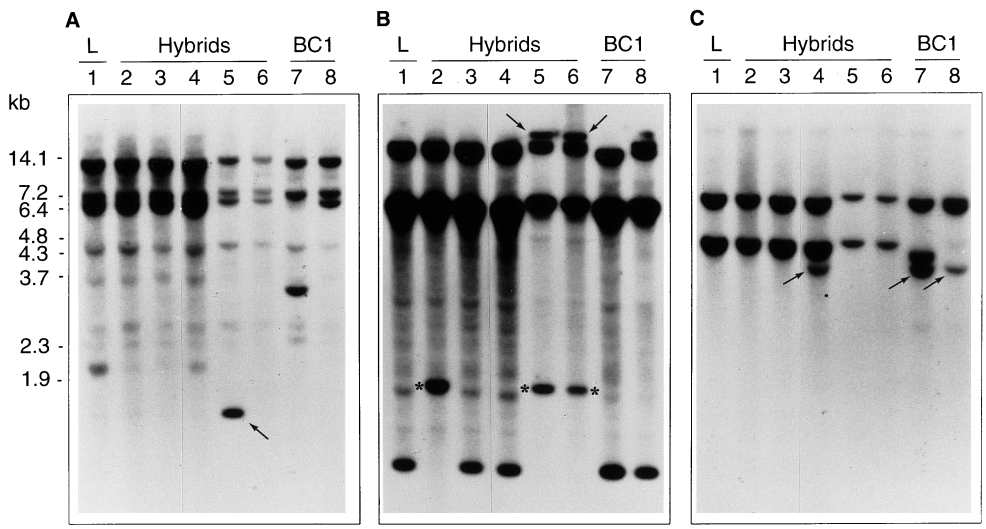

Fig. 6A–C Southern hybridization with probes for potato/tomato chromosome 3 (TDs369) (**A**), chromosome 4 (TG123) (**B**) and chromosome 5 (TG23) (**C**) to DNA of *L. peruvianum* (*lane 1*, *L*), *DNA of microprotoplast hybrid plants* 4-3-3 (*lane 2*), 5-1-10 (*lane 3*), 5-3-20 (*lane 4*), 5-5-5 (*lane 5*), 5-5-20 (*lane 6*) and DNA of the BC1 plants C2-29 (*lane 7*), and C7-17 (*lane 8*), digested with *Hin*dIII (**A** and **C**) or *Eco*RV (**B**). The potato chromosome-specific hybridization bands are marked with *arrows*. The hybridization bands marked with *asterix* belong to *L. peruvanum*

showed only the band representing chromosome 4, suggested that the missing segment of chromosome 3 in 5-5-20 harbours the marker genes *nptII* and *uid*A. The hybridization bands that were marked with an asterix (Fig. 6B) belong to the recipient *L. peruvianum* genome because they were not present in the donor potato line 413 (Fig. 5B).

Figure 6C shows that the probe for potato chromosome 5 gave a hybridization band in lanes 4, 7 and 8 (arrow) which was absent in *L. peruvianum* (lane L). These data reveal that the microprotoplast hybrid plant 5-3-20 (lane 4) and BC_1 plants C2-29 (lane 7) and C7-17 (lane 8), which were monosomic additions ($2n = 24$ *L. peruvianum* chromosomes + one potato chromosome), contained potato chromosome 5, harbouring one *uid*A and one *nptII* copy. Also, the pattern of the hybridization bands for BC_1 plants C2-29 (lane 7) and C7-17 (lane 8) conformed with the hybridization band observed in microprotoplast hybrid plant 5-3-20 (lane 4) because they were derived from the backcrosses of plant 5-3-20 with *L. peruvianum* cross-compatible genotypes LA7 and LA6, respectively. All 14 *uid*A-positive plants, 13 of which also contained the *nptII* gene, showed only 24 *L. peruvianum* chromosomes, i.e. the potato chromosome was absent as revealed by GISH.

Further, Fig. 6A and C showed differences in hybridization patterns between *L. peruvianum* (lane 1: L) and BC_1 (lanes 7 and 8) due to backcrossing with different *L. peruvianum* genotypes as compared to the genotype used for fusion.

Discussion

The data obtained in the present study showed that the *nptII* and *uid*A copies introduced into donor potato line 413 through *Agrobacterium*-mediated transformation were distributed in three integration sites. These consisted of 1) one copy each of *uid*A (7.0 kb) and *nptII* (6.1 kb), (2) one copy of *uid*A (8.4 kb) and two of *nptII* (3.7 kb and 8.4 kb), and (3) one *uid*A copy only (4.0 kb). These three integration sites were located on three different chromosomes, i.e. the first on chromosome 3, the second on chromosome 5 and the third on a chromosome carrying the *uid*A gene but without *nptII*. After the fusion of potato donor microprotoplasts with recipient *L. peruvianum* protoplasts, monosomic-addition hybrid plants containing 24 *L. peruvianum* chromosomes and one potato chromosome, i.e. chromosome 3 or 5, were obtained, as clearly demonstrated by our previous studies using GISH (Ramulu et al. 1996a). Hybrids with a potato chromosome carrying *uid*A without *nptII* could not be recovered as the fusion products were selected on the basis of kanamycin resistance. Studies with potato, tomato and tobacco, which possess small chromosomes (Ramulu et al. 1977; Pijnacker and Ferwerda 1984; Narayan 1987), and with other species suggest that GISH is a useful technique for chromosome identification (Leitch and Heslop-Harrison 1993; Schwarzacher and Heslop-Harrison 1993; Ramulu et al. 1996b).

The results of the present study also demonstrated that chromosome-specific markers are highly useful for the identification of the donor potato chromosomes present in microprotoplast hybrid plants. The use of a combined analysis of GISH and chromosome-specific RFLPs for the identification of chromosomes, as demonstrated for potato (+) *L. esculentum* somatic hybrids (Jacobsen et al. 1995), and for potato (+) *L. peruvianum* microprotoplast hybrids (Ramulu et al. 1996a; this study), offers attractive possibilities for monitoring chromosome and gene introgression between *Solanaceae*.

Further, our results revealed the stable transfer of a single potato chromosome carrying the *nptII* and *uid*A genes to tomato by microprotoplast fusion, and to seed progeny after backcrossing (see also Ramulu et al. 1996b). Several microprotoplast hybrids and the BC_1 progeny which expressed kanamycin resistance or *uid*A contained a donor potato chromosome as revealed by GISH. This suggests that the expression of *nptII* and *uid*A genes is linked to the presence of a specific donor chromosome. In addition, a few BC_1 plants contained the *nptII* and *uid*A genes in the absence of the potato addition chromosome, i.e. with only 24 tomato chromosomes, indicating that the marker genes have been integrated into the tomato genome. Various mechanisms for donor DNA integration, such as homoeologous chromosome pairing and recombination in meiosis, or somatic transfer mechanisms occurring in pre- or post meiotic cells, have been discussed previously (Sybenga 1989, 1992; Ramulu et al. 1996b). The transfer and introgression of alien genes through specific chromosomes between sexually non-crossable species, as demonstrated for the first time through microprotoplast fusion technology, offer several advantages for plant breeding and genome analysis, eg. gene synteny and chromosome evolution in various taxa (Bennett 1988; Sybenga 1989; Tanksley et al. 1992; Fedak 1992; Heslop-Harrison et al. 1993). When chromosome tagging with selectable markers, such as kanamycin or hygromycin resistance through transposable elements (Pereira et al. 1992) is used, the microprotoplast fusion technique would facilitate the construction of a complete set of monosomic addition plants. The direct production of monosomic additions and the occurrence of donor DNA integration in sexually non-hybridizing, but related species will be potentially useful for the transfer of economically important traits, such as disease resistance, which are controlled by polygenes clustered within blocks on specific chromosomes (Tanksley et al. 1992), and also non-host resistances or other characters that are not yet cloned, eg. apomixis (Hanna 1995). Intergeneric monosomic additions are useful for speeding up introgressive breeding, gene mapping and expression (Bennett 1988; Sybenga 1992; Heslop-Harrison et al. 1993) as well as for the construction of chromosome-specific DNA libraries (Schondelmaier et al. 1993).

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