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Tomato protoplast DNA transformation: physical linkage and recombination of exogenous DNA sequences

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Abstract

Tomato protoplasts have been transformed with plasmid DNA's, containing a chimeric kanamycin resistance gene and putative tomato origins of replication. A calcium phosphate-DNA mediated transformation procedure was employed in combination with either polyethylene glycol or polyvinyl alcohol. There were no indications that the tomato DNA inserts conferred autonomous replication on the plasmids. Instead, Southern blot hybridization analysis of seven kanamycin resistant calli revealed the presence of at least one kanamycin resistance locus per transformant integrated in the tomato nuclear DNA. Generally one to three truncated plasmid copies were found integrated into the tomato nuclear DNA, often physically linked to each other. For one transformant we have been able to use the bacterial ampicillin resistance marker of the vector plasmid pUC9 to 'rescue' a recombinant plasmid from the tomato genome. Analysis of the foreign sequences included in the rescued plasmid showed that integration had occurred in a non-repetitive DNA region. Calf-thymus DNA, used as a carrier in transformation procedure, was found to be covalently linked to plasmid DNA sequences in the genomic DNA of one transformant. A model is presented describing the fate of exogenously added DNA during the transformation of a plant cell. The results are discussed in reference to the possibility of isolating DNA sequences responsible for autonomous replication in tomato.

Introduction

Thus far, the isolation of genuine origins of replication has only been successful for yeast (10, 31, 32) and for the green unicellular alga *Chlamydomonas reinhardtii* (25), but not for higher organisms like plants. To test whether tomato DNA sequences, which promote autonomous replication in yeast (35), are capable of conferring on a plasmid the ability to replicate autonomously in plant cells, tomato protoplasts were transformed with a non-replicating plasmid and a series of plasmids containing the preselected tomato DNA segments. This approach for detecting autonomous replication in tomato cells was based on the idea of a distinct difference in transforming ability of autonomously replicating plasmids and non-replicating plasmids. Until recently, cultivated tomato (*Lycopersicon esculentum* Mill.) did not possess a potential for protoplast culture and regeneration as efficient as that of tobacco or petunia, though some improvements have been reported recently (28). The development of the new tomato genotype MsK93 (12), combining the excellent regeneration

characteristics of *L. peruvianum* with crossability with *L. esculentum*, enabled us to use tomato for protoplast DNA transformations.

Direct gene transfer to plant cells using simple *E. coli* vectors, which harbor a chimeric dominant selectable marker gene, has been described employing several different procedures (18, 9, 7, 29). In general, calcium phosphate precipitates are being used in conjunction with polymers like PEG (18) or PVA (9). However, none of the previous publications on direct gene transfer have provided explicit accounts of the patterns of integration of all exogenously supplied DNA sequences. Cotransformation of plasmid and carrier DNA has been shown to occur in plants (19). Genetic analyses of the progeny of transformed plants revealed that some of the integrated DNA fragments inherited as one block, suggesting that integration occurred in one locus (19, 21). Actual recombination between foreign DNA sequences has not been demonstrated, however. For mammalian cells much more work has gone into unraveling the mechanisms of integration (8, 27, 26, 11), providing clear evidence for recombination between non-homologous exogenous sequences (20, 2). In this paper we report a detailed biochemical analysis of plasmid DNA integrations into the nuclear DNA of seven MsK93 tomato transformants and present evidence showing linkage in plants between plasmid and carrier DNA.

Materials and methods

Selection of tomato ars elements

Chromosomal tomato DNA sequences which promoted the autonomous replication of the hybrid plasmid YIp5 in *Saccharomyces cerevisiae* YNN27 were isolated and characterized as described in detail by Zabel *et al.* (35). *Eco*RI and *Hin*dIII restricted tomato DNA was inserted into the *Eco*RI and *Hin*dIII site of YIp5 and the respective pools of hybrid molecules were used to transform YNN27 to the Ura3$^+$ phenotype. DNA from the yeast transformants was used to transform *E. coli* to ampicillin resistance. The tomato DNA inserts in those shuttle plasmids were subcloned into the plant vector plasmid pneoΔ18 (3) resulting in the respective pCTW plasmids (Fig. 1).

Plant material and protoplast isolation

The origin, properties and the isolation of protoplasts from callus of the tomato genotype MsK93 has been described (12).

DNA isolations

All plasmids were isolated using the alkaline extraction procedure (4), followed by one CsCl-ethidium bromide gradient banding. Non-transformed MsK93 tomato leaves and transformed calli, maintained on R3B medium (15), were used for plant DNA isolations as described (17). At this stage, selection for kanamycin resistance was omitted in order to detect possible loss of the kanamycin resistance gene.

DNA transformation of protoplasts

Two methods of transformation were applied, both slightly modified compared to the original procedures of (i) Krens *et al.* (13) and (ii) Hain *et al.* (9):

(i) Transformants TMJ22-1, -2, -3 and -6 and TMJ78-1: To 1 ml of 5×10^5 protoplasts in ATp medium (1) 10 μg plasmid DNA and 50 μg calf-thymus DNA was added and gently mixed with the protoplasts. Thereafter, 0.5 ml of 40% (w/v) polyethylene glycol (PEG-6000) dissolved in F-medium (13) was added and the mixture was left for 10 min. F-medium was then added at 10-min intervals (2×1 ml, 2×2 ml, 1×3 ml) followed by pelleting and culturing.

(ii) Transformants TMJ22-4 and -5: First a calcium phosphate-DNA coprecipitate of plasmid DNA with or without carrier DNA was formed. Either 50 μg plasmid DNA (leading to transformant TMJ22-4) or 10 μg plasmid DNA and 50 μg calf-thymus DNA (leading to TMJ22-5) were dissolved in 1 ml HEPES-buffered saline (NaCl, 140 mM;

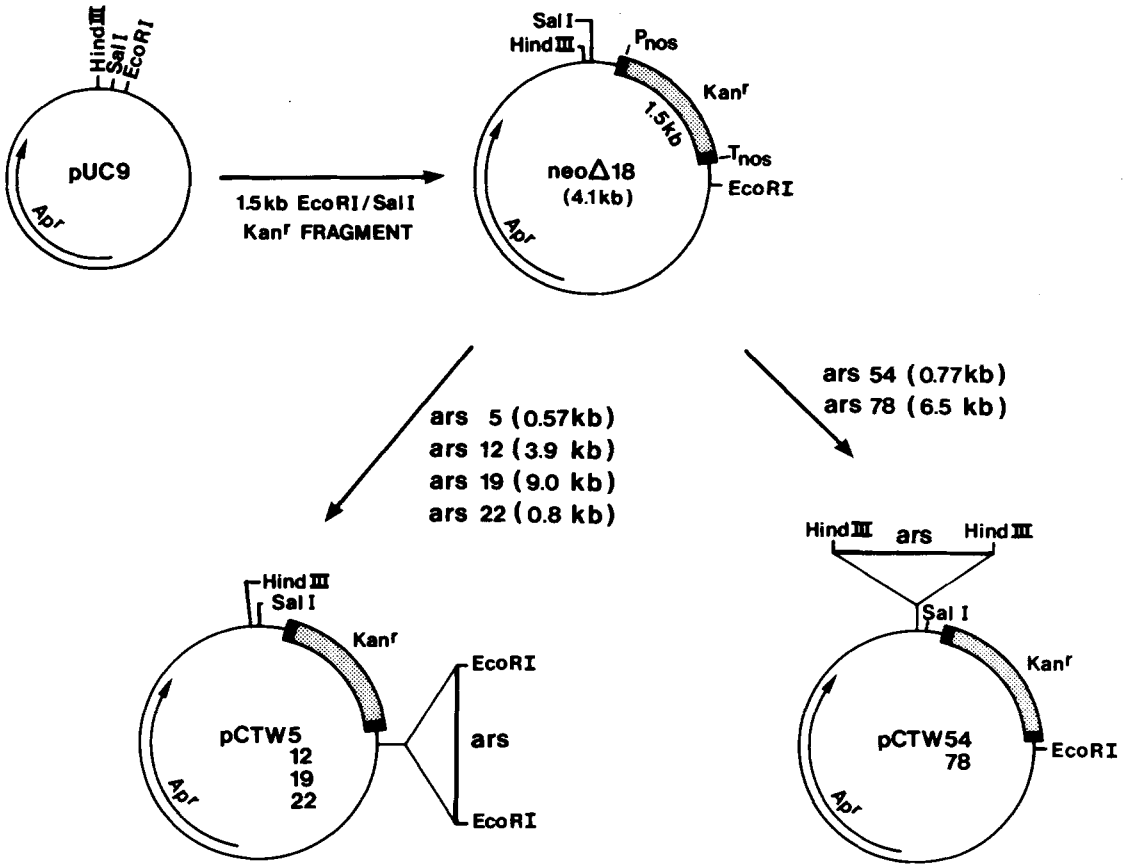

Fig. 1. Construction of plasmids containing a dominant selectable marker for plant cell transformation. *Eco*RI (ars5, 12, 19 and 22) and *Hin*dIII (ars54 and 78) restricted chromosomal tomato DNA fragments, selected for autonomous replication in yeast (35), were inserted in pneoΔ18 (3) as shown, resulting in the respective pCTW plasmids. Kan^r, kanamycin resistance gene from Tn5; P_{nos} and T_{nos}, promoter (P) and polyadenylation signals (T) derived from the nopalin synthase gene (nos).

KCl, 5 mM; $K_2HPO_4 \cdot 2H_2O$, 0.55 mM; glucose, 5 mM; HEPES, pH 7.05, 21 mM). The DNA solution was adjusted to 125 mM $CaCl_2$ using a 2 M $CaCl_2$ solution and left for 20 min to allow the formation of a calcium phosphate-DNA coprecipitate at room temperature. Subsequently, 10^6 MsK93 callus protoplasts in 0.25 ml ATp protoplast culture medium were added to the DNA solution and incubated for 10 min. A solution of 20% (w/w) polyvinyl alcohol (PVA, degree of polymerization 300, 88% hydrolyzed) in 0.4 M mannitol was added dropwise to a final concentration of 10%. After 10 min, F-medium was added at 10-min intervals (2×1 ml, 2×2 ml, 1×3 ml) followed by pelleting and culturing.

Protoplast culture and selection; culture of transformed cell lines

Protoplasts were cultured in liquid ATp medium (1) at a density of 5×10^4 protoplasts/ml and at 25 °C. After 4–6 days, when the first divisions could be observed, the cultures were diluted 1:1 with fresh ATp medium and transferred from the dark to low light conditions (approximately 200 lux). Selection for kanamycin resistance (50 mg/l) was started at about two weeks after protoplast isolation. After repeated dilutions with ATp medium containing 5.5% mannitol and 50 mg/l kanamycin, transformed microcalli were transferred to solid ATc medium (1) at 7 to 11 weeks after protoplast

isolation. From this medium they were transferred to ATs medium (1) and subsequently to 2Z medium (33) in order to lower the osmotic value for sustained growth of the microcalli and to change the hormonal balance to favor shoot regeneration. Kanamycin selection was maintained at a level of 50 mg/l for about six months.

Southern blot analysis

For Southern blot analysis, 10 μg of total plant DNA was digested overnight with 100 units of restriction endonucleases as specified in the figure legends in 100 μl of the appropriate restriction buffer. The DNA was precipitated by adding ethanol and the dried DNA pellet was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Restriction fragments were separated on 1% agarose gels and transferred to Gene Screen Plus membrane filters employing an alkaline blotting procedure (22). Probes were either labeled from an M13 template (Kan^r gene) as described (16) or synthesized by nick-translation (pUC9, ars22, calf thymus DNA) as described (24). When calf-thymus DNA was used as a probe, 1 μg instead of the usual 50 ng was labeled. Unreacted nucleotides were removed by chromatography on a Sephadex G50 column. The specific activity of the probes was approximately 10^8 cpm/μg. Prehybridization was performed for 24 hours at 65 °C in 10 × Denhardt solution (14), 40 mM Tris-HCl pH 7.0, 5 × SSC, 1 mM EDTA, 1% SDS and 100 μg/ml of sonicated denatured salmon sperm DNA. Hybridization was carried out for 40 hours at 65 °C in fresh prehybridization solution containing about 2 ng/ml of denatured ^{32}P-labeled DNA probe, prepared as described above. Filters were washed for 10 min in 5 × SSC, 1 mM EDTA at room temperature and for 60 min in 1 × SSC, 0.1% SDS at 65 °C and finally autoradiographed at −80 °C for 2–4 days using an intensifying screen. Probe was removed by washing at 42 °C in 0.4 M NaOH for 30 min. After subsequent neutralization in 0.2 M Tris-HCl pH 7.5, 2 × SSC and overnight exposure to check the removal of probe, the full hybridization procedure was carried out again with a different probe.

Plasmid rescue

In a rescue experiment, 10 μg genomic DNA was digested overnight in a volume of 100 μl with 100 units of restriction endonuclease *Eco*RI. The reaction mixture was ethanol precipitated and centrifuged. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in 1 ml ligation buffer ($MgCl_2$, 5 mM; dithiotreitol, 5 mM; BSA, 0.1 mg/ml; Tris-HCl, pH 7.4, 50 mM; ATP, 0.5 mM) containing 400 units of T4 ligase. The mixture was incubated overnight at 15 °C and the DNA was again ethanol precipitated, centrifuged, washed, dried and dissolved in 100 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. This batch of circularized cellular DNA was used to transform 5 ml of competent cells of *E. coli* strain MH1 using a $CaCl_2$ procedure (5). After the transformation procedure, the cells were pelleted and resuspended in 5 ml of broth media; 200 μl of this suspension was added to each Petri dish of broth media agar containing ampicillin at 50 μg/ml.

Results

Protoplast transformation

Autonomous replication in tomato cells (referred to as art activity) was meant to lead to an increase in the transformation frequency. We obtained six transformants for plasmid pCTW22 and one for pCTW78, but none for the original vector plasmid pneoΔ18 without a tomato DNA insert (Table 1). For these three plasmids similar numbers of viable protoplasts were tested, but experimental circumstances were not always identical and never more than two transformants were recovered in any one experiment. In order to check whether the transformed phenotype was maintained by free or integrated plasmids the transformed microcallus was subcultured on medium without kanamycin. No segregation or loss of the resistant phenotype was observed, when after one month kanamycin selection was applied again. Stable kanamycin resistance was observed in all cases, strongly suggesting that integration was the predominant form of transfor-

Table 1. Transformation of tomato protoplasts with plasmids containing a tomato DNA segment selected for autonomous replication in yeast.

Plasmid	Carrier DNA	No. of experiments/ transformation procedure[a]	No. of treated protoplasts ($\times 10^6$)[b]	No. of microcalli[c]	No. of kanamycin resistant calli
pneoΔ18	+	-	1.8	24800	-
	−	-	0.5	12300	-
pCTW5	+	-	0.5	300	-
	−	-	-	-	-
pCTW12	+	-	0.5	200	-
	−	-	-	-	-
pCTW19	+	-	1.6	5200	-
	−	-	1.5	10000	-
pCTW22	+	3 (i)	3.1	17400	4 (TMJ22-1, -2, -3, -6)
	+	1 (ii)	1.8	1200	1(TMJ22-5)
	−	- (i)	2.0	9700	-
	−	1 (ii)	1.0	6600	1 (TMJ22-4)
pCTW54	+	-	0.5	500	-
	−	-	-	-	-
pCTW78	+	1 (i)	1.5	11700	1[d] (TMJ78-1)
	−	-	0.5	400	-

[a] Indicated are the number of experiments that resulted in kanamycin resistant calli, carried out according to transformation procedures (i) or (ii) as described in the Materials and methods section.

[b] All experiments were carried out using 0.3 − 1.0 million protoplasts resulting in transformation frequencies of approximately 3×10^{-4} transformants per microcallus or 1×10^{-6} based on the number of protoplasts treated. The column represents cumulative figures.

[c] The number of microcalli represents the number of protoplasts surviving the protoplast isolation and the DNA transformation procedure.

[d] Initially 11 transformants were identified in one Petri dish. DNA analysis revealed that they were in fact 11 identical subclones.

mation. Most other plasmids (pCTW5, 12, 19 and 54) were not tested on significant numbers of protoplasts due to the relatively low viability (0.1 − 2.0% survival) of the protoplasts at that time.

Only one of the six pCTW22 transformants was obtained without the use of carrier DNA even though approximately the same number of protoplasts was screened. This confirms earlier findings (29) that the addition of carrier DNA may increase the frequency of transformation of plant protoplasts by a factor of three.

DNA analysis of transformants TMJ22-1 to -6

In order to be able to carry out a detailed analysis of the integrated DNA copies of plasmid pCTW22, we chose the restriction enzymes *Eco*RI and *Hin*dIII to distinguish between (i) full length single copy integrations, (ii) tandem repeats, (iii) inverted repeats and (iv) the extent of degradation or scrambling. *Hin*dIII cuts only once in the multilinker between pUC9 and the Kan^r gene, resulting in a full length copy, which would enable us to distinguish between different types of repeats. *Eco*RI has two sites at either side of the ars22 tomato DNA insert, thus differentiating between intact pneoΔ18 and ars22. The double digestion with *Eco*RI and *Hin*dIII creates the three separate segments of Kan^r, pUC9 and ars22. When only part of a segment was found integrated into the genome, the relative intensities of the radio-active bands on the autoradiographs served to indicate the extent of degradation or fragmentation. The three probes used – the 1.5 kb *Eco*RI-*Sal*I segment comprising the kanamycin re-

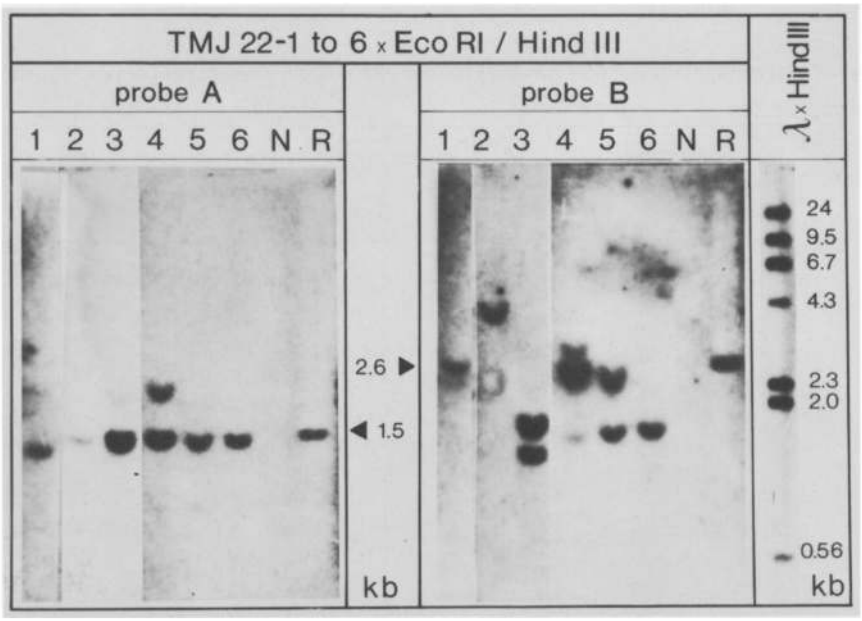

Fig. 2. Detection of the Kan^r gene and pUC9 sequences in the DNA of callus lines transformed with plasmid pCTW22. After double digestion with restriction enzymes *Eco*RI and *Hin*dIII and electrophoresis of 10 μg DNA per lane, DNA of the TMJ22 transformants was analyzed according to the Southern procedure. After hybridization to the Kan^r probe, A, the blot was washed to remove the probe and rehybridized with the pUC9 probe, B. Lanes 1–6, DNA from callus of transformants TMJ22-1 to -6; lane N. DNA from non-transformed MsK93 leaves; lane R, single copy reconstruction of plasmid pCTW22 by mixing 10 pg pCTW22 with 10 μg salmon sperm DNA after double digestion with *Eco*RI/*Hin*dIII. The phage λ DNA restricted with *Hin*dIII was end-labeled with radio-active α-^{32}P-ATP prior to electrophoresis. The arrows indicate the presence of an intact integrated copy of the Kan^r gene (1.5 kb) and the pUC9 sequences (2.6 kb).

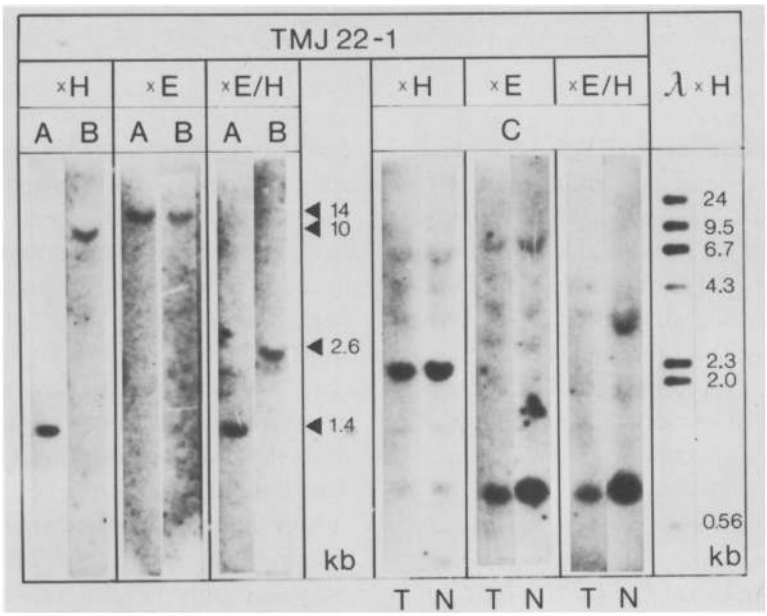

Fig. 3. Detection of all pCTW22 sequences in the DNA of transformant TMJ22-1. After restriction with either *Hin*dIII (xH), *Eco*RI (xE) or *Eco*RI/*Hin*dIII (xE/H) of 10 μg DNA per sample and subsequent electrophoresis, DNA was treated according to the Southern procedure and successively hybridized with probes of firstly, the Kan^r gene (A), secondly, pUC9 DNA (B) and thirdly, ars22 (C). In the case of probe C the transformant TMJ22-1 lane (T) as well as the non-transformed MsK93 lane (N) is shown, because probe C is a tomato-derived DNA sequence so that only additional bands in T indicate an integrated copy (see also the legend of Fig. 2).

sistance gene, plasmid pUC9 (2.6 kb) and the 0.8 kb *Eco*RI ars22 segment – covered the full length (4.9 kb) of pCTW22 and thus in principle any integrated part of the plasmid DNA would be detected.

Five out of six transformants (Fig. 2) possessed the Kan^r gene intact as a 1.5 kb segment. In transformant TMJ22-1 the Kan^r gene has lost at least 0.1 kb of its original gene sequence. On either side of the gene there are non-coding regions, however, whose absence does not interfere with the production of a functional protein. In fact, it was shown (23) that enzyme activity of the Kan^r gene product is not impaired when substantial parts of the original N- or C-terminus are deleted. Based on the number of bands in combination with band intensity (Fig. 2), two or three copies of the Kan^r gene were found to be integrated in most transformants. When we probed for pUC9 DNA sequences, only callus lines TMJ22-1, TMJ22-4 and TMJ22-5 were shown to contain the intact 2.6 kb segment (Fig. 2).

The autoradiographic details of transformant TMJ22-1 (Fig. 3) were interpreted in the following way: Double digestion with *Eco*RI and *Hin*dIII showed pUC9 to be intact (probe B: 2.6 kb) but the Kan^r gene to be slightly shorter (probe A: 1.4 kb). *Hin*dIII digestion showed an identical Kan^r fragment (probe A: 1.4 kb), implying that the Kan^r gene had lost the *Eco*RI site and gained a second *Hin*dIII site on a slightly smaller fragment. *Eco*RI digestion showed both pUC9 and the Kan^r gene to be located on the same DNA fragment (probe A, B: 14 kb). From this information we deduced that the Kan^r gene and pUC9 had remained physically linked through the original *Hin*dIII site and that recombination with either carrier or plant DNA had occurred near the C-terminus of the Kan^r gene. The other end of the linear integrated plasmid consisting of the ars22 DNA sequence between *Eco*RI sites and the C-terminal end of the Kan^r gene was not detected using the Kan^r probe. However, more significantly, the ars22 necessarily contained in the 10 kb band of *Hin*dIII did not show up either, meaning that besides the terminal DNA sequence of the Kan^r gene also most of the ars22 DNA sequence had disappeared. As the *Eco*RI site was preserved in the pUC9 segment some of the ars22 DNA must have remained intact, though.

This interpretation of TMJ22-1 is drawn in Fig. 4 together with those of the other five transformants. When we consider all integrations separately, approximately 60% of the selected Kan^r sequences and 40% of the non-selected pUC9 and ars22 sequences are found as intact integrated segments. The patterns of integration were also checked with the restriction endonucleases *Pvu*II, *Pst*I and *Hae*III (results not shown). TMJ22-4, for example, the only transformant obtained without carrier DNA, was thus unambiguously shown to possess two integrated plasmids linked by a stretch of 2.5 kb tomato DNA. Using total calf-thymus DNA as a probe carrier DNA was detected in none of the TMJ22 lines, but may nevertheless have cointegrated in TMJ22-1, -2, -3, -5 and -6, as non-repetitive calf-thymus DNA escapes detection by a calf-thymus DNA probe. Surprisingly, the close physical linkage of exogenous sequences via short stretches of undefined DNA was a pattern recurring in four out of six analyzed transformants. We were able to connect only those sequences possessing overlapping restriction fragments and detectable DNA sequences. Therefore, the extent of coupled integration may in fact be greater and include copies that have now been drawn as separate or single integrations.

Plasmid rescue from transformant TMJ22-4

The presence of the bacterial plasmid pUC9 sequence in the genomic DNA of transformant TMJ22-4 (Fig. 2), as part of the integrated plasmid pCTW22, enabled us to rescue this DNA sequence in *E. coli* as a recombinant plasmid including the unknown flanking DNA region. The rescued plasmid TMJ22-4 represented a 7.4 kb *Eco*RI restriction fragment (Fig. 4), consisting of 3.4 kb tomato DNA linked to approximately 1.4 kb of the Kan^r gene and 2.6 kb pUC9 sequence. Restriction analysis revealed that the tomato DNA fragment did not contain any *Hae*III sites, possibly implying integration in an AT-rich region of the genome. In order

TMJ22

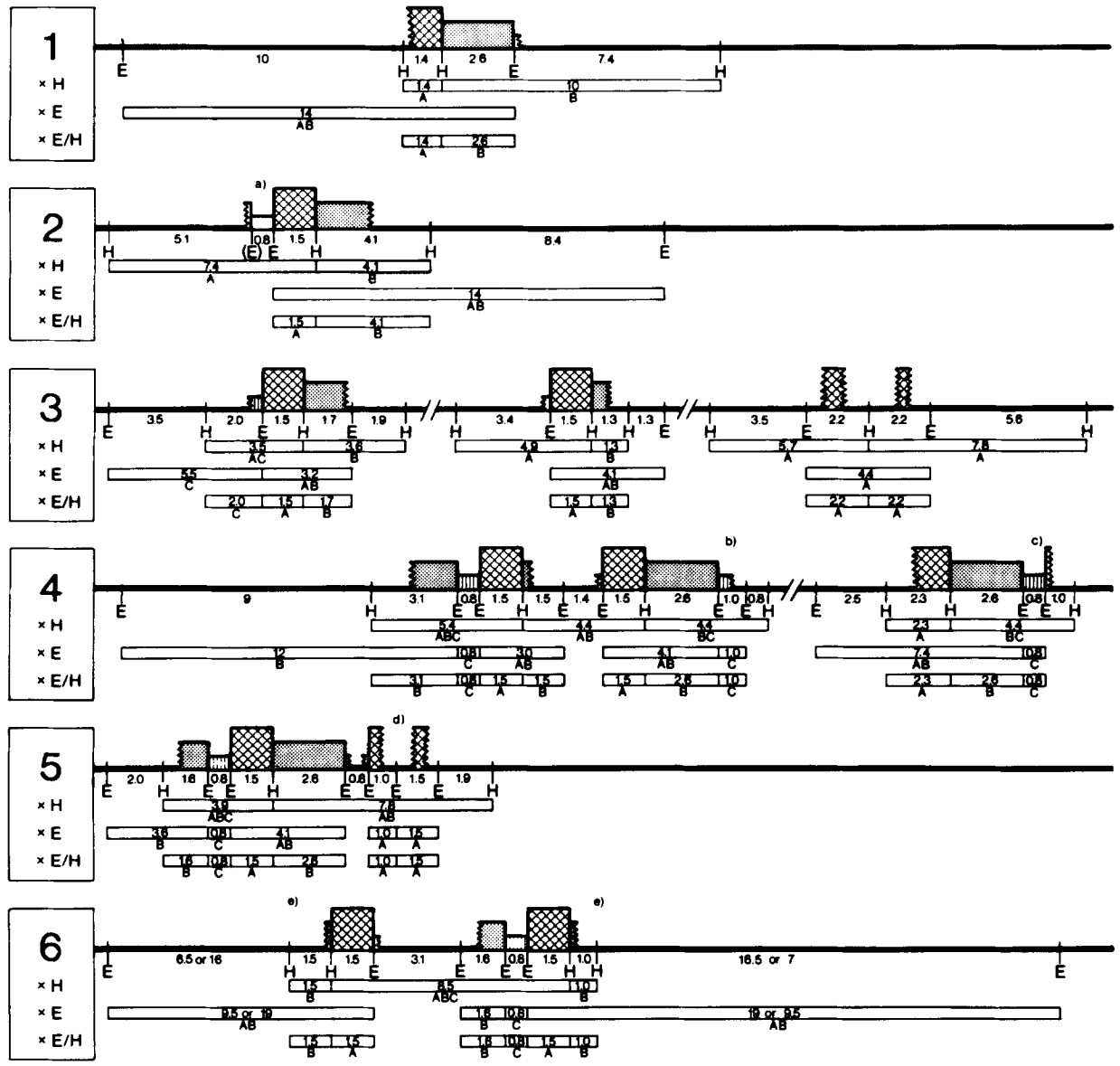

Fig. 4. Restriction endonuclease map of the exogenous pCTW22 DNA sequences incorporated into the nuclear DNA of transformants TMJ22-1 to -6. Data have been derived from autoradiographs similar to the one shown in Fig. 3. The heavy line next to the TMJ numbers indicates either tomato or carrier DNA. Boxes on top of this line represent segments of the plasmid pCTW22 with shadings corresponding as follows: Kan^r, ▩ pUC9, ▨ ars22 ▥. Boxes below this line represent the pattern of probed restriction fragments. The interruption sign signifies physically separate integration. Jagged edges represent recombination with bordering DNA sequences. E and H denote the restriction sites for *Eco*RI and *Hin*dIII and the numbers indicate the length of the restriction fragments in kb. The respective probes correspond to those in the other figures: Kan^r (A), pUC9 (B) and ars22 (C). In the left margin the restriction endonucleases used, resulting in the pattern of fragments to the right, are indicated.

a) Endogenous sequences detected by probe C, prevented confirmation of this stretch of DNA.
b) the 1 kb fragment detected by probe C may also belong to the third integrated fragment (c).
c) It is not clear from the autoradiograph whether the 4.4 kb band detected by probe C represented a double hybridization or not. When it would be a single band the indicated fragment ends in the 0.8 kb section.
d) These two *Eco*RI fragments detected by probe A may be located anywhere within the 7.8 kb *Hin*dIII fragment.
e) These two *Hin*dIII fragments detected by probe B may exchange positions.

to determine the nature of the rescued fragment of tomato DNA the entire plasmid was nick-translated and hybridized to non-transformed tomato DNA (data not shown). The hybridization intensity was equivalent to the single copy reconstruction which suggests that the flanking tomato DNA only contained unique DNA sequences in this case.

Recombination of pCTW78 sequences with carrier DNA

Transformant TMJ78-1 presented a special case compared to the various TMJ22 transformants for three reasons: (i) both the Kan^r gene and pUC9 DNA sequences had undergone modifications, (ii) no exogenous ARS78 sequences could be detected due to the high background hybridization of the endogenous sequences already present and (iii) cotransfer of carrier DNA during transformation was detected. The combination of (i) and (ii) made a full interpretation of the autoradiographs not feasible. However, we are able to give a qualitative description of the integration event due to the fact that we detected highly repetitive co-integrated carrier DNA sequences which showed an identical electrophoretic migration pattern compared to endonuclease fragments containing plasmid pCTW78 sequences (Fig. 5). Double digestion of DNA from transformant TMJ78-1 with *Eco*RI/*Hin*dIII gave rise to four restriction fragments, each of which hybridized to two of the three probes used (Kan^r, pUC9 and calf-thymus DNA).

These restriction fragments would imply recombination between (i) Kan^r and carrier DNA, (ii) pUC9 and carrier DNA and (iii) Kan^r and pUC9. A similar pattern of supposedly fused DNA fragments reappeared after digestion with *Hin*dIII. Larger DNA fragments were observed, which hybridized with two or all three of the probes used, suggesting that leaving out one restriction enzyme created larger concatenate fragments containing all three types of DNA. These results suggest that in the particular case of TMJ78-1 extensive degradation and/or fragmentation of pCTW78 DNA sequences had occurred, followed by fusion with cotransferred carrier DNA sequences into a larger concatenate DNA fragment and subsequent integration of this intermediate into the genomic DNA.

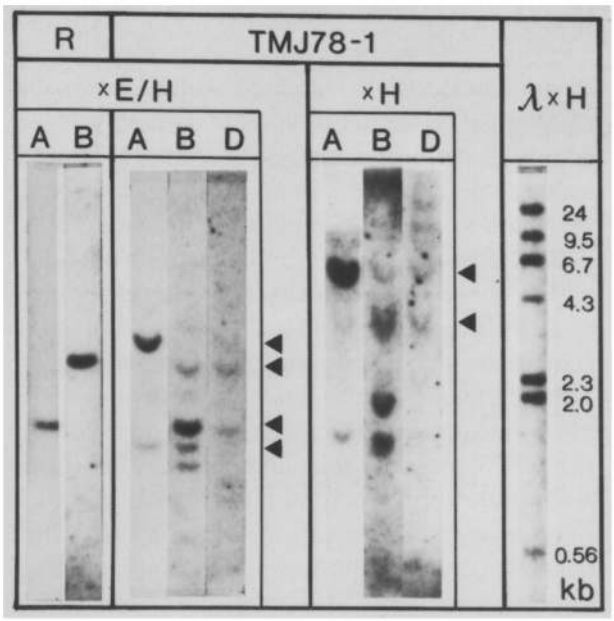

Fig. 5. Detection of pCTW78 sequences and carrier DNA in transformant TMJ78-1. After restriction with either *Eco*RI/*Hin*dIII (xE/H) or *Hin*dIII (xH) of 10 μg DNA per sample and electrophoresis, DNA was treated according to the Southern procedure and subsequently hybridized to the Kan^r probe (A), pUC9 probe (B) and calf thymus DNA probe (D). The arrows indicate DNA fragments which are fusions of carrier DNA and/or Kan^r and/or pUC9 (see also the legend of Fig. 2). The reconstruction lanes (R) are only given for the internal Kan^r and pUC9 fragments.

The selection for transformants was carried out in liquid medium. In the case of TMJ78-1, the transformed microcallus could therefore disintegrate prior to identification resulting in the appearance of 11 different micro calli resistant to kanamycin. Southern blot analysis (data not shown) of these subclones after independent maintenance on non-selective medium for six months revealed identical DNA integration patterns. Apparently, no rearrangements, deletions or amplifications of inserted DNA sequences had occurred at the early stage of callus development, as otherwise a major fraction of the cells of one or more subclones might have possessed a deviating structure.

Discussion

Using a new tomato genotype which carries the regeneration potential of *L. peruvianum*, we have addressed the question whether tomato DNA elements, selected for autonomous replication in yeast, act as origins of replication in tomato cells.

Generally, protoplast survival (0.1–2.0%) following the calcium phosphate-DNA transformation procedure remained too low for obtaining statistically significant numbers of transformants. This interfered with the initial objective of comparing the frequencies of transformation of the different pCTW plasmids containing putative origins of replication with the control plasmid pneoΔ18 without a tomato DNA insert. However, a higher frequency of transformation would not in itself have proved autonomous replication of the plasmid, as the transforming ability of the plasmids may also have a bearing on other factors like the mechanism or frequency of integration and the size or code of the insert. In order to differentiate between these factors a detailed DNA analysis of the transformants was still required. Recently, it was shown (29) that the use of carrier DNA in the transformation procedure resulted in a 3-fold increase in the transformation frequencies of plant cells. This fact prompted us to use carrier DNA as well. From our results it was apparent that carrier DNA may not be neutral in function, however, and may also play an active role in stabilizing, fusing or integrating plasmid DNA sequences. These possible additional functions of carrier DNA would clearly interfere with the aim of discerning between the properties encoded on tomato derived ars elements.

For autonomous replication it is necessary that the plasmids remain circular in order to be able to fully replicate and to withstand any exonuclease activity. Finding full length plasmids which integrated in a single cross-over event would thus support the plausibility of autonomous replication. However, the frequent degradation and fragmentation of the plasmids into smaller fragments, which in some cases were linked with carrier DNA to form a larger concatenate prior to integration, suggests that they did not arrive intact in the nucleus. The few plasmids that would have remained intact and replicated autonomously may then very well have passed by unnoticed.

One potential problem associated with our approach of screening for art activity by testing plasmids containing tomato DNA for a higher transformation frequency, may be the high frequency with which integration of foreign DNA sequences is prone to occur in plants independent of the properties encoded on the plasmid. Shillito *et al.* (29) employing the electroporation technique have standard transformation frequencies with tobacco protoplasts of 2% and Crossway *et al.* (6) attained 6% using cytoplasmic and 14% using intranuclear microinjection (injecting 2000 copies/cell), suggesting that the bottleneck for plant cell transformation is mainly concerned with passing all the physical barriers and not with the event of integration.

The stable physical presence of the kanamycin resistance gene was evidenced when no loss or segregation of the resistant phenotype was observed after one month of subculture on non-selective medium. We have subsequently shown by Southern procedure that tomato protoplasts transformed by plasmid pCTW22, carrying a 0.8 kb tomato DNA insert, contain up to three copies of the kanamycin resistance gene integrated into the tomato nuclear DNA. Generally, less than full length plasmid copies integrated into the nuclear DNA. Nuclease activity probably linearized and degraded the plasmid DNA to different extents prior to integration. Tandem integrations as reported with liposome-protoplast fusion (7) were not found.

We found evidence for physical linkage of integrated plasmid DNA fragments in those transformants containing more than one integrated fragment. However, we were not able to account for the linkage of all fragments in those transformants. Evidence for a single genetic location of DNA insertions in the plant genomic DNA and against the hypothetical possibility that our lines represent mixed cell populations, can only be derived from analyses of the progeny of regenerated fertile plants. Thus Potrykus *et al.* (21), studying tobacco transformants obtained with a transformation procedure similar to the one described here, showed that several non-functional gene copies and bacterial plasmid sequences behaved as a single genetic lo-

cus together with the functional gene. Our difficulty with regenerating these particular transformed tomato callus lines prevented us from performing a similar experiment.

The presence of a copy of pneoΔ18 bordered on both sides by an intact ars22 sequence would have served to indicate homologous recombination. No such pattern was found and we therefore conclude that homologous recombination between our exogenously supplied tomato DNA sequences and the resident copies did not occur. In mammalian cells such events are known to happen only at very low frequency (30).

The overall pattern of integration suggests that recombination with plant genomic DNA occurs at random sites in the plasmid DNA. The analysis of a recombinant plasmid from total cellular tomato DNA, which, apart from a complete plasmid pUC9 sequence, included a recombinant kanamycin resistance gene fused to an unknown DNA sequence, indicated that recombination with genomic DNA may occur in non-repetitive regions of the genome. This contrasts with recent findings for mammalian cells employing a similar calcium phosphate transformation procedure (11).

In the TMJ22 transformants we were not able to detect any carrier DNA sequences (results not shown). This does not mean, however, that no such sequences have actually been cotransferred and integrated into the tomato genome. Firstly, Southern blots were coated with salmon sperm DNA which harbors sequences homologous to calf-thymus DNA. If such calf-thymus DNA sequences would have been integrated in the transformants these sequences would be masked by the salmon sperm DNA coat. Secondly, calculations carried out by Peerbolte *et al.* (19) show that only highly repetitive integrated calf-thymus DNA sequences (1–10% of the genome) will be detected by a calf-thymus DNA probe. This leads to a chance of 10–20% of detecting any calf-thymus DNA, only, when one monomer of a highly repetitive DNA sequence has integrated into the plant genome. When more carrier DNA is integrated, the chance of encountering one of these highly repetitive sequences increases proportionally. Cotransformation of carrier DNA is therefore clearly not ruled out in these cases, but may not be as extensive as was found by Peerbolte *et al.* (19). This would agree with the observation that in many TMJ22 transformants, fragments of plasmid DNA were linked via short stretches of undefined DNA. In that respect, it is tempting to suggest that the simple model put forward for DNA transformation of mammalian cells (20) and the fungus *Aspergillus nidulans* (34) applies for plant cells as well. According to this model exogenous DNA is preferably processed by subsequent (i) degradation and/or fragmentation, (ii) ligation to a more stable intermediate and (iii) integration of the concatenate molecule into the genome. In the case of TMJ78-1 we provide evidence supporting this hypothesis by showing that it is indeed carrier DNA linking those sequences, similar to what has been demonstrated for mammalian cells (20). Transformant TMJ22-4 was transformed without using any carrier DNA, but, nevertheless, also in this case we show that two major fragments of plasmid DNA are linked by a stretch of 2.5 kb tomato DNA. A similar phenomenon was also witnessed in mouse cells (2). In terms of the model this may be explained by the fact that during the isolation and transformation of protoplasts, many protoplasts are ruptured and may release their DNA content into the medium. As no carrier DNA was used in this experiment, any alternative carrier would have been accepted quite readily, possibly resulting in a linkage of plasmid sequences by tomato carrier DNA prior to integration.

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